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Influence of lactuside B on the expression of AQP4 and TRPM7 mRNAs in the cerebral cortex after cerebral ischemia injury

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Abstract. – AIM: This study aimed to determine the effect of Lactuside B isolated from *Pterocypsela alata* on the expression of AQP4 and TRPM7 mRNAs after cerebral ischemic injury.

MATERIALS AND METHODS: Brain ischemia injury was established by occluding the MCA (middle cerebral artery) for 2 h, followed by reperfusion in rats. The neurologic deficit scores were used to determine the success of the model. All drugs were intraperitoneally administered once a day (5 ml/kg). Eight animals from each group were investigated for the Na^+ level, and the others were examined for AQP4 and TRPM7 mRNA changes.

RESULTS: Compared with the model group, the neurologic deficit scores and Na^+ levels decreased in the lactuside B groups ($p < 0.05$ vs. $p < 0.01$). All lactuside B groups had significantly decreased AQP4 and TRPM7 mRNA expression compared with the model group ($p < 0.05$ vs. < 0.01). Dose dependence was observed between low and medium doses.

CONCLUSIONS: Lactuside B protected against cerebral edema and nerve cell damage caused by cerebral ischemic injury by decreasing the expression of AQP4 and TRPM7 mRNAs in the cerebral cortex of rats.

Key Words:
Pterocypsela elata, Lactuside B, MCAO, Cerebral cortex, Na^+ level, TRPM7, AQP4.

Introduction

Cerebral ischemia injury is a primary cause of human death and long-term disability. Presently, drugs that can effectively treat cerebral ischemia without a therapeutic time window and severe adverse drug reaction are unsatisfactory to a significant extent[1-2] because of the complexity of the pathogenesis of cerebral ischemia injury. The basic mechanism of cerebral ischemia injury is associated with the cascade reaction induced by intracellular calcium overload, and is mainly related to the toxic action of excitatory amino acid and some glutamate-independent mechanism. In recent years, the ionic imbalance and cell death induced by TRPM7 (transient receptor potential cation channel, subfamily M, member 7) channel activation in the glutamate-independent mechanism have gained people's attention[3-4]. Sun et al[5] found that mice died after complete removal of TRPM7. However, in the event of cerebral ischemia injury, the reduced expression of TRPM7 may have a protective effect on nerve cells[6].

The cerebral edema induced by cerebral ischemia injury has a fatal effect on humans. Cerebral edema is one of the morphological changes induced by local cerebral infarction in the event of cerebral ischemic injury. The complications caused by brain edema, such as brain volume expansion, increased intracranial pressure, and cerebral hernia, are key fatal and disabling factors[7]. The occurrence of cerebral edema is closely associated with the expression of AQP4 (Aquaporin 4) in brain tissues after cerebral ischemia injury[8-9]. Its pathophysiological mechanism mainly includes cytotoxic and vasogenic edema, which occur in the cerebral ischemia reperfusion injury process[10]. Cytotoxic edema is associated with an intracellular ionic imbalance induced by brain tissue ischemia and hypoxia. The occurrence of vasogenic edema is associated with the breakage of integrality of the blood-brain barrier (BBB). Damage to the BBB does not only promote the formation of cerebral edema, but also tends to increase the risk for cerebral hemorrhage after ischemnia[11-12].

Lactuside B is a monomeric compound extracted and separated from the roots of *Pterocypsela elata*. Our previous studies have shown that Lactuside B can reduce the cerebral infarction volume in rats after cerebral ischemia injury, increase the ratio of Bcl-2 (B-cell lymphoma 2)

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to Bax (Bcl-2-associated X protein), and induce resistance to the cerebral ischemia effect[13]. However, its mechanism of action is rarely studied. The neuroprotective mechanism of glutamic acid receptor antagonist in cerebral ischemia injury is frequently studied, but the involved drugs have shown insufficient neuroprotective effects in clinical trials (TRPM7). Thus, in the present work, we determined the influence of Lactuside B on the expression of TRPM7 and AQP4 mRNAs in the cerebral cortex of rats after cerebral ischemia injury, the anti-cerebral ischemia effect of Lactuside B, and the mechanism underlying this effect.

