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# Elaidic acid-induced inhibition of mitophagy activated renal tubular cell necroptosis *via* ROS/RIPK3/MLKL pathway

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## ABSTRACT

Elaidic acid (EA) is a typical trans fatty acid (TFA) that emerges during the processing of various fatty foods. In this study, we found that EA induced renal injury with necroptosis. Pretreatment with a reactive oxygen species (ROS) inhibitor and a RIPK3 inhibitor alleviated EA-induced necroptosis. The data indicated that EA induced renal necroptosis through ROS/RIPK3/MLKL pathway. In mechanistic studies, we explored how EA induced ROS production. Results indicated that EA caused mitochondrial damage by testing MMP, MFN1, VDAC, and FIS1. Further, EA suppressed mitophagy by testing the levels of LC3, p62, PINK1, Parkin, colocalization of LC3 and Mito-Tracker Red. Mitophagy is a process of selective degradation of damaged mitochondria. A large number of damaged mitochondria couldn't be cleared by mitophagy in time, which increased ROS levels in renal cells. Pretreatment with a mitophagy activator decreased EA-induced ROS levels and mitochondrial damage. Taken together, our data identified that EA induced renal necroptosis by destroying mitochondria and inhibiting mitophagy, thereby activating the ROS/RIPK3/MLKL pathway.

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## 1. Introduction

Trans fatty acids (TFAs) are produced during the hydrogenation of vegetable oils<sup>[1]</sup>, and up to 200 g/kg of TFAs were found in food in some areas<sup>[2]</sup>. Globally, approximately 645 000 deaths were linked to high-TFAs diet each year<sup>[3]</sup>. It is necessary to reevaluate the hazards of TFAs. Elaidic acid (EA) is a kind of typical TFA and has multiple toxicities. Studies showed that EA induced atherosclerosis in human umbilical vein endothelial cells<sup>[4]</sup>. Other research indicated that excessive intake of EA accelerated atherosclerosis by promoting inflammation and oxidative stress (OS) in a mouse model of hyperlipidemia<sup>[5]</sup>. It is known that excessive dietary intake of TFAs was one of the causes of chronic kidney disease<sup>[6]</sup>, and researchers

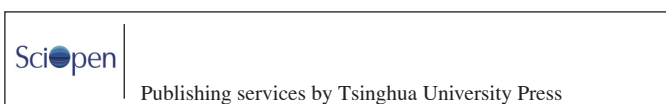
have observed increased plasma EA content in patients with chronic kidney disease<sup>[7]</sup>. Further research is still needed to fully understand the mechanisms of EA nephrotoxicity.

Recent studies found that necroptosis was a key mechanism of renal injury<sup>[8]</sup>. Renal tubular epithelial cell necroptosis promotes tubulointerstitial fibrosis in patients with chronic kidney disease<sup>[9]</sup>. RIPK3-MLKL-mediated necroinflammation contributes to acute kidney injury (AKI) progression to chronic kidney disease (CKD)<sup>[10]</sup>. Necroptosis is a programmed death mediated by necrosomes and characterized by organelle swelling, cell membranes rupture, and a decrease in intracellular adenosine triphosphate (ATP)<sup>[11]</sup>. Necroptosis is caused by the activation of receptor-interacting protein kinase 1 (RIPK1), which can activate receptor-interacting serine threonine kinase 3 (RIPK3) and form a heterodimeric complex with RIPK3. RIPK3 recruits and phosphorylates mixed lineage kinase domain-like protein (MLKL), which causes cell membrane rupture and executes necroptosis<sup>[12]</sup>. Mitochondrial reactive oxygen species (ROS) have long been considered to be a major force in necroptosis. The generation of ROS was the important trigger for necroptosis

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activation<sup>[13]</sup>. RIPK1 autophosphorylation promoted by ROS was essential for RIPK3 recruitment to the necrosome<sup>[12]</sup>. These studies demonstrated that excessive ROS participated in the regulation of necroptosis.

ROS are mainly produced in mitochondria, especially damaged mitochondria. Damaged mitochondria are usually removed by mitochondrial quality control system such as mitochondrial dynamics and mitophagy<sup>[14]</sup>. Mitochondrial dynamics are mainly regulated by the dynamin-like GTPases Mitofusin 1 (MFN1) and mitochondrial fission protein 1 (FIS1)<sup>[15]</sup>. Changes in mitochondrial dynamics led to diseases, including diabetes and acute kidney injury<sup>[15]</sup>. Mitophagy is a process of selective degradation of damaged mitochondria. Autophagosomes recognize damaged mitochondria with specific cohesive molecules, which are then delivered to lysosomes<sup>[16]</sup>. The accumulation of damaged mitochondria promoted cytotoxicity and induced cellular senescence<sup>[16]</sup>. The study showed that the elimination of damaged mitochondria by mitophagy regulated ROS production<sup>[17]</sup>. Inhibition of mitophagy led to ROS production<sup>[18]</sup>. Mitophagy also plays a protective role in renal tubular epithelial cell injury<sup>[19]</sup>. The PINK1/Parkin pathway is the most studied mitophagy pathway in mammalian cells<sup>[20]</sup>. Activation of the PINK1/Parkin pathway promotes mitophagy.

This study focused on the mechanisms by which EA-induced inhibition of mitophagy may ultimately lead to renal cell necroptosis. Our research provided theoretical support for the reduction of TFA damage in the organism.

## 2. Materials and methods

### 2.1 Reagents and antibodies.

EA (CAS: 112-79-8) and urolithin A (UA, CAS: 1143-70-0) were obtained from Aladdin Inc. (Shanghai, China). Uric acid (C012-2-1), urea nitrogen (C013-2-1), creatinine (C011-2-1), and lactate dehydrogenase (LDH) (A020-2-1) test kits were products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mito-Tracker Red (CAS: 167095-09-2) was a product of Beyotime (Beijing, China). Mito-TEMPO (MT, 1569257-94-8) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP, HY-100941) were obtained from Med Chem Express (MCE, Shanghai, China). MitoSOX Red (M36008) was purchased from Thermo Fisher Scientific Co. (USA). GSK'872 (S8465) was a product of Selleck (Shanghai, China).

Antibodies against GAPDH (10494-1-AP), LC3A/B (14600-1-AP), Parkin (14060-1-AP), MFN1 (13798-1-AP), FIS1 (10956-1-AP), and secondary antibodies were products of Proteintech Group (Wuhan, China). Antibodies against PINK1 (DF7742), VDAC (DF6140)), MLKL (DF7412), and RIPK3 (AF7942) were purchased from Affinity Biosciences Co. (Jiangsu, China). Antibody against SQSTM1/p62 (ARG55040) was brought from Arigo Company (Taiwan, China). Antibody against MLKL (phosphor S345) (ab196436) was obtained from Abcam (Cambridge, UK).

### 2.2 Animal experiments

All animal experiments were approved by the Animal Welfare Committee of Jilin University (approval No. SY202306041;

Date 2023.6.28). Male C57BL/6J mice (purchased from Liaoning Changsheng Biotechnology Co., Ltd., Shenyang, China) aged 6 to 8 weeks were randomly divided into 6 groups ( $n = 6/\text{group}$ ). Control group, low EA group (50 mg/kg), high EA group (100 mg/kg), EA (100 mg/kg) + GSK'872 group, EA (100 mg/kg) + MT group, EA (100 mg/kg) + UA group. EA was treated by gavage for 1 month. GSK'872 (2 mg/kg) and UA (5 mg/kg) were dissolved with DMSO and then diluted with normal saline before intraperitoneal injections. MT (1 mg/kg) was dissolved with normal saline before intraperitoneal injections. After the animal experiments, mice are deeply anesthetized by intraperitoneal injection of sodium pentobarbital, and are sacrificed by cervical dislocation after removal of eyeballs for blood<sup>[21-22]</sup>. The collected blood is allowed to stand for 30 min and then centrifuged at 3 500 r/min for 10 min to collect the serum. Mice are dissected to collect kidneys, followed by long-term storage of kidneys and serum in a  $-80^{\circ}\text{C}$  refrigerator. After separating the kidney tissue, the weight of the kidney is recorded. Calculate the renal/body mass index.

### 2.3 Kidney histopathology

A kidney on the same side was removed from a mouse and immobilized by soaking it in a 10% formalin solution. The fixed tissues were dehydrated and embedded in paraffin by the Institute of Zoonoses of Jilin University. The samples were stained with hematoxylin-eosin (HE) staining solution. The pathological changes in kidney tissue were observed and photographed with a microscope.

### 2.4 Detection of renal function

Uric acid, urea nitrogen, and creatinine are serum markers of renal function. Serum levels of uric acid, urea nitrogen, and creatinine were measured according to kit instructions.

### 2.5 Cell culture and drug treatment

NRK-52E cells (ATCC CRL-1571) were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NRK-52E cells were cultured with complete culture medium (10% fetal bovine serum, 1% penicillin-streptomycin, 89% DMEM, Inner Mongolia Wanrui Biotechnology Co., Ltd.) at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . The drug was administered when cell fusion reached 50%–60%. EA was prepared into EA-BSA solution system according to the previous method<sup>[23]</sup>. GSK'872 and CCCP were dissolved in DMSO, and MT was dissolved in DMEM. NRK-52E cells were treated with EA at different concentrations (0, 0.2, 0.4, 0.8 mmol/L) for 24 h. GSK'872 (1  $\mu\text{mol/L}$ ), MT (50 nmol/L), and CCCP (1  $\mu\text{mol/L}$ ) were pretreated for 1 h and then treated with 0.8 mmol/L EA for 24 h.

