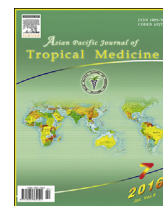




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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2016.05.001>The protective effect and underlying mechanism of Hainan papaya water extract against neuronal apoptosis induced by A β 40Jiu-Hong Zhao^{1,#}, Hai-Ying Zhang^{1,#}, Xian-Fang Zhang¹, Xu Dong², Qi-Bing Liu³, Yue-Li Liu³, Yi-Di Huang¹, Quan-Peng Zhang¹, Gang Luo¹, Zhi-Jian Ma^{1,2}, Xi-Nan Yi^{1,2*}¹Department of Anatomy, Hainan Medical College, Haikou 571199, China²Neuroscience Research Institute, Hainan Medical College, Haikou 571199, China³School of Pharmacy, Hainan Medical College, Haikou 571101, China

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ABSTRACT

Objective: To investigate whether Hainan papayas has protective effects in an A β 40-induced primary neuron injury model and elucidate the underlying molecular mechanism.**Methods:** Cultured primary neurons from the dorsal root ganglia (DRG) of Sprague–Dawley (SD) rats were treated with 20 μ M A β 40 peptide, 100 μ g/L Hainan papaya water extract, peptide plus extract, or culture medium for 24 h. Cell viability was measured by MTT assay, and neuronal apoptosis was evaluated by DAPI staining. ERK signaling pathway-associated molecule activation and changes in Bax expression were analyzed by Western blotting and immunofluorescence.**Results:** A cell viability rate of (44.11 \pm 6.59)% in the A β 40 group was rescued to (79.13 \pm 6.64)% by adding different concentrations of the extract. DAPI showed pyknotic nuclei in 39.5% of A β 40-treated cells; the fraction dropped to 17.4% in the 100 μ g/L extract group. ERK phosphorylation was observed in the A β 40 group but was ameliorated by pretreatment with 100 μ g/L extract. Hainan papaya water extract also prevented A β 40-induced phosphorylation of MEK, RSK1 and CREB associated with ERK signaling and downregulated Bax expression in the neurons.**Conclusion:** The results suggest that Hainan papaya water extract has protective effects on neurons; the mechanism may be related to suppression of ERK signaling activation.

1. Introduction

Hainan papaya water extract has strong antioxidative effects and can delay the aging process by clearing the abundant reactive oxygen species that are present in the liver and blood of mice [1–3]. In a previous large-scale mouse behavioral study, we found that Hainan papaya water extract could improve the learning and memory capacity of Mo/Hu APPswe PS1dE9

double-transgenic Alzheimer's disease (AD) model mice and decrease the levels of intracerebral A β (manuscript in preparation). However, whether Hainan papaya water extract shows neuronal protective effects remains unknown. AD is associated with pathogenic changes that are caused by many factors, and there are multiple pathogenic mechanisms [4–6]. Oxidative damage and cellular apoptosis are extremely important in the pathogenesis of AD [7,8]. The ERK1/2 signaling pathway, a classical mitogen-activated protein kinase (MAPK) signaling pathway, plays a key role in regulating A β -induced apoptosis and toxic synapse injury in PC12 cells [9,10]. In the present study, we injured primary cultured neurons using A β 40 and then evaluated whether Hainan papaya water extract exhibited neuroprotective effects. We also investigated whether this effect correlated with phosphorylation of molecules associated with the ERK1/2 signaling pathway. This study will facilitate future studies of the anti-AD effects of Hainan papaya constituents and provides evidence for its pharmaceutical value.

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These authors contributed equally to this work.

2. Materials and methods

2.1. Experimental animals

Sprague–Dawley (SD) rats, aged 1–2 weeks, were purchased from the Hunan Agricultural University (Changsha, Hunan, P.R. China) and housed in standard rat cages with 12 h light/–dark cycles (the lights switched on at 6:00 am) and with water and rat chow available *ad libitum*. Animals were sacrificed and tissues were collected with the approval of the animal ethics committee of Hainan Medical College. All studies were performed in strict accordance with the guidelines of the Chinese animal protection and management law.

