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Involvement of Inflammasome Activation in Lipopolysaccharide-induced Mice Depressive-like Behaviors

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SUMMARY

Aims: The NLRP3 inflammasome is a cytoplasmic multiprotein complex of the innate immune system that regulates the cleavage of interleukin-1β and interleukin-18 precursors. It can detect a wide range of danger signals and trigger a series of immune-inflammatory reactions. There were plenty of studies indicating that activation of the immune system played pivotal roles in depression. However, the underlying mechanisms of immune-depression interactions remained elusive and there was no report about the involvement of inflammasome activation in depression. **Methods:** We established an acute depression mouse model with lipopolysaccharide to explore the involvement of inflammasome activation in depression. **Results:** The lipopolysaccharide-treated mice displayed depressive-like behaviors and pro-inflammatory cytokine interleukin-1β protein and mRNA levels significantly increased. The NLRP3 inflammasome mRNA expression level also significantly elevated in depressed mice brain. Pretreatment with the NLRP3 inflammasome inhibitor Ac-YVAD-CMK significantly abrogated the depressive-like behaviors induced by lipopolysaccharide. **Conclusion:** These data suggest for the first time that the NLRP3 inflammasome is involved in lipopolysaccharide-induced mice depressive-like behaviors. The NLRP3 inflammasome may be a central mediator between immune activation and depression, which raises the possibility that it may be a more specific target for the depression treatments in the near future.

Introduction

Depression makes a significant contribution to the global burden of disease, being at third place worldwide, and according to the World Health Organization, it is predicted to be the leading cause of burden of disease by 2030 [1]. Major depressive disorder, also known as clinical depression or major depression, is a mental disorder characterized by depressed mood, low self-esteem, anhedonia and disrupted sleeping, eating and cognition, which significantly affects a person's family and personal relationships, work or school life, sleeping and eating habits, and general health.

Although there are a lot of literatures regarding depression in the last decades, the underlying pathophysiological mechanisms of major depressive disorder are not well delineated. Recent researches suggested that immune activation and cytokines might be involved in depressive symptoms in some patients and administration of lipopolysaccharide (LPS) or interleukin-1 (IL-1) to animals could induce depressive-like behaviors, which resembled the symptoms of depression [2]. Elevation of brain IL-1 levels found in many medical conditions was both necessary and sufficient for producing the high incidence of depression [3].

Inflammasomes are multimolecular platforms assembled and activated upon detection of damage-associated danger molecules and pathogen-associated danger molecules. These platforms drive the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18 to engage innate immune defenses and regulate adaptive immune responses [4]. The NLRP3 inflammasome is the most well-characterized inflammasome, which could be activated by a wide range of divergent invading pathogens and cellular damages [5,6]. It includes three constituents, the intracellular pattern recognition NLRP3 receptor, the adaptor protein ASC, and the effector protein caspase-1 [7]. Formation of this multiprotein platform results in the autocatalysis and activation of caspase-1, which processes precursor IL-1β and IL-18 into its secreted biologically active form. Recently, inflammasomes and IL-1β activity had been suggested as contributors of some common human diseases such as gout, type II diabetes, nonalcoholic steatohepatitis, atherosclerosis, Alzheimer's disease, and cancer [8–11].

However, whether the NLRP3 inflammasome is involved in progression and development of major depression disorder remains elusive. Therefore, we established an acute depression mouse model with a low dosage of LPS administered intraperitoneally (i.p.) to explore whether the NLRP3 inflammasome is involved in the pathogenesis of acute activation of peripheral immune system-induced depression.

Methods

Reagents

Lipopolysaccharide from *Escherichia coli* 0111:B4 (Product Number: L2630) were obtained from Sigma-Aldrich (St. Louis, MO, USA) Co., LLC. The irreversible caspase-1 inhibitor Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK, Product Number: ALX-260-028) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). RNAiso Plus (TaKaRa Code: D9108B), PrimeScript® RT Master Mix (TaKaRa Code: DRR036A) and SYBR® Premix Ex Taq™ (TaKaRa Code: DRR420A) were purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd. Real-time reverse transcription–polymerase chain reaction (RT-PCR) primers were designed and synthesized by Sangon Biotechnology (Shanghai, China) Co., Ltd.

