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Celecoxib aggravates atherogenesis and upregulates leukotrienes in *ApoE*$^{-/-}$ mice and lipopolysaccharide-stimulated RAW264.7 macrophages

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HIGHLIGHTS

- The COX-2-selective inhibitor celecoxib aggravates atherogenesis but has no effect on hyperlipidemia.
- Leukotrienes are upregulated in the celecoxib-treated atherosclerosis (AS) model.
- Leukotrienes upregulation is due to the a 5-LO pathway shunt in the celecoxib-treated AS model.

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ABSTRACT

Background and aims: COX-2-selective inhibitors have been associated with an increased risk of cardiovascular complications, and their impact on atherosclerosis (AS) remains controversial. The proinflammatory COX-2 and 5-LO pathways both play essential roles in AS and related cardiovascular diseases. Previous clinical studies have provided evidence of the ability of COX-2-selective inhibitors to shunt AA metabolism from the COX-2 pathway to the 5-LO pathway. In this study, the effects of celecoxib, a selective COX-2 inhibitor, on AS and the COX-2 and 5-LO pathways were investigated *in vivo* and *in vitro*.

Methods: Male *ApoE*$^{-/-}$ mice fed a western-type diet for 18 weeks and cultured mouse RAW264.7 macrophages stimulated with 1 μg/mL LPS for 24 h were used in this study.

Results: In *ApoE*$^{-/-}$ mice, intragastric administration of celecoxib (80 mg/kg/d) for 18 weeks significantly increased aortic atherosclerotic lesion area but had no effect on hyperlipidemia. In addition, celecoxib significantly lowered TNF-α and PGE_2 levels but increased both LTB_4 and CysLTs levels in aortic tissues. In LPS-stimulated RAW264.7 macrophages, pretreatment with 8 μmol/L celecoxib for 1 h significantly lowered the TNF-α, NO, and PGE_2 levels but increased the LTB_4 and CysLTs levels. Celecoxib also decreased the protein and mRNA expression of COX-2 but increased the expression of 5-LO and LTC_4S in both *ApoE*$^{-/-}$ mouse aortic tissues and LPS-stimulated RAW264.7 macrophages.

Conclusion: The COX-2-selective inhibitor celecoxib can aggravate atherogenesis, an effect that may be related to upregulation of LTs via a 5-LO pathway shunt.

1. Introduction

Atherosclerosis (AS) is the main pathological basis of cardiovascular and cerebrovascular disease (CVD) [1]. It is widely accepted that AS is a complex chronic inflammatory vascular disease [2], and cyclooxygenase (COX)-2, the inducible form of COX, is an enzyme that plays a key role in atheroinflammatory processes [3,4]. Thus, in theory, COX inhibitors such as nonsteroidal anti-inflammatory drugs (NSAIDs) and, in particular, COX-2-selective inhibitors might provide protective effects within the cardiovascular system or may even reduce AS due to their COX-2-inhibiting activities and anti-inflammatory effects. However, recent clinical studies have indicated that some COX-2-selective inhibitors and even some nonselective NSAIDs are associated with an increased risk of cardiovascular complications [5–8], which has

Abbreviations: AS, atherosclerosis; COX-2, cyclooxygenase-2; 5-LO, 5-lipoxygenase; LTs, leukotrienes; LTB_4, leukotriene B_4; LTC_4S, LTC_4 synthase; CysLTs, cysteinyl leukotrienes; ApoE, apolipoprotein E; LPS, lipopolysaccharide; TG, total triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TNF-α, tumor necrosis factor-α

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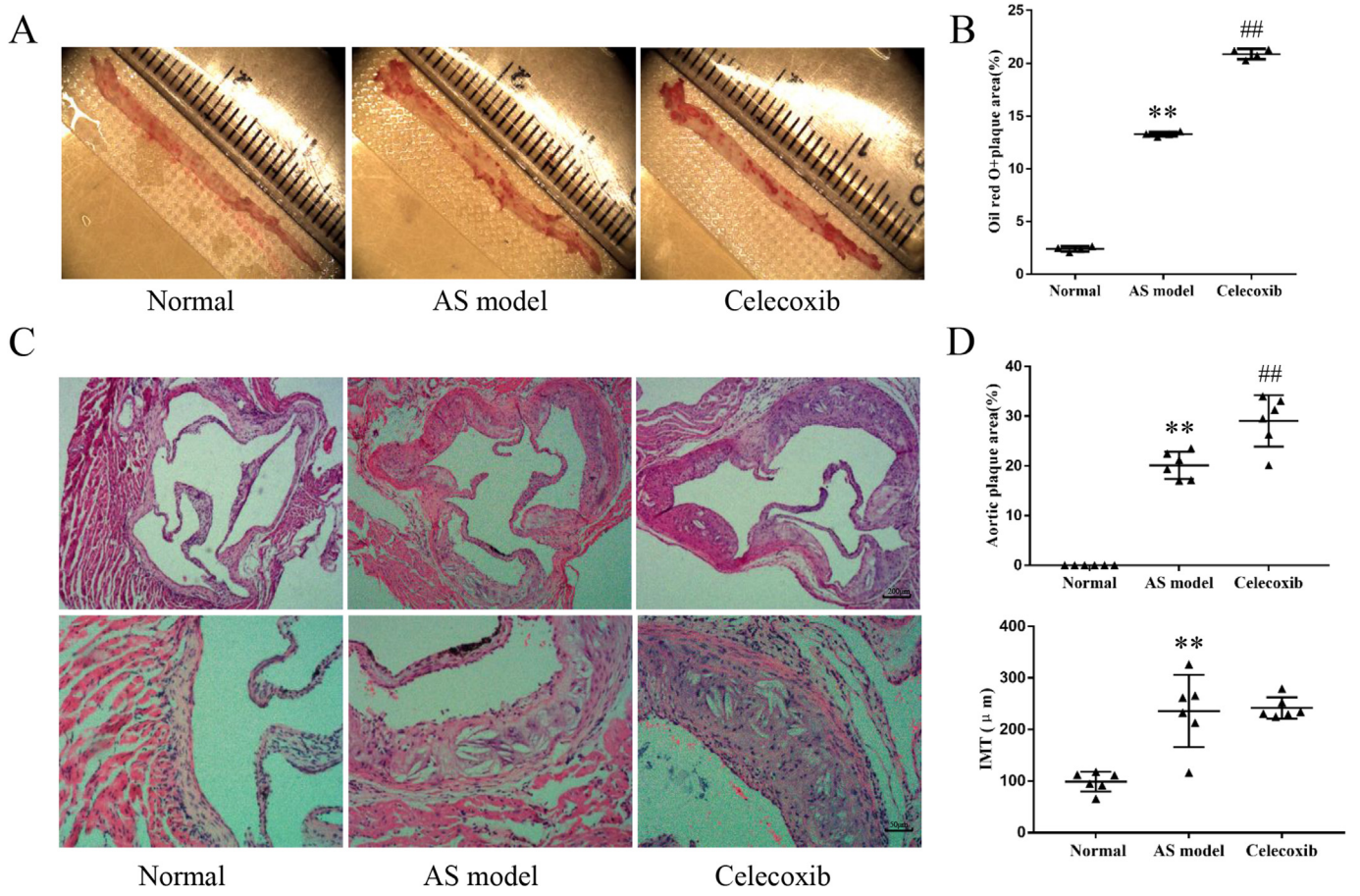