Materials and Methods

Experimental Animal, Grouping, and Administration of Drugs

Lactuside B is a yellowish white amorphous powder (> 98% purity) provided by the Department of Medicinal Chemistry, Xinxiang Medical College, China. The experimental animals were male Sprague-Dawley rats (clean grade II, body weights = 280-320 g) purchased from Henan Experimental Animal Center, China, with Animal Certificate No.: SYXK (Henan) 2005-0012. The rats were treated following the Guidance on Kind Treatment of Laboratory Animals promulgated by the Ministry of Science and Technology of the People's Republic of China in 2006, and the Institutional Animal Care and Use Committee of Xinxiang Medical College. The rats were randomly divided into five groups with 16 rats in each sham group and 28 rats in each of the other groups (successful and survival rate of animal model was approximately 60%-70%). Sixteen rats in the model group and 18 rats in the low-dose group of lactuside B (LL group; 12.5 $mg \cdot kg^{-1}$) survived. Eighteen rats in the medium-dose group of Lactuside B (LM group, 25 $mg \cdot kg^{-1}$) and 17 rats in the high-dose group of Lactuside B (LH group, 50 $mg \cdot kg^{-1}$) survived. Eight living rats in each group were used to determine the Na^+ level in the brain tissue, and the remaining rats were used to detect the expression level of TRPM7 and AQP4 mRNAs in the cerebral cortex. The sham and model groups were administered with equal volume of normal saline. The low-, medium-, and high-dose groups of lactuside B were administered with the corresponding doses of lactuside B. After reperfusion, all animals were administered with 5 $ml \cdot kg^{-1}$ drugs twice every day through intraperitioneal injection. Then, 24 and 72 h after drug administration, the animals in each group were killed and their brains were collected.

Model Preparation

Model No. 1.5 (produced by Japanese DaDong Yang) had a diameter of 0.2 mm and was cut into several 5 cm segments. The end was burnt into a circle near the flame, marked at the length of 2.0 cm, and then immersed in heparin sodium solution.

The ischemia-reperfusion injury model of the middle cerebral artery (MCA) in rats was prepared following the ligation method of Longa et al[14] (animals were anesthetized using 10% chloral hydrate) as follows. The prepared choke line was inserted from the external carotid artery (ECA) through the internal carotid artery (ICA) to a depth of 2.0 cm into the middle cerebral artery. The choke line was inserted to a depth of about 0.5 cm into the ICA of the sham group, and the remaining operations were all the same as those in the other groups. After ligation for 2 h, the choke line ball was pulled back to ECA for reperfusion of all animals. After awaking, the animals were scored for their neural deficiency symptoms according to the Longa et al method. Animals with scores of 1-4 were included in the group. The animals were scored again for their neural deficiency symptoms before being killed to determine evaluate the effect of the drugs against cerebral ischemia.

Determination of the Na^+ Level in the Brain Tissue

The rats were decapitated and their brains were collected. At 4°C, the right cerebral tissues were homogenated on ice and the protein content was determined with Coomassie brilliant blue. The absorbance of Na^+ in the brain tissue of animals in each group was determined using SbK $(OH)_6$ (antimony potassium hydroxide). The Na^+ content in the animal brain tissue was calculated according to the following formula: Na^+ content in the tissue = (absorbance of the colorimetric tube / absorbance of the standard colorimetric tube) × concentration in the standard colorimetric tube / content of the homogenized protein.

RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

Rats were decapitated and their brains were collected. Then, 80 mg of cerebral cortex around the cerebral infarction area was ground in liquid nitro-

Table I. Upstream and downstream primers sequence of TRPM7, AQP4 and β-actin.

Gene	Sequence	Production
TRPM7	F: 5′-CTGAAGAGGAATGACTACAC-3′	
	R: 5′-ACAGGGAAAAAGAGAGGGAG-3′	661bp
AQP4	F: 5′-GGGTTGGACCAATCATAGGCG-3′	
	R: 5′-GCAGGAAATCTGAGGCCAGTTCTAGG-3′	330bp
β-actin	F: 5′-CCTTCCTGGGCATGGAGTCCTG-3′	
	R: 5′-GGAGCAATGATCTTGATCTTC-3′	208bp