### 2.6 Thiazolyl blue (MTT) assay

NRK-52E cells were cultured in 96-well plates for 6 h with  $5 \times 10^3$  cells per well. Different concentrations of EA were treated for 20 h. MTT (5 mg/mL) was applied for 4 h. After discarding the culture solution and fully dissolving it with DMSO, absorbance at 570 nm was measured with an enzyme label instrument.



## 2.7 LDH release detection

LDH is an important indicator of cell membrane rupture, and when the cell membrane ruptures, LDH is released from the cell, resulting in elevated LDH in the culture medium. Culture medium was collected after drug treatment. LDH levels were tested according to the kits.

## 2.8 Propidium iodide (PI) staining

NRK-52E cells ( $1 \times 10^4$  cells per well) were cultured in 24-well plates. The cells were treated with different concentrations of EA for 24 h. Under the condition of no light at 37 °C, PI (10 ng/mL) was added to 24-well plates for 20 min. Cells were photographed with a fluorescence microscope.

## 2.9 Mitochondrial membrane potential (MMP) detection

NRK-52E cells ( $1 \times 10^4$  cells per well) were cultured in 24-well plates. The cells were treated with different concentrations of EA for 24 h. Under the condition of no light at 37 °C, a JC-1 probe (Beyotime, Shanghai, China) was added to 24-well plates for 25 min, and then cells were photographed with a fluorescence microscope.

## 2.10 ROS detection

NRK-52E cells ( $1 \times 10^4$  cells per well) were inoculated in 24-well plates. When the cell fusion rate was 50%–60%, the cells were treated with drugs for 24 h, then washed with DMEM 3 times, and stained with MitoSOX at 37 °C for 30 min. The MitoSOX (5  $\mu$ mol/L) fluorescent probe was dissolved in DMEM. Intracellular ROS were imaged using fluorescence microscopy. A total of  $1 \times 10^6$  cells were inoculated in 9-cm petri dishes. After drug treatment, the cells were collected and treated with the MitoSOX solution at 37 °C for 30 min. The ROS level was detected by flow cytometry.

## 2.11 Western blot analysis

Kidney tissue or cells are lysed with lysates containing protease inhibitors and protein phosphatase inhibitors. After cracking, it was centrifuged at 10 000 r/min for 10 min at 4 °C. The supernatant was separated, and the protein content was determined by the BCA assay kit. SDS loading buffer to adjust the loading amount. The proteins were isolated by SDS-PAGE gel electrophoresis and then transferred to PVDF membranes. Sealed with 5% BSA or skim milk powder for 2 h, incubated with primary antibody at 4 °C overnight, then incubated with secondary antibody at room temperature for 2 h. The gray values of protein bands were quantified by ImageJ.

## 2.12 LC3 and mitochondrial colocalization

NRK-52E cells ( $5 \times 10^5$  cells per dish) were inoculated in 2 cm confocal culture dishes. When the fusion rate was 50%–60%, the cells were pretreated with 1  $\mu$ mol/L CCCP for 1 h and treated with 0.8 mmol/L EA for 24 h. After drug treatment, Mito-Tracker Red was stained at 37 °C for 30 min, fixed with 4% paraformaldehyde,

and treated with 0.5% Triton X-100 for 15 min to permeate the cells. The cells were then sealed with 5% goat serum at room temperature for 1 h and incubated overnight with LC3A/B antibodies at 4 °C. The cells were incubated with the fluorescein II antibody for 2 h. The co-localization of LC3 and Mito-Tracker Red was observed by laser confocal microscopy.

## 2.13 siRNA transfection of NRK-52E Cells

The siRIPK3 sequence (sense: 5'-GGAAGCAUCAUUUGGGCAUTT-3'; antisense: 5'-AUGCCCAAUGAUGCUUCCTT-3') and the siRNA sequence of negative control (siNC) (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') were provided by Genepharma Co., (Shanghai, China). A total of  $2 \times 10^5$  NRK-52E cells/well were inoculated in six-well plates. For siRNA transfection, siRNA (150 pmol) was transfected into cells using GP-transfect-Mate reagents (6  $\mu$ L) according to the operating instructions. After 48 h siRNA transfection, proteins and LDH were detected<sup>[24]</sup>.

## 2.14 Statistical analysis

Data from at least 3 independent experiments are displayed as means  $\pm$  standard deviation (SD). Western blot analysis of protein bands, ROS fluorescence, and colocalization was performed by ImageJ software. The GraphPad Prism software was used to analyze the differences between groups of data; the *t*-test was used to compare the statistical differences between the 2 groups of data; and the one-way analysis of variance (ANOVA) was used to compare the statistical differences between multiple groups of data. *P* < 0.05 indicated that the difference between groups was statistically significant.

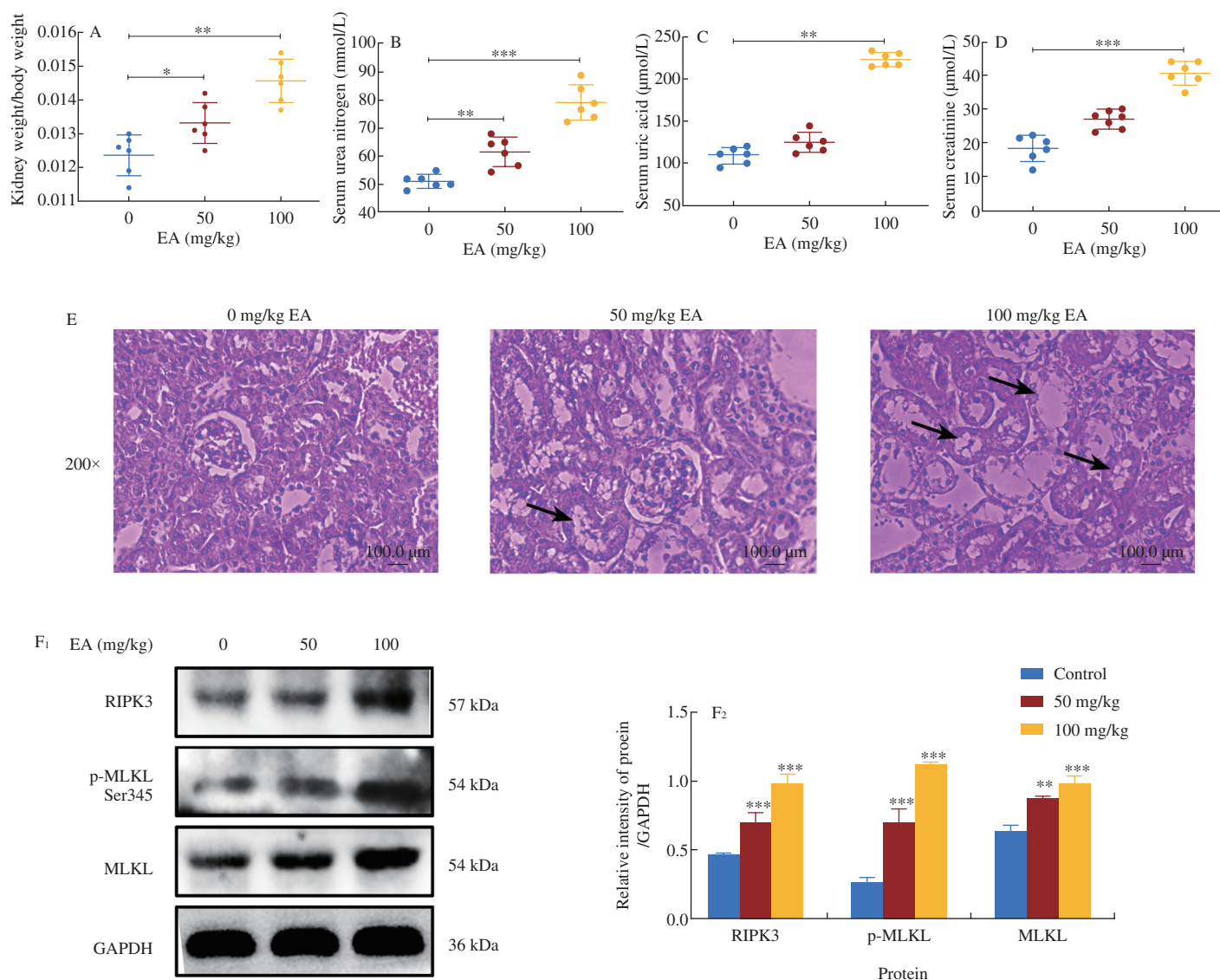
# 3. Results

## 3.1 EA induced renal injury and necroptosis in mice

To study the effect of EA on renal function in mice. Mice were given different doses of EA (0, 50, 100 mg/kg) for 1 month. The relative kidney weight of mice was significantly increased (Fig. 1A), which could be caused by renal injury such as congestion or edema. Data showed that levels of serum urea nitrogen, serum creatinine, and serum uric acid all increased in a dose-dependent manner with EA, indicating impaired renal function (Figs. 1B–D). Histopathologic analysis revealed that EA induced tubular cell swelling, rupture, nucleus detachment, and glomerular volume reduction, which were aggravated with the increase in EA dose (Fig. 1E). Consistent with previous studies that linked renal injury to necroptosis, we found that the protein levels of RIPK3, MLKL, and p-MLKL all increased with higher EA exposure doses in the kidneys of mice (Fig. 1F). These results showed that EA induced renal injury and necroptosis in mice.

