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ALA/LA Inhibited Renal Tubulointerstitial Fibrosis of DKD db/db Mice Induced by Oxidative Stress

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ABSTRACT

Background: Diabetic kidney disease (DKD) is a leading cause of end-stage renal disease. Its progression is caused by various pathological mechanisms, including oxidative stress (OS), inflammation, and fibrosis. This study aimed to explore the effects of alpha-linolenic acid (ALA)/ linoleic acid (LA) on preventing and delaying the progression of interstitial fibrosis and improving OS in DKD mice.

Methods: Male eight-week-old db/db mice were randomly allocated to either the DKD model group, the low-dose ALA/LA group (250 mg/kg·d), the high-dose ALA/LA group (500 mg/kg·d), or the control group, consisting of db/m mice. After 12 weeks of ALA/LA intervention, blood urea nitrogen, blood glucose, and urine protein levels were significantly lower in db/db mice than in the control group.

Results: ALA/LA enhanced SOD and CAT levels and reduced reactive oxygen species and MDA production. Furthermore, db/db mice in the intervention group had lower mRNA and protein expression levels of p38, p-p38, ERK, p-ERK, /transforming growth factor-β1 (TGF-β1), and type IV collagen (ColIV) than did the model group ($P<0.05$).

Conclusion: ALA/LA improved recovery from injury in db/db mice by reducing OS and alleviating kidney fibrosis, especially in the tubules. The potential mechanism was that ALA/LA inhibited renal tubulointerstitial fibrosis and OS via the P-P38, P-ERK/ TGF-β1/ColIV signaling pathway.

INTRODUCTION

Diabetic kidney disease (DKD) is the leading cause of chronic kidney disease and end-stage renal disease (ESRD) worldwide and accounts for approximately 50% of all ESRD cases Barrera-Chimal et al. (2020). DKD is a major complication among patients with type 1 and type 2 diabetes, even in the presence of adequate glycemic control, and it serves as the pri-mary cause of morbidity and mortality in this patient population Doshi et al. (2017). It is character-ized by capillary injury, mesangial cell expansion, extracellular matrix (ECM) accumu-lation, thickening of the glomerular basement membrane, and podocyte and glomeru-lar injury, leading to glomerular sclerosis and tubulointerstitial fibrosis Anders et al. (2018), Thomas et al. (2015). Renal fibrosis, the main pathological characteristic of DKD, is characterized by collagen depo-sition and fibroblast stimulation in the diseased kidney Roberts et al. (2016), Guan et al (2020).

DKD has multiple etiologies and underlying mechanisms; notably, it has been suggested that fibrosis contributes to its pathogenesis. Furthermore, increasing evidence from experimental and clinical studies suggests a strong association between hyperglycemia, oxidative stress, and DKD Lin et al. (2018). High glucose levels stimulate several fi-brogenic pathways, triggering the production of reactive oxygen species (ROS), stimu-lating neurrohormone responses, and activating growth factor cascades. The mitogen-activated protein kinases (MAPK) signaling pathway, including JNK, Erk, and

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P38, is involved in producing pro-fibrotic mediators. Specifically, the MAPK pathway is activated by multiple stimulations, in-cluding transforming growth factor-beta 1 (TGF-β1) and ECM proteins Fan et al. (2020); notably, the former is an important pro-fibrotic cytokine significantly elevated in diabetes that induces ECM production in mesangial cells Dai et al. (2004). TGF-β1 activates the downstream Smad3 pathway to induce ECM collagen IV (ColIV) and fibronectin secretion, leading to renal fibrosis Navarro-Gonzalez et al. (2008), Lv et al. (2015), Forouhi et al. (2016).

Little is known about the effects of the consumption of plant-derived n-3 polyun-saturated fatty acid (PUFA) alpha-linolenic acid (ALA) and the major n-6 PUFA, lino-leic acid (LA) on OS and fibrosis. However, the consumption of ALA and LA has been associated with reduced glycemia in diabetes and diabetic kidney disease Fan et al. (2020), Dos Santos, et al. (2018).