2.2. Hainan papaya water extract

Hainan papayas were purchased from Beijing Tong Ren Tang Chinese Medicine Co. Ltd in May 2014, which were identified by Prof. Niankai Zeng. A voucher specimen (No. HP201405) was deposited in the herbarium of School of Pharmaceutical Science, Hainan Medical University. Approximately 5 kg of fresh Hainan papaya fruits were cut into slices, dried for 12 h at 50 °C in an air-drying oven, mashed, covered with 5 L of purified water and then digested in a water bath at 60 °C for 12 h. The water extract was filtered through absorbent gauze, and the filtrate was concentrated under reduced pressure to remove as much water as possible using a Buchi rotary evaporator (Flawil, Switzerland). The remaining water was then removed by freeze drying. A dark brown block of Hainan papaya water extract (8.7 g) was obtained and stored at –20 °C until use.

2.3. Neuronal cultures

The DRG were isolated from 3-week-old SD rats that had been sacrificed by dissociation of the cervical spinal cord under sterile conditions and were then cultured as previously reported [11]. The culture medium was changed every other day. A β 40 (1 mg) was dissolved in 2 mL of saline, mixed well, aliquoted and stored at 4 °C until use. Freeze-dried Hainan papaya water extract powder was dissolved in fresh culture medium for each experiment. The cultured neurons were divided into 4 groups, which were subsequently treated with 20 μ M A β 40 peptide (Sigma, St. Louis, MO, USA), 100 μ g/L Hainan papaya water extract, 20 μ M A β 40 peptide plus 100 μ g/L Hainan papaya water extract or culture medium (CON).

2.4. Cell viability and apoptosis measurements

Neuron cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay according to methods previously reported [12]. Briefly, 20 μ L of MTT solution was added to cultured neurons in a 96-well plate before incubation for 4 h at 37 °C. Next, the culture medium was carefully removed from the wells, and 100 μ L of formazan solution (Boster) was added to the cells to help dissolve the precipitated formazan crystal products. After incubating for 15 min with gentle shaking to dissolve the precipitated crystals, the plates were immediately analyzed by measuring the absorbance at 570 nm using an ELISA microplate reader.

Neuronal apoptosis in each treatment group was analyzed by DAPI (4',6-diamidino-2-phenylindole) staining. After performing the desired treatment for 24 h, neurons grown on culture slides were fixed, stained with DAPI (Boster) in the dark for 1 min and then washed with phosphate-buffered saline (PBS) 3 times. The samples were observed under 365 nm excitation using an immunofluorescence microscope. The number of apoptotic cells was calculated based on DNA staining with the DAPI fluorescence probe. Apoptotic neurons were characterized by the presence of fragmented or condensed nuclei. Cells in 10 different fields of view were counted per treatment in triplicate under 200 \times magnification on the fluorescence microscope. Approximately 100 neurons were counted in each field of view. The results are expressed as the percentage of apoptotic cells among the total number of cells counted.

2.5. Immunofluorescence assay

Immunofluorescence staining was performed as reported previously [8]. Neurons grown on culture slides were fixed, blocked with 5% donkey serum at room temperature for 1 h, incubated with primary antibodies at 4 °C overnight, and finally incubated with secondary antibodies at room temperature for 1 h. The primary antibodies used in this study were chicken anti-rat neuron-specific enolase (NSE) (1:100, Abcam) and mouse anti-rat p-ERK-1/2 (1:100, Sigma). The secondary antibodies were Alexa Fluor 488-conjugated donkey anti-chicken IgG (1:200, Jackson) and Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:200, Jackson). Slides stained with 2% donkey serum instead of primary antibody were used as a negative control. Positive staining was visualized using a confocal fluorescence microscope (Nikon 901 Japan).

2.6. Western blotting

Western blotting was performed as reported previously [8]. After digestion with trypsin, neurons from each treatment group were mixed with protein lysis buffer, incubated for 15 min, placed on ice for 25 s, and then vortexed for 40 min. After centrifugation, the supernatant was harvested as the protein lysate, and its concentration was determined using the Bradford method. The protein lysates (20 μ g/well) were separated using 8% SDS-polyacrylamide gel electrophoresis and then transferred electrophoretically onto nitrocellulose membranes. The membranes were blocked with 5% fat-free milk for 1 h, incubated with primary antibodies at 4 °C for 12 h, and then incubated with secondary antibodies at room temperature for 2 h. The color was developed using 3,3'-diaminobenzidine (DAB) solution. The primary antibodies used in this study were mouse antibodies against rat p-ERK, p-MEK, p-RSK1, p-CREB, and Bax (all were used at a 1:1000 dilution; Abcam). After scanning the results using an HP Scanjet G4050, the levels of total ERK, MEK, RSK1, and CREB were quantified with the Quantity One Software using β -actin as an internal reference.