## 3.2 EA induced necroptosis in NRK-52E cells

*In vitro*, renal tubular epithelial cells (NRK-52E cells) were selected to further study the mechanism of renal injury. We first evaluated the effect of EA on NRK-52E cell activity by MTT. The



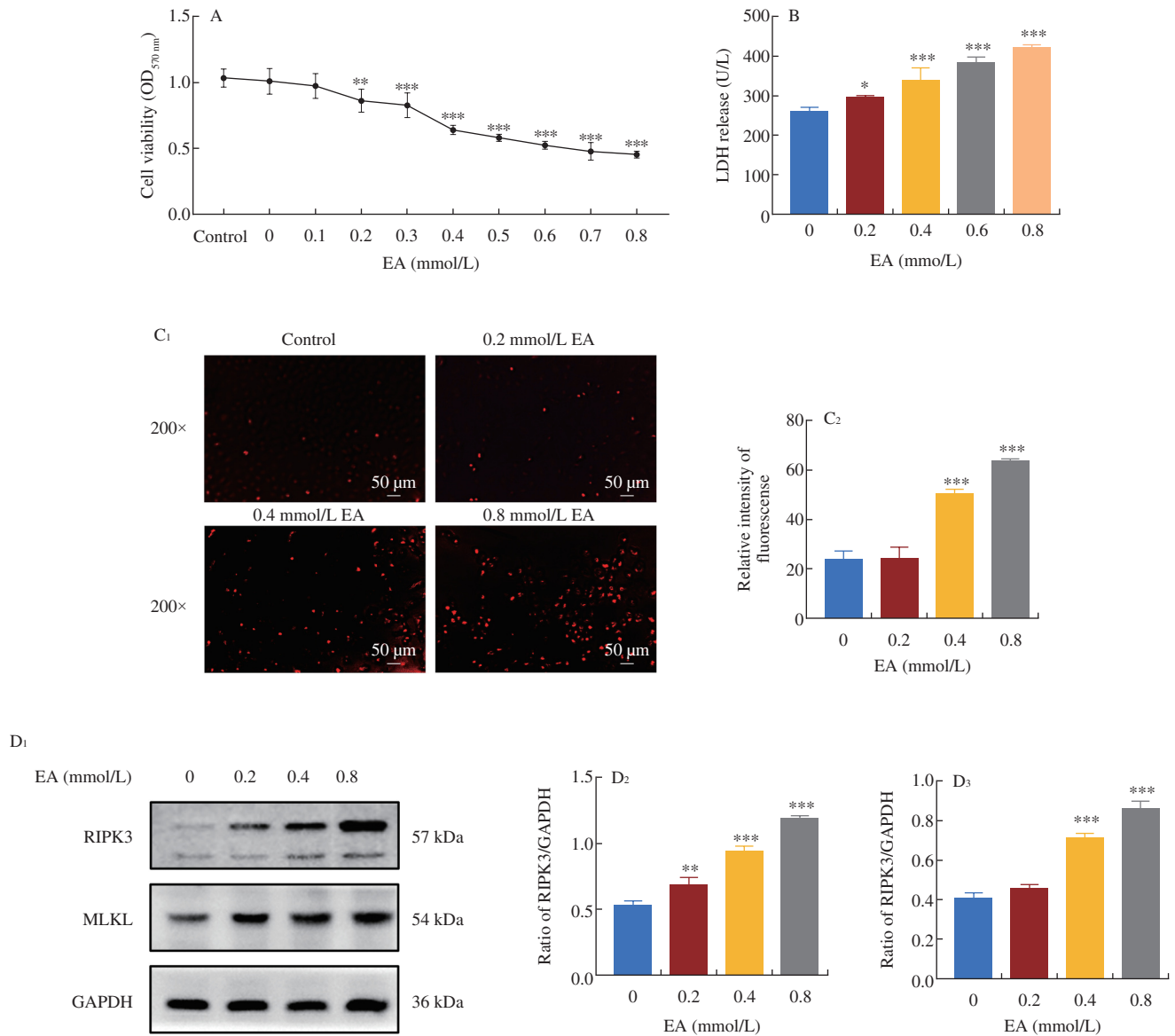
**Fig. 1** EA caused renal injury and necroptosis in mice. (A) Relative kidney weight in mice. (B) Serum urea nitrogen, (C) serum uric acid, (D) serum creatinine levels were detected by kits. (E) HE staining (200× magnification). (F) Expression levels of MLKL, p-MLKL, and RIPK3 were assessed by Western blot in mice kidneys. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control.

results showed that EA decreased cell activity in a dose-dependent manner (Fig. 2A). LDH activity and PI staining proved that EA disrupted the cell membrane of NRK-52E cells (Figs. 2B and C). In order to show that necroptosis leads to cell death, we tested the effects of different concentrations of EA on RIPK3 and MLKL levels. After the treatment of EA (0, 0.2, 0.4, 0.8 mmol/L), the levels of RIPK3 and MLKL were increased (Fig. 2D). These results indicated that EA caused necroptosis in NRK-52E cells.

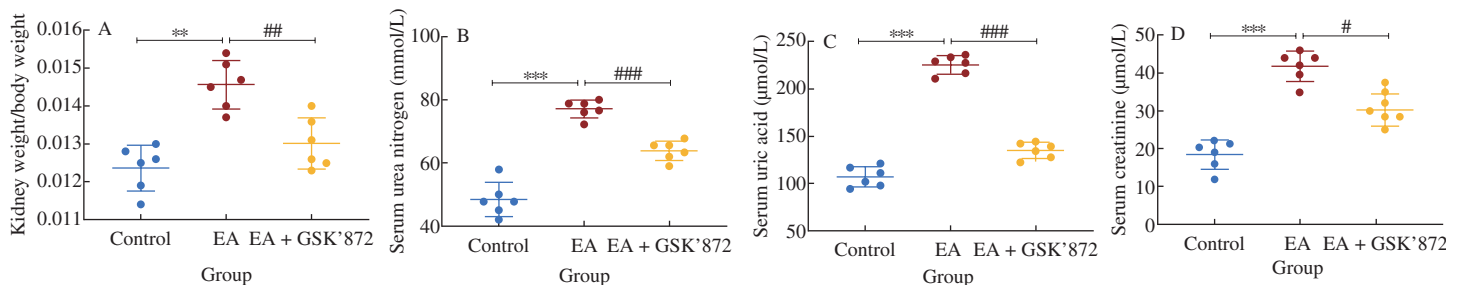
### 3.3 EA caused kidney injury and cell death through RIPK3/MLKL necroptosis

Our results suggested that EA caused kidney injury and cell death with necroptosis. To demonstrate that necroptosis is the main mechanism of EA-induced kidney injury and cell death, RIPK3 inhibitor GSK'872 was used. GSK'872 was used to evaluate the relationship between the mechanism of EA toxicity and RIPK3-

mediated necroptosis and the usage of GSK'872 based on previous studies<sup>[25]</sup>. *In vivo*, the data showed that compared with the EA group, the relative kidney weight, serum urea nitrogen, serum uric acid, serum creatinine levels, and RIPK3, MLKL, p-MLKL protein levels in the EA + GSK'872 group were decreased, suggesting that GSK'872 alleviated EA-induced kidney injury and necroptosis (Figs. 3A-D and F). HE staining results further demonstrated the effects of GSK'872 on kidney injury (Fig. 3E). *In vitro*, compared with the EA group, pretreatment of GSK'872 decreased the expression of RIPK3 and MLKL (Fig. 3G). The levels of PI staining and LDH release were also reduced (Figs. 3J and K). RIPK3 expression was significantly down-regulated after transfection of siRIPK3 in NRK-52E cells (Fig. 3H). In contrast to the EA group, RIPK3 and MLKL protein levels, PI staining levels, and LDH activity in the EA + siRIPK3 group were decreased (Figs. 3I-K). The above results manifested that EA caused necroptosis *via* the RIPK3/MLKL pathway in NRK-52E cells.



**Fig. 2** EA induced necroptosis in NRK-52E cells. NRK-52E cells were incubated with various concentrations of EA for 24 h, (A) cell viability was detected by MTT assay. (B) Relative LDH release in NRK-52E cells treated with EA for 24 h. (C) PI staining was used to indicate the damage degree of the cell membrane (200× magnification). (D) The expression levels of MLKL and RIPK3 were assessed by Western blot in NRK-52E cells under the treatment with EA for 24 h. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control.



**Fig. 3** EA caused kidney injury and cell death through necroptosis. (A) Relative kidney weight in mice. (B) Serum urea nitrogen, (C) serum uric acid, (D) serum creatinine levels were detected by kits. (E) HE staining (200× magnification). (F) Expression levels of MLKL, p-MLKL, and RIPK3 were assessed by Western blot in mice kidneys. (G) Expression levels of MLKL and RIPK3 were assessed by Western blot in NRK-52E cells. (H) After transfection with siNC or siRIPK3 for 48 h, RIPK3 proteins were examined by western blot. (I) After transfection with siRIPK3 for 48 h, NRK-52E cells were exposed to 0.8 mmol/L EA for 24 h. RIPK3 and MLKL proteins were examined by western blot. (J) NRK-52E cells were transfected with siRIPK3 for 48 h or pretreatment with GSK'872 for 1 h, and then exposed to 0.8 mmol/L EA for 24 h. PI staining was detected (200× magnification). (K) NRK-52E cells were transfected with siRIPK3 for 48 h or pretreatment with GSK'872 for 1 h and then exposed to 0.8 mmol/L EA for 24 h. LDH activity was detected. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. EA or siNC.