gen. Total RNA was extracted using the Trizol method, and its purity and concentration were tested under the premise that the extracted RNA was neither decomposed nor contaminated. cDNA template was synthesized using the TaKaRa pimeScript TM RT-PCR kit; 1 μL of cDNA was collected as a template for amplification with 50 μL of the PCR solution and 0.5 μL of (100 μmol·L^{-1}) each primer (Table I). The PCR conditions were as follows: TRPM7 was pre-denatured at 95°C for 5 min, denatured at 95°C for 30 s, annealed at 51°C for 30 s, and extended at 72°C for 1 min. All operations were repeated 30 times. Upon completion of the last repetition, TRPM7 heating was extended for 10 min at 72°C, and then stored at 4°C. AQP4 was annealed at a temperature of 59°C. The other conditions were the same as those for TRPM7 with β-actin gene as an internal reference. The PCR products of TRPM7 and AQP4 were electrophoresized on 1.5% and 1.8% agarose gels, respectively, and photographed with a gel image analyzer. The gray scale of each strip was determined with Quantity One image analysis software (SPSS Inc., Chicago, IL, USA), and the ratio of the gray value of the specifically amplified fragment to the gray value of the simultaneously amplified β-actin fragment for internal reference was taken as the semi-quantitative test value of the specifically amplified fragment.

Statistical Analysis

The obtained results were expressed as the mean ± standard deviation ($\bar{x} \pm s$), and treated using SPSS17.0 statistical software. Comparison between groups was analyzed with the one-factor analysis of variance (using the LSD method in the event of homoscedasticity, and using Dunnett's T3 in the event of heterogeneity of variance). $p < 0.05$ was considered statistically significant.

Results

Score of the Neural Deficiency Symptoms

2.1 Lactuside B reduces the score of the neural deficiency symptoms in rats with cerebral ischemia injury. The score of neural deficiency symptoms in animals of the model group significantly increased and significantly differed from that in the sham group ($p < 0.01$, Table II). Each dose group of lactuside B can reduce the score of neural deficiency symptoms in animals. One day after drug administration, the scores the groups were as follows: 3.50 ± 0.58 for the model group, 2.50 ± 0.58 for the LL group, 2 ± 0.82 for the LM group, and 2.05 ± 0.96 for the LH group. Three days after the administration of drugs, the scores of the groups were as follows: 3.40 ± 0.50

Table II. Influence of Lactuside B on the score of neural deficiency symptoms in rats with cerebral ischemia injury ($\bar{x}$ ± s, score before killing the animals).

Group	n	Dose/mg▪kg^{-1}	Administed for 1d	Administed for 3d
Sham	16	–	0.00 ± 0.00§	0.00 ± 0.00§
Model	16	–	3.50 ± 0.58*	3.40 ± 0.50*
LL	18	12.5	2.50 ± 0.58$^{\#}$	2.25 ± 0.50$^{\#,\dagger}$
LM	18	25	2.00 ± 0.82§	1.50 ± 0.58$^{\S,\Diamond,\ddagger}$
LH	17	50	2.05 ± 0.96$^{\#}$	1.75 ± 0.50§

*$p < 0.01$, comparison of the model group and the sham group; $^{\#}p < 0.05$, $^{\S}p < 0.01$ comparison of each dosage group of Lacuside B and the model group; $^{\Diamond}p < 0.01$, comparison of the LM group, LL group and LH group; $^{\dagger}p < 0.05$; $^{\ddagger}p < 0.01$ comparison of 1d and 3d after administration of drugs.

Table III. Influence of Lactuside B on the Na^+ level in brain tissues of rats with cerebral ischemia injury ($\bar{x} \pm s$, n=8, mmol/gprot).