Animals

Male, 8-week-old BALB/c mice (Chinese Academy of Sciences, China) were housed in groups and kept in standard housing conditions (lights on 7 a.m.–7 p.m.; room temperature 22 ± 2°C). Food and water were provided *ad libitum*. All procedures were approved by the local animal care committee and carried out in accordance with related regulations and laws. After 2 weeks of adaptation to the new environment and the sucrose solution (1%, weight/volume), the mice were randomly assigned to control group, YVAD group, LPS group, and YVAD + LPS group.

Drug Administration

The control group mice were injected (i.p.) with phosphate-buffered saline (PBS) at 10 mL/kg body weight. The YVAD group mice were injected (i.p.) with Ac-YVAD-CMK at a dose of 8 mg/kg body weight. The LPS group mice were injected (i.p.) with the bacterial antigen LPS at a dose of 0.8 mg/kg body weight. The YVAD + LPS group mice were administrated with Ac-YVAD-CMK (8 mg/kg body weight) 30 min before LPS (0.8 mg/kg body weight) injection. Both Ac-YVAD-CMK and LPS were prepared in PBS at a volume of 10 mL/kg body weight. Depressive behavioral tests were performed 24 h post-LPS injection according to our previous studies (Y.-L. Peng, Y.-N. Liu, Y. Zhang, T.-Y. Wu, C.-L. Jiang, Y.-X. Wan, unpublished data) and previous reports that sickness behaviors gradually resolved and evolved into depressive behaviors 24 h post-LPS administration [12].

Behavioral Tests

Sucrose preference was tested using a sucrose solution and tap water two-bottle free-choice method [13]: Mice were presented with two bottles containing 1% sucrose solution or tap water for 1 h during the dark phase in the home cage after 20 h water deprivation, and intake volume was measured. Sucrose preference was calculated as the percentage of sucrose solution ingestion relative to the total amount of liquid consumption [14].

The forced swim test was performed at night as described previously [15]. The apparatus is a transparent cylindrical polypropylene tank (height: 40 cm, diameter: 30 cm) containing 35 cm height of 25 ± 1°C tap water. Mice were placed in the tank individually and forced to swim for 6 min. Immobility was defined as the mice floated in an upright position with its heads above water and without any other body movements. Immobility time during the last 5 min of the test was recorded.

Sacrifice

After the depressive behavioral tests, mice were injected (i.p.) with pentobarbital at a dose of 40 mg/kg in a volume of 10 mL/kg body weight. Brains were dissected out and frozen at −80°C until the levels of IL-1β and components of the NLRP3 inflammasome could be measured and further detected.

Interleukin-1β Detection

IL-1β protein concentration of mice brain was analyzed with a Bio-Plex Mice Cytokine 6-Plex panel (Bio-Rad Laboratories Inc., Hercules, CA, USA), and it was performed by the Miao Tong (Shanghai, China) Biological Science & Technology Co., Ltd. This assay is a suspension-based bead array system, where in sets of microspheres are internally dyed with different ratios of fluorophores and each conjugated to a different cytokine specific antibody capture probe.

Real Time RT-PCR

Total RNA was extracted from the brain tissue using the RNAiso Plus. For reverse transcription, 1 μg of total RNA was used to synthesize the first-strand cDNA using the PrimeScript RT Master Mix. Total of 2 μL of the first-strand cDNA solution was used in combination with the SYBR® Premix Ex Taq™ solution for real-time RT-PCR. The primers used for this study were listed in Table 1. All experiments were run in triplicate. The real-time RT-PCR was run on Applied Biosystems 7500 (Life Technologies Corporation., Carlsbad, CA, USA) with initial activation for 30 second at 95°C and followed by 40 cycles of denaturation (95°C, 5 second), annealing (60°C, 34 second), and extension (72°C, 30 second). The threshold cycle (CT) of each target product was determined and normalized to internal standard β-actin. Difference in CT values (CT) of two genes was calculated using the $\Delta\Delta C_t$ method, $\Delta\Delta C_t$ = target group (CT of target genes – CT of β-actin) – control group (CT of target genes – CT of β-actin), and data are expressed as fold change of control.