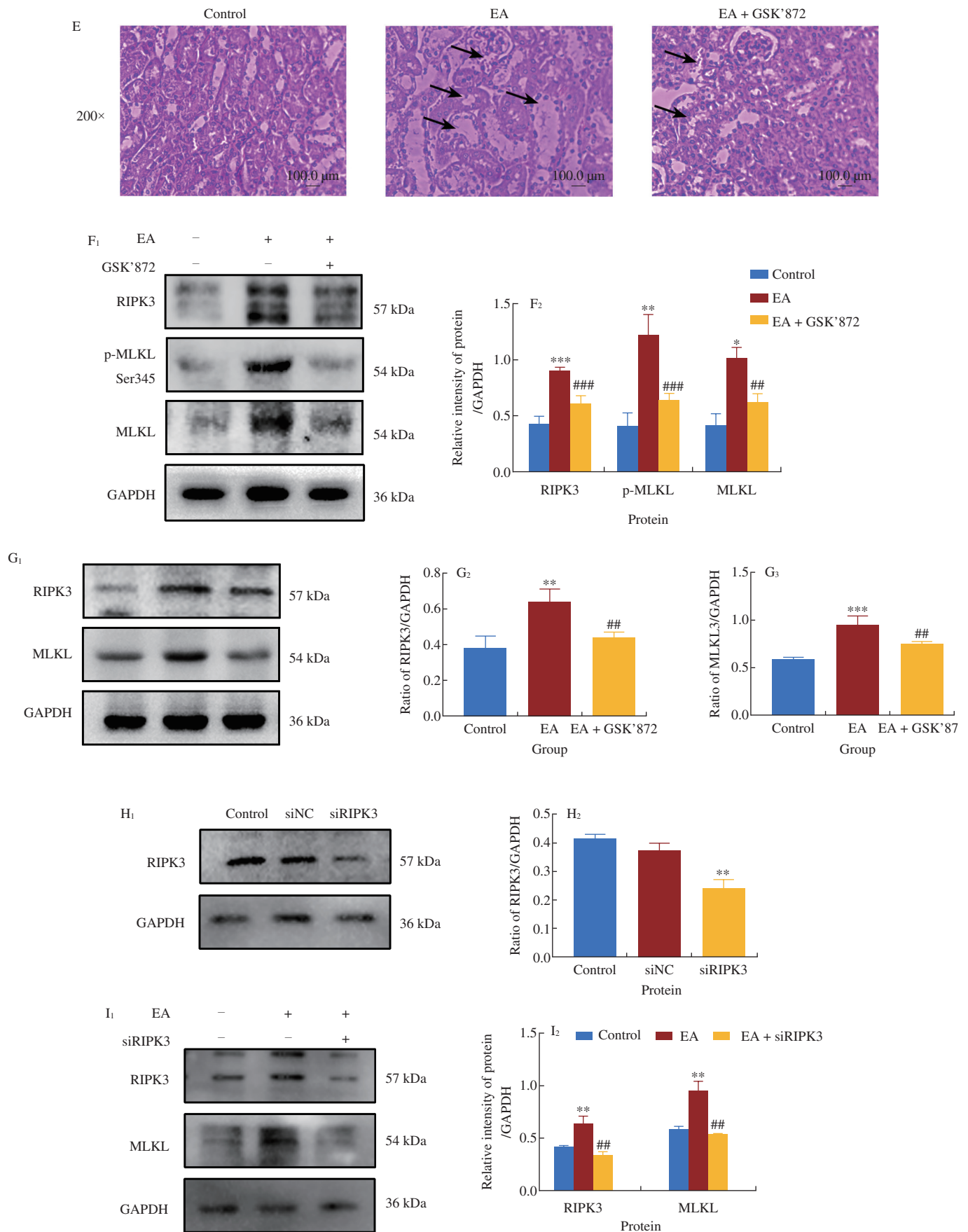


Fig. 3 (Continued)



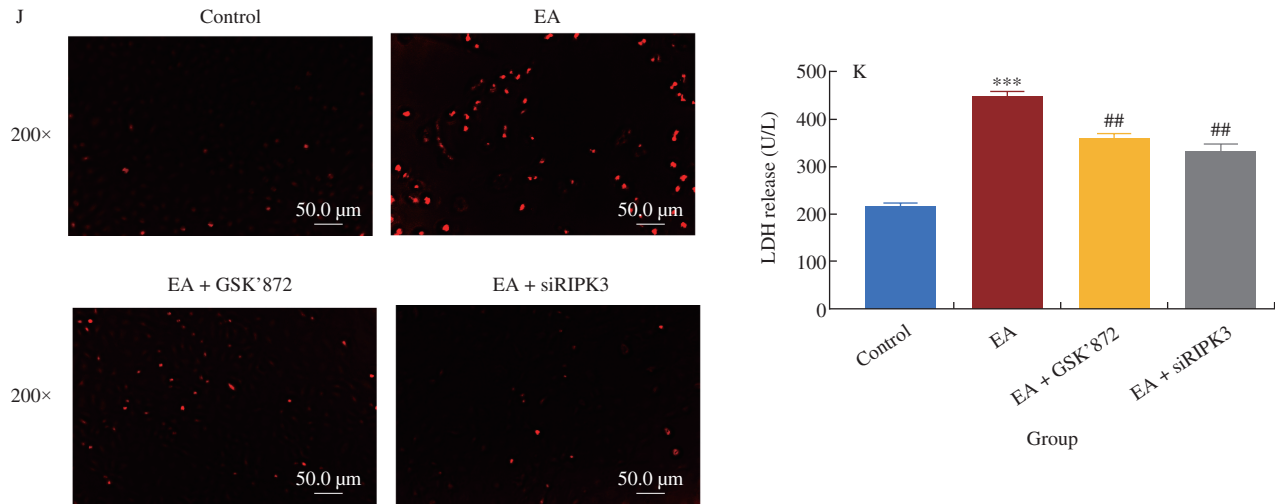
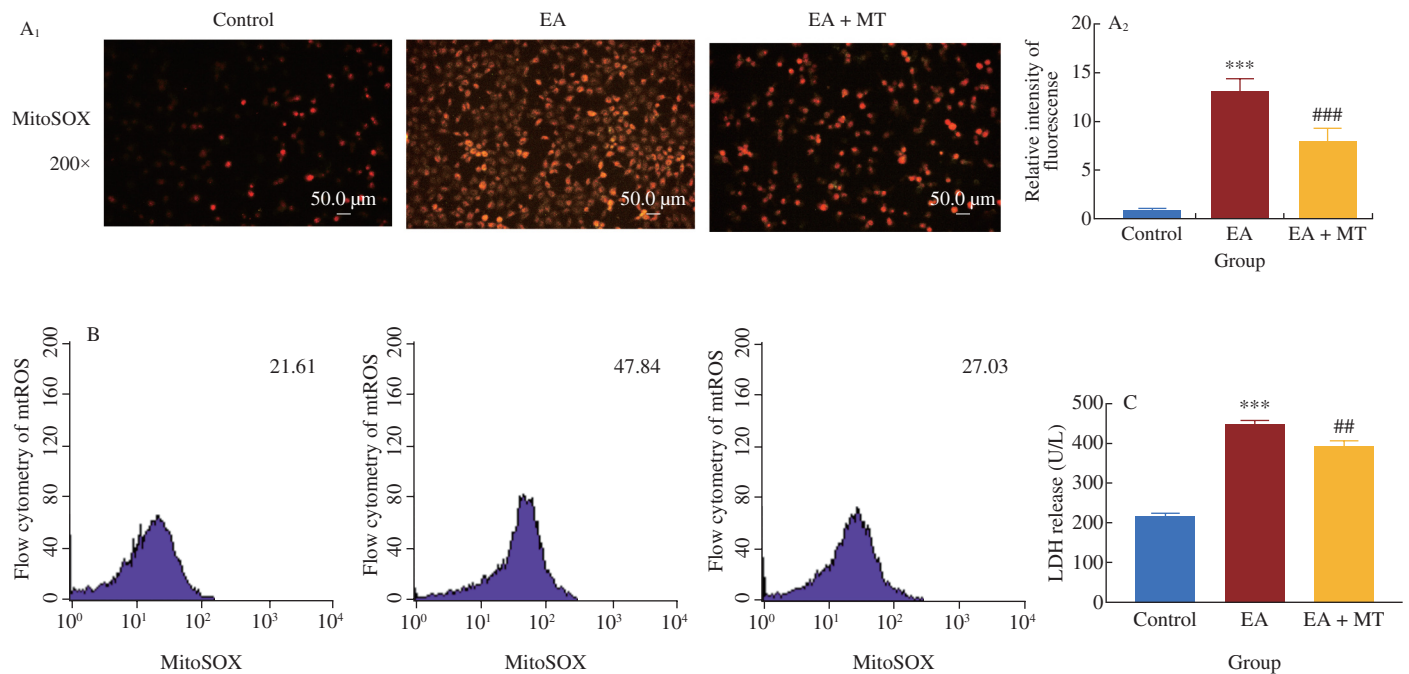


Fig. 3 (Continued)

### 3.4 EA induced necroptosis via the ROS/RIPK3/MLKL pathway *in vivo* and *in vitro*

ROS is a key regulatory factor of necroptosis signal transduction and can activate necroptosis through RIPK3. In order to prove the relationship between ROS and necroptosis, the ROS scavenger MT was used and the usage of MT was based on previous studies<sup>[26]</sup>. The data showed that EA increased ROS levels compared with the control group, and the addition of MT reduced ROS levels compared with the EA group (Fig. 4A). This was also confirmed by flow cytometry (Fig. 4B). In addition, LDH activity, RIPK3, and MLKL levels were

also lower after pretreatment with MT than those without treatment (Figs. 4C and D). These results have also been demonstrated *in vivo*. The data showed that compared with the EA group, the relative kidney weight, serum urea nitrogen, serum uric acid, serum creatinine levels, and RIPK3, MLKL, and p-MLKL protein levels in the EA + MT group were decreased (Figs. 4E and G). HE staining results also demonstrated the effects of MT on kidney injury (Fig. 4F). The above results suggested that ROS was involved in the regulation of necroptosis and EA induced necroptosis through ROS/RIPK3/MLKL *in vivo* and *in vitro*.