2.7. Statistical analysis

All results are presented as the means \pm S.E.M. Statistical analysis was performed using SPSS 15.0 software. The differences between groups were evaluated using a one-way ANOVA, and $P < 0.05$ was considered a significant difference.

3. Results

3.1. Hainan papaya water extract prevents A β 40-induced declines in neuronal viability

A preliminary study revealed that A β 40 at concentrations ranging from 10 to 80 μ M led to cell death in a dose-dependent manner, with 20 μ M A β 40 inducing a mild extent of cell injury. Therefore, this concentration was used for all further experiments (data not shown). To characterize the protective effects of Hainan papaya water extract on cell viability after A β -induced neuronal cell injury, cells were pre-incubated with various concentrations (200, 100, 50, 25 μ g/L) of Hainan papaya water extract for 10 min before being treated with 20 μ M A β 40. Cells' viability in different groups was evaluated using the MTT assay. The result showed that neurons exposed to 20 μ M A β 40 for 24 h had a cell viability of (44.11 \pm 6.59)%, which was significantly decreased compared to CON. In contrast, pretreatment of the cells with Hainan papaya water extract at 200, 100, 50, or 25 μ g/L rescued cell survival remarkably, to (76.20 \pm 5.38)%, (79.13 \pm 6.64)%, (65.50 \pm 3.67)%, and (58.44 \pm 3.58)%, respectively. Furthermore, the cells did not show obvious signs of toxicity after treatment with 100 μ g/L of Hainan papaya water extract [cell viability: (95.36 \pm 5.39)%]. These results suggest that Hainan papaya water extract has a protective effect against A β 40-induced neuronal cell injury.

3.2. Hainan papaya water extract inhibits A β 40-induced neuronal apoptosis

To assess the effect of Hainan papaya water extract on the growth of cultured neurons, apoptosis was analyzed by observing the morphology of the bodies and nuclei of neurons after staining with NSE and DAPI. As shown in Figure 1, compared with CON group, the A β 40 group contained fewer neurons, and the neurons exhibited shrunken bodies that stained densely and unevenly (Figure 1C). DAPI staining revealed pyknotic nuclei (Figure 1G). The percentage of cells with pyknotic nuclei was significantly higher in the A β 40 group

(39.5%) than in CON group (3.5%) or the A β 40 plus Hainan papaya water extract group (17.4%) ($P < 0.05$). There was no significant difference in the percentage of cells with pyknotic nuclei between the Hainan papaya water extract group (5.7%) and CON group (3.5%).

3.3. Hainan papaya water extract prevents the A β 40-induced activation of ERK1/2

The ERK1/2 signaling pathway plays a pivotal role in regulating cellular apoptosis. Phosphorylation of ERK1/2 leads to its translocation into the nucleus [13]. To assess the effect of Hainan papaya water extract on ERK1/2 phosphorylation, we stained p-ERK1/2 in the neurons from each group. The data revealed that p-ERK1/2 localized mainly in the cytoplasm, but not the nuclei, of normal neurons (Figure 2D). After treatment with A β 40 for 24 h, the p-ERK-1/2 levels increased, and positive staining was observed in the nucleus (Figure 2A). In neurons pretreated with Hainan papaya water extract for 10 min before being treated with A β 40 for 24 h, p-ERK-1/2 staining was not observed in the nucleus (Figure 2C). This result suggests that Hainan papaya water extract prevents the A β 40-induced nuclear translocation of ERK1/2. Next, Western blotting was used to assess the levels of phosphorylated and total ERK1/2, with total ERK1/2 being used as a loading control. The data revealed that the A β 40 group had the highest level of phosphorylated ERK-1/2; it was nearly 2.53 times that of CON group. Compared with A β 40 group, the level of phosphorylated ERK-1/2 in the A β 40 plus Hainan papaya water extract group was significantly decreased ($P < 0.05$) (Table 1).