Statistical Analysis

All analyses were performed with SPSS 17.0 (SPSS Science, Chicago, IL, USA). The Student's t-test was used for two experimental group data analysis. One-way analysis of variance was used for multi-experimental group data analysis, followed by post hoc

Table 1 Primers used in this study for real-time RT-PCR

Genes	Forward primer	Reverse primer
β-Actin	5-GATTACTGCTCTGGCTCCTAGC-3	5-GACTCATCGTACTCCTGCTTGC-3
IL-1β	5-TGAAATGCCACCTTTTGACAG-3	5-CCACAGCCACAATGAGTGATAC-3
NLRP3	5-AGAAGAGTGGATGGGTTTGCT-3	5-GCGTTCCTGTCCTTGATAGAG-3
ASC	5-GTCACAGAAGTGGACGGAGTG-3	5-CTCATCTTGTCTTGGCTGGTG-3
Caspase-1	5-CGTGGAGAGAAACAAGGAGTG-3	5-AATGAAAAGTGAGCCCCTGAC-3

Student–Newman–Keuls test. Differences were considered significant, while the *P*-value was <0.05. All data were presented as mean ± SEM.

Results

Levels of IL-1β Protein and mRNA Significantly Elevated in LPS-Treated Mice Brain

We established an acute depression mouse model with a low dosage (0.8 mg/kg) of LPS administered intraperitoneally. Depressive behavioral tests were performed to confirm that the mice developed depressive-like behaviors 24 h post-LPS administration. Anhedonia is one of the core symptoms of major depression disorder, so we first tested sucrose preference as a reflection of anhedonia. LPS group showed significantly lower sucrose preference compared with control group (Figure 1A). Forced swim test has been widely used in the pharmaceutical industry for antidepressants screening as one of the most commonly used depressive behavioral tests. Mice typically display an immobile posture when they are confined in a cylinder of water without an opportunity to escape, which is considered as a reflection of helpless or "behavioral despair" state. We measured the immobility time of mice in forced swim test. LPS group had significantly longer immobility time versus control group (Figure 1B). Both sucrose preference test and forced swim test confirmed that the mice developed depressive-like behaviors after LPS administration.

IL-1β is an important pro-inflammatory cytokine of which stimulation and secretion can result in the activation of monocytes, macrophages, and neutrophils and subsequently induce Th1 and Th17 adaptive cellular responses [16]. Production of brain IL-1β and the resultant glucocorticoid secretion mediate the development of depressive symptoms associated with exposure to acute and chronic immunological and psychological challenges [17]. Koo et al.[14] reported that IL-1β was a critical mediator of depressive-like behavior caused by acute and chronic stress. Therefore, we measured IL-1β concentration and mRNA expression level in our acute depression mouse model. IL-1β protein concentration (Figure 1C) and mRNA expression level (Figure 1D) in LPS group mice brain showed a significant elevation compared with control group mice. These data suggested that IL-1β might have pivotal roles in LPS-induced mice depressive-like behaviors.

NLRP3 Inflammasome mRNA Expression Level Increased Significantly in LPS-Treated Mice Brain

IL-1β precursor requires proteolytic cleavage to be secreted in its active form [18]. Caspase-1 is a member of the caspase family of cysteine proteases, also known as IL-1β-converting enzyme, and specifically cleaves the 31-kd precursor IL-1β to produce the mature 17-kd biologically active IL-1β [19]. A wide variety of stimuli, such as bacterial compounds, viruses, and endogenous molecules released from injured cells trigger the activation of caspase-1 through the NLRP3 inflammasome-a key player in caspase-1 activation [20–22]. Serial stimulation with toll-like receptor ligands, plus adenosine triphosphate or pore-forming agents could activate the NLRP3 inflammasome [23]. The significant elevation of IL-1β protein concentration in LPS-treated mice brain (Figure 1C), and further proved by a triple elevation of IL-1β mRNA expression level in LPS group mice brain (Figure 1D), indicated that the NLRP3 inflammasome might be activated in the brain. We postulated that there were higher expression levels of NLRP3 inflammasome constituents. As we predicted, all three components of the NLRP3 inflammasome had significantly higher mRNA expression levels measured by real-time RT-PCR in the LPS group mice brain compared with the control group mice, including NLRP3 (Figure 2A), ASC (Figure 2B), and caspase-1 (Figure 2C). These data illustrated that the NLPR3 inflammasome might have core roles in the LPS-induced mice depressive-like behaviors.