To the best of our knowledge, few studies have evaluated PUFAs in relation to the histo-pathological features of the kidneys Eide et al. (2018). Robust large-scale evidence indicates that circulating plasma phospholipid n-3 PUFA (ALA) and n-6 PUFA (LA) concentrations are associated with a lower incidence of type 2 diabetes mellitus (T2DM). Moreover, lower intake of PUFAs, especially ALA and LA, is associated with chronic kidney disease in patients with T2DM Xi et al. (2023), Zhu et al. (2020).

Over the last two decades, no new drugs have been approved to specifically prevent DKD or improve kidney functions Breyer et al. (2016), Srivastava et al. (2021). Therefore, it is tempting to hypothe-size that the protection afforded by agents known to delay the progression of renal disease in diabetic patients may be mediated, at least in part, by the attenuation of fi-brosis Tuleta et al. (2021).

Our previous findings showed that ALA/LA ameliorates glucose toxicity in HK-2 cells by attenuating oxidative stress and apoptosis through the ROS/p38/TGF-β1 pathway Jiang et al. (2017). However, the effects and mechanisms of action of ALA/LA in renal fi-brosis remain poorly understood. This study aimed to assess whether ALA/LA intake is associated with the prevention and development of interstitial fibrosis in DN db/db mice and the relationship between tubular interstitial fibrosis and OS.

METHODS

Animal Experimental Design

Eight-week-old diabetic male db/db mice from the Academy of Military Sciences (Tianjing, China) with serum glucose levels >11.1 mM was randomly divided into three groups: the DKD model group (n=10), the low-dose group (n=10), and the high-dose group (n=10). Eight-week-old male db/m mice served as controls (n=10). The mice in the low-dose group were fed with 250 mg/kg·d ALA/LA (1:4), and the mice in the high-dose group were fed with 500 mg/kg·d ALA/LA (1:4). All the procedures were performed in accordance with the relevant institutional guidelines of the South-eastern University Animal Experimentation Ethics Committee.

Metabolic Data

Weight was measured weekly. Plasma glucose, urine volume, and 24-hour urine protein levels were measured every 4 weeks. Plasma glucose levels were meas-ured after an overnight fast. Water intake, food intake, blood urea nitrogen (BUN), creatinine (Scr), and urinary acid (UA) levels were measured at 8, 12, 16, and 20 weeks of age. Kidneys were harvested after 20 weeks. Serum BUN, Scr, and UA levels were measured using a fully enzymatic method. Analysis of serum and urine markers of nephrotoxicity Serum creatinine and BUN levels were estimated using commercially available kits following the manufacturer's instructions. Urinary albumin levels were measured by the turbidimetry method using a kit, according to the manufacturer's instructions.

ROS Expression Detection

To evaluate the effects of ALA/LA on ROS production, superoxide anion radicals were detected using dihydroethidium (DHE) staining (Beyotime Institute of Biotech-nology, Nantong, China). Briefly, the kidney sections were incubated with DHE (2 mmol/l) at 37°C in a humidified chamber protected from light for 45 min. DHE fluo-rescence intensity was analyzed using BIOZERO software (Keyence). Superoxide dismutase (SOD), Catalase (CAT), and malondialdehyde (MDA) levels were measured using UV-Visible Spectrophotometry, and 8-OHDG levels were determined using ELISA.

Quantitative Real-time polymerase chain reaction (PCR)

Total RNA was extracted from the renal tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). According to the manufacturer's instructions, an equal amount of total RNA (1 μg) was reverse-transcribed into cDNA using a Prime Script RT Reagent Kit (Perfect Real Time, Takara, Japan). The primer sequences used in this study are listed in Table 1. Quantitative real-time PCR was performed in triplicates using an Applied Biosystems 7500 quantitative PCR system (Applied Biosystems, Foster City, CA, USA). The Ct values obtained from different samples

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Table 1: Primer sequences of GAPDH, TGF-β1, P38, ERK and COLIV

Primer	Sequence	Length(bp)
GAPDH	F 5' AATGGATTTGGACGCATTGGT 3'	21
	R 5' TTTGCACTGGTACGTGTTGAT 3'	21
TGF-β1	F 5' CTCCCGTGGCTTCTAGTGC 3'	19
	R 5' GCCTTAGTTTGGACAGGATCTG 3'	22
P38	F 5'CCTATCCTGGAAGAGCCATACT 3'	22
	R 5'ACTTTGTCACGCTGACCAGAT 3'	21
ErK	F 5' GGTTGTTCCCAAATGCTGACT 3'	21
	R 5' CAACTTCAATCCTCTTGTGAGGG 3'	23
COLIV	F 5' CTGGCACAAAAGGGACGAG 3'	19
	R 5' ACGTGGCCGAGAATTTCACC 3'	20

were compared using the 2-ΔΔCt method. Glyceraldehyde-3-phosphate dehydrogenase served as an internal ref-erence gene.

