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Research Article

Protective Effects of Phytic Acid on CCl_4-Induced Liver Fibrosis in Mice

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Liver fibrosis is the main pathological feature of various chronic liver diseases that can progress to cirrhosis. However, no effective treatment strategy for liver fibrosis is available. Phytic acid (PA) is a natural plant compound found in cereals, legumes, and rapeseed. Currently, there are few reports on its relationship with liver fibrosis. Herein, we explored the effects of PA on liver fibrosis. In this study, the liver fibrosis model was constructed by intraperitoneal injection of CCl_4 and intragastric administration of sodium phytate (100 mg/kg) or silymarin (100 mg/kg) five times a week. The CCl_4 injection could significantly increase the content of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum and induce the damage of liver cells, infiltration of inflammatory factors, and deposition of collagen fibers in the liver tissue. Compared to the CCl_4 group, PA significantly reduced AST and ALT. Also, PA alleviated liver damage and reduced inflammatory cell infiltration, collagen deposition area, and hydroxyproline (Hyp) content in the liver tissue, as well as downregulated α-smooth muscle actin (α-SMA) and collagen I (Col-1). Mechanistically, PA downregulated PI3K, p-AKT, p-p65, and p-IκBα in the liver tissue and reduced the release of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). In conclusion, PA might ameliorate liver fibrosis by inhibiting the NF-κB and PI3K/AKT signaling pathways.

1. Introduction

Hepatic fibrosis is a pathological liver change caused by wound healing response to various chronic injuries and is considered reversible [1–3]. However, liver fibrosis will worsen when chronic injury persists in the liver, leading to cirrhosis, liver failure, and other serious liver diseases [4]. Thus, reversing hepatic fibrosis progression is significant for chronic liver disease patients. Due to the complex pathological mechanisms of liver fibrosis, no antifibrosis therapy is currently approved [5, 6]. Hence, exploring effective antifibrosis treatment strategies is crucial.

Natural products have attracted increasing attention because of their advantages, such as low toxicity and few side effects [7]. Phytic acid (PA) is a natural phytochemical widely found in cereals, legumes, and oilseeds and has been considered an antinutritional factor. However, increasing *in vitro* and *in vivo* studies have shown the positive role of PA in preventing and treating various diseases [8]. Moreover, PA can inhibit the proliferation of human breast cancer, colorectal cancer, and other cancer cell lines, promote their apoptosis, and inhibit their invasion and metastasis ability [9–11]. PA can also induce the polarization of bone marrow-derived macrophages (BMDMs) into the M2a-like subtype and reduce the endotoxin-induced proinflammatory response of macrophages by upregulating genes related to anti-inflammatory response and inflammatory pathway regression [12]. Additionally, PA can improve sucralose-induced fatty liver by regulating liver lipid-forming genes and the intestinal flora [13]. By inhibiting lipid peroxidation, PA reduces tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)

expression in the liver tissue, improving liver injury induced by iron overload in mice [14]. Besides, PA can ameliorate the fatty liver of mice on a high-fat diet by restoring the homeostasis of the gut-liver axis and improving liver inflammation and oxidative stress [15]. The abovementioned evidence suggests that PA has anti-inflammatory and liver-protective effects.

The activation of hepatic stellate cells (HSCs) is crucial for hepatic fibrosis occurrence. Activated HSCs produce extracellular matrix (ECM) in the liver and intensify the hepatic fibrosis process. Inflammation occurs before fibrosis and might lead to its deterioration [1]. Moreover, the NF-κB and PI3K/AKT signaling pathways regulate hepatic fibrosis progression [16, 17]. The hepatoprotective and anti-inflammatory effects of PA have been previously reported, but its potential molecular mechanisms remain unknown, and few studies have investigated the hepatoprotective effects of PA in chemical liver injury. Herein, we constructed a CCl_4-induced liver fibrosis model and observed the changes of PI3K/Akt and NF-κB signaling pathways and inflammation in mouse liver tissues, aiming to investigate the protective effects of phytic acid on the liver of CCl_4-induced liver fibrosis mice and the possible mechanisms of antiliver fibrosis and to reveal the potential targets of antifibrosis, in order to lay the foundation for its subsequent in-depth study.

2. Materials and Methods

2.1. Main Reagents. Phytic acid sodium salt hydrate, carbon tetrachloride, and olive oil were purchased from Macklin Biochemical (Shanghai, China). Silymarin was obtained from Psaitong (Beijing, China), Western blot reagents from Solarbio (Beijing, China), and primary antibodies from Zen-Bioscience (Chengdu, China). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Absin (Shanghai, China). All other reagents used were high-grade, commercially available reagents.