Fig. 1. Effects of intragastric administration of 80 mg/kg/d celecoxib for 18 weeks on aortic atherosclerotic lesions in *ApoE*$^{-/-}$ mice fed a western diet. (A) Oil red O staining of the entire aorta (magnification: 8 ×). (B) Proportion of the oil red O-positive plaque area (%) in the entire aorta (n = 4). (C) HE staining of atherosclerotic plaques in the aortic root (magnification: 40 × , scale bar: 200 μm; 100 × , scale bar: 50 μm). (D) Proportion of the aortic plaque area (%) and IMT in the aortic root (n = 6). All values are shown as the mean ± SEM. $^{**}p < 0.01$ *vs.* the normal control group, $^{\#\#}p < 0.01$ *vs.* the AS model group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Effects of intragastric administration of 80 mg/kg/d celecoxib for 18 weeks on serum lipids in *ApoE*$^{-/-}$ mice fed a western diet (n = 10).

Groups	Mean values of serum lipids levels ± SEM (mmol/L)			
	TG	TC	LDL-C	HDL-C
Normal	1.32 ± 0.13	3.66 ± 0.36	0.85 ± 0.37	1.36 ± 0.05
AS model	1.53 ± 0.50	30.61 ± 7.74**	12.93 ± 2.44**	3.54 ± 1.24*
Celecoxib	1.22 ± 0.23	28.53 ± 3.85**	11.71 ± 1.39**	3.04 ± 0.55*

$^{*}p < 0.05$ or $^{**}p < 0.01$ *vs.* the normal control group.

subsequently raised concerns regarding whether COX-2-selective inhibitors may contribute to an increased risk for AS development.

Results from animal experimental studies have shown that the impact of COX-2-selective inhibitors on AS remains controversial [9–15], and the mechanisms underlying the risk of COX-2-selective inhibitor-associated cardiovascular events remain to be clarified. COX-2 catalyzes the conversion of arachidonic acid (AA) into various prostaglandins (PGs) and thromboxanes (TXs), which have different or even opposite effects on vascular wall biology [16]. It was previously assumed that selective COX-2 inhibition would cause an imbalance between thromboxane A_2 (TXA_2) produced in platelets via COX-1 and prostacyclin (PGI_2) synthesized via COX-2 and thus accelerate AS and increase the risk of thrombosis and other cardiovascular complications [17,18]. Notably, however, a similar or even higher cardiovascular risk has been observed with some nonselective NSAIDs, indicating that other mechanisms may also play a role in the cardiovascular complications of these drugs.

Lipoxygenase (5-LO) is another enzyme involved in the metabolism of AA that catalyzes leukotriene (LT) production. The final biologically active metabolites of the 5-LO pathway include leukotriene B_4 (LTB_4) and cysteinyl LTs (CysLTs: LTC_4, LTD_4, and LTE_4) [19], and LTC_4 synthase (LTC_4S) is the rate-limiting enzyme for CysLTs generation [20]. A large number of clinical studies and basic studies have revealed that the 5-LO pathway also plays a crucial role in promoting the pathogenesis and rupture of atherosclerotic plaques and the pathogenesis of other cardiovascular diseases [21–24]. When COX is inhibited, AA metabolism may be transferred from the COX pathway to the 5-LO pathway, resulting in increased production of LTs; this transfer is termed the 5-LO shunt theory. Previous studies on the treatment of active cigarette smokers with celecoxib have provided clinical evidence for the ability of COX-2-selective inhibitors to shunt AA from the COX-2 pathway to the 5-LO pathway and upregulate LT production [25–27]. Considering the role of LTs in the pathogenesis and progression of cardiovascular diseases, this finding may provide fresh insights into the mechanisms underlying the cardiovascular toxicity of COX-2-selective inhibitors [28]. To further analyze these potential mechanisms, we investigated the effects of celecoxib, a selective inhibitor of COX-2, on atherogenesis and its influences on the proinflammatory COX-2 and 5-LO pathways in apolipoprotein E (*ApoE*)$^{-/-}$ mice fed a western diet and in cultured mouse RAW264.7 macrophages stimulated by lipopolysaccharide (LPS) *in vivo* and *in vitro*.