Group	Dose/mg·kg^{-1}	Administed for 1d	Administed for 3d
Sham	–	35.63 ± 2.13	29.83 ± 5.01
Model	–	68.14 ± 3.34*	70.68 ± 5.06*
LL	12.5	56.23 ± 9.64$^{\#}$	49.93 ± 10.14$^{\#}$
LM	25	44.14 ± 5.37§	42.56 ± 9.05$^{\S,\Diamond}$
LH	50	58.51 ± 7.42$^{\#}$	56.97 ± 3.61$^{\#}$

*$p < 0.01$, comparison of the model group and the sham group; $^{\#}p < 0.05$, $^{\S}p < 0.01$ comparison of each dosage group of Lacuside B and the model group; $^{\Diamond}p < 0.01$, comparison of the LM group, LL group and LH group.

for the model group, 2.250 ± 0.50 for the LL group, 1.50 ± 0.58 for the LM group, and 1.75 ± 0.50 for the LH group, which significantly differed from that in the model group ($p < 0.05$ *vs.* $p < 0.01$; Table II). The animals in the LM group showed the best drug effect, and a dose-effect relationship was observed between the LL and LM groups. Three days after drug administration, the score of the neural deficiency symptoms in each dose group of Lactuside B more significantly decreased ($p < 0.05$ *vs.* $p < 0.01$; Table II).

Na^+ Content in the Brain Tissue

After the rats in the cerebral ischemia injury model were killed, their right brain tissue were visually observed to have significant edema, whereas the animals in other groups had milder or no edema on the same site. Na^+ content in the brain tissue of animals in the model group significantly increased, which significantly differed from that in the sham group ($p < 0.01$; Table III). At a different time point after ischemia reperfusion, each dose group of lactuside B reduced the Na^+ content in brain tissue. One day after drug administration, the scores of the groups were as follows: 68.14 ± 3.34 for the model group, 56.23 ± 9.64 for the LL group, 44.14 ± 5.37 for the LM group, and 58.51 ± 7.42 for the LH group. Three days after drug administration, the scores of the groups were as follows: 70.68 ± 5.06 for the model group, 49.93 ± 10.14 for the LL group, 42.56 ± 9.05 for the LM group, and 56.97 ± 3.61 for the LH group, which significantly differed from that in the model group ($p < 0.05$ *vs.* $p < 0.01$; Table III). Animals in the LM group showed an evident drug effect, and a dose-effect relationship was observed between the LL and LM groups. However, the drug effect in each dosage group of lactuside B did not statistically differ 1 and 3 d after administration ($p > 0.05$; Table III).

mRNA Expression in the Cerebral Cortex

After cerebral ischemia reperfusion injury, AQP4 mRNA in the brain cortex of rats sharply increased, which significantly differed from that in the model group ($p < 0.01$; Table IV and Figure 1). Each dose group of lactuside B reduced the expression of AQP4 mRNA. One day after drug administration, the scores of the groups were as follows: 1.32 ± 0.04 for the model group, 0.94 ± 0.03 for the LL group, 0.68 ± 0.02 for the

Table IV. Influence of Lactuside B on the expression of AQP4 and TRPM7 mRNA in cerebral cortex of rats with cerebral ischemia injury ($\bar{x} \pm s$).

Group	n	Dose/mg·kg^{-1}	Administed for 1d		Administed for 3d	
			AQP4	TRPM7	AQP4	TRPM7
Sham	8	–	0.24 ± 0.02	0.97 ± 0.02	0.26 ± 0.02	0.96 ± 0.02
Model	8	–	1.32 ± 0.04*	1.36 ± 0.01‡	1.30 ± 0.03*	1.25 ± 0.02‡
LL	10	12.5	0.94 ± 0.03$^{\#}$	1.05 ± 0.04$^{\#}$	0.90 ± 0.01$^{\#}$	1.03 ± 0.06$^{\#}$
LM	10	25	0.68 ± 0.02$^{\S,\Diamond}$	0.87± 0.04$^{\#,\Diamond}$	0.52 ± 0.01$^{\S,\Diamond}$	0.89 ± 0.06$^{\#,\Diamond}$
LH	9	50	0.67 ± 0.01$^{\#,\Diamond}$	1.11 ± 0.07	0.44 ± 0.02$^{\#,\Diamond}$	0.96 ± 0.04$^{\#}$

*$p < 0.01$, comparison of the model group and the sham group; $^{\#}p < 0.05$, $^{\S}p < 0.01$ comparison of each dosage group of Lacuside B and the model group; $^{\Diamond}p < 0.01$, comparison of the LM group and LL group.

