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ORIGINAL ARTICLE

Roflumilast Reduces Cerebral Inflammation in a Rat Model of Experimental Subarachnoid Hemorrhage

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Abstract—Roflumilast, a selective inhibitor for PDE4, is approved by FDA as an anti-inflammation drug for treatment of chronic obstructive pulmonary disease (COPD). This study investigates the effects of roflumilast on cerebral inflammation in the rat SAH model. Here, we show that subcutaneous administration of roflumilast (3 mg/kg) significantly improved the neurological deficits. Measurement of evans blue extravasation and brain water content revealed a significant reduction of blood-brain barrier permeability and brain edema. Importantly, roflumilast treatment remarkably decreased levels of IL-1β, IL-6, and TNF-α and the number of apoptotic neurons in the brain after SAH. These results indicate that roflumilast is effective in treating cerebral inflammation following SAH.

KEY WORDS: subarachnoid hemorrhage; roflumilast; cerebral inflammation.

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INTRODUCTION

Subarachnoid hemorrhage (SAH) is a form of stroke, mainly caused by rupture of intracranial blood vessels leading often to long-lasting neurological impairment or death. Inflammation is known to contribute to brain edema, blood-brain barrier (BBB) permeability, cerebral vasospasm, and neuronal apoptosis following SAH [1, 2]. In patients, numerous studies have shown that levels of inflammatory cytokines increased not only within cerebrospinal fluid (CSF) but also in blood after SAH [3–5] and associated with vasospasm, hyperthermia, and unfavorable outcome [3, 6, 7]. Noticeably, this systemic increase of inflammatory cytokines following SAH is related to a late inflammatory response and a predictive marker for poor outcome [1]. Animal studies show elevated serumal cytokine concentration, accumulation of vascular neutrophils and increase of adhesion molecules within hours following SAH, and these responses are associated with early brain injury [8]. It is therefore possible that anti-inflammatory therapy in peripheral blood may suppress serum inflammatory mediators from entering the brain parenchyma.

Phosphodiesterase-4 (PDE-4) is the predominant enzyme that degrades the second messenger cAMP in many

Table 1. Behavior Scores

Category	Behavior	Score
Appetite	Finished meal	0
	Left meal unfinished	1
	Scarcely ate	2
Activity	Active, walking, barking, or standing	0
	Lying down, walk and stand with some stimulations	1
	Almost always lying down	2
Deficits	No deficits	0
	Unstable walk	1
	Impossible to walk and stand	2

group were perfused transcardially *via* left ventricle with PBS followed by 4% paraformaldehyde in PBS. The brains were carefully removed and postfixed overnight in PBS containing 4% paraformaldehyde.

For frozen sectioning, brains were placed in PBS containing 30% sucrose at 4 °C until buoyancy was lost coronal sections (10 μm thick) were cut on a Leica CM1950 cryostat (Leica, Germany), permeabilized with 1% Triton X-100 for 5 min on ice, blocked in 5% goat serum for 2 h at room temperature, and then incubated with one of the primary antibodies [IL-1β (1:200, sc-7884), IL-6 (1:200, sc-1265), TNF-α (1:200, sc-1351), Santa Cruz Biotechnology; cleaved caspase-3 (1:200, ab2302), Abcam, USA; NeuN (1:200, MABN140, Millipore, USA)] overnight at 4 °C. Sections were washed with PBS, and incubated with an appropriate secondary antibody [anti-Rabbit IgG-FITC (1: 200, F9887), anti-mouse IgG-TRITC (1:200, T5393), anti-goat-IgG-TRITC (1:200, T7028), Sigma] for 2 h at room temperature. Sections were finally washed with PBS buffer and cover-slipped with the anti-fading medium. In addition, TUNEL staining of sections were performed using the *in situ* Cell Death Detection Kit with Fluorescein (Cat #11684795910, TUNEL, Roche, Germany) according to the manufacturer's instruction. All images were captured on a fluorescence microscope (Olympus BX51, Japan).