2.2. Animal Models and Experimental Design. Male C57BL/6 mice ($n = 36$, six-week-old) were purchased from Speyford Biotechnology Co. LTD (Beijing, China). Animals were humanely cared for according to the agency's guidelines. The feeding environment was SPF, with 12 h light/darkness cycles, at room temperature (20–24°C), 50–60% humidity, and free access to food and sterile water. After one week of adaptive feeding, animals were randomly divided ($n = 9$) into control (C), model (M), PA intervention (PA), and silymarin positive control (S) groups. The C group was intraperitoneally injected with olive oil, while M, PA, and S groups were intraperitoneally injected with CCl_4 1 mL/kg/d (1: 4 dissolved in olive oil) twice a week for eight consecutive weeks. The C and M groups received normal saline gavage, and the PA group received 100 mg/kg/d PA gavage five days a week. The S group received silymarin 100 mg/kg/d by gavage five days a week. We performed intraperitoneal injections of CCl_4 on Thursday and Sunday of each week and PA gavage on Monday, Tuesday, Wednesday, Friday, and Saturday at 3 pm. We recorded the mice's weight weekly. At the end of the experiment, mice were anesthetized, the blood and liver were collected, and the liver was weighed. The Experimental Animal Ethics and Welfare Committee of Qingdao University approved this study (20211116C575420220116085).

2.3. Serum and Liver Tissue Biomarkers. The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum and the content of hydroxyproline (Hyp) in the liver were determined using the Nanjing Jiancheng commercial kit (Jiancheng Institute of Biotechnology, Nanjing, China).

2.4. Liver Histopathology and Immunohistochemical. First, mice's livers were collected and fixed with 4% paraformaldehyde for 24 h, followed by paraffin embedding. Next, the embedded liver was cut into 5 μm thick slices and taped to glass slides. Hematoxylin and eosin (H&E), Sirius red, and Masson stainings were performed for histological evaluation. Liver pathology scoring was conducted using the Ishak scoring system. For immunohistochemical, the primary antibody of CD68 was first incubated, then the secondary antibody was incubated, and the DAB substrate was used for detection. The nuclei were restained with hematoxylin. For all semiquantitative analyses, different visual fields were randomly selected from each slice under 200x magnification, and the analysis was performed using Image J.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA). IL-1β, IL-6, and TNF-α were detected using ELISA kits (Wuhan Boster Bio-engineering Co. Wuhan, China). Experiments were carried out following the manufacturer's instructions.

2.6. Western Blot Analysis. In brief, liver tissue samples were lysed with RIPA buffer containing benzene sulfonyl fluoride (PMSF) and phosphatase inhibitors. Then, samples were centrifuged at 4°C/12000g for 15 min, and the supernatant was collected. Protein concentration was measured using the bicinchoninic acid (BCA) assay. Next, protein denaturation was conducted, and the protein extract was separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Then, the membrane was sealed with 5% milk for 2 h and cleaned with TBST three times (10 min each). The primary antibody was incubated at 4°C overnight and then cleaned with TBST three times (10 min each). Next, samples were incubated with the secondary antibody at room temperature for 1.5 h. The bands were displayed using enhanced chemilescence (ECL), and target protein levels were analyzed using Fusion FX7 software. Image J was used for statistical analysis.

2.7. Statistical Analysis. Data are expressed as means ± SEM. The least significant difference (LSD) test in the analysis of variance (ANOVA) was used for pairwise comparison

between the groups. IBM SPSS v. 22.0 was used for analysis, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of PA on Body Weight and the Liver Index in CCl_4-Induced Mice. The mice's weight in each group presented an increasing trend (Figure 1(a)). Compared to the C group, the weight gain was significantly reduced in the M group. In PA and S groups, the weight gain increased compared to the M group (Figure 1(b)) ($p < 0.05$). The liver index (liver/body weight) of the M group significantly increased compared to the C group. Meanwhile, compared to the M group, the liver index of the PA and S groups significantly decreased (Figure 1(c)) ($p < 0.05$). These results indicated that PA intervention and silymarin could improve the liver index increase induced by CCl_4.

3.2. Effects of PA on Liver Injury in CCl_4-Injected Mice. Serum AST and ALT levels were significantly higher in the M group than in the C group. On the other hand, AST and ALT were significantly lower after PA or silymarin intervention compared to the M group (Figures 2(a) and 2(b)) ($p < 0.05$). The H&E staining showed that the liver cells in the C group were normal, the liver lobule structure was complete, they were neatly arranged around the central vein, there were no steatosis and evident lymphocyte infiltration, and they were arranged in a radial or cord-like manner. Compared to the C group, the liver tissue of the M group showed spot-like necrosis, shrinkage, fragmentation, and dissolution of liver cells, accompanied by abundant lymphocyte infiltration, loose and light staining of the cytoplasm, and abundant hepatocytes steatosis with round vacuoles of varying sizes in the cytoplasm, as well as significantly higher necroinflammatory scores (Figure 2(c)) ($p < 0.05$). The liver lesions of PA and S groups were mild, cell necrosis was improved (Figure 2(d)), lymphocyte infiltration was reduced, and necroinflammatory scores were significantly lower. Notably, PA almost achieved the same effects as silymarin.

3.3. Effects of PA on Liver Fibrosis of CCl_4-Injected Mice. Hyp is one of the main characteristic components of collagen. Its content in the liver tissue can indirectly reflect collagen accumulation, indicating the severity of liver fibrosis. Sirius and Masson trichrome stainings also reflect the collagen deposition area in the liver tissue. After CCl_4 induction, Hyp levels and the collagen deposition area significantly increased in the liver tissue, demonstrating that the hepatic fibrosis model was successfully established. Compared to the M group, the Hyp level significantly decreased in the PA group (Figure 3(d)) ($p < 0.05$), and collagen deposition was inhibited in the liver, similar to the silymarin intervention (Figures 3(a)–3(c)) ($p < 0.05$). Meanwhile, liver fibrosis improved and the collagen content significantly reduced in the two intervention groups compared to the M group. Moreover, CCl_4 injection increased α-SMA (a marker of hepatic stellate cell activation) and collagen I (ECM component) protein levels (Figure 3(e)) ($p < 0.05$), while PA or silymarin intervention reduced this increase.