Tissue Preparation and Histological Analysis

Immediately after sacrificing the animals, their kidneys were stored in 10% neu-tral-buffered formalin and embedded in molten paraffin to prepare tissue blocks. Sub-sequently, tissue sections of 5 µm thickness were cut using a microtome (Leica RM2235, Germany). These sections were stained with hematoxylin and eosin (H&E) for histopathological changes. Tissue sections were subjected to Masson's trichrome (MT) and periodic acid-Schiff (PAS) staining to demonstrate fibrosis and glomerulosclerosis in the renal tissues and visualized under a light microscope (Olympus CX31, Japan).

Statistical Analysis

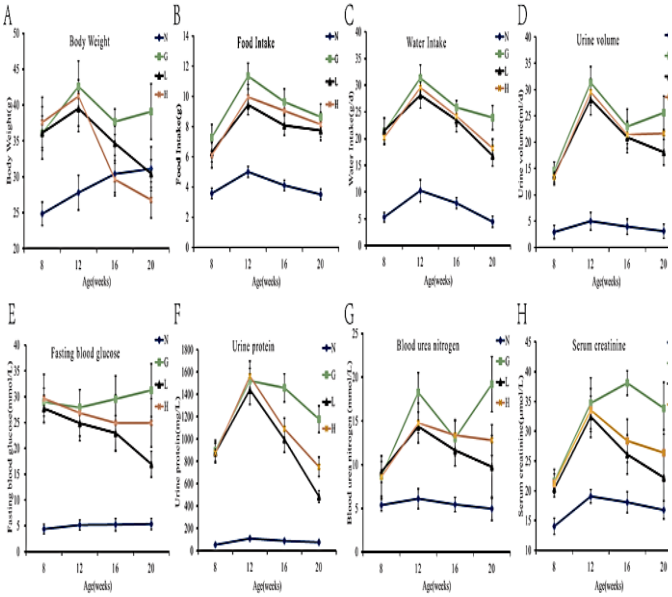
Data are presented as means ± SEM. Differences among different groups were evaluated by one-way analysis of variance, followed by least significant difference tests (using SPSS 18.0). Differences yielding P<0.05 were considered statistically signif-icant.

RESULTS

Effect on Renal Function Related Parameters.

The weight gain of db/db mice with DKD was significantly greater than that of db/m mice. When administered both doses of ALA/LA (250 mg/kg·d and 500 mg/kg·d) at a1:4 ratio over 12 weeks, the weight of db/db mice was lower not only compared to DKD mice but also to db/m mice (P<0.01). DKD db/db mice demonstrated significant-ly increased levels of blood glucose, blood urea nitrogen (P<0.01), and urinary protein (P<0.01), indicating the development of diabetic kidney disease (Fig.1 E, F, G, H). Treatment with both 250 mg/kg·d ALA/LA (1:4) and 500 mg/kg·d ALA/LA (1:4) sig-nificantly normalized their levels as compared to those of DKD db/db mice.

Figure 1: Effects of ALA/LA on db/db mice from age week 8 to 20 on body weight, food intake, water intake, urine volume, fasting blood glucose, urine protein, blood urea nitrogen and serum creati-nine. * P<0.05 vs. db/m mice; # P<0.05 vs. DKD db/db mice.



Effects of ALA/LA on db/db Mice on OS Markers

Compared to the normal control group, the DKD model group demonstrated a significant (P<0.001) increase in the levels of MDA and 8-OHDG and a reduction in the levels of antioxidants (SOD and CAT). The lower doses of ALA/LA (250 mg/kg·d with ratio of 1:4) significantly (P<0.01) augmented the levels of the antioxidant en-zymes and reduced the level of MDA of DKD db/db mice. The higher doses of ALA/LA (500 mg/kg·d with a ratio of 1:4) had no significant effect on the oxidant-antioxidant status of DKD db/db mice (Fig. 2).

