Pharmacogn. Mag.
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ORIGINAL ARTICLE

Costunolide Attenuates Oxygen-glucose Deprivation/ Reoxygenation-Induced Apoptosis in Mouse Brain Slice through Inhibiting Caspase Expression

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Submitted: 09-Aug-2020 Revised: 08-Dec-2020 Accepted: 24-Feb-2021 Published: 12-Jul-2021

ABSTRACT

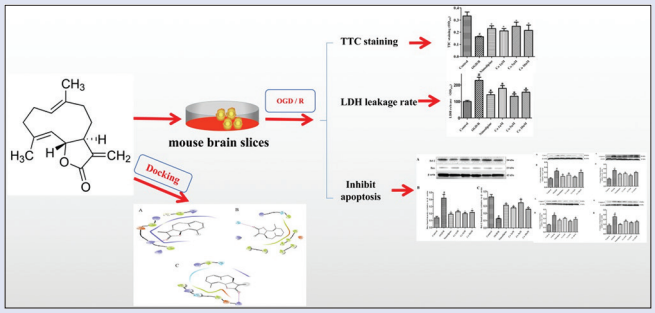
Background: Costunolide (Co) has anti-tumor, anti-inflammation, and anti-ulcer effects, it has the budding effect of anti-apoptosis. This study was intended to explicate its inhibitory effect on caspase in a mice brain slices injury model. **Materials and Methods:** The maestro 11.1 software was employed to envisage the binding sites of Co with Caspase-3, Caspase-9, and Caspase-7. Oxygen-glucose deprivation/reoxygenation (OGD/R) method was employed to persuade mouse brain slice injury *in vitro*. Purpose of lactate dehydrogenase (LDH) in culture medium and 2,3,5-triphenyl-tetrazolium chloride (TTC) staining of brain slices for the assessment of injury degree. The expression of Cytochrome c, Caspase-9, Caspase-7, Caspase-3, Bcl-2, and Bax was studied by Western blot method. **Results:** The results of docking displayed that Co had binding sites withe Caspase-9, Caspase-7, and Caspase-3. Compared with OGD/R, Co could diminution the LDH levels, upsurge the TTC staining intensity, augment Bcl-2 expression level and inhibit Caspase-3, Caspase-9, Caspase-7, Bax, and Cytochrome c expression levels. **Conclusion:** These results recommended that Co has latent neuroprotective activities by inhibiting caspase expression.

Key words: Apoptosis, brain slice, Caspase-3, costunolide, ischemic stroke

SUMMARY

- Costunolide (Co) has binding sites with Caspase-9, Caspase-7 and Caspase-3. Oxygen-glucose deprivation/reoxygenation (OGD/R) method was employed to persuade mouse brain slice injury *in vitro*. Compared with OGD/R, Co could decline the Lactate dehydrogenase levels, surge the 2,3,5-triphenyl-tetrazolium chloride staining intensity. Compared with OGD/R, Co could augment Bcl-2 expression level and inhibit Cytochrome c, Bax, Caspase-3, Caspase-7, and Caspase-9 expression levels.

Abbreviations used: Co: Costunolide; TTC: 2,3,5-triphenyl-tetrazolium chloride; OGD/R: Oxygen-glucose Deprivation/reoxygenation; Cyt-c: Cytochrome c; LDH: Lactate dehydrogenase; aCSF: Artificial cerebral spinal fluid; PDB: Protein data bank; PBS: Phosphate buffer saline.



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DOI: 10.4103/pm.pm_360_20

Access this article online
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INTRODUCTION

Ischemic stroke is one of three types of stroke. It is also called to as brain ischemia and cerebral ischemia. It is a deadly ailment all over the world. Patients with ischemic stroke may agonize long-term paralysis, cognitive deficits, and high mortality.[1-3] Inflammation, glutamate toxicity, calcium overload, and apoptosis are vital pathogenesis of ischemic stroke.[1] Tissue plasminogen activator (TPA) can be employed in patients with acute stroke.[4] However, TPA can cause blood-brain barrier disruption, bleeding, and other side effects.[5] It is an urgent job to look for drugs to treat ischemic stroke. Many old-style Chinese herbal medicines can be employed to treat ischemic stroke.[6] Therefore, we can invention and study the actual chemical constituents in these herbs to treat ischemic stroke.