3.4. Effects of PA on Liver Inflammation of CCl_4-Injected Mice. Due to the close relationship between liver fibrosis and inflammation, we detected the contents of proinflammatory factors IL-6, IL-1β, and TNF-α in liver tissues. Intraperitoneal CCl_4 injection significantly increased IL-6, IL-1β, and TNF-α levels compared to the C group. Meanwhile, IL-6, IL-1β, and TNF-α levels significantly decreased in the liver tissue of the PA and S groups compared to the M group (Figures 4(a)–4(c)) ($p < 0.05$). The immunohistochemistry analysis showed that compared with the C group, the CD68 positive area increased in the liver of mice in the M group. In contrast, the CD68 positive area in the liver of mice decreased in the two intervention groups compared with the M group (Figure 4(d)). These results demonstrated that PA intervention reduced CCl_4-induced liver inflammation in mice.

3.5. Effects of PA on the NF-κB and PI3K/AKT Pathway Activity in the Liver of CCl_4-Injected Mice. The Western blot showed that CCl_4 injection activated the NF-κB signaling pathway, and phosphorylated NF-κB P65 and IκB-α were significantly upregulated compared to the C group. PA intervention significantly inhibited the phosphorylation of NF-κB P65 and IκB-α, inhibiting the NF-κB signaling pathway activity (Figures 5(d)–5(g)) ($p < 0.05$). Compared to the C group, PI3K and the phosphorylated AKT content significantly increased in the M group, the opposite of PA intervention, and the activity of the PI3K/AKT signaling pathway was inhibited (Figures 5(a)–5(c)) ($p < 0.05$).

4. Discussion

Liver cirrhosis and its syndrome cause 1.16 million deaths yearly, and liver cancer causes 788,000 deaths, ranking as the 11th and 16th most common death causes, respectively [18]. Liver fibrosis is a necessary stage in chronic liver disease progression to cirrhosis and liver cancer, but it can be reversed [1]. Hence, reversing liver fibrosis to stop the progression of these diseases is of great importance to chronic liver disease patients.

Furthermore, PA is naturally found in cereals, legumes, and oilseeds and is well known for its anti-inflammatory, antioxidant, and antitumor effects [8]. PA can be an antinutritional factor because it chelates minerals in the gastrointestinal tract, preventing the absorption of iron, copper, zinc, and calcium. However, the intake of PA at appropriate doses does not negatively affect the nutritional status in well-balanced and well-nourished subjects [19–21]. The dose of PA intervention used in this experiment did not significantly affect the nutritional status of mice, consistent with the results of a previous study [22].

Herein, the liver fibrosis animal model was constructed by intraperitoneal CCl_4 injection. CCl_4 is the most commonly used modeling method for liver fibrosis. Its advantages lie in the high and stable modeling rate and the similar

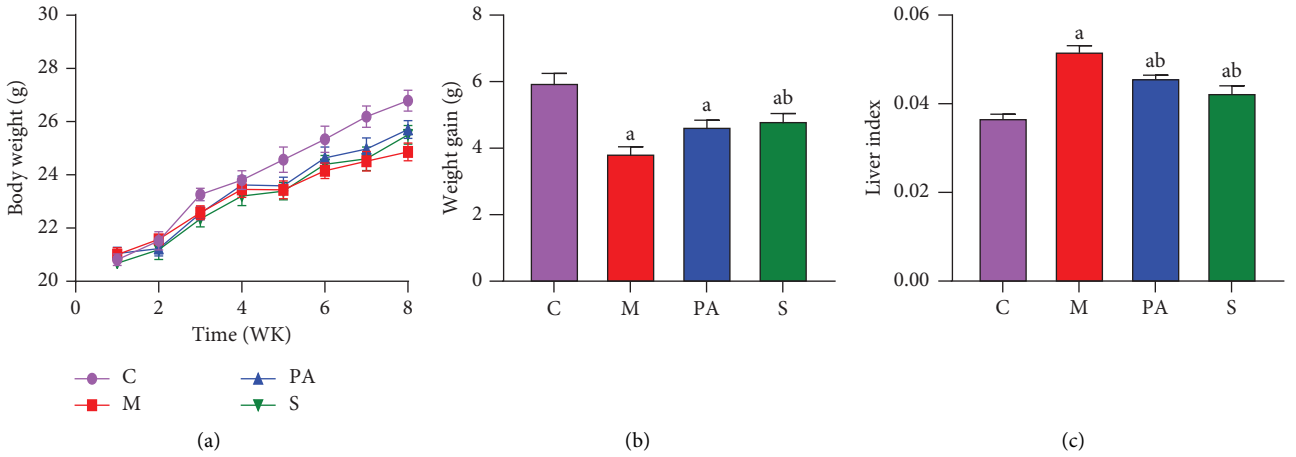


Figure 1: Effects of PA on body weight and the liver index in CCl_4-induced mice. (a) Weekly weight curves of mice, $n = 9$; (b) the final weight gain of the mice, $n = 9$; and (c) liver index (liver/body weight) of mice, $n = 9$. C: control group, M: CCl_4-induced liver fibrosis group, PA: phytic acid intervention group, and S: silymarin positive control group. $^a p < 0.05$ as compared with C, and $^b p < 0.05$ as compared with M.

