**Original Research**

**Cdc42 Promotes Axonogenesis of Primary Hippocampal Neurons by Inhibiting Glycogen Synthase Kinase-3β**

Yu-Ting Li1,†, Fang-Zheng Chen1,†, Wei Chen1,†, Hui-Ming Zhu1,†, Yu Chen3, Zhen-Lin Li1, Fang Yan1, Zhong-Ying Liu1, Wei-Ren Dong3, Lin Zhang1,2,*, Hai-Hong Wang1,2,*

1Department of Histology and Embryology, School of Basic Medical Sciences, Southern Medical University, 510515 Guangzhou, Guangdong, China
2NMPA Key Laboratory for Safety Evaluation of Cosmetics, 510515 Guangzhou, Guangdong, China
3Experimental Education & Administration Center, School of Basic Medical Sciences, Southern Medical University, 510515 Guangzhou, Guangdong, China

*Correspondence: zlilyzh@126.com (Lin Zhang); haihwang@163.com (Hai-Hong Wang)
†These authors contributed equally.

Abstract

**Background:** Progressive axon degeneration is a common pathological feature of neurodegenerative diseases. Cdc42 is a member of the Rho GTPase family that participates in axonogenesis. GSK-3β is a serine/threonine kinase highly implicated in neuronal development and neurodegeneration. This study aimed to examine whether cdc42 promotes axonogenesis by regulating GSK-3β activity. **Methods:** Hippocampal neurons were isolated from neonatal Sprague-Dawley rats and transfected with designated plasmid vectors to alter the activities of cdc42 and GSK-3β. LiCl treatment was used to inhibit the GSK-3β activity in primary neurons. **Results:** The activity of GSK-3β and induced axonogenesis in primary rat hippocampal neurons, while transfection with a negative cdc42 mutant (cdc42F28L) resulted an opposite effect. Moreover, transfection with plasmid vectors carrying wild-type GSK-3β or a constitutively active GSK3/β mutant (GSK-3/β S9A) increased the activity of GSK-3β and attenuated axonogenesis of primary hippocampal neurons with excessive cdc42 activity, whereas inhibition of GSK-3β by LiCl abolished the inhibitory effect of the negative cdc42 mutant on axonogenesis. **Conclusions:** This study suggests that cdc42 induces axonogenesis of primary rat hippocampal neurons via inhibiting GSK-3β activity. These findings support further investigation into the mechanisms of cdc42/GSK-3β-mediated axonogenesis.

**Keywords:** cdc42; axonogenesis; hippocampal neurons; GSK-3β; CRMP-2

1. **Introduction**

Axons are the elongated portion of the neuron that transport various molecules between the soma and axon terminals and transmit signals between neurons or between neurons and effector cells by forming synapses [1]. Axonogenesis, referring to the formation and outgrowth of axons during neuronal development and nerve regeneration, is essential for the establishment and maintenance of neuronal polarity [2]. Progressive axon degeneration is a dynamic process resulting in the loss of communication between neurons, which has been recognized as a common pathological feature of neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease [3]. A better understanding of the physiological mechanisms of axonogenesis may facilitate the development of therapeutic strategies to block axon degeneration in these diseases.

Cdc42 is a member of the Rho GTPase family that plays important roles in multiple cellular processes, such as cytoskeletal organization, cell cycle progression, vesicle transport, and cell survival [4]. Cdc42 is widely expressed in the cell body and neurites of neurons, participating in the establishment of neuronal polarity and the formation of axons, dendrites, and dendritic spines [5]. Previous evidence has shown that knockout of cdc42 inhibited axonogenesis of primary murine hippocampal neurons [6]. Collapsin response mediator protein-2 (CRMP-2) is a cytosolic protein that acts as a key regulator of axonogenesis [7]. Overexpression of full-length CRMP-2 was shown to promote axon elongation and induced the formation of multiple axons, while transfection of CRMP-2 dominant negative mutants inhibited axonogenesis [8]. CRMP-2 can bind directly to the tubulin heterodimers, promoting microtubule assembly and axon growth [9]. Moreover, we previously demonstrated that cdc42 promotes axonogenesis of rat hippocampal neurons by reducing CRMP-2 phosphorylation at Thr514 and stabilizing microtubules [10].