Costunolide (Co) is largely from the *Aucklandia lappa* Decne,[7] *Laurus nobilis* L.,[8] *Magnolia grandiflora*,[9] and *Michelia floribunda*.[10] Co has anti-carcinogenesis, anti-inflammation, and other pharmacological effects.[11,12] Co can hinder the interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and other inflammatory factors in BV2 microglial cells stimulated with lipopolysaccharide.[12] Within 12–24 h of ischemic stroke, a huge number of pro-inflammatory factors (such as IL-6 and TNF-α) are unconfined, and thrombo-inflammation is the dynamic force

Cite this article as: Ma H, Zhu Y, Zhang Z, Zhang X, Zhao Q. Costunolide attenuates oxygen-glucose deprivation/reoxygenation-induced apoptosis in mouse brain slice through inhibiting caspase expression. Phcog Mag 2021;17:231-5.

of ischemic stroke.[13] Inflammation leads to apoptosis and death of brain cells and the caspase pathway is a significant pathway of apoptosis.[14] Panaxatriol saponins could decrease inflammation and apoptosis after glucose-oxygen deprivation in BV2 Cells.[15] However, whether Co has an anti-apoptotic effect is unidentified.

Thus, we planned to reconnoiter whether Co had an anti-apoptotic role by inhibiting caspase expression on mouse brain slice injury-induced oxygen-glucose deprivation/reoxygenation (OGD/R).

MATERIALS AND METHODS

Molecular docking

Finding the mol2 format of Co from PubChem. Co and its interacting protein crystals were familiarized into the Maestro 11.1 software ligprep module. The energy optimization of Co in the first field (OPLS-2005) was diminished. By querying the protein data bank, the structures of Caspase-9 (RCSB ID, 3D9T), Caspase-7 (RCSB ID, 4JR2), Caspase-3 (RCSB ID, 2DKO), BAX (RCSB ID, 4ZIE) and Cytochrome c (RCSB ID, 5XTE) were attained. Using the Maestro 11.1 software Glide module, the target protein is altered, dehydrated and hydrogenated, under default parameters. The active site of docking is created by centring on the original ligand. Finally, Co is molecularly docked with the target protein.

Preparation of mice brain slices

Male ICR mice (weight 20 ± 2 g) were acquired from the Laboratory Animal Center of Ningxia Medical University (No. 1160 Shengli Street, Xingqing District Yinchuan City, 750004, China). The preparation of mice brain slices and oxygen-glucose deficiency reperfusion methods denote to our published article.[16] The mice's brains were quickly detached after anesthetized with 0.3 mg/kg chloral hydrate, placed in a beaker with 0°C artificial cerebral spinal fluid (aCSF) for 2 min. The 0°C aCSF was soaked with 5% CO_2/95% O_2 in advance.[16] The brains were coronally cut from the bregma at 0°C with a vibration slicing machine (LEICA VT 1000S), each slice was 350 μM thick.

Oxygen-glucose deprivation/reoxygenation injury model

Positioned the brain slices in 35°C oxygenated aCSF for 1 h and relocated them to the culture dish (six slices per dish). For oxygen-glucose deprivation, the brain slices were moved into the medium without glucose, gassed with 95% N_2 and 5% CO_2 for 30 min. Afterward, the brain slices were transferred into a new culture dish cultured for 1 h.[16] The brain slices were alienated into Co (Batch No. AF8082206, 1, 5 and 10 μM), its content was 99%, was delivered by Chengdu Alfa Biotechnology (Chengdu, China), nimodipine (10 μM) was attained from Bayer (Germany, batch number: 12301323), OGD and control group. Each group compromised 48 slices. Nimodipine and Co were added during oxygen-glucose deficiency. The slices of the control group were cultured with aCSF that was soaked with 5% CO_2/95% O_2, 35°C all the time.