Enzyme-Linked Immunosorbent Assay

After anesthesia with chloral hydrate, 1 ml of blood was drawn from the femoral vein in animals of the sham, SAH, and SAH+roflumilast group using a syringe without any anticoagulant. The blood was centrifuged for 10 min at 4 °C at 3000 rpm and the supernatant stored in a −80 °C refrigerator. One hemisphere of the brain was taken and weighed immediately, and homogenized in PBS using a handheld homogenizer. The homogenate was centrifuged for 10 min at 4 °C at 13000 rpm and the protein concentration of the supernatants was determined using the Protein Assay kit (Tiangen, China). Levels of IL-1β, IL-6, and TNF-α were measured using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions (MultiSciences, China) and normalized to that of the sham group.

Statistical Analysis

All data obtained were expressed as mean ± SD (standard deviation). Comparisons among the sham, SAH, and SAH+roflumilast group were made by two-way analysis of variance (ANOVA) using the GraphPad Software Prism 6.0. Statistical significance was considered at the 95% confidence interval.

RESULTS

Roflumilast Improved Neurological Deficits Following SAH

To evaluate possible neuroprotective role of roflumilast on experimental SAH, neurological assessments were conducted at 48 and 72 h after SAH using a battery of three behavioral tests (Table 1). As shown in Fig. 1, the neurological score in the SAH group was obviously increased when compared to the sham group, while neurological score in the SAH+roflumilast group significantly reduced as compared with the SAH group. The mortalities of the sham, SAH, and SAH+roflumilast at 72 h were 0% (0 of 24 mice), 22.5% (7 of 31), and 20.0% (6 of 30), respectively.
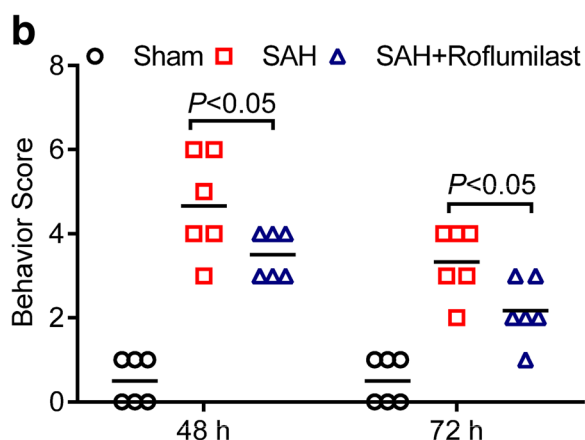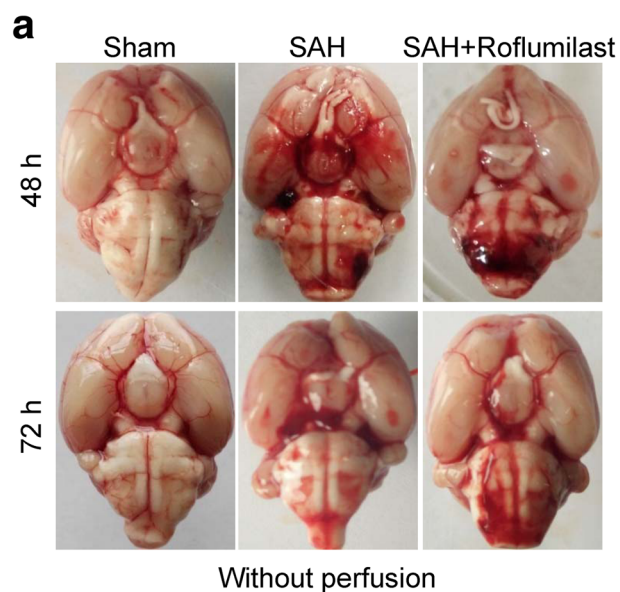
Fig. 1. **a** Representative images of brain in the sham, SAH, and SAH+roflumilast group at 48 and 72 h. **b** The neurological score was performed at 48 and 72 h in the sham, SAH, and SAH+roflumilast group. Values were expressed as mean ± SD ($P < 0.05$; two-way ANOVA with Tukey's multiple comparison test; $n = 6$ in each group).