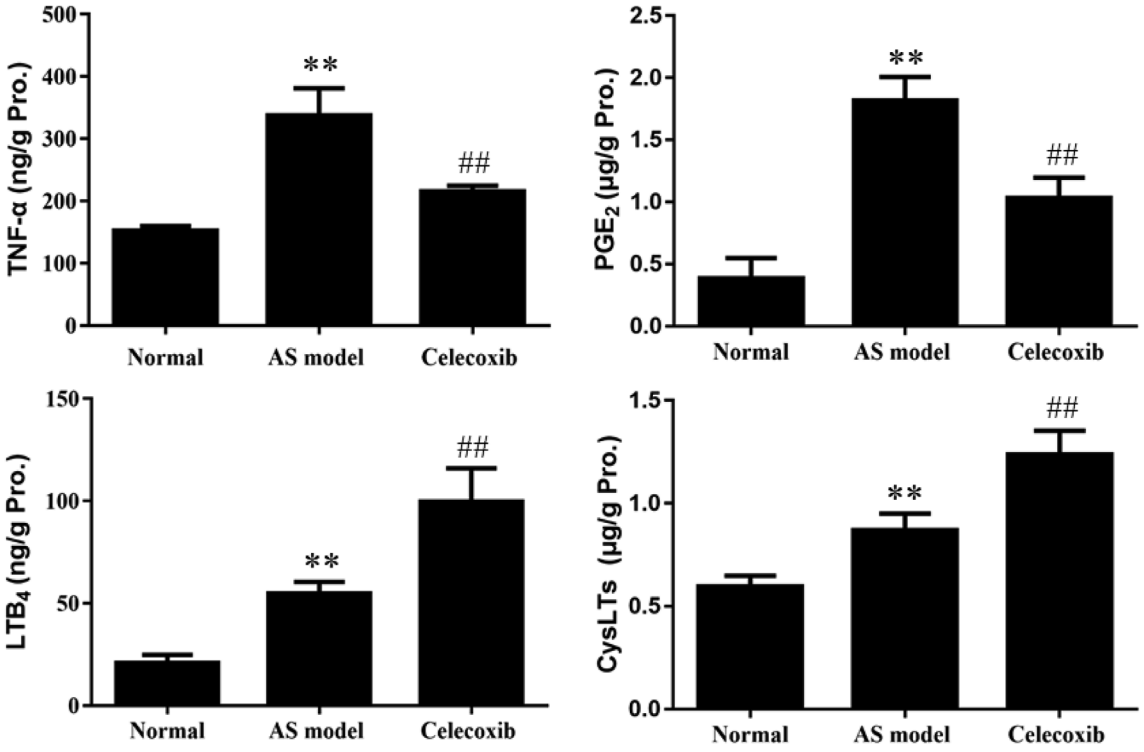

Fig. 2. Effects of intragastric administration of 80 mg/kg/d celecoxib on the levels of TNF-α, PGE_2, LTB_4 and CysLTs (n = 6) in aortic tissues of $ApoE^{-/-}$ mice fed a western diet.
All values are shown as the mean ± SEM. $^{*}p < 0.05$ or $^{**}p < 0.01$ *vs*. the normal control group, $^{\#\#}p < 0.01$ *vs*. the AS model group.

2. Materials and methods

2.1. Animals and treatment

All animal studies were conducted under the approval of the Animal Care and Use Committee of Guangxi Medical University (approval *No*. 201701009). Six-week-old male $ApoE^{-/-}$ mice and wild-type (WT) C57BL/6J mice were obtained from the Beijing Vital River Laboratory Animal Technology Company (Beijing, China). After two weeks of adaptive feeding, the $ApoE^{-/-}$ mice were fed a western-type diet containing 21% fat and 0.15% cholesterol (Beijing Keaoxieli Animal Feed Co., Ltd, China) and randomly divided into two groups, which were orally treated with celecoxib (celecoxib group, 80 mg/kg/d) or sodium carboxymethylcellulose (CMC-Na; AS model group) daily for 18 weeks. The WT C57BL/6J mice were fed a normal diet as a normal control group. Celecoxib [99.6% (HPLC), Hubei Xinghengkang Chemical Technology Co., Ltd, China] was suspended in 0.5% CMC-Na before administration. The dose of 80 mg/kg/d corresponds to a human maximum recommended daily dose of 400 mg of celecoxib for a 60-kg person. At the end of the 18-week celecoxib treatment, blood samples and aortas were collected from mice sacrificed by an overdose of phenobarbital after 12 h of fasting.

2.2. Culture and treatment of RAW264.7 macrophages

Murine RAW264.7 macrophages were obtained from the Chinese Academy of Sciences in Shanghai (originally from the American Type Culture Collection) and cultured in high-glucose DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA, USA) and 1% penicillin/streptomycin in a CO2 incubator (5% CO2 at 37 °C). The LPS model group (DMSO + LPS) and celecoxib group (celecoxib + LPS) cells were pretreated with DMSO or 8 μmol/L celecoxib (Sigma-Aldrich, Milwaukee, WI, USA, dissolved in DMSO) for 1 h and further stimulated with 1 μg/mL LPS (L2880, Sigma-Aldrich) for 24 h under serum-starved conditions. The normal control group cells were treated with only serum-free DMEM as the control. The 8-μmol/L concentration of celecoxib was the maximal nontoxic concentration for LPS-stimulated RAW264.7 cells determined in a cytotoxicity test using the MTT method (data not shown). All the cell experiments were repeated at least three times in triplicate.

2.3. Measurement of serum lipids

Blood samples were collected at the end of the 18-week celecoxib treatment, and serum lipids, including total triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), were measured with commercially available kits (Zhicheng, Shanghai, China).

2.4. Evaluation of aortic atherosclerotic lesions

At the end of the 18-week celecoxib treatment, the atherosclerotic lesions in the aorta were estimated by oil red O staining of the entire aorta and by hematoxylin and eosin (HE) staining of the aortic root as described previously [29]. The aorta was stained with oil red O from the proximal ascending aorta to the iliac bifurcation, and then the proportion of the oil red O-positive atherosclerotic plaque area (%) was measured with ImageJ software (NIH, Bethesda, MD, USAhttp://www.rab.info.nih.gov/ij)). The upper portion of the heart together with 2 mm of the aortic root was embedded in paraffin and then sectioned (5 μM) serially from the start of the aortic valve to the start of the ascending aorta until the valve cusps were no longer visible. The proportion of the atherosclerotic plaque area (%) and the intima-media thickness (IMT) in the aortic root were calculated with ImageJ software.