Measurement of lactate dehydrogenase release

When cell membranes are interrupted, lactate dehydrogenase (LDH) releases to extracellular. The higher the LDH content in the culture medium, the greater the degree of cell injury. We employed the LDH kit (Nanjing Jiancheng, China) to assess the content of LDH. The culture medium was composed and centrifuged for 5 min at 3000 rpm. The supernatant was verified using a spectrophotometer (Thermo Fisher 1510) at 450 nm.[16]

2,3,5-triphenyl-tetrazolium chloride staining

After reoxygenation was finished, the mice brain slices were located in 2% 2,3,5-triphenyl-tetrazolium chloride (TTC) solution and stained at 37°C for 10 min. Then, eroded the brain slices with standard saline three times. The TTC stained brain slices were mined with DMSO and ethanol (1:1) (20uL/mg) for 24 h in the dark, centrifuged for 5 min at 3000 rpm. The supernatant was confirmed using a spectrophotometer (Thermo Fisher 1510) at 490 nm.[16]

Western blot analysis

The total protein extraction kit (Keygen Biotech. China) was employed to extract protein of mice brain slices, and the BCA protein analysis kit (Keygen Biotech., China) was employed to assess the protein content. 10% SDS-PAGE gel was used to detached the proteins, and then transferred the separated proteins to the PVDF membranes. The PVDF membranes were eroded three times with PBST and then blocked for 1 h in the PBST of 5% skimmed milk. The primary antibodies were incubated on the consistent position of membrane overnight at 4°C. The information of the primary antibodies was as follows: Caspase-9 (1:1000), Caspase-3 (1:500) and Caspase-7 (1:500) were attained from Abcam. Bax (1:300), Cytochrome c (Cyt-c) (1:1000), Bcl-2 (1:1000), and β-actin (1:2000) were acquired from Cell Signaling Technology. Incubated the second antibodies on the position of the primary antibody for 2 h. The PVDF membranes were eroded three times with PBST before and after incubating the second antibody exposure of proteins with chemiluminescence reagent (Thermo Fisher).

Statistical analysis

The data were accessible as the mean ± standard deviation, analyzed using one-way ANOVA using SPSS17.0 software (IBM, USA). Significant differences were allotted $P < 0.05$.

RESULTS

Results of molecular docking

We employed molecular docking technology to envisage the possible target of Co anti-apoptosis effect. The docking outcomes

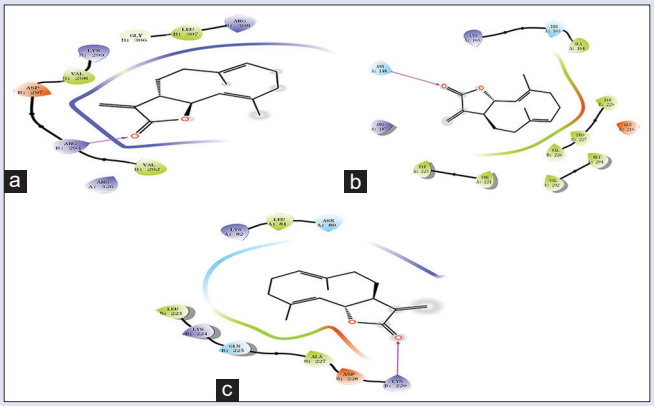

Figure 1: Docking results of costunolide and apoptotic protein. (a) Caspase-9, (b) Caspase-7, (c) Caspase-3. The amino acids binding to costunolide were expressed as droplets. Abbreviations for amino acids are represented by capital letters. The numbers represent the numbering of amino acids in proteins. The line represents the costunolide is in the protein's cavity. The arrow mark indicates hydrogen-bonding interactions between costunolide and protein domain residues

exposed a high affinity of Co towards Caspase-9 (docking score 4.2), Caspase-7 (docking score 4.6), and Caspase-3 (docking score 3.9) [Figure 1]. Caspase-9 exhibited interactions with Co and Gly306, Leu307, Arg308, Yal292, Arg294, Asp297, Yal298, and Lys299 forming a hydrogen bond effect, Co and active site with residues Arg294 forms a hydrogen bond outcome. Caspase-7 was found to be interacting with Co through Val226, Pro227, Tyr229, Lys160, Thr163, Ala164, Tyr223, and Phe221. Co and active site with residues Asn148 forms a hydrogen bond consequence. Co was found to be interacting with the Lys82, Leu81, Asn80, Lbu223, Lys224, Gln225, Ala227, and Asp228 residues of Caspase-3 form a durable hydrophobic effect, Co and active site with residues Lys229 forms a hydrogen bond result.