**Fig. 4** EA induced necroptosis via the ROS/RIPK3/MLKL pathway *in vivo* and *in vitro*. (A) After pretreatment with 50 nmol/L MT for 1 h, NRK-52E cells were incubated with 0.8 mmol/L EA for 24 h. MitoSOX was used to examine ROS levels (200× magnification). (B) Flow cytometry examined ROS levels. (C) Relative LDH release in NRK-52E cells. (D) Levels of RIPK3 and MLKL were assessed by Western blot in NRK-52E cells. (E) Relative kidney weight, serum urea nitrogen levels, serum uric acid levels, and serum creatinine levels in mice. (F) HE staining (200× magnification). (G) Levels of MLKL, p-MLKL, and RIPK3 were assessed by Western blot in mice kidneys. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. EA.

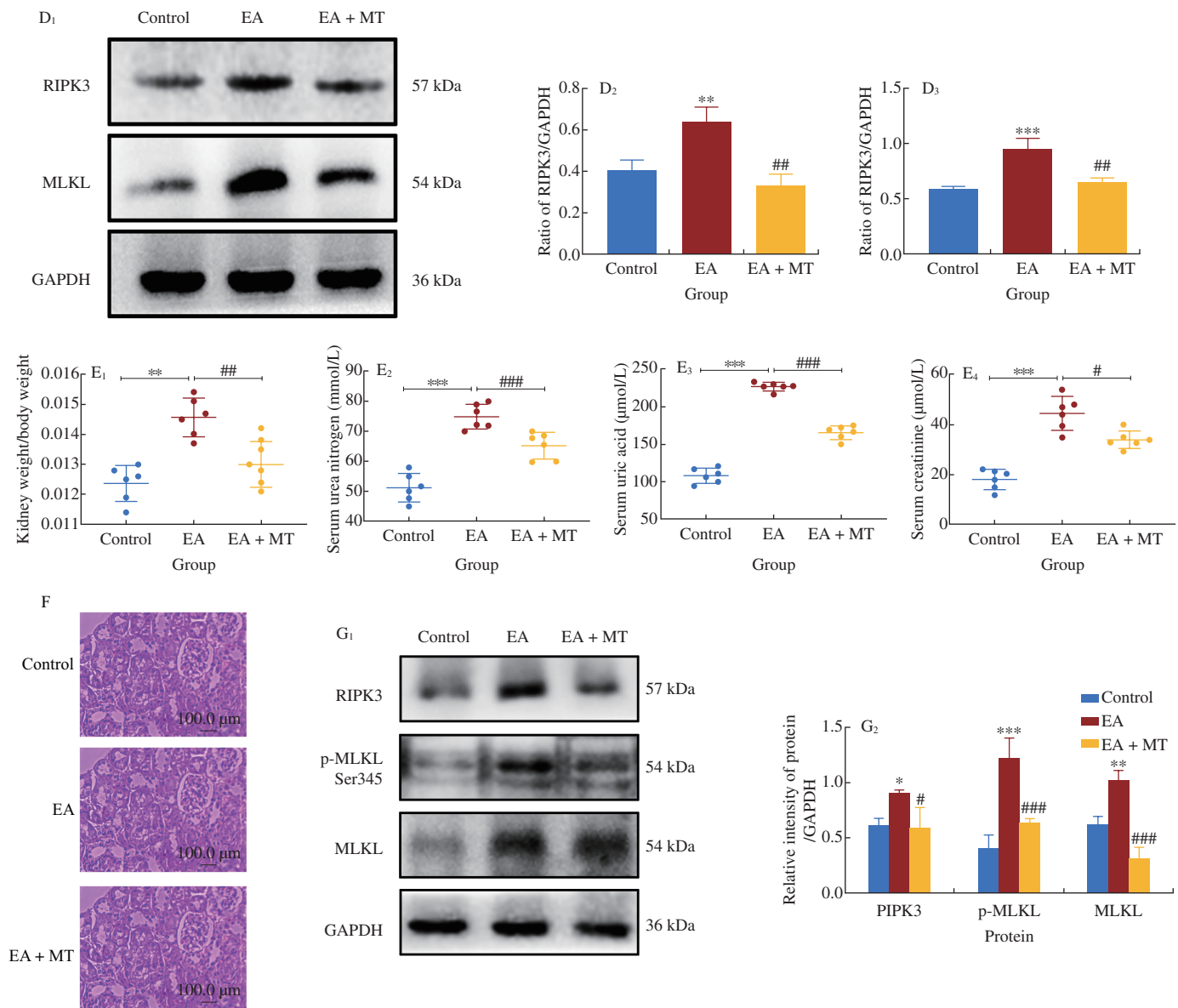


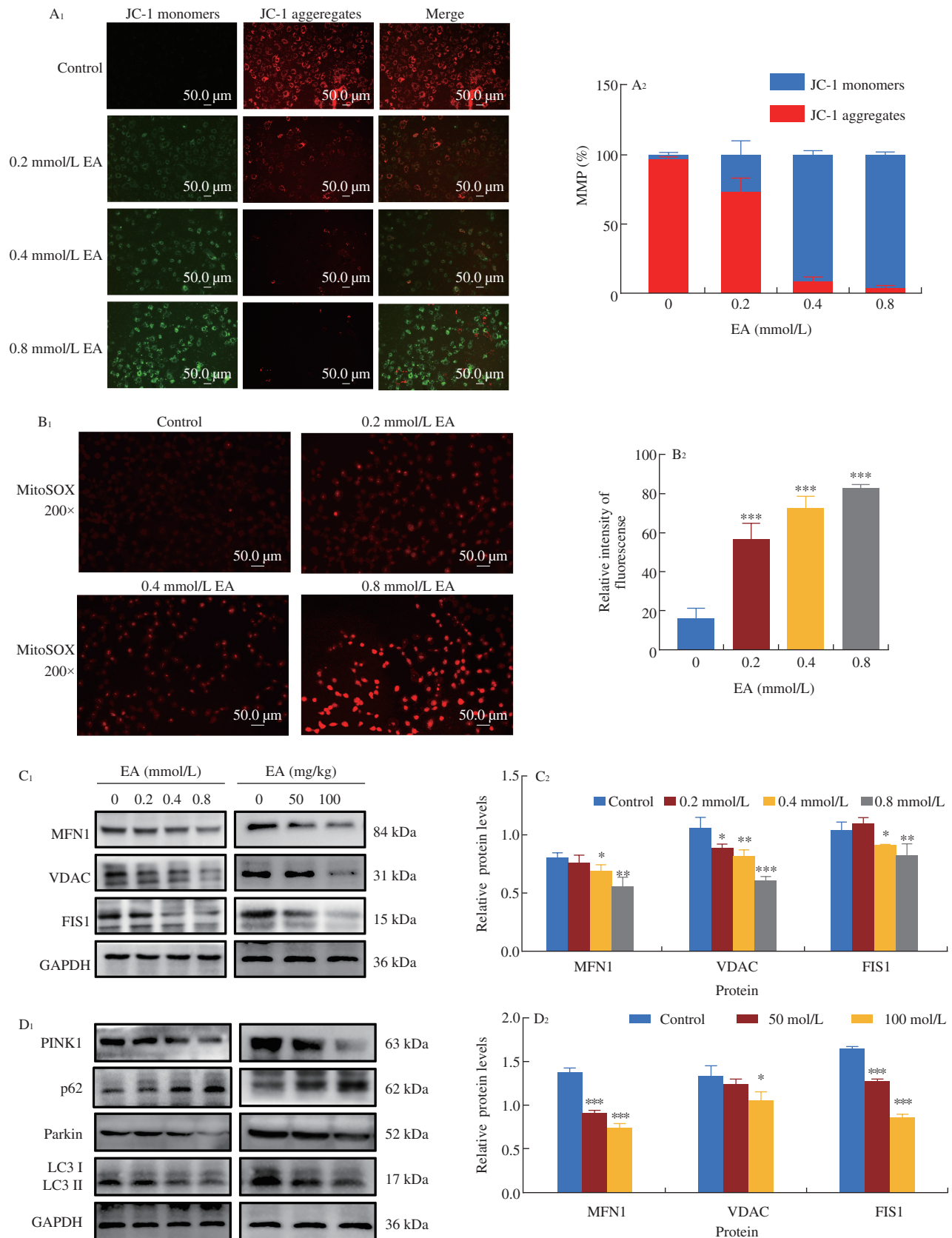
Fig. 4 (Continued)

### 3.5 EA led to ROS production via damaging mitochondria.

Mitochondria have been proposed as multifaceted regulators of cell death, including necroptosis, ferroptosis<sup>[27]</sup>, and pyroptosis. In addition, damaged mitochondria can lead to the production of ROS, which is the most important method of ROS production. MMP and ROS were detected to assess mitochondrial damage (Figs. 5A and B). The results showed that MMP decreased and ROS increased with increased EA. Besides, MFN1, FIS1, and voltage-dependent anion channel (VDAC) were detected to evaluate mitochondrial dynamics and mitochondrial function (Fig. 5C). Results showed that the expressions of MFN1, FIS1, and VDAC decreased significantly with the increase in EA dose *in vivo* and *in vitro*. Mitophagy-associated proteins Parkin, PINK1, p62, and LC3 II/I were also detected *in vivo* and *in vitro*, and the data indicated that EA inhibited mitophagy (Fig. 5D). The above results indicated that EA dose-dependently caused mitochondrial damage and ROS production.