As illustrated in Fig. 2A and F, stimulation withfi high glucose concentrations significantly increased ROS levels in the kidneys of DKD db/db mice. Immunofluores-cence assays revealed that two doses of ALA/LA (250 mg/kg·d

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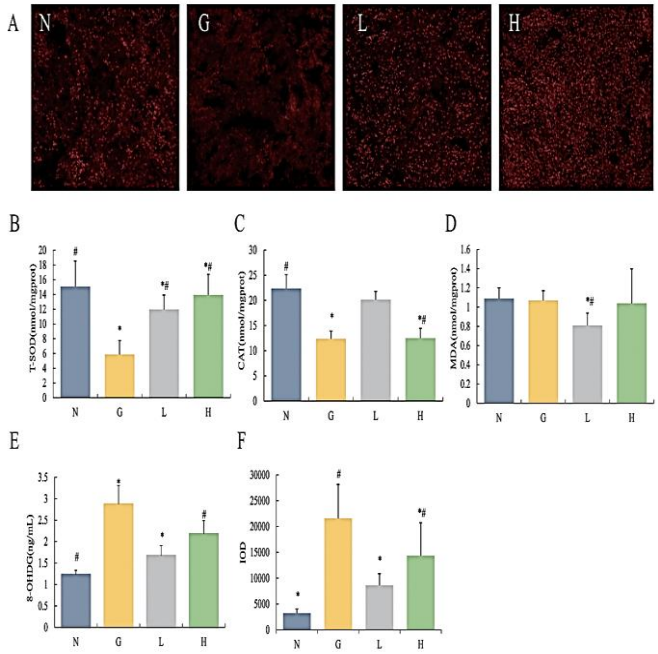
Figure 2: Effects of ALA/LA on db/db mice on oxidative stress markers. (A, F) DCF-DA staining for ROS production in kidneys of mice(n=5) (B-E) Assessments of SOD, CAT, MDA, 8-OHDG level in kidney of mice. (F) effect of ALA/LA on ROS production in kidney of db/db mice. * P<0.05 vs. db/m mice; # P<0.05 vs. DKD db/db mice.



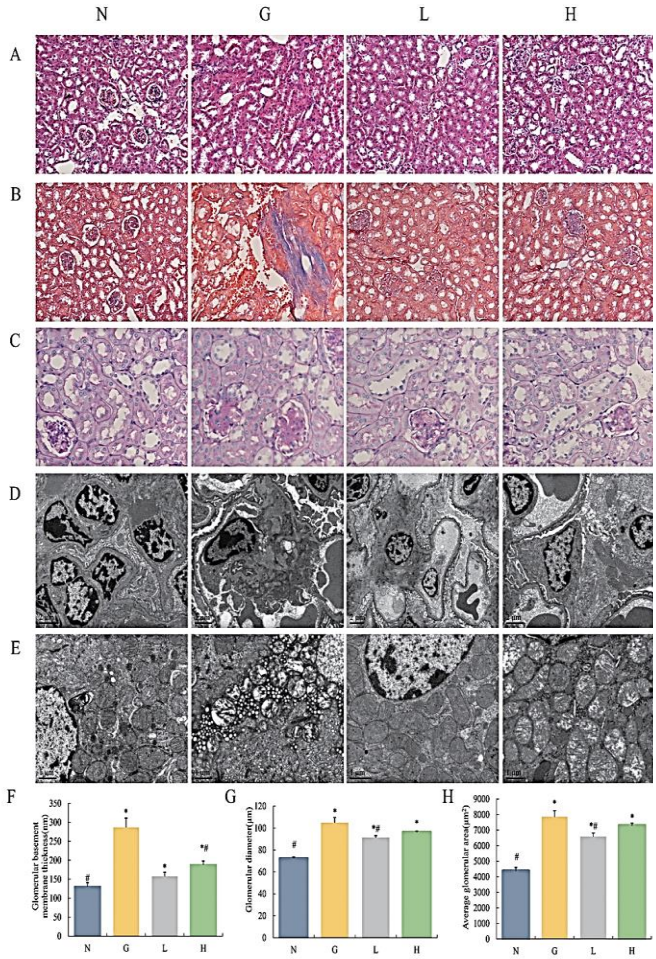
with a ratio of 1:4 and 500 mg/kg·d with a ratio of 1:4) significantly reduced the level of ROS in the kidney of DKD db/db mice.