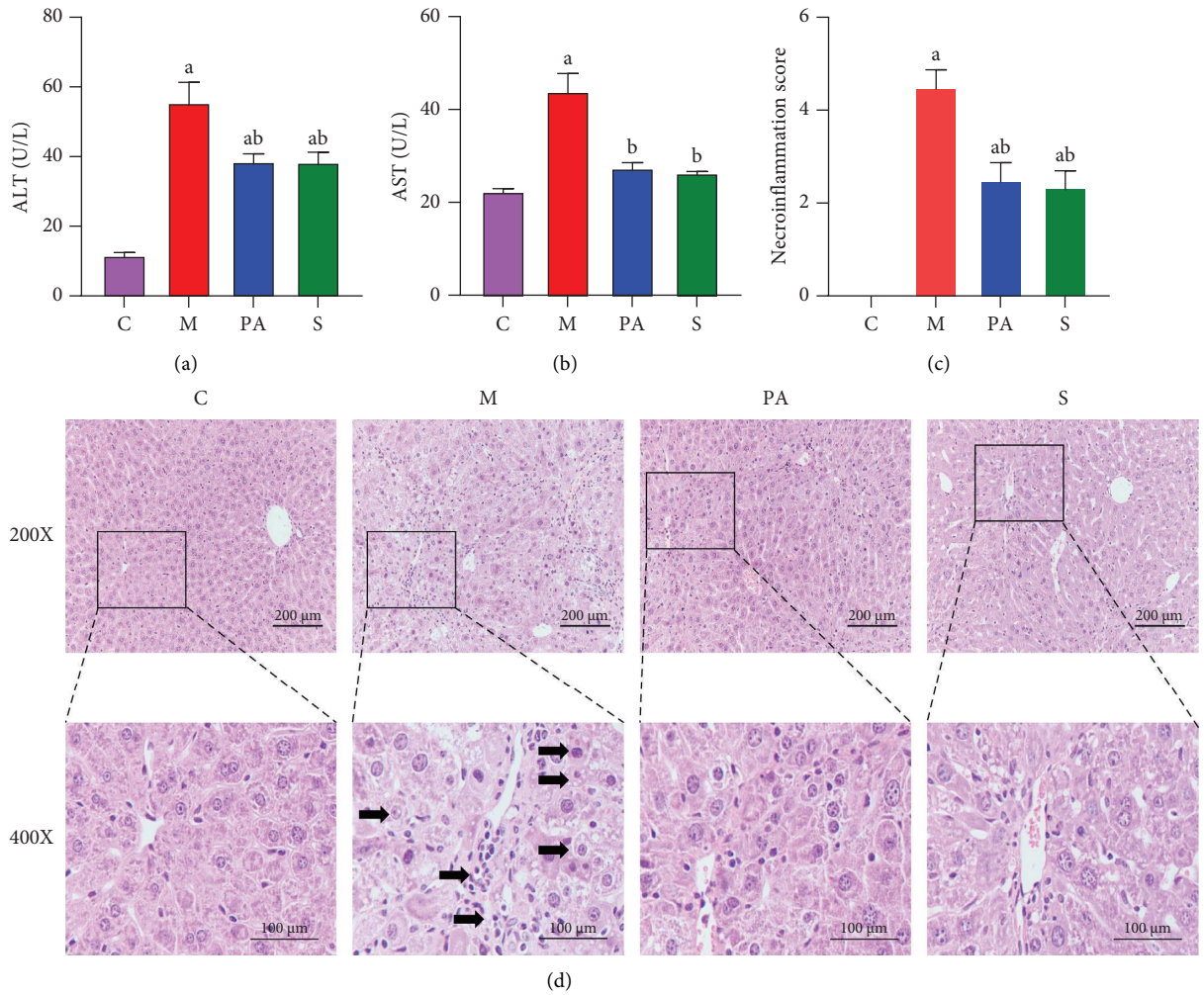


Figure 2: Effects of PA on liver injury in CCl_4-injected mice. (a) ALT in serum, $n = 6$; (b) AST in serum, $n = 6$; (c) necroinflammatory scores, $n = 6$; and (d) hematoxylin and eosin (HE) staining of the liver. C: control group, M: CCl_4-induced liver fibrosis group, PA: phytic acid intervention group, and S: silymarin positive control group. $^a p < 0.05$ as compared with C, and $^b p < 0.05$ as compared with M.

