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Non-steroidal mineralocorticoid receptor antagonist finerenone ameliorates mitochondrial dysfunction via PI3K/Akt/eNOS signaling pathway in diabetic tubulopathy

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ABSTRACT

Diabetic tubulopathy (DT) is a recently recognized key pathology of diabetic kidney disease (DKD). The mitochondria-centric view of DT is emerging as a vital pathological factor in different types of metabolic diseases, such as DKD. Finerenone (FIN), a novel non-steroidal mineralocorticoid receptor antagonist, attenuates kidney inflammation and fibrosis in DKD, but the precise pathomechanisms remain unclear. The role of mineralocorticoid receptor (MR) in perturbing mitochondrial function via the PI3K/Akt/eNOS signaling pathway, including mitochondrial dynamics and mitophagy, was investigated under a diabetic state and high glucose (HG) ambiance. To elucidate how the activation of MR provokes mitochondrial dysfunction in DT, human kidney proximal tubular epithelial (HK-2) cells were exposed to HG, and then mitochondrial dynamics, mitophagy, mitochondrial ROS (mitoROS), signaling molecules PI3K, Akt, Akt phosphorylation and eNOS were probed. The above molecules or proteins were also explored in the kidneys of diabetic and FIN-treated mice. FIN treatment reduced oxidative stress, mitochondrial fragmentation, and apoptosis while restoring the mitophagy via PI3K/Akt/eNOS signaling pathway in HK-2 cells exposed to HG ambiance and tubular cells of DM mice. These findings linked MR activation to mitochondrial dysfunction via PI3K/Akt/eNOS signaling pathway in DT and highlight a pivotal but previously undiscovered role of FIN in alleviating renal tubule injury for the treatment of DKD.

1. Introduction

Diabetic kidney disease (DKD) is a high-burden complication that progresses into end-stage kidney disease (ESKD) and triggers severe cardiovascular (CVS) events [1,2]. An accumulation of studies elucidates these consequences [3,4]. Tubular hypoxia due to high energy demands and reduced blood perfusion and non-hypoxia-related factors drive the rapid progression of interstitial fibrosis and tubular atrophy, resulting in DKD [4]. Since the proximal tubule is enriched in mitochondria for its high energy demand and is dependent on aerobic metabolism, the mitochondria-centric view of primary DT is regarded in DKD [3,5]. In a few previous studies, hyperglycemia disrupted mitochondrial function in the renal tubules, causing decreased bioenergy production, faulty mitophagy, and abnormal mitochondrial dynamics, and triggering oxidative stress, apoptosis, and metabolic irregularities [6,7]. However, the pathogenesis of mitochondrial dysfunction and the primary stage of tubulopathy in diabetes mellitus (DM) are unknown and remain to be comprehended [8].

DKD and its related cardiovascular events pose significant risks despite treatments with angiotensin-converting enzyme inhibitors (ACEIs) [9], angiotensin II receptor blockers (ARBs) [10], and sodium-glucose cotransporter-2 inhibitors (SGLT2is) [11]. Aldosterone (Aldo) breakthrough/escape occurs when a renin-angiotensin-aldosterone system (RAAS) inhibitor is used continuously. As a result, Aldo suppression fails, partially contributing to the progression of DKD [12,13]. In recent clinical trials, the protective

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effects of finerenone (FIN), a new non-steroidal mineralocorticoid receptor antagonist (MRA) were emphasized, specific to alleviating proteinuria and regaining renal function in individuals with DKD [14,15]. Furthermore, in the FIDELIO-DKD [14] trial that included 5700 individuals with T2DM and CKD who took maximum tolerable dosages of an ARB or ACE inhibitor, FIN substantially slowed down the development of CKD by 18 % compared to placebo in a mean follow-up period of 2.6 years.

FIN and other MRAs exhibited antioxidant, anti-inflammatory, and anti-fibrotic properties, and reversed metabolic abnormalities in DKD in a previous study [16]. Insulin resistance is commonly associated with MR activation in DT and is significantly linked to a decline in GFR [17]. In insulin resistance, the physiological action of insulin is impaired, disrupting mitogen-activated protein kinase (MAPK), insulin receptor substrate/phosphatidylinositol 3-kinase (PI3K) signaling pathway, and others. The downstream candidatures of the PI3K/protein kinase B (AKT)/endothelial nitric oxide synthase (eNOS) signaling directly regulate mitochondria, controlling mitochondrial function, oxidative stress, and apoptosis [18,19]. Mitochondrial dysfunction, including irregular dynamics, was involved in glomerular podocyte injury in DKD patients and mouse models [20,21]. However, the involvement of the mitochondrial axis and the MR-PI3K/AKT/eNOS signaling pathway in DKD, especially with DT manifestation, has not yet been reported. Hence, this study aimed to elucidate the effects of MR activation on mitochondrial dysfunction in the renal tubules in DKD and highlight the mechanisms by which activation of MR in epithelial cells perturbs mitochondrial homeostasis via the PI3K/Akt/eNOS signaling pathway.