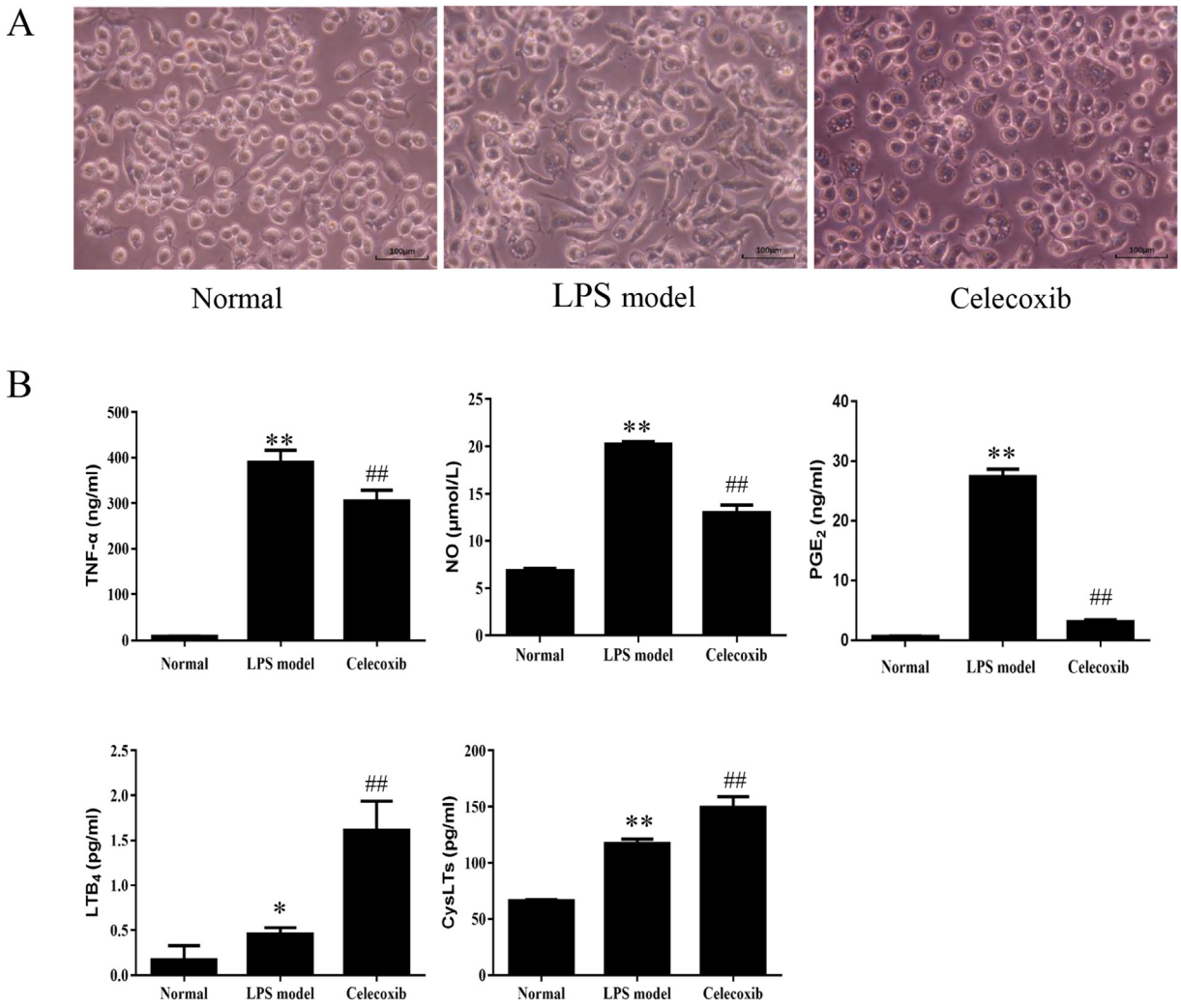

Fig. 3. Effects of celecoxib on cellular morphology (A) and the levels of TNF-α, NO, PGE_2, LTB_4, and CysLTs (B) in LPS-stimulated RAW264.7 macrophages. Magnification: 400 ×, scale bar: 100 μm. All values are shown as the mean ± SEM, n = 6 independent experiments. $^{*}p < 0.05$ or $^{**}p < 0.01$ *vs*. the normal control group, $^{\#\#}p < 0.01$ *vs*. the LPS model group.

2.5. PGE_2, CysLTs, LTB_4, TNF-α, and NO measurement in $ApoE^{-/-}$ mouse aortic homogenates and RAW264.7 macrophages

Aortas were crushed in 300 μL of homogenization buffer (0.1 M NaH_2PO_4, 1 mM EDTA and 10 μM indomethacin). Then, the supernatants were obtained to detect the protein content with a BCA protein quantification kit (Beyotime, Shanghai, China) and stored at −80 °C until the assays. Commercially available ELISA kits were used to determine the concentrations of tumor necrosis factor-α (TNF-α) (Boster Biological, Wuhan, Hubei, China), prostaglandin E_2 (PGE_2) (Cayman, Ann Arbor, Michigan, USA), LTB_4 (ENZO, New York, NY, USA) and CysLTs (Cayman) in mouse aortic homogenates and RAW264.7 cells together with medium according to the manufacturers' instructions. In addition, the levels of NO in RAW264.7 cells together with medium were detected with Griess reagent (Beyotime, Shanghai, China).

2.6. Western blot analysis of $ApoE^{-/-}$ mouse aortic homogenates and immunocytochemical staining of RAW264.7 macrophages

Aortas were lysed in RIPA lysis buffer (Beyotime) containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein (60 μg) for each sample were separated on 10%–15% denaturing SDS gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with blocking buffer (Beyotime, China) for 60 min and then incubated with specific primary antibodies overnight at 4 °C as follows: rabbit anti-COX-1 (1:100 , Boster), rabbit anti-COX-2 (1:100, Abcam, Cambridge, Cambridgeshire, UK), rabbit anti-5-LO (1:200, Abcam), rabbit anti-LTC_4S (1:100, Santa Cruz, Santa Cruz, CA, USA) or mouse anti-GAPDH (1:2000, Vazyme, Nanjing, Jiangsu, China). The primary antibody signals were detected by IRDye 680RD goat anti-rabbit or goat anti-mouse secondary antibodies (1:10000, LI-COR, Lincoln, Nebraska, USA). The blots were visualized with the Odyssey Infrared Imaging System (LI-COR) and normalized to GAPDH.