ALA/LA Treatment Attenuates the Pathological Changes in the Kidneys of DKD db/db Mice

H&E and periodic acid-Schiff (PAS) staining were used to evaluate pathological changes. As shown in Figs. 3A-C, morphological changes in the renal tissue were iden-tified by H&E, Masson, and PAS staining. The control group exhibited normal glo-meruli without any pathological changes. In group G, glomerular hypertrophy, fibro-sis, mesangial cell proliferation, mesangial matrix expansion, and inflammatory cell infiltration were observed in the kidneys of db/db mice with DKD. In addition, com-pared to the control group, DKD db/db mice had the highest injury scores based on the above ratings. Further, the DKD db/db mice treated with a dose of 500 mg/kg·d, at a ratio of 1:4 ALA/LA (high-dose group, group H), exhibited comparable injuries, including glomerular hypertrophy, mesangial matrix expansion, and inflammation, to those of the DKD control group. Conversely, the DKD db/db mice administered a dose of 250 mg/kg·d, at a ratio of 1:4 ALA/LA (low-dose group, group L), showed a reduction in the extent of glomerular hypertrophy, fibrosis, mesangial matrix expan-sion, and inflammation. Additionally, the injury score in the mice of group L was significantly lower than that in the DKD model group (P<0.05).

The results from scanning electron microscopy (SEM) revealed that in the normal control group (group N), the glomerular structure of db/m mice was well-maintained, with neatly arranged Sertoli cells, clearly visible foot processes, and distinct mito-chondrial structures in renal tubular epithelial cells; the basement membrane was also clear and uniformly textured.

Figure 3: ALA/LA treatment improved the pathological changes in the kidneys of DKD db/db mice. The pathological analysis of kidneys was conducted using (A) H&E staining (Scale bars =100 μm), (B) Masson staining (Scale bars = 100 μm), (C) PAS staining (Scale bars =100 μm) and (D, E) SEM assays (Scale bars =2 μm, 1 μm). Quantification of (F) glomerular basement membrane, (G) glo-merular diameter, (H) average glomerular area were exhibited. Data are presented as the mean ± SEM (n = 5 in each group). * P<0.05 vs. db/m mice; # P<0.05 vs. DKD db/db mice.



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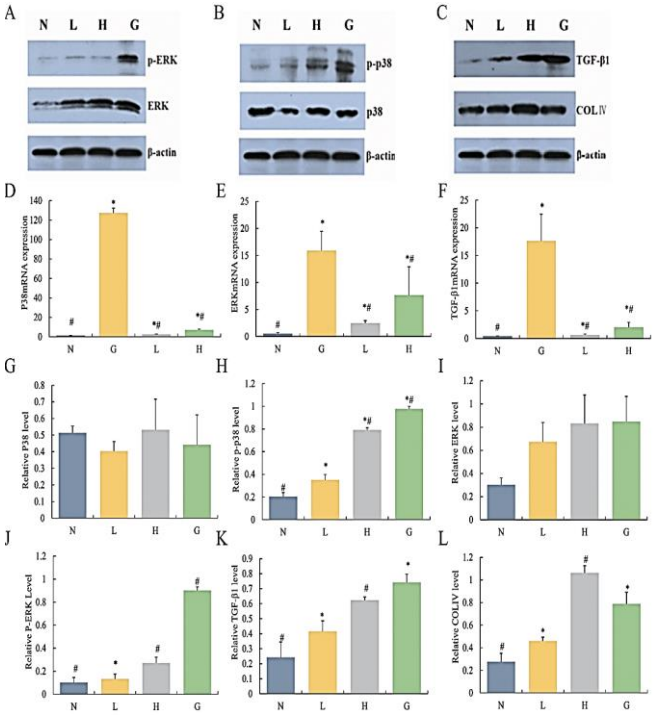
In contrast, the DKD model group (group G) featuring db/db mice exhibited mild thickening of the glomerular capillary basement membrane, increased mesangial matrix, and disordered arrangement of foot processes. Additionally, mitochondrial swelling and cristae disappearance were observed (Fig.3D, E). In the low and high doses of ALA/LA intervention groups (groups L and H), the glomerular capillary basement membrane thickening of the mild glomerular mesangial area matrix increased slightly. SEM results showed slight changes in endoplasmic reticulum and mitochondrial swelling and hyperplasia of interstitial collagen fibers in renal tubular epithelial cells. Compared with that of the DKD model group, glomerular damage was notably reduced in the low-dose group, which demonstrated superior intervention effects relative to the high-dose group ($P < 0.05$).