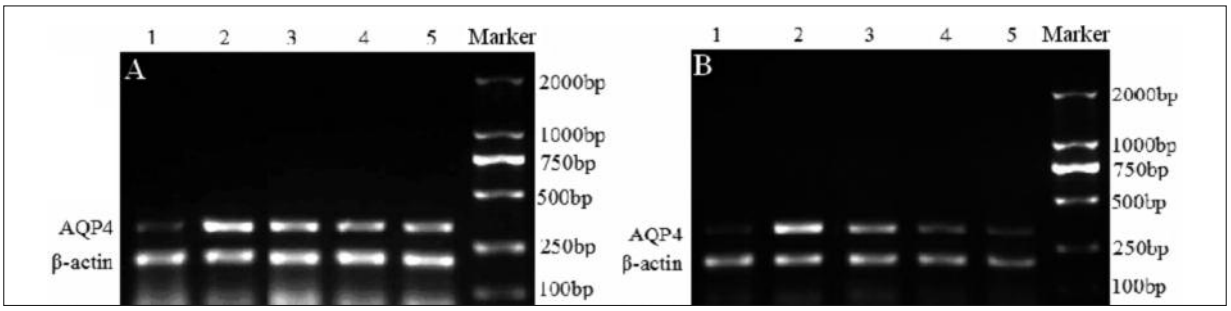

Figure 1. Effect of Lactuside B on the AQP4 mRNA expression of the cerebral cortex in rats. TRPM7 mRNA expression were detected by RT-PCR for cerebral cortex. ***A,*** Administered for 1 d. ***B,*** Administered for 3 d. 1: sham group; 2: I/R model group; 3: I/R model+Lactuside B 12.5 mg·kg^{-1}; 4: I/R model+Lactuside B 25 mg·kg^{-1}; 5. I/R model+Lactuside B 50 mg·kg^{-1}.

LM group, and 0.67 ± 0.01 for the LH group. Three days after drug administration, the scores of the groups were as follows: 1.30 ± 0.03 for the model group, 0.90 ± 0.01 for the LL group, 0.52 ± 0.01 for the LM group, and 0.44 ± 0.02 for the LH group, which significantly differed from that in the model group ($p < 0.01$; Table IV and Figure 1). Dose dependence appeared to exist between various groups, but no significant difference was observed 1 and 3 d after drug administration ($p > 0.05$; Table IV and Figure 1).

Results showed that the expression of TRPM7 mRNA in the cerebral cortex of animals in the model group increased, which significantly differed from that in the sham group ($p < 0.01$; Table IV and Figure 2). Each dose group of lactuside B reduced the expression of TRPM7 mRNA. One day after drug administration, the scores of the groups were as follows: 1.36 ± 0.01 for the model group, 1.05 ± 0.04 for the LL group, 0.87 ± 0.04 for the LM group, and 1.11 ± 0.07 for the LH group. Three days after drug administration, the scores of the groups were as follows: 1.25 ± 0.02 for the model group, 1.03 ± 0.06 for the LL group, 0.89 ± 0.06 for the LM group, and 0.96 ± 0.04 for the LH group, which significantly differed from that in the model group ($p < 0.05$; Table IV and Figure 2). The expression of TRPM7 mRNA most significantly decreased in the LM group. A dose-effect relationship existed between the LL and LM groups, but no significant difference was observed 1 and 3 d after drug administration ($p > 0.05$, Table IV and Figure 2).

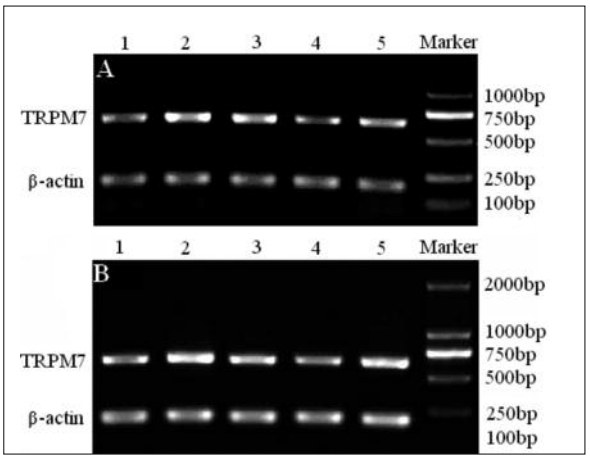

Figure 2. Effect of Lactuside B on the TRPM7 mRNA expression of the cerebral cortex in rats. TRPM7 mRNA expression were detected by RT-PCR for cerebral cortex. ***A,*** Administered for 1 d; ***B,*** Administered for 3 d. 1: sham group; 2: I/R model group; 3: I/R model+Lactuside B 12.5 mg·kg^{-1}; 4: I/R model+Lactuside B 25 mg·kg^{-1}; 5. I/R model+Lactuside B 50 mg·kg-^{1}.