### 3.6 EA increased ROS production via inhibition of mitophagy in NRK-52E cells.

Mitophagy plays an important role in mitochondrial quality control. Damaged mitochondria are removed by mitophagy, thereby reducing ROS accumulation. Mitophagy maintains ROS homeostasis by clearing excess ROS and then the cell survives. To demonstrate the role of mitophagy in EA-induced necroptosis, the mitophagy activator CCCP was used and the usage of CCCP was based on previous studies<sup>[28]</sup>. The level of colocalization of LC3 and mitochondria is an important feature of mitophagy. The results showed that LC3 and mitochondrial colocalization levels were reduced in the EA group compared with the control group, while LC3 and mitochondrial colocalization levels were increased in the EA + CCCP group compared with the EA group (Fig. 6A). These results indicated that EA inhibited mitophagy, and CCCP alleviated the inhibition effect of EA on mitophagy. Increased levels of LC3 II/I, PINK1, Parkin



**Fig. 5** EA led to ROS production *via* damaging mitochondria. (A) NRK-52E cells were incubated with various concentrations of EA for 24 h. MMP levels were examined (200× magnification). (B) ROS levels were detected. (C) The levels of MFN1, VDAC, and FIS1 were assessed after treatment with different doses of EA *in vivo* and *in vitro*. (D) The levels of Parkin, PINK1, p62, and LC3 II/I were assessed after treatment with different doses of EA *in vivo* and *in vitro*. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control.



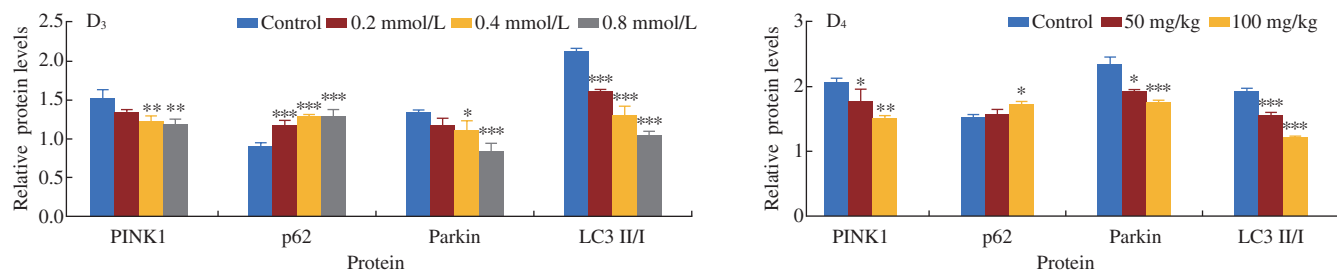
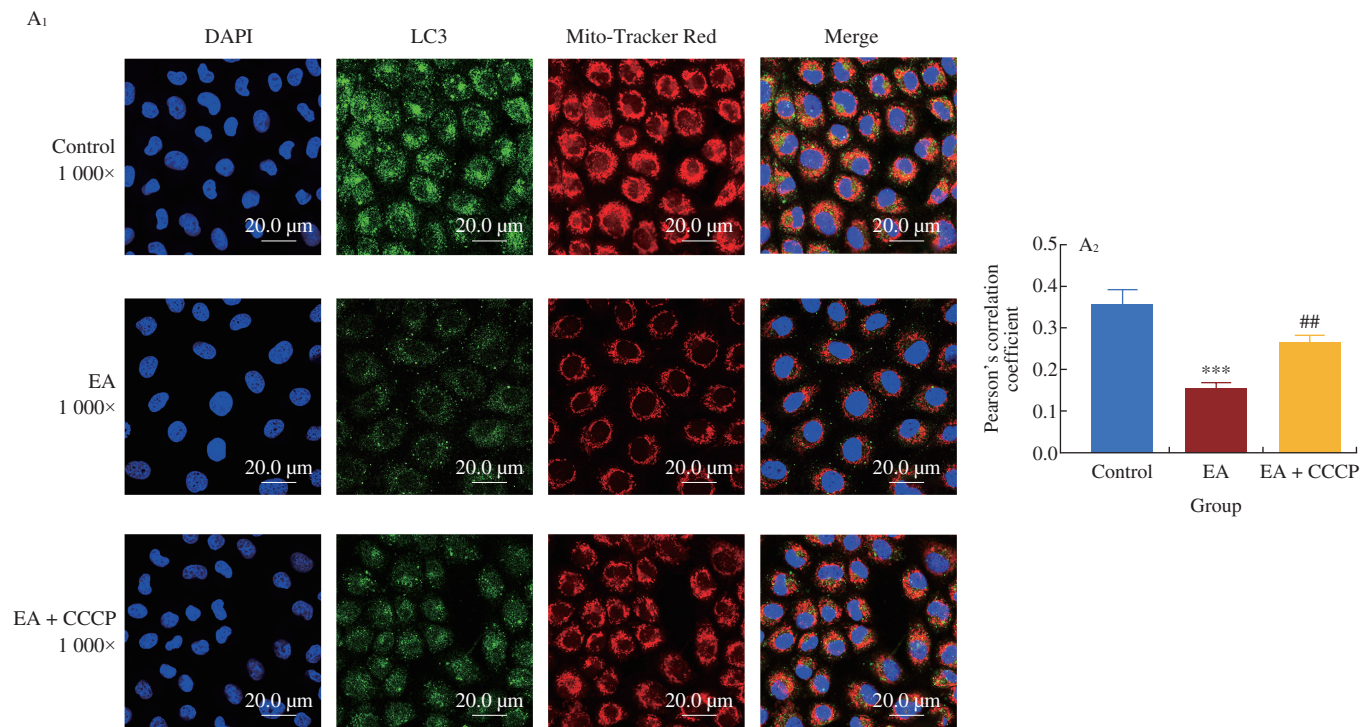


Fig. 5 (Continued)

and the decreased level of p62 also implied that CCCP activated mitophagy (Fig. 6B). Further exploring the effect of mitophagy on ROS, we found that the level of ROS in the EA + CCCP group decreased compared with that in the EA group (Fig. 6C). This was also confirmed by flow cytometry (Fig. 6D). These results indicated that CCCP reduced ROS by activating mitophagy. EA increased ROS by inhibiting mitophagy in NRK-52E cells. EA exacerbated mitochondrial damage, as indicated by reduced expression of MFN1, VDAC, and FIS1. However, after pretreatment with CCCP, the expression of MFN1, VDAC, and FIS1 rebounded significantly, indicating that mitophagy alleviated the mitochondrial damage caused by EA (Fig. 6E). The levels of MLKL and RIPK3 were measured to evaluate the relationship between necroptosis and mitophagy. The results showed that the levels of MLKL and RIPK3 in the EA + CCCP group were significantly lower than those in the EA group, suggesting that EA could cause necroptosis by inhibiting mitophagy in NRK-52E cells (Fig. 6F).

### 3.7 EA exacerbated necroptosis via inhibition of mitophagy in mice.

UA was used to activate mitophagy *in vivo* and the usage of UA was based on previous studies<sup>[29]</sup>. We found that UA alleviated the renal damage caused by EA. The results showed that relative kidney weight, serum urea nitrogen, serum uric acid, and serum creatinine were significantly decreased in the EA + UA group compared with the EA group (Figs. 7A-D). HE staining results also indicated that the degree of renal injury in the EA + UA group was lower than that in the EA group (Fig. 7E). After treatment with UA, the expressions of Parkin, PINK1, and LC3 II/I were significantly increased, while the expression of p62 was significantly decreased compared with the EA group, suggesting that UA activated the mitophagy inhibited by EA (Fig. 7F). Compared with the control group, the expressions of MFN1, VDAC, and FIS1 were decreased in the EA group, while the expressions of MFN1, VDAC, and



**Fig. 6** EA increased ROS production *via* inhibition of mitophagy in NRK-52E cells. (A) After pretreatment with 1  $\mu$ mol/L CCCP for 1 h, NRK-52E cells were incubated with 0.8 mmol/L EA for 24 h. The colocalization of LC3 and Mito-Tracker Red was imaged by a confocal microscope (1 000 $\times$  magnification). (B) Levels of Parkin, PINK1, LC3 II/I, and p62 were assessed by Western blot in NRK-52E cells. (C) MitoSOX was used to detect ROS levels in NRK-52E cells. (D) Flow cytometry examined ROS levels. (E) Levels of MFN1, VDAC, and FIS1 were assessed by Western blot in NRK-52E cells. (F) Levels of MLKL and RIPK3 were assessed by Western blot in NRK-52E cells. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 vs. control. # $P$  < 0.05, ## $P$  < 0.01, ### $P$  < 0.001 vs. EA.