Effect of ALA/LA on Fibrotic Markers of db/db Mice

The levels of fibrotic markers, TGF-β1, and ColIV were analyzed because of their association with the development of DKD. In DKD model mice, the levels of TGF-β1 and ColIV were significantly elevated compared to those of the normal control ani-mals ($P<0.001$). Interestingly, ALA/LA treatment (500 mg/kg) significantly reduced the levels of the fibrotic markers (Fig.4).

Figure 4: Effects of ALA/LA onp38, p-p38, ERK, p-ERK, TGF-β1and COLIV mRNA and protein expression in kidney of db/db mice（n=5）. * $P<0.05$ vs. db/m mice; # $P<0.05$ vs. DKD db/db mice.



As shown in Fig.4D and Fig.4H, compared to those of the normal control mice, p38 mRNA expression and relative p-p38 levels were enhanced in the DKD model mice (group G) and low- and high-dose groups (group L and group H) (group N) ($P<0.05$). However, with the treatment of 1:4 ALA/LA, the expression of p38 mRNA and relative p-p38 was improved, not only in the 250 mg/kg·d dose (group L) but also in the 500 mg/kg·d dose (group H) ($P<0.05$). Similar results were obtained for ERK, a MAPK signaling molecule. However, only in group L was the expression of ERK mRNA and relative p-ERK lower than that in the DKD model group ($P<0.05$).

DISCUSSION

The therapeutic use of polyunsaturated fatty acids (PUFAs) in preserving insulin sensitivity and prevention of cardiovascular disease and cancer has gained interest in recent decades; however, the roles of ALA and LA remain poorly understood. Our data demonstrated that treatment with 250 mg/kg·d and 500 mg/kg·d ALA/LA resulted in a more pronounced decline in weight and FBG in db/db mice than it did in the DKD model group ($P<0.01$). An n–3 PUFA-rich diet is correlated with decreasing or preventing adipose tissue fat accumulation, insulin resistance, hypertension, obesity, cardiovascular diseases, and T2DM Huang et al. (2016), Lalia et al. (2016), Lepretti et al. (2018). Furthermore, Zhang et al. reported that long-term intake of LA (for women only) and ALA might have a protective effect against the development of T2DM in obese/overweight subjects Zhuang et al. (2018).

Increasing atten-tion is being paid to mechanistic studies on the effects of n-3 PUFAs on metabolic dis-eases. Specifically, N-3 PUFAs have been reported to improve both glucose and lipid metabolism in obesity by modulating neuropeptides in the hypothalamus Ma et al. (2016). David reported that a high dietary intake of n-3 PUFA increased beneficial microorganisms, primarily Bacteroidetes to Firmicutes ratios, as well as Actinobacteria and Proteobac-teria species. These bacterial species are associated with increased short-chain fatty ac-id production, which prevents and reduces obesity and its related metabolic dysbiotic effects Machate et al. (2020). However, further studies are needed to verify how ALA/LA works on weight loss and glucose reduction in db/db mice.

Further, ALA/LA improves kidney function in db/db mice with DKD. In our data analysis, ALA/LA intake improved blood urea nitrogen and urinary protein levels in DKD db/db mice. The results of the meta-analysis suggested that using n-3 PUFA supplements reduced urine

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protein excretion but not the glomerular filtration rate.

More importantly, high plasma glucose levels directly damage renal tubular cells, resulting in various metabolic and cellular dysfunctions Forbes et al. (2018). SEM results showed that, compared to those of db/m mice, GB MT increased, foot processes fused, mitochondria enlarged, and cristae disappeared in db/db mice with DKD. The effect of prolonged exposure to high glucose and the activation of signaling pathways leads to the impairment of mitochondrial structure and function in kidney cells, leading to increased ROS synthesis Zhang et al. (2018). Similarly, our previous study showed that overproduction of ROS, activation of apoptosis, and p38 MAPK signaling pathways are interlinked mechanisms that play pivotal roles in the progression of DKD Jiang et al. (2017). In contrast, ALA/LA treatment significantly enhanced the levels of SOD and CAT, decreased MDA, inhibited the overproduction of ROS ($P<0.05$), and attenuated kidney OS (Fig.2).