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase originally identified as a key regulator of glucose metabolism. Increasing evidence has suggested that GSK-3β is highly implicated in neuronal development and neurodegeneration [11]. Phosphorylation of GSK-3β has been found to inhibit phosphorylation of CRMP-2 at...
Thr514 and promote axonogenesis [12]. Our previous findings revealed that cdc42 promoted CRMP-2 dephosphorylation in rat hippocampal neurons by enhancing GSK-3\(\beta\) phosphorylation [10]. However, whether GSK-3\(\beta\) is involved in cdc42-mediated axonogenesis remains uncertain.

In this study, primary rat hippocampal neurons were used to investigate whether cdc42 promotes axonogenesis by inhibiting GSK-3\(\beta\) activity. Specifically, we sought to determine the effects of increased GSK3\(\beta\) activity and GSK3\(\beta\) inhibition on axonogenesis.

2. Materials and Methods

2.1 Isolation of Rat Hippocampal Neurons

The use of animals was approved by the Animal Care and Use Committee of the Southern Medical University and all protocols were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. Primary hippocampal neurons were isolated from neonatal Sprague-Dawley rats as previously described [13]. Briefly, rats (male:female = 1:1) on postnatal day 1 were obtained from the Experimental Animal Center of Southern Medical University. The pups were euthanized by decapitation and the head was separated from the body. The intact brain was immediately removed and quickly placed into dissection medium. The hippocampus was carefully separated under a microscope and placed in pre-chilled D-Hank’s solution (8 g NaCl, 0.4 g KCl, 0.06 g KH\(_2\)PO\(_4\), 0.35 g NaHCO\(_3\), 0.12 g Na\(_2\)HPO\(_4\)·12H\(_2\)O in 1 L ddH\(_2\)O; Guangdong Guanghua Sci-Tech Co., Ltd., Guangzhou, China). Subsequently, the hippocampus was cut into small pieces and digested with 0.25% trypsin-EDTA (1:5 v/v; Gibco, Grand Island, NY, USA) at 37 °C for 10 min. DMEM/F12 medium (Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 10% newborn calf serum (NBCS; Gibco) were added to stop digestion. Cells were centrifuged at 80 × g for 5 min at room temperature. The supernatant was discarded, and cell pellets were resuspended in Neurobasal-A medium (Gibco, Grand Island, NY, USA) supplemented with 2% B27 (Gibco), 1% 100× GlutaMAX-I (Gibco), 25 μM Glutamate, and 0.5 μg/L Plasmocin at 24 and 24 h after transfection.

2.2 Cell Transfection

Primary rat hippocampal neurons were transfected with designated plasmid vectors using the Amaxa™ Rat Neuron Nucleofection Kit (Cat. # VPG-1003, Lonza, Basel, Switzerland) as previously reported [14]. Plasmid vectors carrying a constitutively active cdc42 mutant (pIRES-EGFP-cdc42F28L) were gifts from Prof. Andreas Püschel (Westfälische Wilhelms-Universität Münster, Germany). Plasmid vectors carrying a dominant negative cdc42 mutant (pIRES-EGFP-cdc42N17) were gifts from Prof. Lin Zhang (Southern Medical University, China). Plasmid vectors carrying a wild-type GSK-3\(\beta\) (pcDNA3.1-GSK-3\(\beta\) wild type) or a constitutively active GSK3\(\beta\) mutant (pcDNA3.1-GSK-3\(\beta\) S9A) were gifts from Prof. James R. Woodgett (University of Toronto, Canada). These plasmids were validated in our previous study [10]. Cells transfected with empty vectors (pIRES-EGFP or pcDNA3.1) were used as the controls. The culture medium was replaced with Neurobasal-A medium containing 2% B27, 1% 100× GlutaMAX-I, 25 μM Glutamate, and 0.5 μg/L Plasmocin at 24 and 24 h after transfection.

2.3 Drug Treatment

At 24-h post-transfection, rat hippocampal neurons were treated with or without phosphate-buffered saline (PBS), 2 mM NaCl (diluted in cultured medium), or 2 mM LiCl (diluted in cultured medium) for an additional 48 h.