Costunolide attenuated the release of lactate dehydrogenase

The more serious OGD/R persuaded cell injury, the more LDH was unconfined. The level of LDH augmented after OGD/R ($P < 0.05$). LDH release reduced after nimodipine (10 μM) and Co (1, 5 and 10 μM) treatment ($P < 0.05$). However, there was no variance in the inhibition of LDH release between nimodipine and Co ($P > 0.05$) [Figure 2].

Costunolide increased the absorbance value of 2,3,5-triphenyl-tetrazolium chloride dyeing

After TTC staining, the diminution of the absorbance value designated that the cell impairment is serious. The absorbance value of TTC dyeing in the OGD/R group diminished ($P < 0.05$). Co (1, 5, and 10 μM) and nimodipine (10 μM) could upsurge the absorbance value ominously ($P < 0.05$) [Figure 3].

Costunolide enhanced Bcl-2 and inhibited bax

After OGD/R injury, the content of Bax augmented and the content of Bcl-2 lessened in mice brain slices. After interference with nimodipine (10 μM) and Co (1, 5 and 10 μM), the content of Bax in brain slices reduced, while the content of Bcl-2 augmented ($P < 0.05$) [Figure 4].

Costunolide inhibited cytochrome c, caspase-9/-3/-7

After OGD/R injury, the expression of Caspase-3, Caspase-9, Caspase-7, and Cyt-c improved in mice brain slices ($P < 0.05$). After intervention with nimodipine (10 μM) and Co (1, 5, and 10 μM), the content of these proteins diminished ($P < 0.05$) [Figure 5].

DISCUSSION

Ischemic stroke has a high occurrence and great destructiveness. Outcome and researching drugs for treating ischemic stroke is the emphasis of research. There are many *in vitro* and *in vivo* models for the study of ischemic stroke.[17] However, the cultured cell experiment absences intercellular connection. The disadvantage of the *in vivo* model is that the stability of the model is deprived and the experimental measure is hefty. However, the brain slice model has the features of intercellular connection and stumpy dosage.[18,19] Therefore, we employed brain slices injury persuaded by OGD/R to mimic cerebral ischemia. Nimodipine may recover neurologic recovery and avert postischemic impairment of cerebral reperfusion in some patients with acute ischemic stroke.[20] Therefore, in our study, we picked nimodipine as a optimistic control drug.

LDH unconfined after cell membranes are interrupted.[21] Our results designated that OGD/R could rise the release of LDH from brain slices, which specified that the cell membrane was devastated. However, Co could decrease the level of LDH. TTC staining is a frequently used method to assess brain injury.[22] Our results show that Co could advance brain slices injury by growing the absorbance of TTC staining.

The pathophysiological mechanism of cerebral ischemia is very complex, which is still being considered.[23] Some researches disguised that apoptosis is required for cerebral ischemia and reperfusion.[24-26] The Bcl-2 family (such as Bax, Bcl-2) is involved in regulating apoptosis in many nervous system ailments.[27] Bcl-2 and Bax altered ominously in ischemic stroke.[28,29] The raised intracellular ratio of Bax to Bcl-2 befalls during cell apoptotic death.[30] In this study, OGD/R persuaded the diminution of Bcl-2 and the increase of Bax in mice brain slices, which was reliable with earlier studies.[31] Bcl-2 can upsurge the leakage of cytochrome c from mitochondria and induce apoptosis.[32] The level

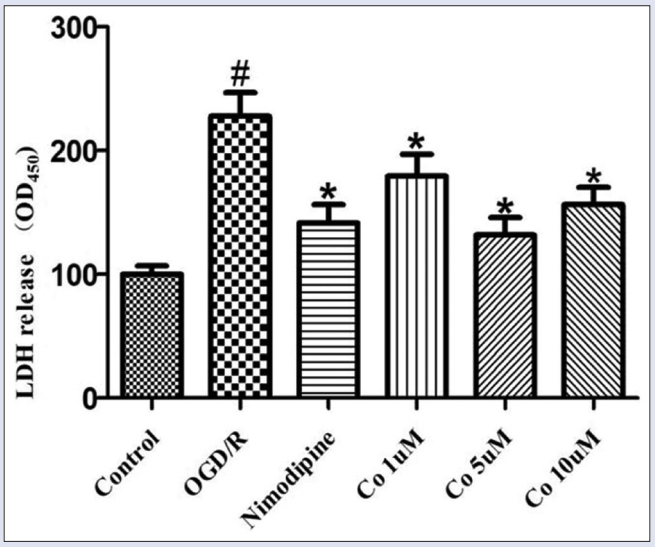

Figure 2: Costunolide (Co) attenuated the release of lactate dehydrogenase. #$P < 0.05$ versus control group, *$P < 0.05$ versus Oxygen-glucose deprivation/reoxygenation group