3.4. Hainan papaya water extract inhibits A β 40-induced MEK activation

MEK, or ERK-1/2 kinase, can activate ERK by phosphorylating MEK. A previous study reported that A β 40 can activate MEK [14]. To evaluate whether Hainan papaya water extract can inhibit A β 40-induced MEK activation, Western blotting was used to detect MEK phosphorylation levels in cultured neurons from each group; the total MEK levels were used as a loading

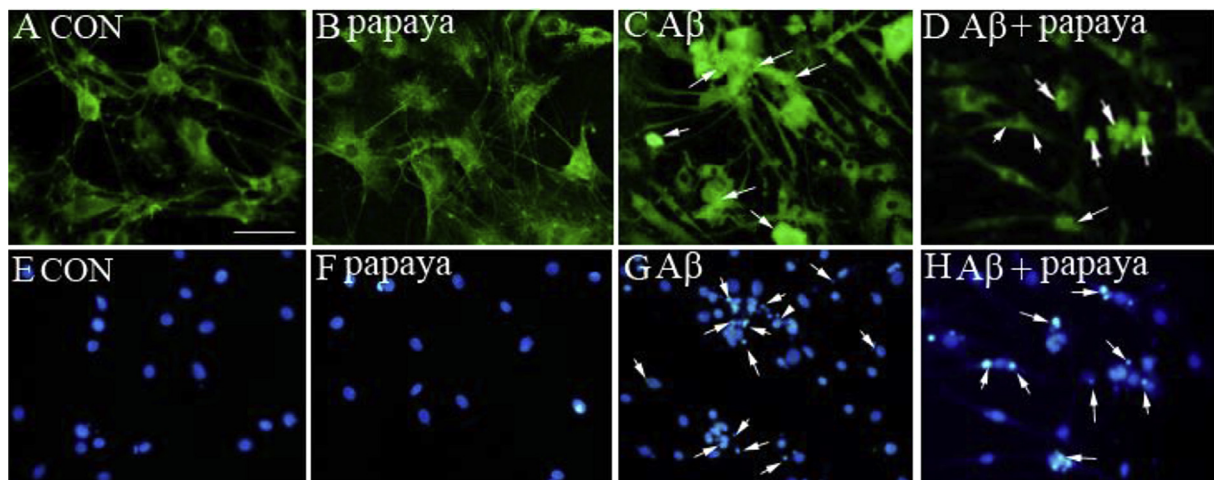


Figure 1. Papaya water extract prevents A β 40-induced neuronal apoptosis. Cultured DRG neurons were treated with 20 μ M A β 40 (C and G), 100 μ g/L Hainan papaya water extract (B and F), a combination of both (D and H) or no treatment (A and E) for 24 h. The cells were immunostained with anti-NSE (A–D) or DAPI (E–H). Positive staining was visualized using a confocal fluorescence microscope. The pyknotic nuclei were counted with Axiovision software version 4.6.3. Arrows indicate a small number of neurons with pyknotic nuclei (scale bar = 100 μ m).

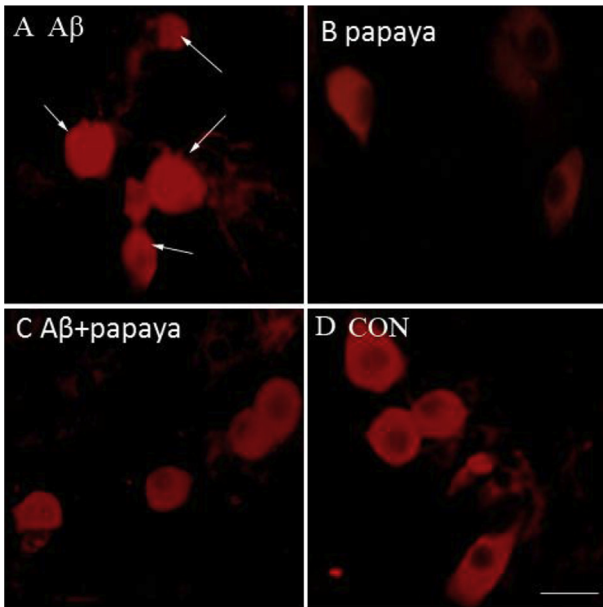


Figure 2. Effect of papaya water extract on A β 40-induced ERK-1/2 activation.