2.4 Measurement of GSK-3\(\beta\) Activity

GSK-3\(\beta\) activity in primary rat hippocampal neurons following different treatments was measured using the GENMED kit (Genmed Sciences Inc., Wilmington, DE, USA) as reported previously [15]. In brief, cells were harvested and centrifuged at 16,000 × g for 5 min at 4 °C. The supernatant was collected, and the protein concentration was determined using the BCA assay (Pierce, USA). Protein samples in triplicates (10 μg each) were added to a 96-well plate and incubated with the reagents from the kit. Optical density was measured at 340 nm using the SpectraMax 190 microplate reader (Molecular Devices, San Jose, CA, USA). GSK-3\(\beta\) activity in the control group (transfected with empty pIRES-EGFP vectors) was set as 1.

2.5 Immunofluorescence

After treatment, rat hippocampal neurons were washed with PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 90 min, and then permeabilized in 1% Triton X-100 (Sigma-Aldrich) for 30 min. After blocking with 3% bovine serum albumin (Genview, League City, TX, USA) in Tris-buffered saline (Sigma-Aldrich) at room temperature for 1 h, neurons were probed with anti-Tau-1 antibody (1:200 dilution; cat no. AB5622, Merck Millipore, USA) at 4 °C overnight. Subsequently, cells were incubated with rhodamine Red™-X goat anti-mouse IgG (1:700 dilution; Invitrogen, USA) and goat polyclonal secondary antibody to rabbit IgG-H&L (Cy5), pre-adsorbed (1:1000 dilution; cat no. ab5622, Abcam, New Temtories, HK, China) for 2 h. The nuclei were stained with Hoechst 33258 (0.5 µg/mL in PBS, Sigma-Aldrich) at 4 °C overnight. The fluorescent signal was observed under a confocal microscope (FV500; Olympus, Japan). Processes with Tau1 immunoreactivity in their distal segments were counted.

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as axons. The longest axon of individual neurons in each group (100 GFP-positive neurons per group) was traced to determine axon length. The number of axons per cell and the percentages of neurons with no axon, a single axon, or multiple axons in individual groups were calculated. The longest axon of each neuron was used to determine the axon length using the Image-ProPlus software (Version 6.0; Media Cybernetics, Silver Springs, MD, USA). One hundred GFP-positive neurons per group were analyzed.

2.6 Statistical Analysis

Data were analyzed using the SPSS software (Version 19.0; IBM, Armonk, NY, USA) and presented as mean ± standard deviation (SD) from three independent experiments. One-way ANOVA followed by Tukey’s test was used to compare data among groups. A p-value of less than 0.05 was defined as statistically significant.

3. Results

3.1 Transfection with GSK-3β wt or GSK-3β S9A Restored GSK-3β Activity in Rat Hippocampal Neurons Overexpressing cdc42F28L

Hippocampal neurons were isolated from neonatal rats and transfected with vectors expressing cdc42F28L (a constitutively active cdc42 mutant) alone or together with vectors carrying GSK-3β wt, GSK-3β S9A (a constitutively active GSK3β mutant), or empty vectors (GSK-3β vector). Cells transfected with empty pIRES-EGFP vectors served as the controls (the Vector group). After 72 h, GSK-3β activity in different groups of cells was measured. Overexpression of cdc42F28L significantly decreased GSK-3β activity in hippocampal neurons compared with the Vector group (p < 0.01). Transfection with vectors carrying either GSK-3β wt or GSK-3β S9A significantly increased GSK-3β activity in neurons overexpressing cdc42F28L (p < 0.01). The delivery of empty GSK-3β vector did not change GSK-3β activity in cdc42F28L-overexpressing neurons (Fig. 1). These findings suggest that transfection of GSK-3β wt or GSK-3β S9A restored GSK-3β activity in hippocampal neurons overexpressing cdc42F28L.