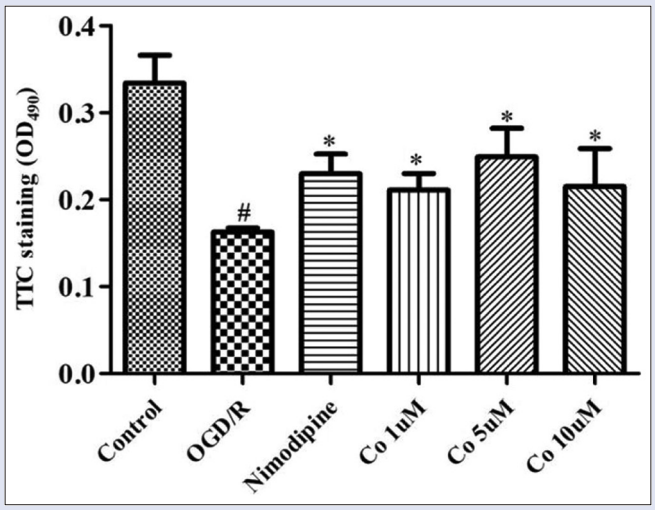

Figure 3: Costunolide (Co) increased the absorbance value of 2,3,5-triphenyl-tetrazolium chloride dyeing. #$P < 0.05$ versus control group, *$P < 0.05$ versus Oxygen-glucose deprivation/reoxygenation group

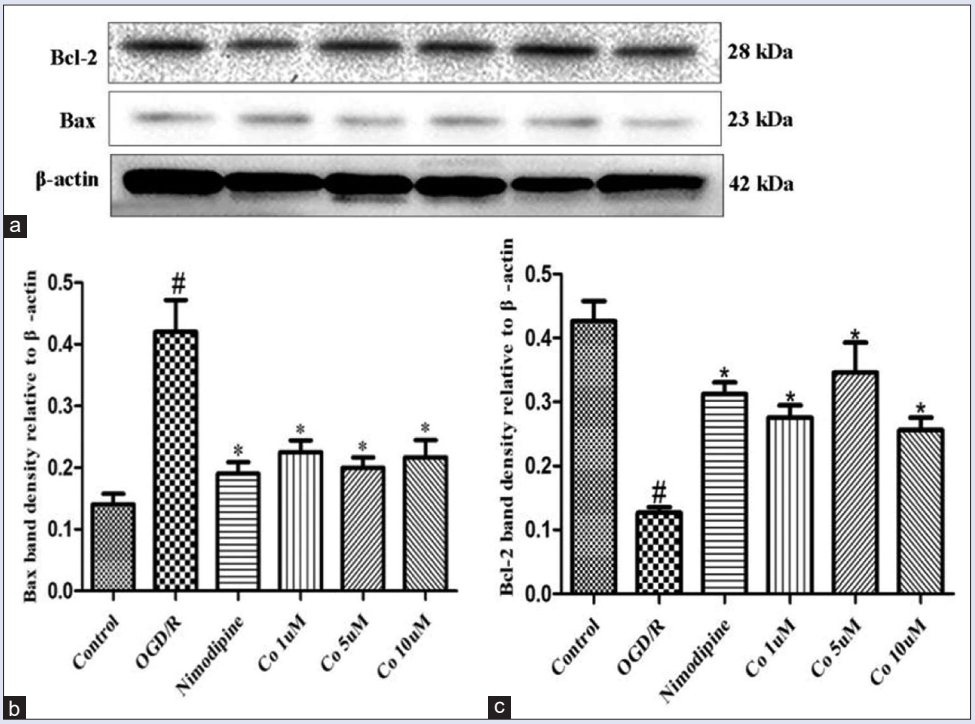

Figure 4: Costunolide (Co) enhanced Bcl-2 and inhibited Bax. The band of Bax and Bcl-2 (a) and quantitative analysis (b and c). $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus Oxygen-glucose deprivation/reoxygenation group