Cultured DRG neurons were treated with 20 μ M A β 40 (A), 100 μ g/L Hainan papaya water extract (B), a combination of both (C) or no treatment (D) for 24 h and were then stained with an anti-p-ERK1/2 antibody. ERK nuclear translocation (arrow) can be observed only in A (scale bar = 50 μ m).

control. As shown in Table 1, MEK phosphorylation was highest in the A β 40 group; it was nearly 1.95 times that of CON group ($P < 0.05$). Compared with A β 40 group, the MEK phosphorylation level in A β 40 plus Hainan papaya water extract group was significantly decreased ($P < 0.05$). This result suggests that Hainan papaya water extract can inhibit A β 40-induced MEK activation.

3.5. Hainan papaya water extract prevents A β 40-induced RSK1 activation

RSK1 (90-kDa ribosomal protein S6 kinase), also known as MAPK kinase 1, is a downstream molecule in the ERK-1/2 signaling pathway that regulates the expression of apoptosis-related proteins. ERK phosphorylates and activates RSK1, which upregulates the expression of such apoptosis-associated molecules as Bax. Western blotting was used to evaluate the levels of phosphorylated RSK1 in each group, which were normalized to the total RSK1 levels. As shown in Table 1, RSK1 phosphorylation was highest in the A β 40 group; it was nearly twice that of CON group ($P < 0.05$). Compared with A β 40 group, RSK1 phosphorylation in the A β 40 plus Hainan papaya water extract group was significantly decreased ($P < 0.05$).

Table 1

The activation level of ERK1/2 pathway-associated molecule in all groups (via Western blotting method).

Groups	p-ERK/ERK	p-MRK/MRK	p-RSK1/RSK1	p-CREB/CREB	Bax/ β -actin
CON	0.32 \pm 0.06	0.29 \pm 0.03	0.18 \pm 0.03	0.21 \pm 0.05	0.16 \pm 0.09
A β 40	0.81 \pm 0.03 [#]	0.57 \pm 0.13 [#]	0.36 \pm 0.03 [#]	0.67 \pm 0.03 [#]	0.50 \pm 0.02 [#]
papaya	0.40 \pm 0.02	0.28 \pm 0.02	0.19 \pm 0.02	0.25 \pm 0.01	0.20 \pm 0.01
A β 40 + papaya	0.52 \pm 0.03 [*]	0.37 \pm 0.02 [*]	0.25 \pm 0.03 [*]	0.31 \pm 0.02 [*]	0.28 \pm 0.02 [*]

#: Compared with CON group, $P < 0.05$; *: compared with A β 40 group, $P < 0.05$.

3.6. Hainan papaya water extract prevents A β 40-induced CREB activation

CREB (cAMP-responsive element-binding protein) can be activated after being phosphorylated by RSK1. Western blotting was used to evaluate the CREB phosphorylation levels in each group, which were normalized to the total CREB levels. As shown in Table 1, CREB phosphorylation was highest in the A β 40 group; it was nearly 3 times that of CON group ($P < 0.05$). Compared with A β 40 group, the CREB phosphorylation level in the A β 40 plus Hainan papaya water extract group was significantly decreased ($P < 0.05$).

3.7. Hainan papaya water extract down-regulates A β 40-induced Bax expression in neurons

Next, to analyze the expression of ERK-1/2 downstream genes, the levels of Bax in each group were measured using Western blotting, with β -actin being used as an internal control. As shown in Table 1, Bax expression was highest in the A β 40 group; it was 3.12 times that of CON group ($P < 0.05$). Compared with A β 40 group, the expression of Bax in the A β 40 plus Hainan papaya water extract group was significantly decreased ($P < 0.05$).