Furthermore, the foot process width, mitochondrial structure, and function of DKD mice were improved by ALA/LA administration (Fig. 2A and E). Although renal tubular epithelial cells (TECs) have the highest mitochondrial content, they may be affected by diabetes-induced mitochondrial injury Forbes et al. (2018). Furthermore, fatty acid oxidation (FAO) also occurs in mitochondria. ALA and LA are essential long-chain fatty acids that are the preferred energy sources for TECs via the FAO. Fatty acid uptake, oxidation, and synthesis are tightly balanced to prevent intracellular lipid accumulation Kang et al. (2015), potentially representing the underlying mechanism by which ALA/LA protects kidney structure in DKD mice.

Renal fibrosis is a histological manifestation of a progressive, irreversible process that causes chronic and end-stage kidney disease Kang et al. (2015). Renal fibrosis is caused by the loss of epithelial function and the expansion of connective tissue (swelling of interstitial myofibroblasts and excess deposition of ECM). Renal fibrosis is caused by the loss of epithelial function and the expansion of connective tissue (swelling of interstitial myofibroblasts and excess deposition of ECM). Additionally, fibrosis is character-ized by the loss of capillary networks and accumulation of fibrillary collagen, activat-ed myofibroblasts, and inflammatory cells Humphreys et al. (2010), Zeisberg et al. (2003). Although TECs may not be the di-rect precursors of myofibroblasts, they play an instrumental role in orchestrating renal fibrosis via multiple mechanisms, including the secretion of various cytokines.

In par-ticular, TGF-β1 is a key mediator of tissue fibrosis; it induces the secretion of fibrillary collagens and promotes cell death and dedifferentiation DeBerardinis et al. (2012). Our data showed that in DKD db/db mice, the levels of TGF-β1 and ColIV were significantly ($P<0.001$) elevated in comparison to those of the control animals. Interestingly, ALA/LA (50 mg/kg) treatment can ameliorate tubular injury, TGF-β1 production, and deposition of the ECM components (ColIV).

MAPK are intracellular signaling molecules that elicit diverse pro-fibrotic effects, both in vitro and in vivo. The anti-fibrotic effect has been recently reported in experi-mental models of renal interstitial fibrosis, showing that blockade of the MAPK pathway ameliorates renal fibrosis Li et al. (2019). Several MAPK inhibitors have been developed and tested in various stages of clinical trials. The P38 pathways play important roles in the production of pro-fibrotic mediators, which are activated by various cellular stresses Ono et al. (2000).

The administration of pharmacological MAPK inhibitors has been shown to suppress the development of glomerulosclerosis and tubulointerstitial fibrosis in various animal models Lim et al. (2011). Similar results reported by Lim et al. were obtained in our study. ALA/LA treatment significantly inhibited the P38/ERK MAPK signaling path-ways and decreased the deposition of TGF-β1and ColIV.

CONCLUSIONS

We confirmed that ALA/LA downregulates OS and metabolic diseases in db/db mice with DKD by reducing ROS production and promoting the expression of antioxidant enzymes, such as SOD and CAT. Moreover, ALA/LA ameliorates renal fibrosis by blocking MAPK signaling pathways, cutting down the deposition of TGF-β1 and reducing ECM accumulation. Our results provide new insights into the antioxidant ef-fects of ALA and LA on DKD. Further studies are needed to clarify the underlying mechanisms and enhance the performance of ALA/LA in protecting against DKD.

DECLARATIONS

Author Contributions

MXJ and CKZ participated in the conception and design of the study, interpretation of the data, and drafting of the article. HS participated in writing the paper and interpreting the data. HFZ and YC participated in the analysis and interpretation of the data. HFZ and HZ participated in the acquisition of the data. CKZ participated in revised the article critically for important

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intellectual content. All the authors read and approved the final manuscript.

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Ethics approval and consent to participate

The animal study protocol was approved by the Ethics Committee of Southeast University (protocol code 20210106039 and date of approval Jan6, 2021).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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