3.2 Transfection with GSK-3β wt or GSK-3β S9A Attenuated Axonogenesis of Rat Hippocampal Neurons with Excessive Cdc42 Activity

To investigate whether transfection of GSK-3β wt or GSK-3β S9A could affect axonogenesis in hippocampal neurons with excessive expression of cdc42F28L, we quantified the number and length of neuronal axons using immunofluorescence. At 72 h after transfection, different groups of neurons were stained with Tau-1 for axons, MAP2 for dendrites, and Hoechst 33258 for nuclei. Cells were then observed under a laser confocal microscope. The distal of the axons and the proximal segments of the dendrites were positively stained with Tau-1 (red). The dendrites and the proximal segments of the axons were stained with MAP2 (purple). As neurons were cultured for only 3 days, the dendrites were relatively short (Fig. 2A). Compared with the Vector group, neurons overexpressing cdc42F28L showed significantly lower numbers of neurons with no axon (14.33% vs. 5.66%; p < 0.01) or a single axon (74.55% vs. 51.67%; p < 0.01) but had a higher number of neurons with multiple axons (11.67% vs. 42.67%; p < 0.01) (Fig. 2B). The number of axons per neuron and the mean axon length of the Vector group were significantly lower than those of the Cdc42F28L group (0.8 vs. 1.58 and 105.47 μm vs. 159.80 μm, respectively; both p < 0.01) (Fig. 2C). In neurons overexpressing cdc42F28L, transfection with vectors carrying GSK-3β wt or GSK-3β S9A significantly increased the number of neurons with no axon (21.00% and 45.67%, respectively; p < 0.01 vs. the Cdc42F28L group) but decreased the number of neurons with multiple axons (6.33% and 2.66%, respectively; p < 0.01 vs. the Cdc42F28L group) (Fig. 2B). GSK-3β wt or GSK-3β S9A also significantly decreased the number of axons per neuron (0.92 and 0.57, respectively; p < 0.01 vs. the Cdc42F28L group) and the mean axon length (66.88 μm vs. 21.00% and 45.67%, respectively; p < 0.01 vs. the Cdc42F28L group) but decreased the number of neurons with multiple axons (6.33% and 2.66%, respectively; p < 0.01 vs. the Cdc42F28L group) but decreased the number of neurons with multiple axons (6.33% and 2.66%, respectively; p < 0.01 vs. the Cdc42F28L group).
Fig. 2. Effects of GSK-3β wt or GSK-3β S9A on axonogenesis of rat hippocampal neurons with excessive cdc42 activity. Primary rat hippocampal neurons were transfected with pIRES-EGFP vectors expressing cdc42F28L alone or together with pcDNA3.1 vectors carrying GSK-3β wt, GSK-3β S9A, or empty pcDNA3.1 vectors (GSK-3β vector) at a ratio of 1:3 [24]. Cells transfected with empty pIRES-EGFP vectors served as the controls (Vector). (A) Immunofluorescence analysis of neurons. Different groups of cultured neurons were stained with Tau-1, MAP2, and fluorescent secondary antibodies, followed by nuclei staining with Hoechst 33258. Images were captured under a laser confocal microscope. Green, GFP; red, Tau-1; purple, MAP2; blue, Hoechst. Bar = 50 µm. (B) Percentage of neurons with no axon, a single axon, and multiple axons. (C) Number of axons per cell. (D) Mean axon length. Data are shown as mean ± SD. **p < 0.01 vs. the Vector group; ##p < 0.01 vs. the Cdc42F28L group.
and 38.03 µm, respectively; *p < 0.01 vs. the Cdc42F28L group) compared with the Cdc42F28L group (Fig. 2C,D). There was no significant difference in the percentage of different subtypes of neurons, the number of axons per cell, or mean axon length between the Cdc42F28 and Cdc42F28 + GSK-3β vector groups (*p > 0.05) (Fig. 2B–D). These results support that GSK-3β wt or GSK-3β S9A attenuated Cdc42F28L overexpression-induced axonogenesis of rat hippocampal neurons.

3.3 Treatment with LiCl Decreased GSK-3β Activity in Rat Hippocampal Neurons Overexpressing Cdc42N17

To determine if cdc42 promoted axonogenesis by inhibiting GSK-3β, we transfected primary rat hippocampal neurons with vectors expressing cdc42N17 (a dominant negative cdc42 mutant) or empty control vectors, and then treated the cells with or without PBS, NaCl, or LiCl (a GSK-3β inhibitor) for 48 h. GSK-3β activity assays showed that transfection with cdc42N17 significantly induced the kinase activity in hippocampal neurons. Treatment with LiCl, however, significantly decreased GSK-3β activity in neurons overexpressing cdc42N17 (*p < 0.01). No significant difference was observed in the Cdc42N17, Cdc42N17 + PBS, and Cdc42N17 + NaCl groups (*p > 0.05) (Fig. 3).