4. Discussion

In this study, primary cultured neurons isolated from rat DRG were used to establish an A β 40-induced neuronal injury model. These cells were pretreated with 100 μ g/L Hainan papaya water extract for 10 min prior to treatment with A β 40 to evaluate its protective effects. The data revealed that treating cells with A β 40 alone for 24 h induced neuronal apoptosis, significantly decreased cell viability, significantly increased the phosphorylation of molecules associated with the ERK1/2 signaling pathway, and induced the translocation of activated ERK1/2 into the nucleus. Treatment with Hainan papaya water extract alone showed no toxic effects on the neurons. Furthermore, Hainan papaya water extract exhibited neuroprotective effects because it was found to suppress A β 40-induced activation of the ERK1/2 signaling pathway by inhibiting the phosphorylation of its targets, to prevent ERK1/2 nuclear translocation, and to significantly decrease A β 40-induced neuronal apoptosis.

The “A β hypothesis” has been widely accepted as one of the primary mechanisms of AD. To date, several studies have established AD cell models by using A β to injure neurons. In most cases, the neurons were isolated from the hippocampus or cerebral cortex of neonatal mice [15,16]. The current study was the first to initiate an AD cell model by injuring neurons isolated from the rat DRG, and the results demonstrated that A β 40 treatment increases DRG neuronal apoptosis and decreases viability. After

culture with A β 40 for 24 h, the level of p-ERK in DRG neurons increased, and nuclear translocation of p-ERK was observed; the phosphorylation levels of the downstream markers MEK, RSK1, and CREB also all increased, and expression of the apoptosis-related protein Bax was enhanced. Young *et al.* found that A β could activate a tyrosine kinase to initiate a signaling cascade that resulted in the activation of the MAP kinase ERK1/2 in AD neurons and caused increased phosphorylation levels among proteins associated with the ERK1/2 signaling pathway [17,18]. These findings are in accord with the present results, which suggest that DRG neurons are sensitive to A β 40 and can be used as an AD cell injury model.

The pathogenic mechanism of AD is complex. In addition to apoptosis, oxidative damage is also extremely important during the early stages of AD. Modern pharmaceutical research has demonstrated that papaya has strong antioxidant effects [1–3]. In theory, its antioxidant activity can help clear the abundant reactive oxygen species in the brain at the early stages of AD, thereby providing neuronal protection. Nonetheless, the present study is the first to confirm that papaya water extract has no toxic effects on neurons. The MTT assay results showed that neuronal viability was lower in the Hainan papaya water extract group compared with the blank control group, but the difference between the two was not statistically significant. This result suggests that papaya water extract is not toxic to neurons. Furthermore, pre-treatment with papaya water extract decreased A β 40 toxicity and increased the viability of the DRG neurons.

The ERK1/2 signaling pathway is active mainly in neuronal dendrites, axons, and activated astrocytes [19,20]. This pathway is important for transducing extracellular signals to the nucleus via cell surface receptors. The ERK1/2 signaling pathway regulates several cellular functions and plays particularly important roles in cell proliferation, differentiation, and apoptosis. Certain extracellular stimuli (e.g., A β 40) can induce the phosphorylation of MEK, which further activates ERK1/2 and thus stimulates translocation of p-ERK1/2 to the nucleus, where it phosphorylates the transcription factors RSK and CREB. p-RSK and p-CREB regulate intracellular signaling and are involved in regulating the transcription and translation of apoptosis-related genes such as Bcl-2 and Bax. A β 40 accelerates cellular apoptosis by upregulating Bax gene expression via the activation of ERK1/2 signaling. The present study revealed that pre-treatment with Hainan papaya water extract could mitigate the A β 40-induced increase in p-ERK levels and inhibit p-ERK translocation into the neuronal nucleus. Further examination of the expression levels of molecules associated with the ERK pathway showed that pre-treatment with Hainan papaya water extract for 10 min led to significant reductions in p-ERK1/2, p-MEK, p-RSK1, p-CREB, and Bax levels (all $P < 0.05$).

In summary, our study demonstrates that the neuroprotective effects of Hainan papaya water extract are associated with the downregulation of ERK1/2 signaling. It has been reported that Hainan papaya water extract contains several components, including phenols, flavone, and polysaccharides [21,22]. However, the components of papaya extract that are responsible for its neuroprotective effects remain to be determined.

Conflict of interest statement

We declare that we have no conflict of interest.

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