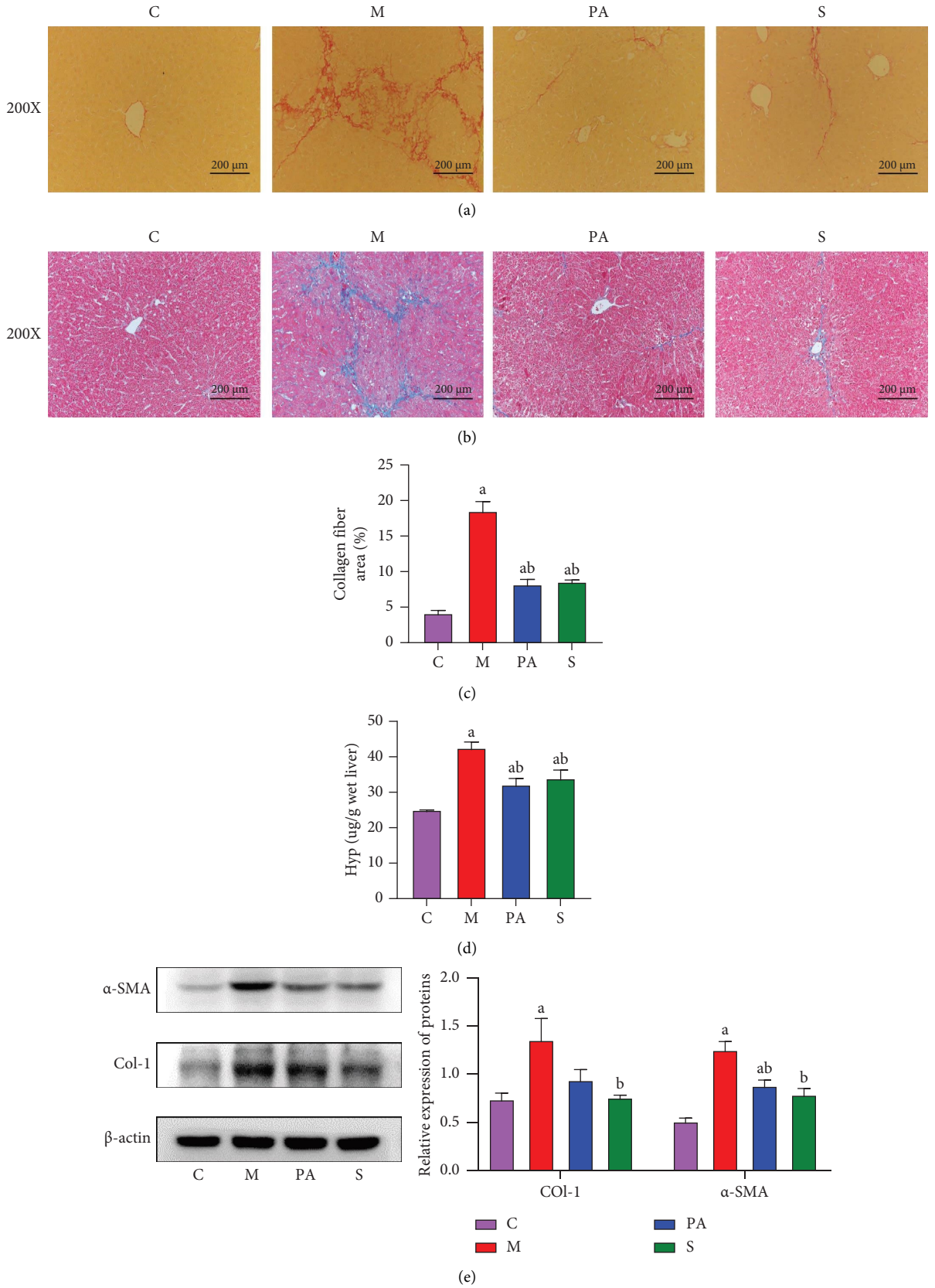


FIGURE 3: Effects of PA on liver fibrosis of CCl_4-injected mice. (a) Sirius red staining. (b) Masson trichrome staining. (c) Collagen fiber percentage, $n = 6$. (d) Hydroxyproline (Hyp) content in the liver tissue, $n = 6$. (e) α-SMA and Col-1 in liver, $n = 3$. C: control group, M: CCl_4-induced liver fibrosis group, PA: phytic acid intervention group, and S: silymarin positive control group. $^a p < 0.05$ as compared with C, and $^b p < 0.05$ as compared with M.