3.4 Treatment with LiCl Abolished the Inhibitory Effect of Cdc42N17 Overexpression on Axonogenesis

We further analyzed the effects of GSK-3β inhibition on the axonogenesis of rat hippocampal neurons. After transfection with vectors carrying cdc42N17 or empty control vectors, neurons were treated with or without PBS, NaCl, or LiCl for 48 h. Immunofluorescence staining of neurons with Tau-1 (for axons), MAP2 (for dendrites), and Hoechst (for nuclei) is shown in Fig. 4A. Compared with the Vector group, transfection with cdc42N17 significantly increased the number of neurons with no axon (14.00% vs. 35.34%; *p < 0.01) and decreased the number of neurons with a single axon (71.00% vs. 60.33%; *p < 0.01) or multiple axons (15.00% vs. 4.33%; *p < 0.01) (Fig. 4B). The number of axons per neuron and the mean axon length of the Cdc42N17 group were significantly lower compared to the controls (0.68 vs. 1.05 and 40.81 µm vs. 113.33 µm, respectively; both *p < 0.01) (Fig. 4C,D). Inhibition of GSK-3β by LiCl treatment effectively reduced the number of axon-free neurons (2.00%) and neurons with a single axon (46.00%) but increased the number of neurons with multiple axons (52.00%) compared with the Cdc42N17 group (all *p < 0.01) (Fig. 4B). Neurons exposed to LiCl also exhibited a significantly higher number of axons per cell (1.60) and mean axon length (147.10 µm) compared to the Cdc42N17 group (both *p < 0.01) (Fig. 4C,D). There was no significant difference in the percentage of different subtypes of neurons, the number of axons per cell, or mean axon length between the Cdc42N17, Cdc42N17 + PBS, and Cdc42N17 + NaCl groups (*p > 0.05) (Fig. 4B–D). The above data demonstrate that inhibition of GSK-3β by LiCl abolished the inhibitory effect of cdc42N17 overexpression on axonogenesis of rat hippocampal neurons.

4. Discussion

Progressive loss of neuronal polarity is a major pathological event in neurodegenerative diseases [16]. The morphological development and functional polarization of axons are key steps in the development and maintenance of neuronal polarity [17]. A better understanding of axonogenesis-related pathways may contribute to the development of novel therapeutic interventions for neurodegenerative disorders. Our previous work has shown that cdc42 promoted axonogenesis of rat hippocampal neurons by enhancing microtubule stabilization and reducing CRMP-2 phosphorylation [10]. Moreover, dephosphorylation of CRMP-2 by cdc42 was mediated through GSK-3β phosphorylation. In the present study, we showed that cdc42 promoted axonogenesis of rat hippocampal neurons by inhibiting GSK-3β.

Axonogenesis is a dynamic process mediated by various effectors and signaling pathways, which ultimately act on the actin and microtubule cytoskeleton [18]. Cdc42 regulates axon specification, elongation, and guidance via interacting with effector proteins, such as cofilin, a key

![Fig. 3. Effect of LiCl treatment on GSK-3β activity in rat hippocampal neurons overexpressing cdc42N17.](image-url)
Fig. 4. Effect of LiCl treatment on the inhibitory effect of cdc42N17 overexpression on axonogenesis. Primary rat hippocampal neurons were transfected with pIRES-EGFP vectors expressing cdc42N17 or empty pIRES-EGFP vectors (Vector) for 24 h, and then treated with or without PBS, NaCl, or LiCl for 48 h. (A) Immunofluorescence analysis of neurons. Different groups of cultured neurons were stained with Tau-1, MAP2, and fluorescent secondary antibodies, followed by nuclei staining with Hoechst 33258. Images were captured under a laser confocal microscope. Green, GFP; red, Tau-1; purple, MAP2; blue, Hoechst. Bar = 50 μm. (B) Percentage of neurons with no axon, a single axon, and multiple axons. (C) Number of axons per cell. (D) Mean axon length. Data are shown as mean ± SD. *p < 0.05 vs. the Vector group; **p < 0.01 vs. the Vector group; ###p < 0.01 vs. the Cdc42N17 group.
factor for sustaining axon outgrowth [19]. Cdc42 ablation increased phosphorylation (inactivation) of coflin in the axonal growth cones of mouse hippocampal neurons, while the active (non-phosphorylated) form was enriched in the wild-type neurons [6]. Schwamborn et al. [20] reported that transfection of a cdc42 active mutant induced the formation of multiple axons in rat hippocampal neurons, whereas knockdown of cdc42 by RNA interference led to complete loss of neuronal polarity. Consistently, our data showed that increasing the activity of cdc42 by transfecting cells with an active cdc42 mutant induced axonogenesis in primary rat hippocampal neurons, while transfection of a negative cdc42 mutant exerted an opposite effect.