Roflumilast Inhibited Blood-Brain Barrier Permeability and Brain Edema Following SAH

Next, we examined the effect of roflumilast on BBB permeability and brain edema at 48 and 72 h after experimental SAH. The evans blue dye extravasation and brain water content were higher in the SAH group than in the sham group, while SAH-induced increase of BBB permeability and brain edema were reduced back to lower level in the roflumilast-treated SAH group (Fig. 2).
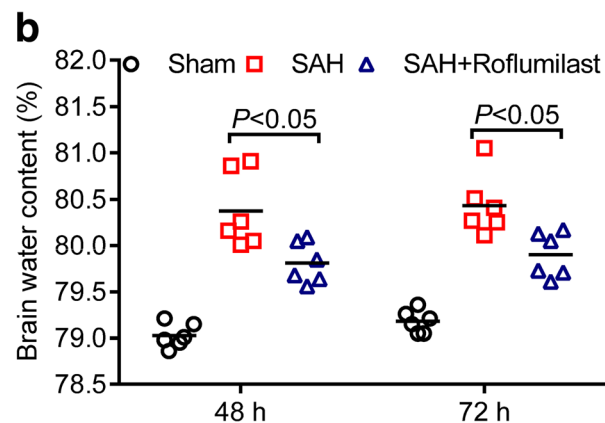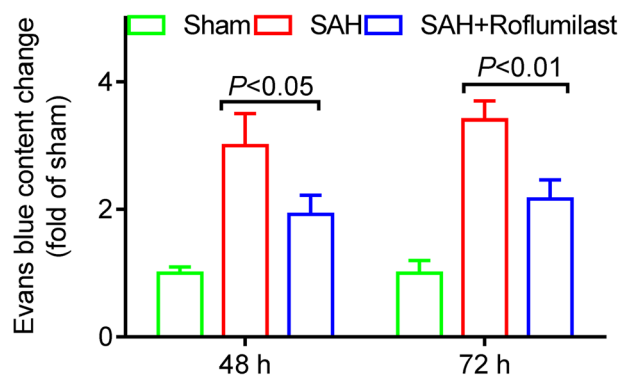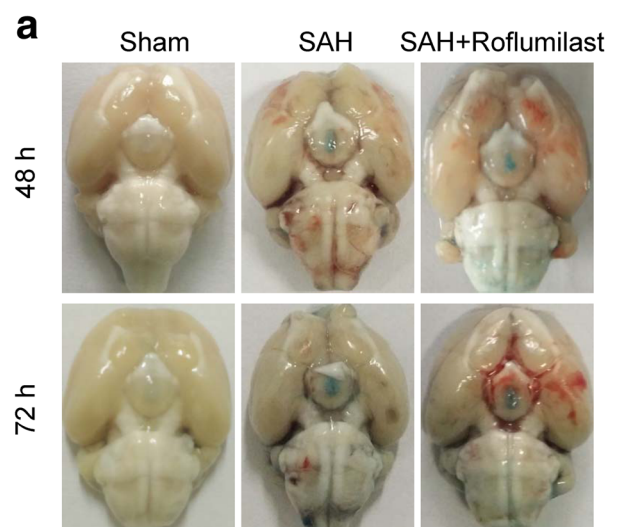
Fig. 2. **a** Representative images showed rat brain with perfusion after Evans blue injection in the sham, SAH, and SAH+roflumilast group at 48 and 72 h. **a** Evans blue content as indices of blood-brain barrier permeability, and the brain water content (**b**) was measured at 48 and 72 h in the sham, SAH, and SAH+roflumilast group. Values were expressed as mean ± SD ($P < 0.05$ or 0.01; two-way ANOVA with Tukey's multiple comparison test; $n = 3$ (**a**) or $n = 6$ (**b**) in each group).

Roflumilast Suppressed the Level of IL-1β, IL-6, and TNF-α Following SAH

We also examined the effect of roflumilast on the expression of IL-1β, IL-6, and TNF-α at 48 and 72 h after SAH. Immunofluorescence staining results were recorded using fluorescence microscope. Statistics results showed that the number of IL-1β, IL-6, and TNF-α-positive cells in the SAH group were significantly increased when compared to the sham group, while administration of roflumilast significantly reduced these positive cells as compared with the SAH group (Fig. 3). Next, ELISA was used to detect the cortical or serumal level of IL-1β, IL-6, and TNF-α at 48 and 72 h after SAH induction. As shown in Fig. 4, our results revealed that the cortical or serumal level of IL-1β, IL-6, and TNF-α obviously increased in the SAH group when compared to the sham group. However, this phenomenon was partially offset by administration of roflumilast. The cortical and serumal expression of IL-1β, IL-6, and TNF-α, which were increased by SAH injury,

were decreased in the roflumilast-treated SAH group (Fig. 4).