NLRP3 Inflammasome Inhibitor Ac-YVAD-CMK Significantly Ameliorated LPS-Induced Mice Depressive-Like Behaviors

To further demonstrate the involvement of NLRP3 inflammasome in LPS-induced mice depressive-like behaviors, the NLRP3 inflammasome inhibitor Ac-YVAD-CMK was administered 30 min before LPS injection. As we predicted, the administration of Ac-YVAD-CMK significantly ameliorated the depressive-like behaviors induced by LPS. LPS group mice displayed a lower percentage of sucrose preference and longer immobility time, while pretreatment with Ac-YVAD-CMK before the LPS injection (YVAD + LPS group) significantly improved the sucrose preference percentage (Figure 3A) and lowed the immobility time (Figure 3B). These data strongly proved that the NLRP3 inflammasome was involved in LPS-induced mice depressive-like behaviors.

Conclusion

Lipopolysaccharide is a potent acute inflammatory stimulus and commonly used as an inflammation activator [24,25]. It is also known to induce a syndrome of behavioral sickness which is considered as a model of depression [26,27]. Acute activation of the immune system post-LPS administration could induce depressive-like behaviors in mice, which was characterized by decreased sucrose consumption and increased immobility in the forced swim

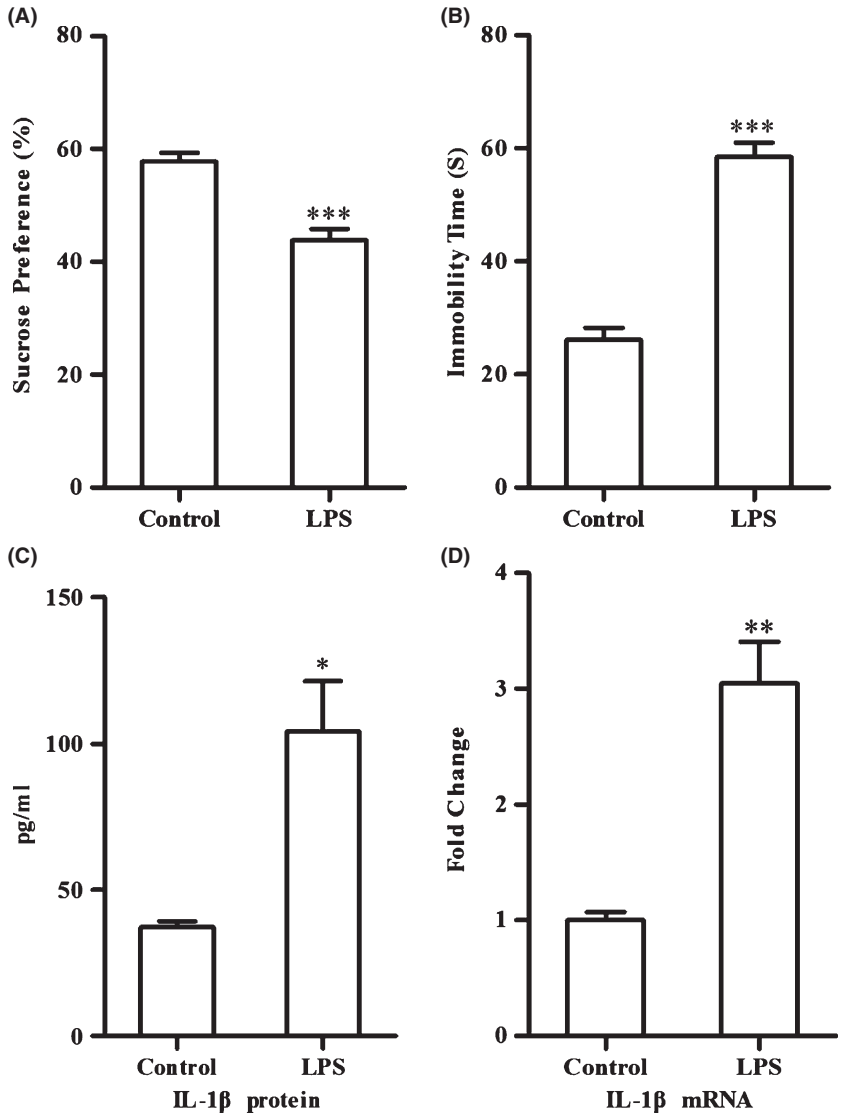

Figure 1 Interleukin-1β protein concentration and mRNA expression level significantly elevated in lipopolysaccharide (LPS)-treated mice brain. Sucrose preference test (**A**) and immobility time of forced swim test (**B**) confirmed depressive-like behaviors post-LPS administration. Interleukin-1β protein concentration (**C**) and mRNA expression level (**D**) were analyzed 24 h after LPS injection. Data are represented as mean ± SEM (n = 8–10 in **A**, **B**; n = 4–6 in **C**, **D**). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for each comparison.