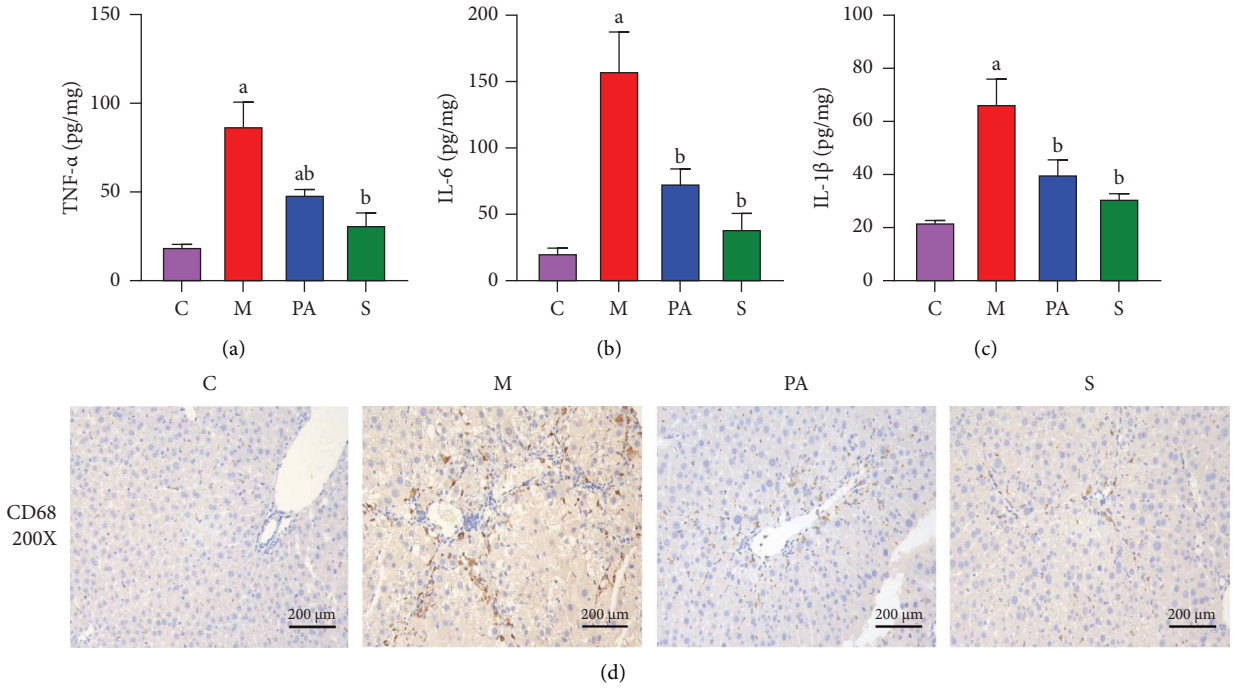
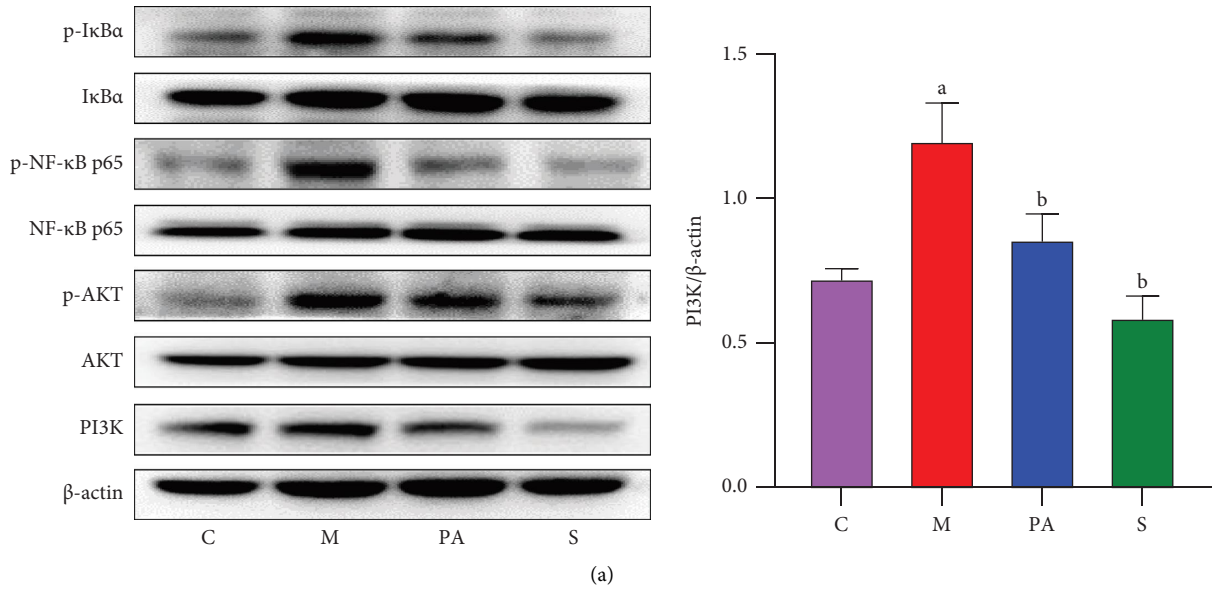


Figure 4: Effects of PA on liver inflammation of CCl_4-injected mice. (a) TNF-α in the liver tissue, $n = 6$. (b) IL-6 in the liver tissue, $n = 6$. (c) IL-1β in the liver tissue, $n = 6$. (d) CD68 immunohistochemistry (×200 magnification. Scale bar: 200 μm). C: control group, M: CCl_4-induced liver fibrosis group, PA: phytic acid intervention group, and S: silymarin positive control group. $^a p < 0.05$ as compared with C, and $^b p < 0.05$ as compared with M.