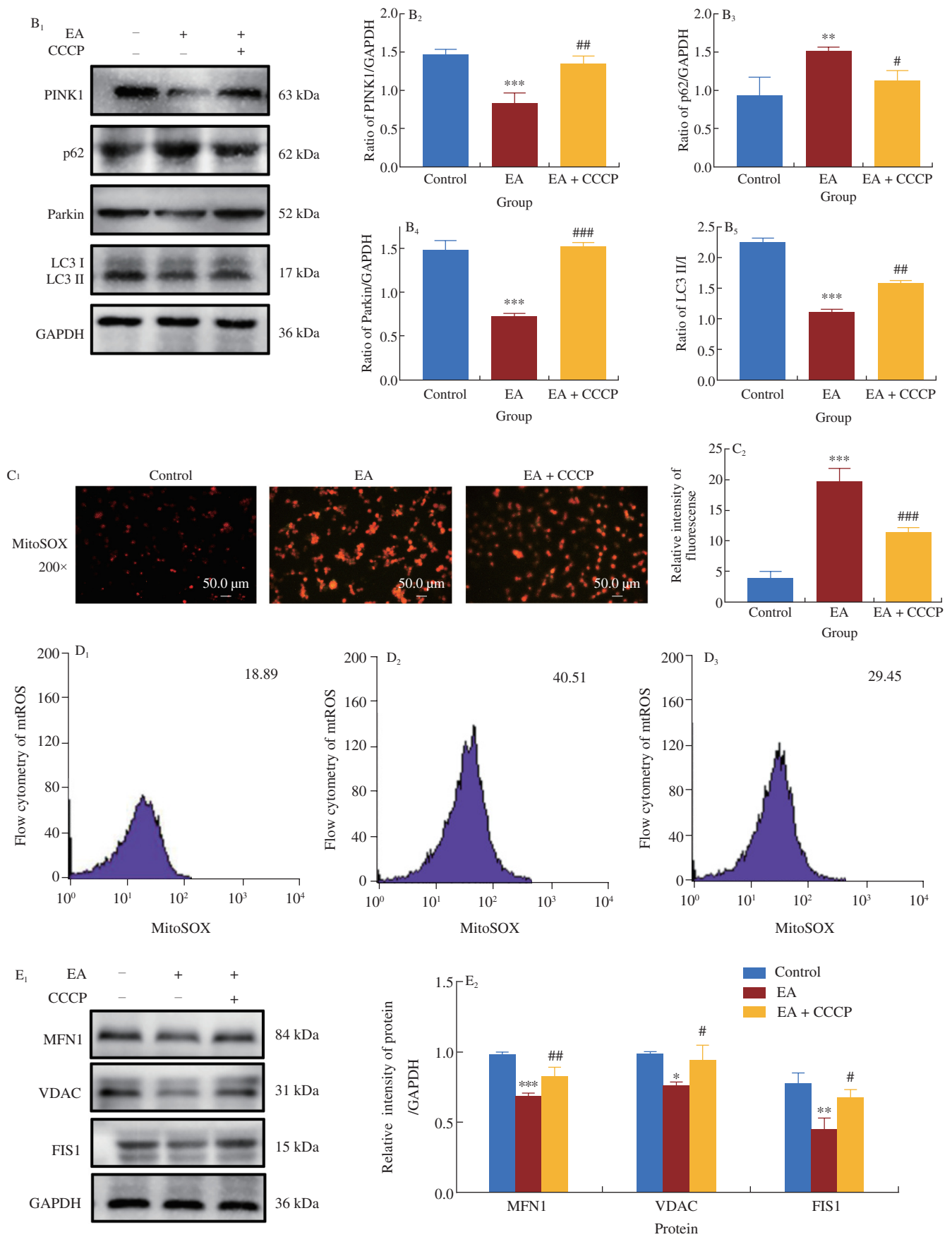
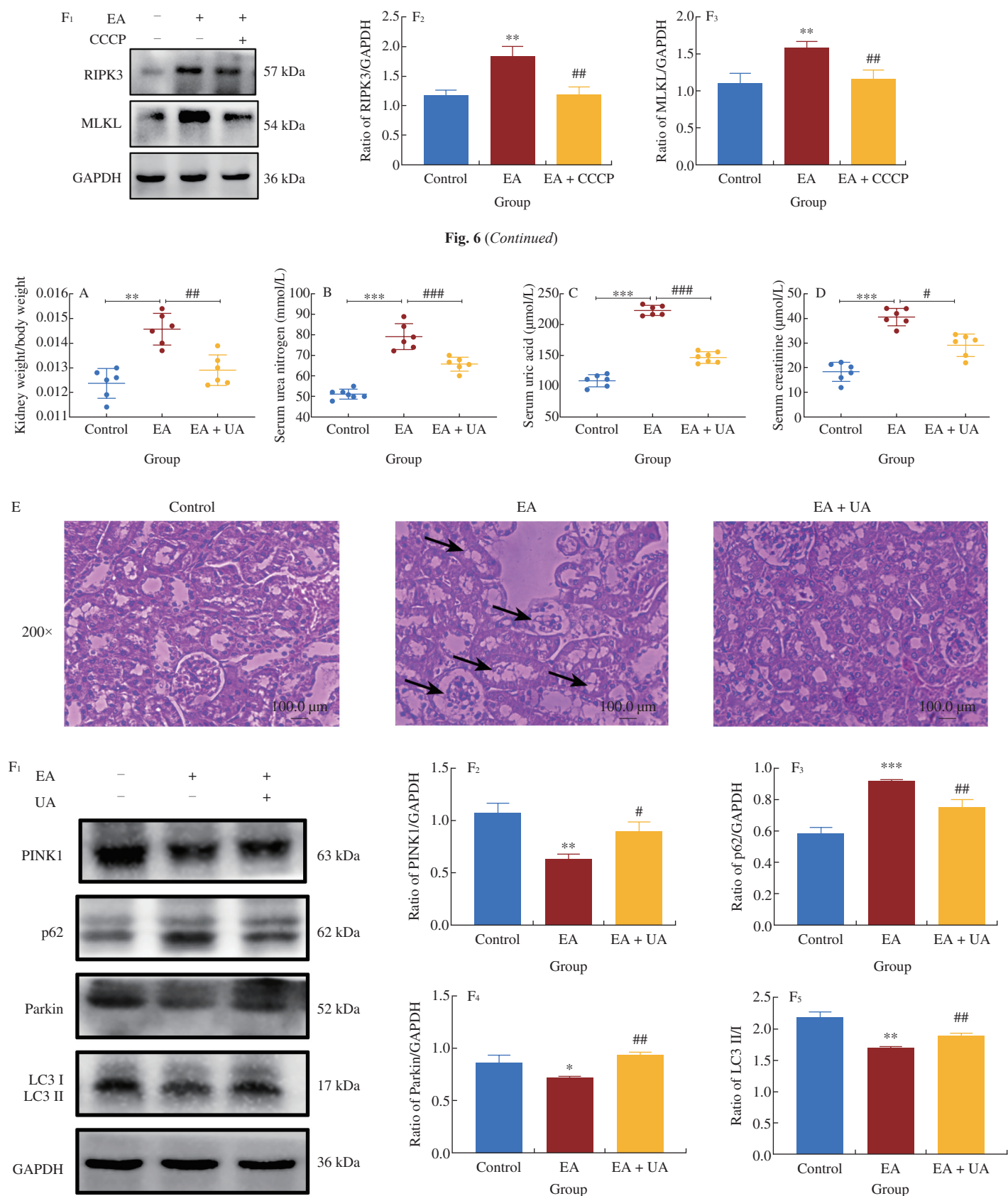


Fig. 6 (Continued)



**Fig. 7** EA exacerbated necroptosis *via* inhibition of mitophagy in mice. (A) Relative kidney weight in mice. (B) Serum urea nitrogen, (C) serum uric acid, (D) serum creatinine levels were detected by kits. (E) HE staining (200× magnification). (F) Levels of Parkin, PINK1, p62, LC3 II/I were assessed by Western blot in mice kidneys. (G) Levels of MFN1, VDAC, and FIS1 were assessed by Western blot in mice kidneys. (H) Levels of MLKL, p-MLKL, and RIPK3 were assessed by Western blot in mice kidneys. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control. # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  vs. EA.