test. Decreased sucrose intake was proposed as a reflection of impaired sensitivity to reward and an imitation of anhedonia, which was a core symptom of major depression disorder [28]. This study confirmed that the mice treated with LPS had significantly lower sucrose solution consumption (Figure 1A) and longer immobility time (Figure 1B).

Cytokines, primarily acting as signaling molecules of the immune system, have recently been postulated that it may provoke or exacerbate mood disorders such as depression [2]. It is mainly based on these observations [2,26,27,29,30]: (1) Elevated levels or production of plasma inflammatory factors, including several cytokines and their soluble receptors, accompany severe depressive illness in humans and in animal models of depression; (2) Pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and bacterial endotoxins such as LPS elicit sickness behaviors, which resembles depression, that may be attenuated by chronic antidepressant treatment; (3) Several cytokines can activate the hypothalamus–pituitary–adrenocortical (HPA) axis, which is commonly activated in depressed patients, and engender neuroendocrine and central neurotransmitter changes; (4) Cytokine treatments of several diseases can act synergistically with stressors to provoke a sensitization effect, so that the effects of later stressor experiences are exacerbated and elicit symptoms of depression. IL-1β may be the first step in the pro-inflammatory response to psychological stress and results in a cascade of inflammatory cytokine responses [31]. It was suggested that depression was associated with increased secretion of cytokines (especially IL-1) by macrophages [32]. Koo et al.[14] found that blockade of IL-1β