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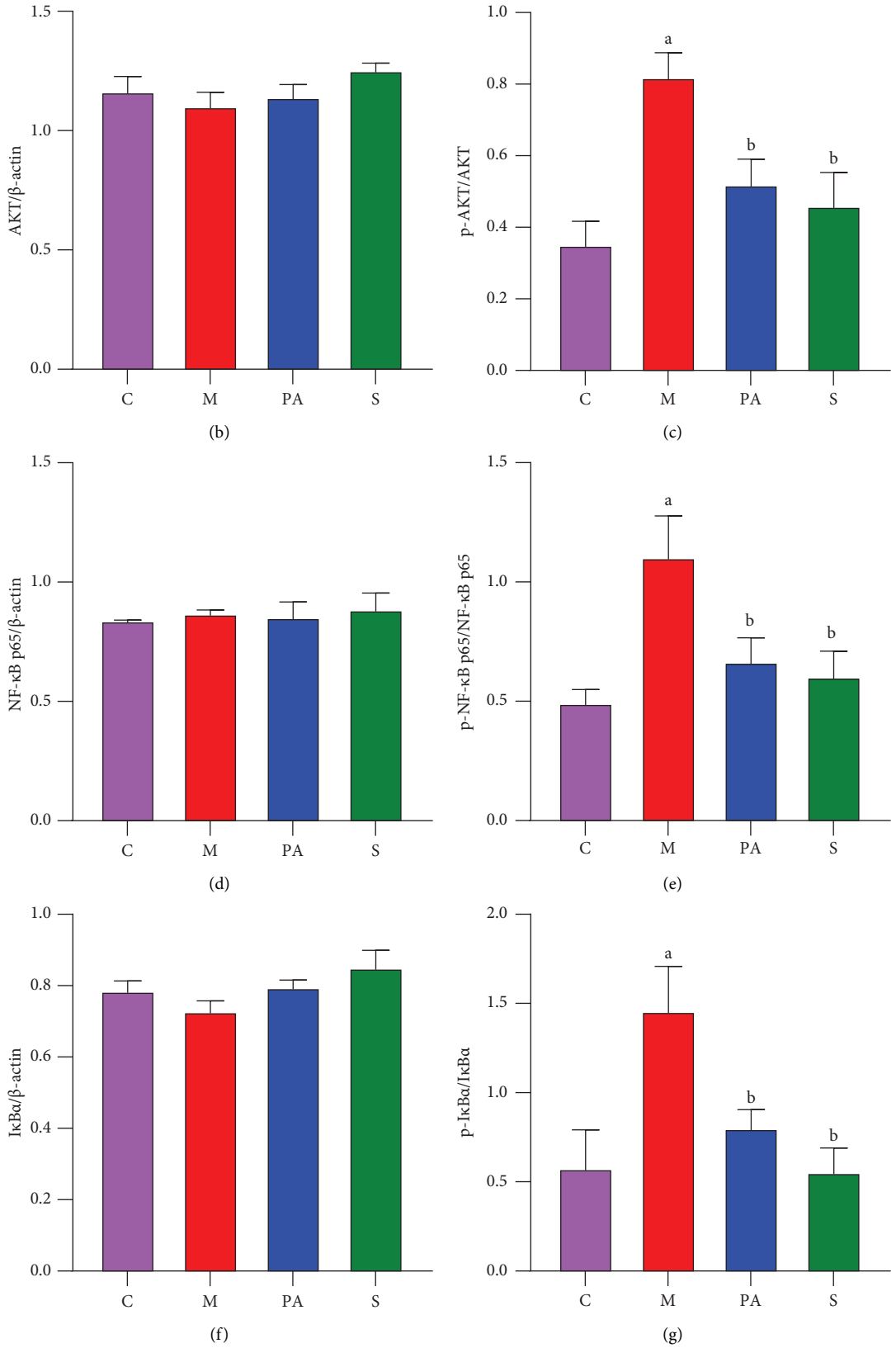

FIGURE 5: Effects of PA on protein expression in the liver tissue of CCl_4-injected mice, $n = 3$. C: control group, M: CCl_4-induced liver fibrosis group, PA: phytic acid intervention group, and S: silymarin positive control group. $^a p < 0.05$ as compared with C, and $^b p < 0.05$ as compared with M.

pathological process to humans [23]. CCl_4 decreased the weight gain, increased the liver index, exacerbated liver injury and inflammatory infiltration, and activated the PI3K/AKT and NF-κB signaling pathways. PA improved liver damage and fibrosis in hepatic fibrosis model mice, reduced the content of inflammatory factors in the liver, and regulated the activity of the PI3K/AKT and NF-κB pathways.

AST and ALT are normally present in hepatocytes, with very low levels in the blood. When hepatocytes are damaged in the initial stage of chronic liver disease, AST and ALT are released, causing an increase in blood levels [24]. Here, AST and ALT levels in the serum of mice were analyzed to assess the liver damage degree. CCl_4 significantly increased serum AST and ALT concentrations, consistent with the previous results [25, 26], while PA significantly reduced these levels. Combined with the HE results, PA intervention reduces necrosis injury and hepatocyte fragmentation.

The activation of hepatic stellate cells is essential for liver fibrosis development. Activated stellate cells release large ECM amounts, and the imbalance between production and breakdown leads to ECM accumulation, resulting in liver fibrosis and even cirrhosis [4]. The inhibition of hepatic stellate cell activation and reduction of ECM production have become two important mechanisms for treating fibrosis [7]. Col-1, a major ECM protein, and α-SMA, a hepatic stellate cell activation marker, are used to evaluate liver fibrosis severity. In the present study, CCl_4 increased α-SMA and Col-1 levels, consistent with the previous studies [27], while PA intervention reduced it.