Discussion

After cerebral ischemia injury, intracellular water and sodium are retained because of the inactivation of the sodium-potassium-dependent ATPase of the cell membrane, resulting in the occurrence of cerebral edema. The experimental results showed that the score of neural deficiency symptoms in rats significantly increased 24 and 72 h after cerebral ischemia injury, and the Na^+ level in the brain tissues also increased. Recently, Morimoto et al[15] reported that after cerebral ischemia injury, the NCX-1 (sodium-calcium exchanger) of Na^+/Ca^{2+} exchanger (NCX) may affect Na^+/Ca^{2+} exchange in nerve cells, and exacerbate cerebral ischemia injury in rats. NCX-1 is an ATP-independent bidirectional transport protein[16], which is exchanged in the form of $3Na^+$: $1Ca^{2+}$. When ATP is exhausted in the event of

brain tissue ischemia and hypoxia, the antiport of NCX-1 is activated, i.e., the transport of Ca^{2+} into the cells is accompanied with the transport of Na^+ out of the cells, thereby resulting in the death of nerve cells induced by Ca^{2+} overload. The experimental study showed that the Na^+ level in the brain cells of animals in the model group significantly increased, which may activate the antiport of NCX-1. Each dose group of lactuside B reduced the Na^+ level in the brain tissues of rats and improved the neural deficiency symptoms in animals. Its neuroprotective effect may be associated with the regulating effect of NCX-1.

Some studies have reported that AQP4 can increase the permeability of astrocytes in brain tissues[17-18] and play an important role in water transport in the central nervous system. The present experimental study showed that after cerebral ischemia injury in rats, the expression of AQP4 mRNA significantly increased in the cerebral cortex of animals in the model group, which was remarkably consistent with the intracellular Na^+ level. This result suggested that the upregulated expression of AQP4 was an important factor affecting the occurrence of cerebral edema and cerebral injury. After treatment, the expression of AQP4 mRNA decreased in the cerebral cortex of animals in each dose group of lactuside B, suggesting that this compound may resist cerebral ischemia-induced cerebral edema and cerebral injury by decreasing the expression of AQP4 mRNA. Nito et al[19] found that 1-3 d after cerebral ischemic injury, the upregulated expression of AQP4 is consistent with the time course of p38 phosphorylation and activation. These results show that lactuside B may also regulate the expression of AQP4 in the cerebral cortex after cerebral ischemia injury by affecting the p38 mitogen-activated protein kinase) pathway.

Some other studies have proven that the cerebral edema reaction of cerebral ischemia is at least partially associated with the activation of the transient receptor potential (TRP) channel. After cerebral ischemia injury, the stress response of the cells can lead to cell membrane depolarization, intracellular calcium accumulation, and cellular swelling[20]. The present study showed that 1 and 3 d after cerebral ischemia injury, the expression of TRPM7 mRNA significantly increased in the cerebral cortex of rats, similar to the findings of another study[21]. After intervention with lactuside B, the expression of TRPM7 mRNA decreased in the cerebral cortex of rats, which significantly differed from that in the model group ($p < 0.01$). A dose-effect relationship existed between the LL and LM groups.

Conclusions

We found that lactuside B can protect against cerebral edema and nerve cell injury induced by cerebral ischemia injury by reducing the expression of AQP4 and TRPM7 mRNAs in the cerebral cortex of rats. After cerebral ischemia injury, three doses of lactuside B were administered to intervene. However, a dose-effect relationship was only observed in the LL and LM groups, and a poor effect was found in the LH group, which may be due to the high-dose setting. The score of neural deficiency symptoms was stronger 3 d after drug administration than 1 d after, and none of the other experimental indices significantly differed, which may be related to some key factors mixed during scoring. The dose setting problems will be improved in future experiments.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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