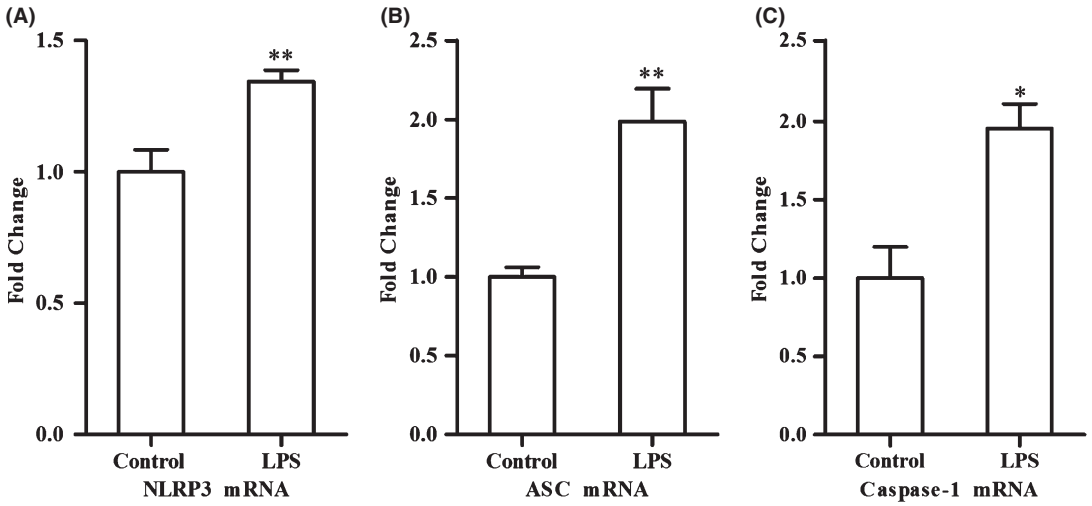

Figure 2 NLRP3 inflammasome mRNA expression level increased significantly in lipopolysaccharide (LPS)-treated mice brain. The mRNA expression levels of NLRP3 inflammasome components NLRP3 (**A**), ASC (**B**) and caspase-1 (**C**) were measured by real-time RT-PCR. Data are represented as mean ± SEM (n = 4–6 in each group). *$P < 0.05$ and **$P < 0.01$ for each comparison.

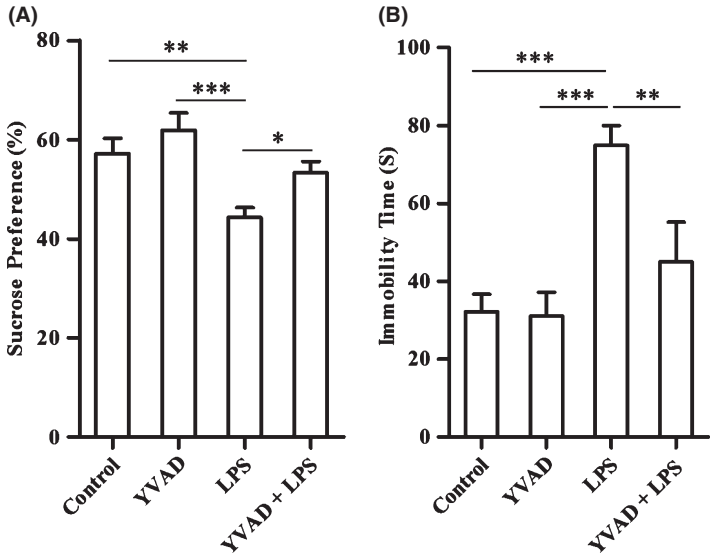

Figure 3 NLRP3 inflammasome inhibitor Ac-YVAD-CMK significantly ameliorated lipopolysaccharide (LPS)-induced mice depressive-like behaviors. There were no significant differences between control group and YVAD group both in sucrose preference test (**A**) and forced swim test (**B**), while the LPS group had significant lower sucrose preference and longer immobility time compared with control group and YVAD group. The behavioral changes elicited by LPS were significantly mitigated by the pre-administration of Ac-YVAD-CMK (YVAD + LPS group). Data are represented as mean ± SEM (n = 8–10). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ for each comparison.

was sufficient to block depressive behavioral and cellular responses resulting from exposure to chronic stress. In our study, IL-1β protein concentration and mRNA expression level significantly increased in LPS group mice brain (Figure 1C–D), which was compatible with a number of earlier findings in depressed patients and animal models [33,34].

The NLRP3 inflammasome is proposed as a bridge between psychological stress and depression, which also provides a bidirectional pathway between depression and comorbid systemic illnesses and suggests novel strategies for treating depression [31]. We examined the mRNA expression levels of NLRP3 inflammasome constituents, which all three components had significant elevations in the LPS group compared with the control group (Figure 2).

To determine the functional significance of the NLRP3 inflammasome activation in LPS group mice, we treated mice with an irreversible, cell-permeable caspase-1-specific inhibitor Ac-YVAD-CMK, which blocked the NLRP3 inflammasome assembly by binding specifically to caspase-1 subunits [35]. Ac-YVAD-CMK was administered (i.p.) at a dose of 8 mg/kg body weight 30 min

before LPS injection [36–38]. It was found that administration of Ac-YVAD-CMK significantly abrogated LPS-induced depressive-like behaviors (Figure 3).

In summary, the present study suggests for the first time that the NLRP3 inflammasome is involved in LPS-induced mice depressive-like behaviors. The NLRP3 inflammasome may be a central mediator between immune activation and development of depression. It raises the possibility that the NLRP3 inflammasome can be a more specific target for the development of novel pharmacological agents for depression treatments in the near future.

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Disclosures

The authors certify that this manuscript have not been published or submitted elsewhere.

Conflict of Interest

The authors declare no conflicts of interest.

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