Hyp is one of the main characteristic collagen components, and its content in liver tissues can indirectly reflect collagen accumulation. Reducing collagen deposition in the liver tissue can effectively improve liver fibrosis [28–30]. Therefore, we evaluated the effects of PA on collagen fibers in the mice's liver using Hyp as the marker. The Sirius red and Masson trichrome stainings were used to detect collagen fiber deposition in the liver. Many collagen fibers were deposited in the liver tissue of the M group. In contrast, PA reduced the liver index, decreased Hyp content in the liver, and reduced the collagen fiber deposition area, demonstrating PA's ameliorative effects on liver fibrosis.

Previous studies have demonstrated that inflammation appears earlier than liver fibrosis during chronic liver injury and can promote each other [31–35]. The NF-κB signaling pathway, important for regulating inflammation, plays a key role in liver fibrosis development. Inhibiting the NF-κB signaling pathway can reduce the expression of proinflammatory cytokines IL-6, IL-18, and TNF-α, CD68 macrophage activation in liver tissue, and decrease collagen deposition in the liver, reducing the expression of the fibrosis marker α-SMA and improving liver damage and fibrosis [17, 27, 36–39]. These results demonstrated that inhibiting the NF-κB pathway activation can effectively ameliorate liver fibrosis. Additionally, NF-κB participates in the liver inflammatory response by controlling the expression of various growth factors and cytokines [38]. IL-6, IL-1β, and TNF-α are among these proinflammatory cytokines and exist with NF-κB signaling pathways in a vicious circle, promoting each other, exacerbating the inflammatory response, and driving liver fibrosis [40–42]. We found that PA can reduce phosphorylated NF-κB p65 and IκB-α, proinflammatory factors (IL-6, IL-1β, and TNF-α), and CD68 macrophage activation. These results suggested that PA might play an anti-inflammatory role in improving liver fibrosis via NF-κB signaling pathway regulation during liver fibrosis.

Hepatic stellate cell activation and ECM synthesis are closely related to the PI3K/AKT signaling pathway [43, 44]. PI3K/AKT signal pathway inhibition can effectively inhibit liver injury, improve liver function, and reduce collagen production and deposition [16, 45–47]. Therefore, PI3K/AKT activity inhibition has become one of the current mechanisms for improving liver fibrosis. PA effectively inhibits the PI3K/AKT signal pathway [48–50], consistent with our results in CCl_4-induced hepatic fibrosis mice. Besides, PA reduced hepatic stellate cell activation and ECM accumulation. These results demonstrated that PA could improve hepatic fibrosis via PI3K/AKT signaling pathway inhibition. PI3K/AKT also regulates the NF-κB signaling pathway. In the present study, both PI3K/AKT and NF-κB pathway activities were inhibited, and crosstalk might have occurred between them.

Silymarin has a good effect against liver fibrosis and is often selected as the positive control in antiliver fibrosis studies [51–53]. Many studies have demonstrated that silymarin can inhibit collagen synthesis and secretion, inhibit hepatic stellate cell activation, and reduce collagen production and deposition. Besides, it can improve liver injury and inflammatory response and protect the liver against fibrosis [53–59], consistent with our current results. Furthermore, silymarin reduced CCl_4-induced hepatic fibrosis by regulating the NF-κB signaling pathway and PI3K/Akt-mediated hepatic stellate cell activation and inflammatory response. These results indicated that silymarin and PA could effectively protect the liver and improve liver fibrosis. However, their antifibrosis mechanisms remain to be further clarified.

5. Conclusion

In summary, PA could effectively improve liver damage, inhibit hepatic stellate cell activation, reduce liver index and collagen deposition area, and improve liver inflammation and fibrosis. The mechanisms might be related to NF-κB and PI3K/AKT signaling pathway inhibition. These results indicated that PA potentially ameliorates hepatic fibrosis, comprising a new possibility for future treatment. However, the mechanisms underlying PA effects against liver fibrosis and to promote PI3K/AKT and NF-κB pathway inhibition need to be further elucidated. More detailed studies are required to understand the relationship between liver fibrosis and inflammation and PA antifibrotic mechanisms.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

This study was approved by the Experimental Animal Ethics and Welfare Committee of Qingdao University (20211116C575420220116085).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Yang Song and Zhen Xu conceptualized the study; Zhen Xu was responsible for the methodology; Zhao Chen was responsible for software; Yang Song, Zhao Chen, and Zhen Xu validated the study; Yang Song performed formal analysis; Zhen Xu, Tongtong Lan, YiSa Han, Ning Yang, and Chuhui Wang performed the investigation; Yang Song was responsible for the resources; Zhen Xu performed the original draft preparation; Zhen Xu and Zhao Chen performed review writing and editing; Meng Tao and Hui Li performed visualization; Yang Song supervised the study; Xuezhen Ma was responsible for project administration; and Yang Song was responsible for funding acquisition. All authors have read and agreed to the published version of the manuscript. Zhen Xu and Zhao Chen contributed equally to this work.

Acknowledgments

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