RAW264.7 cells on slides were fixed in 4% paraformaldehyde for 20 min at room temperature and then depleted of endogenous peroxidase activity with 3% H_2O_2 for 15 min and blocked with 10% normal goat serum for 20 min. The cell slides were then incubated with rabbit anti-COX-2 (1:500, Abcam), anti-5-LO (1:500, Abcam) and anti-LTC_4S (1:400, Santa Cruz) primary antibodies at 4 °C overnight followed by incubation with a biotinylated secondary antibody for 30 min at room temperature. Then, the cells were stained with a DAB Horseradish Peroxidase Color Development Kit for 5 min. Secondary antibodies and an immunohistochemistry (IHC) kit were purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China). Images of each slide and their integrated optical density (IOD) values were obtained and quantitatively analyzed with Image-Pro Plus 6.0 software.

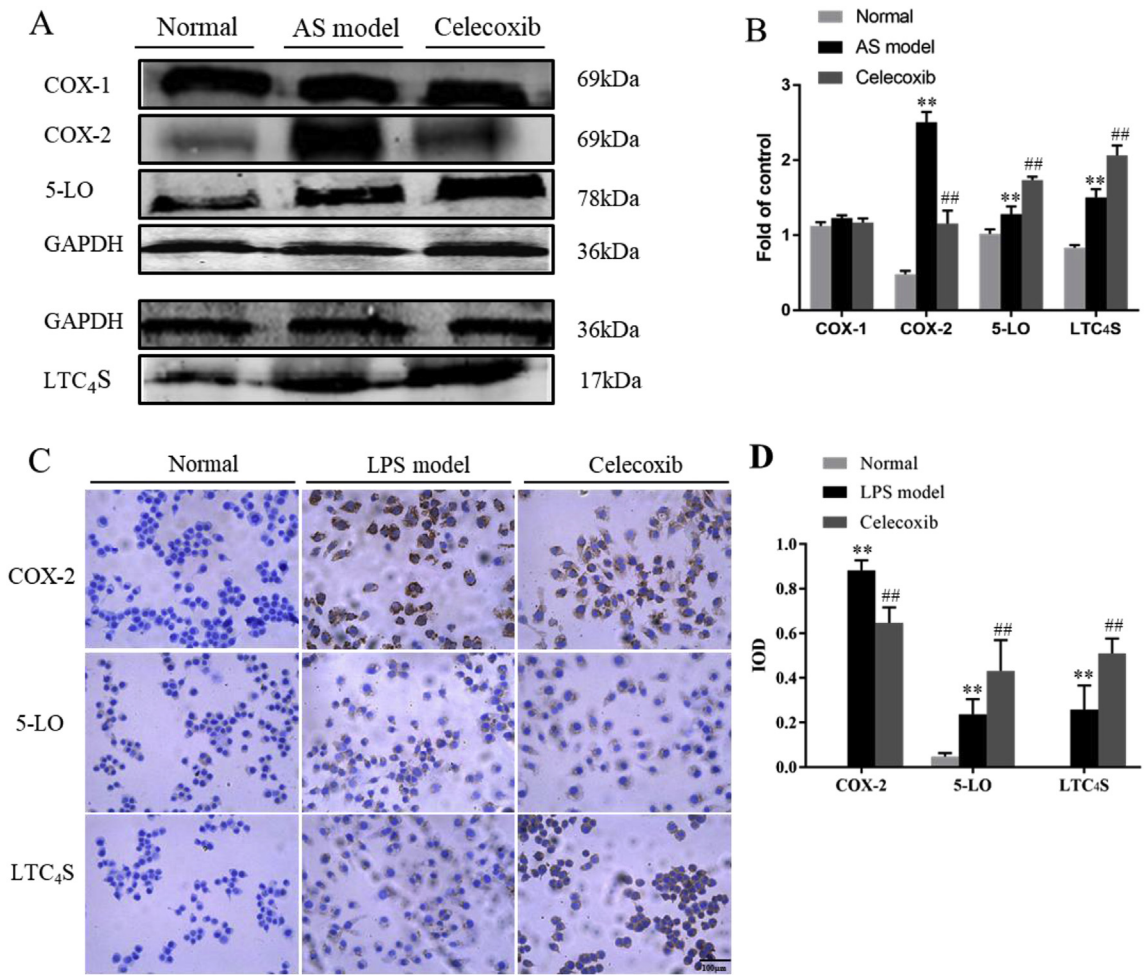

Fig. 4. Effects of celecoxib on the protein expression of COX-2, 5-LO and LTC_4S in $ApoE^{-/-}$ mouse aortic tissues and RAW264.7 macrophages.
(A and B) Immunoblots and densitometric values for COX-1, COX-2, 5-LO and LTC_4S in aortic tissues of $ApoE^{-/-}$ mice (n = 6). (C and D) Protein expression of COX-2, 5-LO and LTC_4S as detected by immunocytochemistry (magnification: 200 ×, scale bar: 100 μm) and their integrated optical density (IOD) values of these proteins in RAW264.7 macrophages (n = 3 independent experiments). All values are shown as the mean ± SEM. $^{**}p < 0.01$ *vs.* the normal control group. $^{\#\#}p < 0.01$ *vs.* the LPS model group.

2.7. Gene expression in mouse aortic homogenates and RAW264.7 macrophages

Total RNA was isolated from the lysates of the aortic tissues and RAW264.7 macrophages using RNAiso Plus Reagent (Takara, Kyoto, Kyoto-fu, Japan) and was reverse transcribed using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). The resulting cDNA was subjected to quantitative real-time PCR analysis with SYBR Premix Ex Taq™ II (Takara) with primers specific for COX-1 (*Ptgs1*), COX-2 (*Ptgs2*), 5-LO (*Alox5*) and LTC_4S (*Ltc4s*).

2.8. Statistical analysis

The values are expressed as the mean ± SEM. Comparisons among multiple groups were performed with one-way ANOVA followed by LSD tests, and comparisons between two groups were performed with independent-sample *t*-tests. *p* values < 0.05 were considered statistically significant.