Roflumilast Inhibited Neuronal Apoptosis Following SAH

We quantified cellular apoptosis by using the active caspase-3/NeuN (a general neuronal marker) immunofluorescence staining and TUNEL/DAPI staining at 48 and 72 h after SAH induction. Statistics results showed that numerous active caspase-3 positive cells were obviously increased in cortex of the SAH group when compared to the sham group. However, quantification showed that active caspase-3 positive cells were significantly reduced with roflumilast as compared with the SAH group (Fig. 5). Similarly, the TUNEL-positive cells were significantly reduced in the SAH+roflumilast group when compared to the SAH group (Fig. 6).

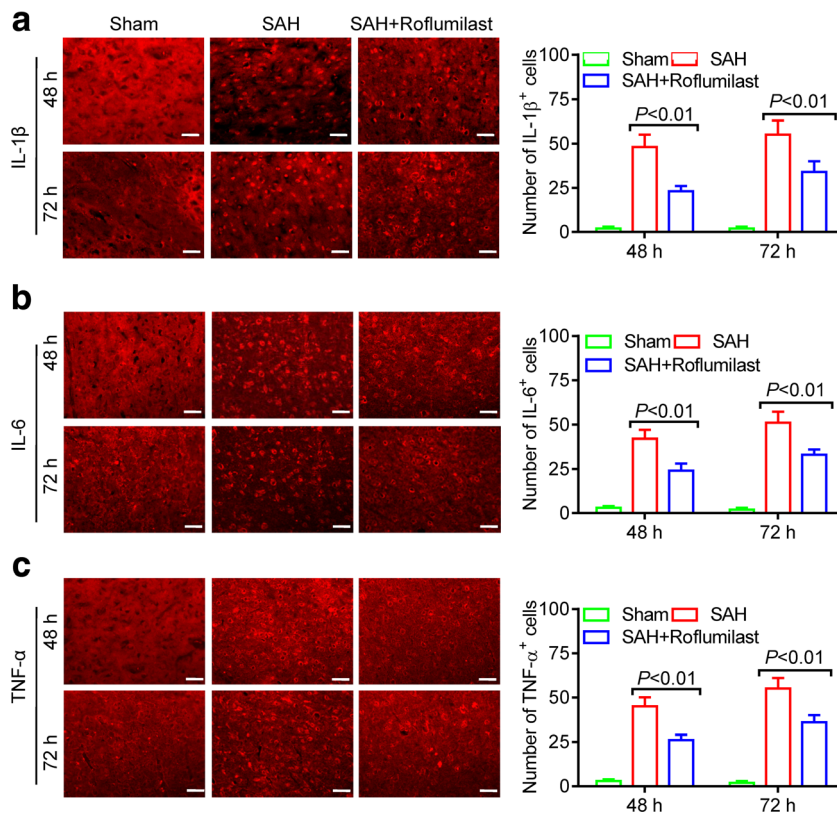**Fig. 3.** Representative immunofluorescence staining slices of **a** IL-1β, **b** IL-6, or **c** TNF-α in the sham, SAH, and SAH+roflumilast group at 48 and 72 h. *Scale bar* is 20 μm. Quantitative analysis of the **a** IL-1β, **b** IL-6, or **c** TNF-α positive cells were expressed as mean ± SD ($P < 0.01$; two-way ANOVA with Tukey's multiple comparison test; $n = 3$ in each group).
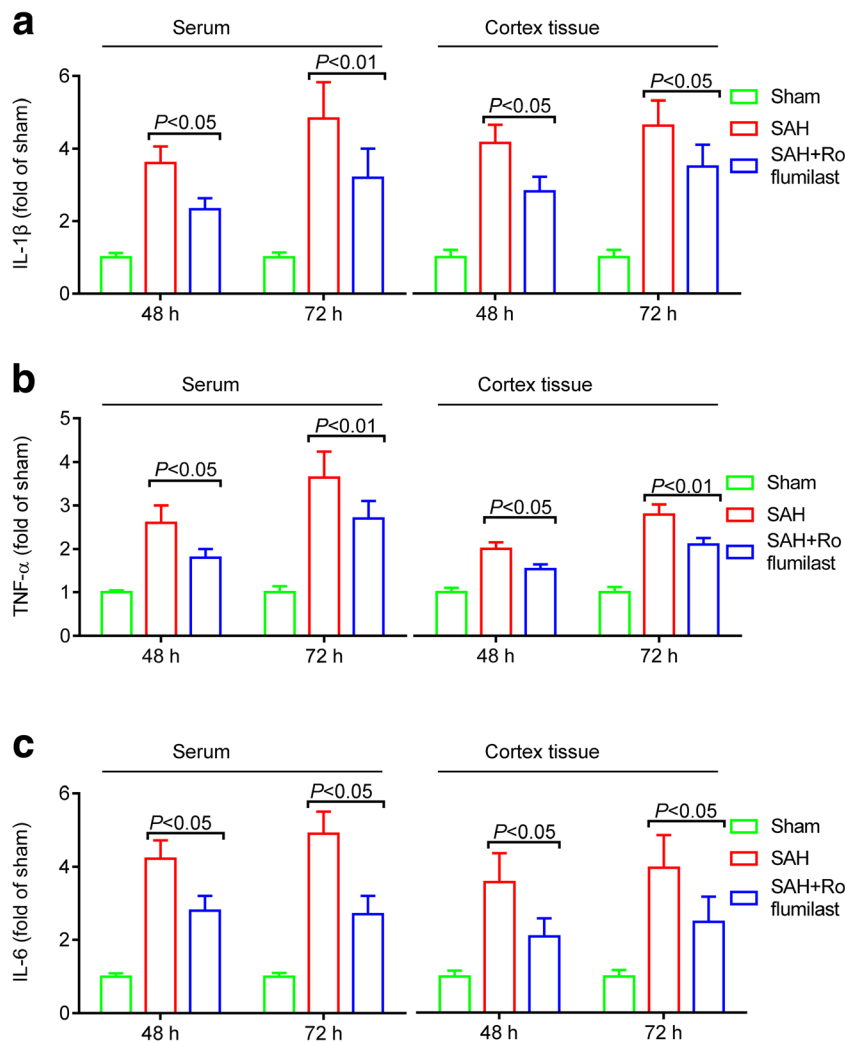
Fig. 4. The cortical or serumal level of IL-1β (**a**), IL-6 (**b**), or TNF-α (**c**) in the sham, SAH, and SAH+roflumilast group at 48 and 72 h. Values were expressed as mean ± SD ($P < 0.05$ or 0.01; two-way ANOVA with Tukey's multiple comparison test; $n = 3$ in each group).