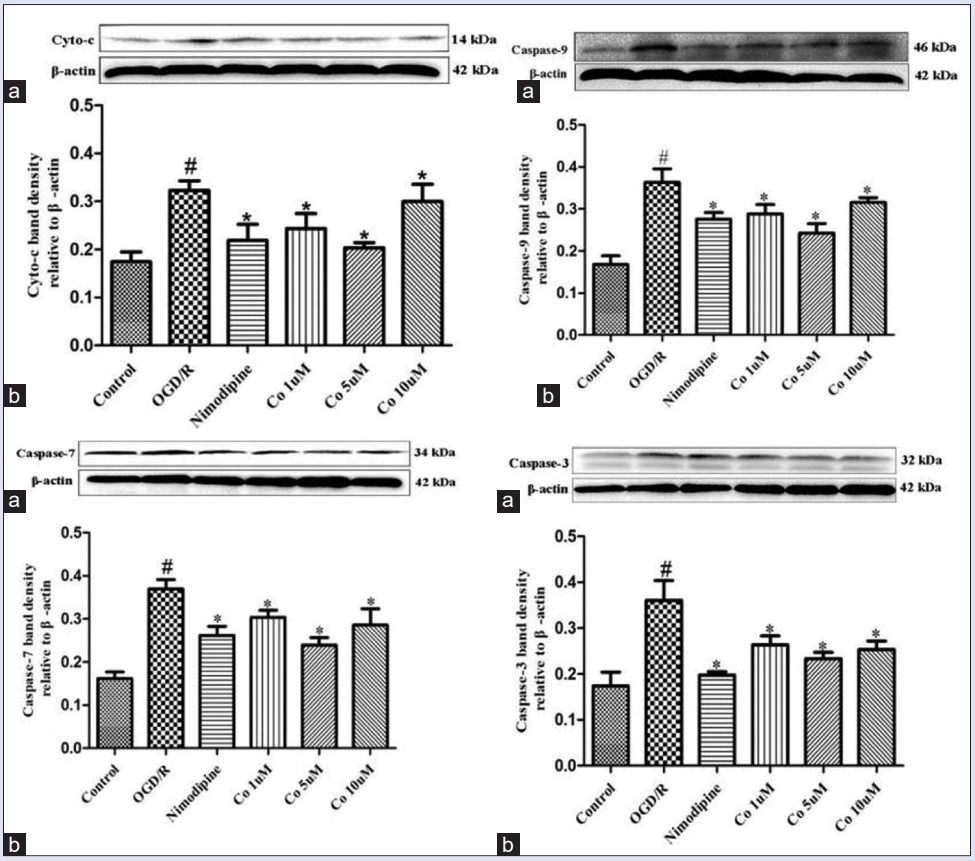

Figure 5: Costunolide (Co) attenuated the expression of Cytochrome c, Caspase-9, Caspase-3, Caspase-7. The band of Cytochrome c, Caspase-3, Caspase-7, and Caspase-9 (a) and quantitative analysis (b). $^{\#}P < 0.05$ versus control group, $^{*}P < 0.05$ versus Oxygen-glucose deprivation/reoxygenation group

of cytochrome C was meaningfully augmented in the brain of rats with focal cerebral ischemia.[33] Caspase-3 is an important executive factor of apoptosis, which is tangled in ischemic stroke.[34,35] In our study, Co could augment Bcl-2 and inhibit Bax, Cyt-c, Caspase-3, Caspase-7, and Caspase-9 expression. It was recommended that Co could adjust mitochondrial apoptosis pathways in OGD/R-induced mice brain slices.

CONCLUSION

Co can defend mice brain injury persuaded by OGD/R by inhibiting the expression of caspase.

Financial support and sponsorship

This study was supported by the Ningxia Natural Science Foundation (2020AAC02017) and the National Natural Science Foundation of China (81660700).

Conflicts of interest

There are no conflicts of interest.

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