3. Results

3.1. Intragastric administration of celecoxib (80 mg/kg/d) for 18 weeks increased aortic atherosclerotic lesion area in $ApoE^{-/-}$ mice

Intragastric administration of 80 mg/kg/d celecoxib to $ApoE^{-/-}$ mice for 18 weeks did not alter body weight gain compared with the AS model group (data not shown). To estimate the atherosclerotic lesions in $ApoE^{-/-}$ mice, oil red O staining of the entire aorta and HE staining of the aortic root were examined.

Oil red O staining of the entire aorta revealed marked lipid deposition in atherosclerotic plaques (dyed red in Fig. 1A) in both the AS model group and the celecoxib group. However, the aortic atherosclerotic lesions in the celecoxib group were deeper than those in the AS model group, and the range of the atherosclerotic lesions in the celecoxib group was larger, extending from the aortic arch and thoracic aorta to the abdominal aorta, than that in the AS model group (Fig. 1A). Quantitative analysis showed that the proportion of the oil red O-positive atherosclerotic plaque area (%) in the celecoxib group was significantly larger than that in the AS model group (Fig. 1B).

HE staining of the aortic root showed marked neointima formation in both the AS model group and the celecoxib group. In these groups, the intima and media were significantly thickened, and a large amount of cholesterol crystals and foam cells were found in plaques, indicating

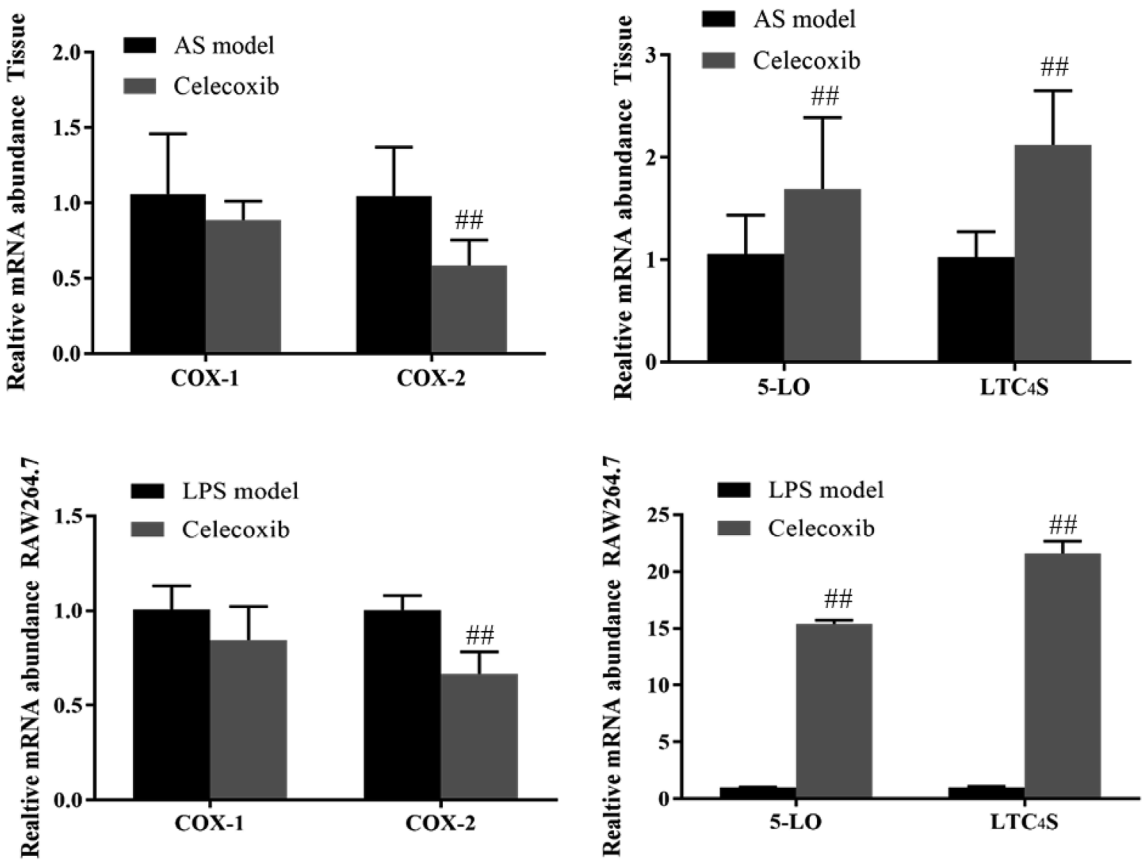

Fig. 5. qPCR analysis of *COX-1*, *COX-2*, *5-LO* and *LTC_4S* mRNA expression in aortic tissues of *$ApoE^{-/-}$* mice (n = 6) and RAW264.7 macrophages (n = 3 independent experiments).
The data were normalized to *GAPDH* expression. The *p* values were analyzed by independent-sample *t*-tests. All values are shown as the mean ± SEM. $^{\#\#}p < 0.01$ *vs.* the AS or LPS model group.

the formation of fibrofatty atherosclerotic lesions (Fig. 1C). Quantitative analysis showed that compared with the AS group, the celecoxib treatment group had a significantly higher proportion of aortic plaque area (%) but did not have a significantly different IMT (Fig. 1D).

3.2. Intragastric administration of celecoxib (80 mg/kg/d) for 18 weeks had no influence on hyperlipidemia in *$ApoE^{-/-}$* mice

To observe the influence of celecoxib on blood lipids, the serum levels of TG, TC, LDL-C and HDL-C were detected at the end of the 18-week celecoxib treatment. The serum levels of TC, LDL-C and HDL-C were higher in both the AS model group and the celecoxib group than those in the normal control group, while the levels of TG showed no significant differences. However, no significant differences were found in the levels of TG, TC, LDL-C, or HDL-C between the AS model group and the celecoxib group (Table 1).

3.3. Effects of celecoxib on COX-2 and 5-LO pathway metabolites in *$ApoE^{-/-}$* mouse aortic tissues

To investigate the mechanism involved in AS aggravation by celecoxib, the levels of PGE_2 (the major metabolite of the COX-2 pathway) and LTB_4 and CysLTs (the two major types of metabolites of the 5-LO pathway) were measured in *$ApoE^{-/-}$* mouse aortic tissues at the end of 18 weeks of celecoxib treatment. In addition, TNF-α, a commonly used inflammatory biomarker for AS, was also measured.