DISCUSSION

In this study, we show that subcutaneous administration of roflumilast ameliorated brain edema and BBB permeability, decreased levels of cortical and serumal proinflammatory cytokines and reduced neuronal apoptosis 48 and 72 h after SAH. These results suggest that roflumilast is neuroprotective during early brain injury after SAH through an anti-inflammatory mechanism.

Neurological deficit and brain edema were important indicators for evaluating the outcome following SAH. Our results indicate that administration of roflumilast improved the neurological deficit and reduced brain edema after

SAH, which is in good accordance with previous studies in ischemic stroke [14, 15]. Our observation in the SAH model is in accordance with previous study describing PDE4 inhibition-induced attenuation of blood-brain barrier permeability after ischemic stroke, an effect that may attribute to decrease of inflammation [14]. It is noteworthy to mention that roflumilast is clearly brain penetrant; plasma and brain concentration of roflumilast and its metabolite (roflumilast-N-oxide) showed linear increase after oral or subcutaneous administration [19, 20].

PDE4 inhibition has been shown to reduce the neutrophils invasion and the expression of proinflammatory cytokines IL-1β and TNF-α after ischemic stroke in

Fig. 5. Representative immunofluorescence staining slices of active caspase-3 plus NeuN in the sham, SAH, and SAH+roflumilast group at 48 and 72 h. *Scale bar* is 20 μm. Quantitative analysis of the active caspase-3 staining positive cells were expressed as mean ± SD ($P < 0.01$; two-way ANOVA with Tukey's multiple comparison test; $n = 3$ in each group).