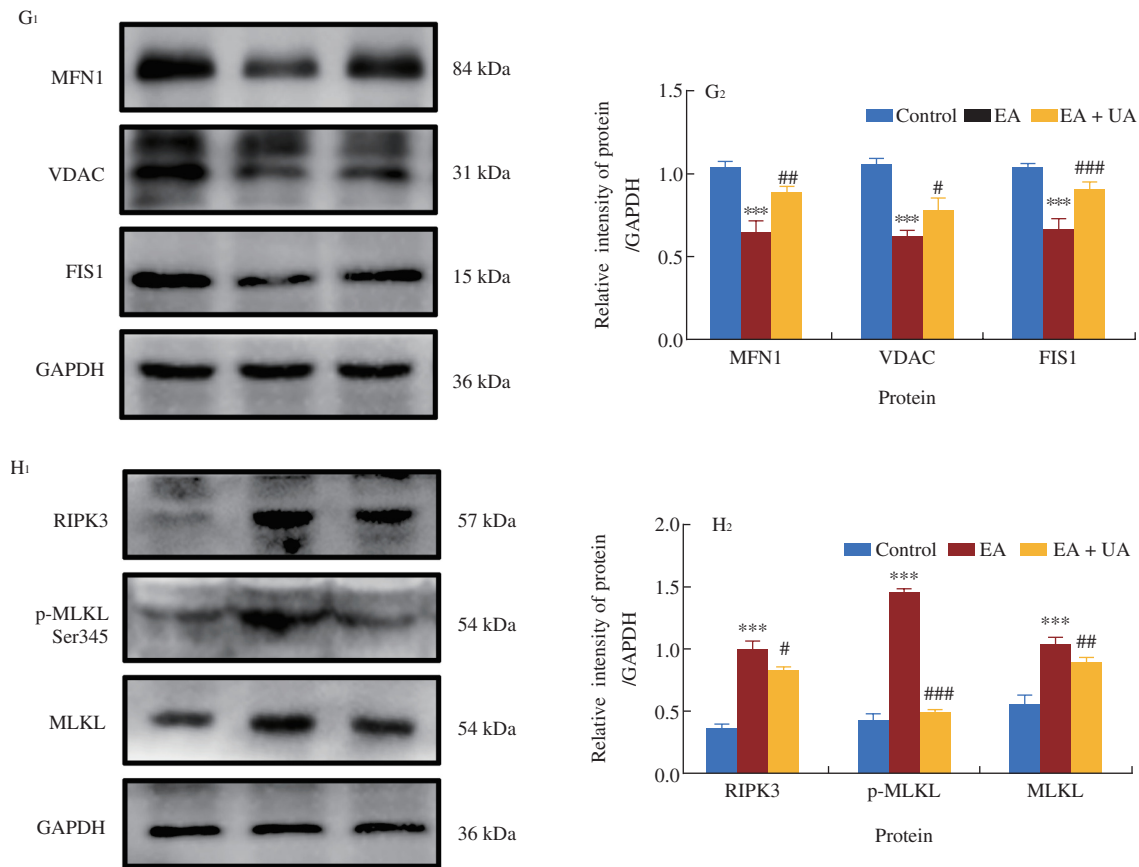


Fig. 7 (Continued)

FIS1 were increased in the EA + UA group compared with the EA group, indicating that mitochondrial damage caused by EA was alleviated after activation of mitophagy (Fig. 7G). This was also consistent with the results of cell experiments. Levels of necroptotic proteins p-MLKL, MLKL, and RIPK3 were detected to explore the relationship between mitophagy and necroptosis. The results showed that activation of mitophagy could alleviate the EA-induced necroptosis *in vivo* (Fig. 7H), which further confirmed the results of cell experiments. Overall, EA induced necroptosis by inhibiting mitophagy in mice.

#### 4. Discussion

TFA have been widely used in the food processing industry to improve the shelf life, stability, texture, and flavor of the products. With the popularity of Western fast food and pastries, consumption of TFAs has soared and the risks of TFAs need to be reassessed. As the most typical TFA, EA was reported to be nephrotoxicity, but the mechanism of nephrotoxicity needs further study. The dose used in mice was converted from the human equivalent dose (HED) based on the human body surface area and a conversion coefficient of 12.3 was used to compensate for the difference between the body surface area of mice and humans<sup>[30]</sup>. TFA intake of urban residents in China was 0.53 g/day<sup>[31]</sup>. A total of 100 mg/kg was calculated as the highest experimental dose in mice. It was found that with the increase in EA dose, renal damage in mice was exacerbated, as shown by increases in relative kidney weight, serum uric acid levels, creatinine levels, and urea nitrogen levels.

Previous studies suggested that chronic kidney injury was closely related to apoptosis<sup>[32]</sup>. Recent studies have reported that necroptosis is the most important cell death mode leading to chronic and acute kidney injury<sup>[33]</sup>. For example, 1,3-dichloro-2-propanol increased RIPK3 and MLKL expression, inducing necroptosis and kidney injury<sup>[24]</sup>. In our study, we found that expressions of RIPK3, p-MLKL, and MLKL were up-regulated in mouse kidneys, which suggested that EA-induced chronic kidney injury was associated with necroptosis. In a further *in vitro* study, we evaluated the effect of EA on NRK-52E cells, and the results showed that EA increased the levels of LDH, RIPK3, and MLKL, suggesting that EA induced necroptosis in NRK-52E cells.

RIPK3 is a key protein in necroptosis. Treatment with the RIPK3 inhibitor GSK'872 reduced the expression of RIPK3 and MLKL, thereby inhibiting necroptosis<sup>[34]</sup>. In our study, GSK'872 was administered intraperitoneally to EA-fed mice. We found that GSK'872 ameliorated EA-induced renal injury in mice, as evidenced by reductions in relative kidney weight, serum uric acid levels, creatinine levels, and urea nitrogen levels. GSK'872 also reduced the expression of RIPK3, MLKL, and p-MLKL and inhibited necroptosis. Similar results were obtained *in vitro* by pretreatment with GSK'872. The levels of LDH, RIPK3, and MLKL were reduced in NRK-52E cells. These results indicated that EA induced necroptosis and renal injury through the RIPK3/MLKL pathway.

Excessive ROS can lead to cell damage in a variety of ways, including oxidative stress and inflammation<sup>[35]</sup>. Studies have shown that ROS cause necroptosis. The production of ROS was the key medium of necroptosis<sup>[36]</sup>, and ROS could promote cell apoptosis to necroptosis<sup>[37]</sup>. These results indicated that ROS was an important

part of necroptosis. To investigate whether ROS was involved in EA-induced necroptosis and renal injury, MT was used. Intraperitoneal injection of MT into EA-fed mice showed that MT alleviated EA-induced renal injury in mice, as evidenced by decreased relative kidney weight, serum uric acid levels, and urea nitrogen levels, as well as decreased the expression of RIPK3, MLKL, and p-MLKL and inhibited necroptosis. *In vitro* experiments, we found that EA increased the production of ROS, and necroptosis was inhibited after the removal of ROS. These results proved that EA induced necroptosis and renal injury through the ROS/RIPK3/MLKL pathway.

Mitochondria, as the source of ROS, play an important role in necroptosis. Studies showed that the accumulation of damaged mitochondria was accompanied by elevated ROS, and elevated ROS aggravated mitochondrial damage, forming a vicious cycle<sup>[38]</sup>. Mitochondria are dynamic organelles whose morphology is controlled by fusion and fission. Mitochondrial fusion and fission are essential for mitochondrial quality control. MFN1, a key component of the mitochondrial fusion mechanism, is located in the outer membrane and mediates the mitochondrial tethering during fusion. The mitochondrial fission mechanism consists of the outer membrane protein FIS1 and the motion-related GTPase Drp1<sup>[39]</sup>. The VDAC is a  $\beta$ -barrel membrane protein located in the outer membrane of the mitochondria. It uses a voltage-sensing mechanism and ligand binding to regulate the entry and exit of key metabolites under physiologically relevant conditions, which is an important basis for assessing mitochondrial function<sup>[40]</sup>. In our study, we found that EA reduced the expression of MFN1, VDAC, and FIS1 *in vivo* and *in vitro*, suggesting that EA affected the normal function of mitochondria, while the accumulation of damaged mitochondria increased the production of ROS. These results were consistent with those of previous studies.

Mitophagy is a selective autophagy that preferentially identifies and eliminates dysfunctional mitochondria, thereby preventing the production of ROS. In the process of mitophagy, damaged mitochondria are wrapped by autophagosomes, which combine with lysosomes to form autolysosomes, thus degrading damaged mitochondria<sup>[41]</sup>. Studies showed that the absence of mitophagy led to the accumulation of ROS<sup>[18]</sup>. The PINK1/Parkin mitophagy pathway was the most studied in mammalian cells<sup>[42]</sup>. It was found that mitophagy was impaired in the kidneys of murine septic AKI mice and that levels of PINK1 and Parkin were elevated from 4 to 8 h after treatment and then decreased from 18 to 24 h<sup>[43]</sup>. During renal injury induced by a high-calorie diet, mitophagy was reduced accompanied by abnormal mitochondrial morphology<sup>[44]</sup>. PINK1/parkin pathway of mitophagy protects against contrast-induced acute kidney injury *via* decreasing mitochondrial ROS and NLRP3 inflammasome activation<sup>[45]</sup>. HIF1 $\alpha$ -BNIP3-mediated mitophagy protected against renal fibrosis by decreasing ROS and inhibiting activation of the NLRP3 inflammasome<sup>[46]</sup>. In order to clarify the effects of EA on mitophagy, we studied the effects of EA on LC3, p62, PINK1, and Parkin proteins and also studied the colocalization of mitochondria and LC3 in NRK-52E cells. The results showed that EA decreased the level of mitophagy. CCCP and UA were used to activate mitophagy and assess the effect of mitophagy on EA-induced necroptosis. We found that EA-induced kidney injury and necroptosis were alleviated with CCCP and UA. We also found that EA-induced mitochondrial damage and ROS were suppressed after the activation of mitophagy. These results proved that EA induced the increase of ROS by

inhibiting mitophagy, and then regulated necroptosis. However, cell death is a complex process that may involve multiple modes of cell death, including necroptosis, pyroptosis, apoptosis, and ferroptosis. In our study, only EA-induced necroptosis was investigated. The effects of EA on other cell death modes and the toxicity mechanisms of the combined effects of different cell death modes need to be further investigated.

In conclusion, we demonstrated that EA could cause kidney injury through necroptosis. EA induced necroptosis and kidney injury by inhibiting mitophagy and activating the ROS/RIPK3/MLKL pathway. The study provided a new perspective on EA-induced nephrotoxicity and a scientific basis for the risk assessment of EA as a food additive.

## Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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