In the AS model group, the levels of TNF-α, PGE_2, LTB_4, and CysLTs were all significantly higher than those in the normal control group. In addition, the TNF-α and PGE_2 levels in the celecoxib group were significantly lower than those in the AS model group, whereas the LTB_4 and CysLTs levels were higher in the celecoxib group than those in the AS model group (Fig. 2).

3.4. Effects of celecoxib on cellular morphology and COX-2 and 5-LO pathway metabolites in LPS-stimulated RAW264.7 macrophages

Monocyte-derived macrophages play key roles in both early atherogenesis and advanced plaque progression [30]. In atherosclerotic plaques, activated macrophages secrete proinflammatory factors, such as TNF-α, NO, PGE_2, interleukin (IL)-1β, IL-6, IL-12, and IL-23, that contribute to the development of the inflammatory response [31].

The normal RAW264.7 cells had round somata with bright edges, and slender tentacles were occasionally observed. After LPS stimulation, RAW264.7 macrophages became bulky and stretched to irregular shapes with large numbers of pseudopodia and intracellular vacuoles, indicating their activation by LPS. After pretreatment with 8 μmol/L celecoxib, LPS-treated RAW264.7 macrophages nearly maintained their normal shape, although they still developed many intracellular vacuoles (Fig. 3A).

In RAW264.7 macrophages, LPS stimulation caused the levels of TNF-α and NO, two commonly used biomarkers for macrophage activation, as well as the levels of PGE_2, LTB_4 and CysLTs to be significantly higher than normal control levels. Compared with the LPS model group, the celecoxib group had significantly lower levels of TNF-α, NO and PGE_2 and higher levels of LTB_4 and CysLTs (Fig. 3B).

3.5. Effects of celecoxib on COX-1, COX-2, 5-LO and LTC_4S protein expression in $ApoE^{-/-}$ mouse aortic tissues and LPS-stimulated RAW264.7 macrophages

We further measured the protein expression levels of COX-2, 5-LO and LTC_4S, the key enzymes involved in the COX-2 and 5-LO pathways, in $ApoE^{-/-}$ mouse aortic tissues and LPS-stimulated RAW264.7 macrophages by western blot analysis and immunocytochemistry, respectively.

In $ApoE^{-/-}$ mouse aortic tissues, the Western blot results showed that the protein expression level of COX-1 showed no significant difference among the three groups, whereas the levels of COX-2, 5-LO and LTC_4S in the AS model group were significantly higher than those in the normal group. After celecoxib treatment, the expression of COX-2 was significantly decreased; however, the expression levels of 5-LO and LTC_4S were further elevated compared with those in the AS model group (Fig. 4A and B).

Strong yellow or yellowish-brown staining was regarded as positive in the immunocytochemistry assay. In normal RAW264.7 cells, no COX-2 or LTC_4S protein and only a small amount of 5-LO protein was expressed. However, clear positive staining for COX-2, 5-LO and LTC_4S protein were observed in the cells of both the LPS model group and the celecoxib group. Analysis of the IOD values in RAW264.7 cells showed that the expression levels of COX-2, 5-LO and LTC_4S protein in the AS and LPS model groups were significantly higher than those in the normal control group; furthermore, celecoxib significantly decreased the expression of COX-2 protein while increasing the expression of 5-LO and LTC_4S compared with the AS and LPS model groups (Fig. 4C and D).

3.6. Effects of celecoxib on COX-1, COX-2, 5-LO and LTC_4S mRNA expression in $ApoE^{-/-}$ mouse aortic tissues and RAW264.7 macrophages

The qPCR results showed the selective inhibitory effect of celecoxib on *COX*-2 mRNA expression. In accordance with the protein expression results, mRNA expression analysis in $ApoE^{-/-}$ mouse aortic tissues and RAW264.7 cells showed that celecoxib treatment selectively inhibited the mRNA expression of *COX-2* while increasing the mRNA expression of *5-LO* and *LTC_4S* (Fig. 5).

4. Discussion

The impact of COX-2-selective inhibitors on AS remains controversial. Results from different animal studies have linked COX-2-selective inhibitors and COX-2-selective inhibitor treatment with increased, reduced, or unaltered atherogenesis [9–15]. In this study, feeding 8-week-old male $ApoE^{-/-}$ mice a western diet for 18 weeks caused the development of marked hyperlipidemia and aortic fibrofatty plaques. Using this AS animal model, we found that intragastric administration of COX-2-selective inhibitor celecoxib (80 mg/kg/d) for 18 weeks significantly increased the aortic atherosclerotic lesion area. This finding is inconsistent with other studies which reported a significant reduction in lesion size by rofecoxib in LDL receptor-knockout ($LDLR^{-/-}$) mice [9] or 900 ppm celecoxib administrated in chow for 16 weeks in $ApoE^{-/-}$ mice [10]; in addition, Vidal et al. [11] reported that treatment with rofecoxib did not modify the neointimal size in rabbits fed a high-fat diet, and similarly, separate studies reported that treatment with 536 ppm celecoxib for 15 weeks [12] or 12 weeks [13] had no effect on the in $ApoE^{-/-}$ mice. Such inconsistencies are most likely related to variations in experimental conditions, including differences in the variety of COX-2-selective inhibitors and, the dosage and duration of treatment, the AS animal model and the diet used, and the stage of AS. Regarding studies involving celecoxib treatment in $ApoE^{-/-}$ mice, unlike our investigation, $ApoE^{-/-}$ mice were fed a standard rodent diet instead of a western or high fat diet [10] or older mice (26 weeks of age) were used to cause the advanced atherosclerotic lesions [12,13]. Furthermore, this finding is in accordance with reports in which treatment with 1000 ppm celecoxib for 5 weeks increased LPS-induced AS [14], and treatment with MF-tricyclic, another COX-2-selective inhibitor, for 3 weeks increased the atherosclerotic lesion area in $ApoE^{-/-}$ mice [15]. Likewise, this finding in our study also supports that celecoxib, a selective COX-2 inhibitor, under the experimental conditions we studied, can aggravate early AS lesion formation.