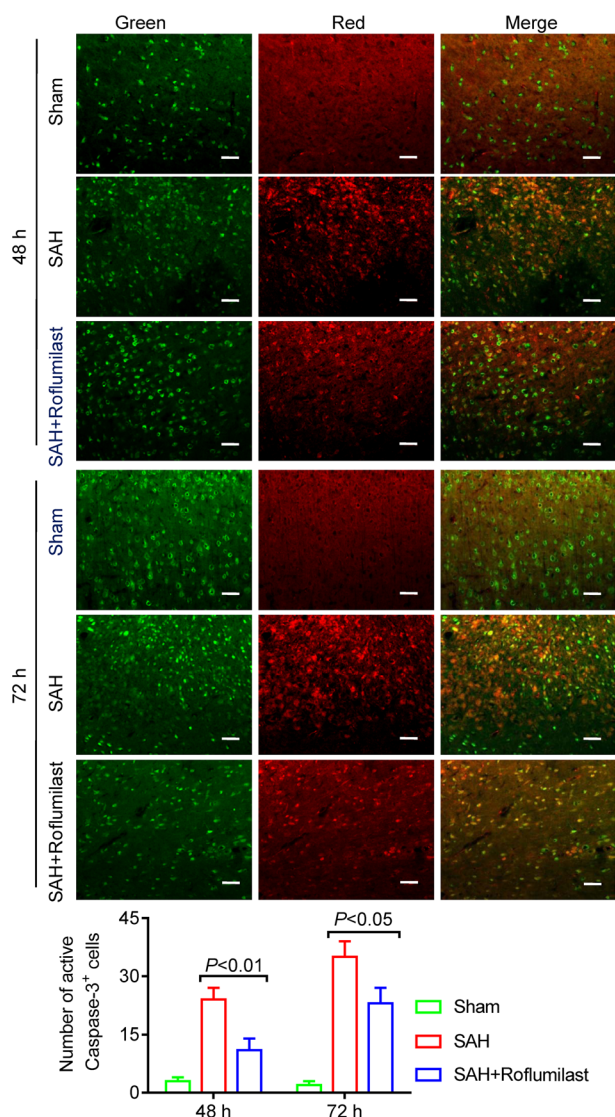**Fig. 6.** Representative immunofluorescence staining slices of TUNEL staining in the sham, SAH, and SAH+roflumilast group at 48 and 72 h. *Scale bar* is 20 μm. Quantitative analysis of the TUNEL staining positive cells were expressed as mean ± SD ($P < 0.01$; two-way ANOVA with Tukey's multiple comparison test; $n = 3$ in each group).

mice [14]. Our observations show that the PDE4 inhibitor roflumilast protects neurons in hemorrhagic stroke in rat by reducing the cortical and serumal level of IL-1β, IL-6, or TNF-α, which are known to aggravate brain injury. It is unclear how roflumilast reduces cerebral cytokines. It is possible that roflumilast inhibits peripheral inflammation and thus reduces the amount of inflammation cytokines entering the brain. Roflumilast may reduce the neutrophils

invading the brain after SAH. Further investigation is warranted to explore the mechanism of roflumilast on cerebral inflammation after SAH.

PDE4 inhibition was also found to attenuate the neuronal apoptosis and increase cell proliferation and survival rate through the CREB signaling pathway in peri-infarct region after ischemic stroke [15]. Our results found that roflumilast administration obviously decreases the number of TUNEL-positive cells and active caspase-3/NeuN-

positive cells after SAH. Even with the limitations of the present study, our results provide information about the effects of roflumilast against SAH-induced cerebral inflammation. Our findings suggested that roflumilast may be an available therapy for treating SAH that inflammation plays an important and direct role in disease progression.

CONCLUSION

The present study shows for the first time that roflumilast against cerebral inflammation in experimental SAH. Subcutaneous administration of roflumilast (3 mg/kg) significantly improved the neurological deficit, reduced the BBB permeability, the level of proinflammatory cytokines IL-1β, IL-6, and TNF-α, and neuronal apoptosis at 48 and 72 h in rat SAH model. These findings indicate that anti-inflammation strategy of using roflumilast may provide neuroprotection after SAH.

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Contributors. BS and JK conceived the project and designed experiments. QW, LQ, HL, LM, MY, RX, and XY performed the experiments; BS and JW analyzed the results, ZZ and BS wrote the manuscript. All authors read and approved the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest

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