Emerging evidence has suggested that GSK-3β plays a key regulatory role in multiple neurodevelopmental events, including neurogenesis, neuronal polarization, axon growth and guidance, and neuronal migration [21]. Phosphorylation of GSK-3β at Ser9 inactivates GSK-3β and dephosphorylates CRMP-2, accelerating microtubule polymerization and stabilization, thereby promoting the formation and elongation of axons [22]. In this study, we observed that GSK-3β attenuated axonogenesis of rat hippocampal neurons with excessive cdc42 activity, whereas inhibition of GSK-3β by LiCl abolished the inhibitory effect of the negative cdc42 mutant on axonogenesis, suggesting that cdc42 induced axonogenesis via inhibiting GSK-3β.

Protein kinase B (Akt) is considered the major regulator of GSK-3β phosphorylation. Constitutive activation of GSK3β has been found to induce Akt-activated axon regeneration [23]. Co-transfection of hippocampal neurons with an active Akt mutant (Myr-Akt) and an active GSK-3β mutant (GSK-3β S9A) partially inhibited Akt-induced formation of multiple axons [22]. However, a study by Gärtner et al. [24] suggested that GSK-3β inhibition promotes the development of neuronal polarity in mice independent of Akt pathway activation, implying that other factors may regulate GSK-3β activity. The Par complex, comprising Par6, Par3, and an atypical PKC (aPKC), is a key factor in cdc42 signaling and plays an essential role in axonogenesis [20]. In migrating astrocytes, cdc42 was shown to regulate the direction of cell protrusion by inducing phosphorylation of GSK-3β at Ser9 via the Par6-aPKC complex [25]. In fibroblasts, cdc42 inactivated GSK-3β (phosphorylation at Ser9) via activating the Par6/aPKC complex, thereby promoting cell polarization. These findings suggest that cdc42 inhibits GSK-3β activation through the Par6/aPKC complex and subsequently inhibits CRMP-2 phosphorylation to promote axonogenesis. Further investigation into the mechanisms of cdc42-mediated axonogenesis is needed.

5. Conclusions

In conclusion, our results suggest that cdc42 induces axonogenesis of rat hippocampal neurons via inhibiting GSK-3β activity. These findings support future exploration of cdc42/GSK-3β-mediated axonogenesis in vivo.

Abbreviations

CRMP-2, collapsin response mediator protein-2; GSK-3β, Glycogen synthase kinase-3β; FBS, fetal bovine serum; NBCS, newborn calf serum; PBS, phosphate-buffered saline.

Author Contributions

YTL, FZC, WC and HMZ contributed equally to this work; HHW designed the research; YTL, FZC, WC and HMZ performed the experiments; YC, ZLL, FY, ZYL, WRD, LZ, and HHW analyzed and interpreted the data; and HHW wrote and reviewed the manuscript.

Ethics Approval and Consent to Participate

The use of animals was approved by the Animal Care and Use Committee of Southern Medical University (The approval date was 26 March 2020) and all protocols were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Acknowledgment

Not applicable.

Funding

This work is supported in part by grants from the National Natural Science Foundation of China (81572222), Guangdong Basic and Applied Basic Research Foundation (2021A1515010529), Science and Technology Projects in Guangzhou (202102080091) and Innovation Training Program for College Students (S202012121131 and 202112121315).

Conflict of Interest

The authors declare no conflict of interest.

References