In $ApoE^{-/-}$ mice, we also examined the impact of celecoxib on serum lipids, and consistent with the results of previous studies, celecoxib had no effect on hyperlipidemia caused by the western diet, indicating that the aggravating effect of celecoxib on atherogenesis is not due to lipid changes.

The 5-LO shunt hypothesis has been proposed for many years and is commonly used to explain the mechanism of aspirin-induced asthma (AIA) [32]. Since only aspirin and traditional NSAIDs, both of which can block COX-1, but not COX-2 selective inhibitors, can induce asthma attacks in patients with aspirin-exacerbated respiratory disease, it was assumed that 5-LO shunting might be COX-1-dependent [33]. However, a previous study performed by Mao et al. [25] showed that oral celecoxib treatment increased LTB_4 level in bronchoalveolar lavage fluid in active smokers. In addition, Duffield-Lillico et al. [26] reported that celecoxib significantly decreased urinary PGE-M (the major urinary metabolite of PGE_2) but increased urinary LTE_4 in smokers, and Mohebati et al. [27] further reported that zileuton, a 5-LO inhibitor, reversed the influences of celecoxib. All these findings have provided clinical evidence that COX-2-selective inhibitors have the ability to shunt AA from the COX-2 pathway to the 5-LO pathway and then upregulate LT production, although these studies did not synchronously detect both the levels of LTB_4 and CysLTs along with PGE_2 and therefor the posttranscriptional mechanism cannot be fully excluded. Moreover, these findings must be confirmed in experiments of animal models as well as clinical parallel studies to test the existence and importance of the increase in LT biosynthesis induced by COX-2-selective inhibitors.

In this study, in western diet-fed $ApoE^{-/-}$ mouse aortic tissues and cultured mouse RAW264.7 macrophages, we evaluated the effects of celecoxib on the two COX-2 and 5-LO pathways. In $ApoE^{-/-}$ mouse aortic tissues, the levels of PGE_2, LTB_4, CysLTs, and TNF-α in $ApoE^{-/-}$ mouse aortic tissues were all significantly elevated in the AS model group, indicating that inflammation was involved in the development of AS and that the COX-2 and 5-LO pathways were both activated during the progression of AS. Expectedly, celecoxib treatment lowered the levels of PGE_2 and TNF-α by selectively inhibiting the COX-2 enzyme and exerting anti-inflammatory effects; however, surprisingly, we found that celecoxib treatment significantly increased the levels of LTB_4 and CysLTs. Vidal et al. [11] reported that refecoxib had no influence on LTB_4 release induced by the physiological stimulation of 10 μM A23187 (10min) in the neutrophils isolated from peripheral blood of healthy rabbits; therefore, a pathological stimuli of 1 μg/mL LPS (24 h) was used to stimulate cultured mouse RAW264.7 macrophages in this study. In RAW264.7 macrophages, LPS stimulation induced obvious cellular morphological changes, distinctly upregulated TNF-a and NO, and markedly increased PGE_2, LTB_4 and CysLTs levels, indicating that the proinflammatory COX-2 and 5-LO pathways were involved in the activation of macrophages induced by LPS stimulation. Similar to the results in $ApoE^{-/-}$ mouse aortic tissues, in LPS-stimulated RAW264.7 macrophages, pretreatment with 8 μmol/L celecoxib, the maximal nontoxic concentration, significantly lowered the levels of TNF-α, NO, and PGE_2 while increasing the levels of LTB_4 and CysLTs. Further investigation of the protein and mRNA expression levels of key enzymes involved in the COX-2 and 5-LO pathways showed that both in the aortic tissues of $ApoE^{-/-}$ mice and in LPS-stimulated RAW264.7 macrophages, celecoxib selectively decreased the protein and mRNA expression levels of COX-2 but increased those of 5-LO and LTC_4S. All these findings reveal that LTs are upregulated in the celecoxib-treated AS model, and upregulation of LTs is due to the a 5-LO pathway shunt.

Recently, LTs have been implicated as mediators, biomarkers, and

possible therapeutic targets in the context of subclinical AS [24]. LTs are potent inflammatory lipid mediators and are mainly produced by macrophages infiltrating atherosclerotic lesions within the vascular wall [34]. LTB_4 is one of the most potent chemoattractants for monocytes/macrophages, neutrophil granulocytes and T lymphocytes and promotes leukocyte adhesion to the vascular endothelium, augments vascular permeability, and promotes vascular smooth cell (VSMC) proliferation and migration [35–37]. LTB_4 also stimulates the release of matrix metalloproteinases (MMPs) from T lymphocytes, neutrophil granulocytes, monocytes, and VSMCs [38]. CysLTs are potent vasoconstrictors that also enhance vascular permeability, furthermore, CysLTs stimulate the proliferation of arterial smooth muscle cells and promote endothelial adhesion molecule P-selectin surface expression, von Willebrand factor secretion, and platelet activating factor (PAF) synthesis in cultured endothelial cells (EC) [39,40]. CysLTs also induce contraction of atherosclerotic human coronary arteries [41]. Thus, considering the proatherosclerotic effects of LTs, there is reason to speculate that the aggravation of AS by celecoxib in our study may be related to the upregulation of LTs.

Overall, our findings reveal that the COX-2-selective inhibitor celecoxib aggravates atherogenesis, an effect that may be related to the upregulation of LTs via the 5-LO pathway shunt. To our knowledge, this report is the first to provide evidence that LT upregulation via the 5-LO shunt exists in a COX-2-selective inhibitor-treated AS model. More importantly, our findings may provide a new understanding of AS and related cardiovascular risks associated with COX-2-selective inhibitor therapy. Certainly, if the cardiovascular risk of COX-2 inhibition is due to increased LT biosynthesis to some extent, whether the combination of celecoxib and LT modifiers, such as the 5-LO inhibitor zileuton or the LT receptor antagonist montelukast, may reduce the proatherosclerotic effects and cardiovascular risks of COX inhibitors should be confirmed by further basic and clinical experiments on AS and other cardiovascular diseases.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Author contributions

Ping He designed the experiments; Yimin Pang, Lu Gan and Xianzhe Wang carried out the experiments and analyzed the experimental results; Qi Su and Cong Liang assisted with the experiments; Ping He, Yinmin Pang and Xianzhe Wang wrote the manuscript.

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