2. Methods

2.1. Animal studies

C57BL/6J male mice at the age of 3–4 weeks, were acquired from the Model Animal Research Center of Nanjing University (Nanjing, China) and reared according to the guidelines of the Animal Research Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Eighteen mice were randomly divided into 3 groups, control mice group (CON), diabetic mice group (DM), and diabetic mice treated with finerenone group (FIN). The Con group received a standard diet, whereas the other 2 groups were provided with a high-fat diet (HFD). After 8 weeks of HFD, mice in the DM group and the FIN group received intraperitoneal injections of streptozotocin (STZ) (50 mg/kg; Sigma-Aldrich, St Louis, MO, USA) for 5 days to induce diabetes mellitus. One week later, blood glucose level was randomly measured in T2DM model mice, and the value was 16.7 mmol/l. The mice in the FIN group were given finerenone (MedChemExpress, USA) orally at 3 mg/kg/d for 12 weeks. Blood and urine samples were collected at specific time points. After sacrificing the mice, the kidneys were collected and stored at suitable storage conditions for further experiments (Supplementary Fig. 1A).

2.2. Cell culture

Human kidney proximal tubular epithelial cells (HK-2 cells) (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium combined with F-12 Ham nutrient mixture (DMEM: F-12, 1:1 mixture) supplemented with 5 % fetal bovine serum (FBS). HK-2 cells were plated at about 70 % confluence, then serum starved for 12 h before exposure to the standard culture conditions containing 5 mM glucose (the NG group) or a DM comprising of 30 mM glucose plus 2 ng/mL TGFβ_1 (the HG group) for 24 h. For positive control of MRAs, HG-cultured HK-2 cells were treated with 5 mM finerenone (the FIN group) simultaneously (Supplementary Fig. 1B).

2.3. Physiological characteristics and renal functions

Body weight was measured, and urine and blood samples were collected biweekly. The Roche Accu-Chek Advantage Meter measured blood glucose levels. Urine and serum creatinine levels were determined using a creatinine assay kit (Jiancheng Bio, Nanjing, China). A mouse albumin ELISA quantification kit (Jiancheng Bio, Nanjing, China) was used to quantify urine albumin. The urinary ACR (mg/g) was determined as follows: Urinary ACR(mg/g) = Urine albumin (mg/dl)/ urine creatinine (g/dl).

2.4. Western blotting

Murine renal tissue or cultured cells was homogenized and lysed in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with phosphatase inhibitor (CW Bio, Beijing, China) and protease inhibitor (CW Bio, Beijing, China). The samples were blotted and tagged with relevant antibodies as previously reported. The antibodies against NR3C2, AKT, p-AKT, PI3K, p62, Atg5, Atg7, Beclin-1, Bax, Cyt C, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibodies against Drp1, Fis1, Mfn2, and OPA1 were purchased from Abcam (Shanghai, China), and anti-LC3-II was purchased from Sigma-Aldrich (Burlington, MA, USA). The antibodies against Mfn1 and eNOS were purchased from proteintech (Wuhan, China). The protein band was quantified using Image J software. Supplementary Table 1 lists the antibody catalog numbers.

2.5. Periodic acid schiff staining and immunohistochemistry

The kidney tissues were paraffin-embedded and cut into 3 μm sections. The sections were stained utilizing the universal SP test Kit (Solarbio, Beijing, China) and primary antibodies against Drp1, p-AKT and MR (listed in Supplementary Table 1). Periodic acid Schiff staining was carried out using a Periodic acid-Schiff (PAS) stain kit (Servicebio, Wuhan, China) according to the supplier's instructions. Briefly, the sections were dewaxed, oxidized with periodic acid, and colored with Schiff's reagent. Hematoxylin dye counter-stained the nucleus, and the stained section was mounted with neutral gum. The images were captured using an Olympus microscope (Tokyo, Japan).

2.6. Transmission electron microscopy (TEM)

Renal cortices were cut into 1 mm^3 sections and transmission electron micrographs of the tissue sections were obtained by using an electron microscope. Briefly, after dehydrating the preserved tissue blocks and embedding them in Epon 812 epoxy embedding medium, thin sections were cut for viewing using electron microscopy to view the mitochondrial fragmentation in renal tubules. Mitochondria with a length of greater than 2 mm were classified as filamentous, whereas those with a length of less than 1 mm with a spherical structure were classified as fragmented [6].

2.7. Immunofluorescence staining and TUNEL assay

HK-2 cells were fixed in 4 % paraformaldehyde for 30 min and then permeabilized in 0.5 % TritonX-100 at room temperature for 15 min. The cells were blocked with 3 % bovine serum albumin (BSA) and labeled with primary antibodies at 4 °C overnight. After rinsing with phosphate-buffered saline (PBS), samples were incubated with the Alexa Fluor 488 or 594 (Invitrogen Life Technologies, Carlsbad, CA, USA). Finally, they were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted using Vectashield mounting media (Vector Laboratories; Burlingame, CA, USA).

TUNEL staining was performed on cells grown on coverslips by using the TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China), following the manufacturer's instructions to assess apoptosis rate. Each treatment

was repeated six times. The cells were viewed using a Zeiss LSM880 confocal microscope (Carl Zeiss, Germany).

2.8. Detection of mitotracker, ROS and ATP

Cell mitochondria were labeled using MitoTracker Red FM (Invitrogen Life Technologies, Waltham, MA, USA). After several treatments, the HK-2 cells were labeled with Mitotracker (50 nM) at 37 °C for 30 min.

To determine the production of mitochondrial and intracellular ROS, HK-2 cells were stained with MitoSOX or H_2-DCFDA (Invitrogen Life Technologies) at 37 °C for 30 min, correspondingly, and observed using Zeiss confocal microscopy. The data from six independent experiments were analyzed.

To assess mitochondrial ATP, kidney tissues were incubated with ATP assay kit (Jiancheng Bio, Nanjing, China).

2.9. Statistical analyses

All *in vitro* and *in vivo* studies were repeated three to six times. The

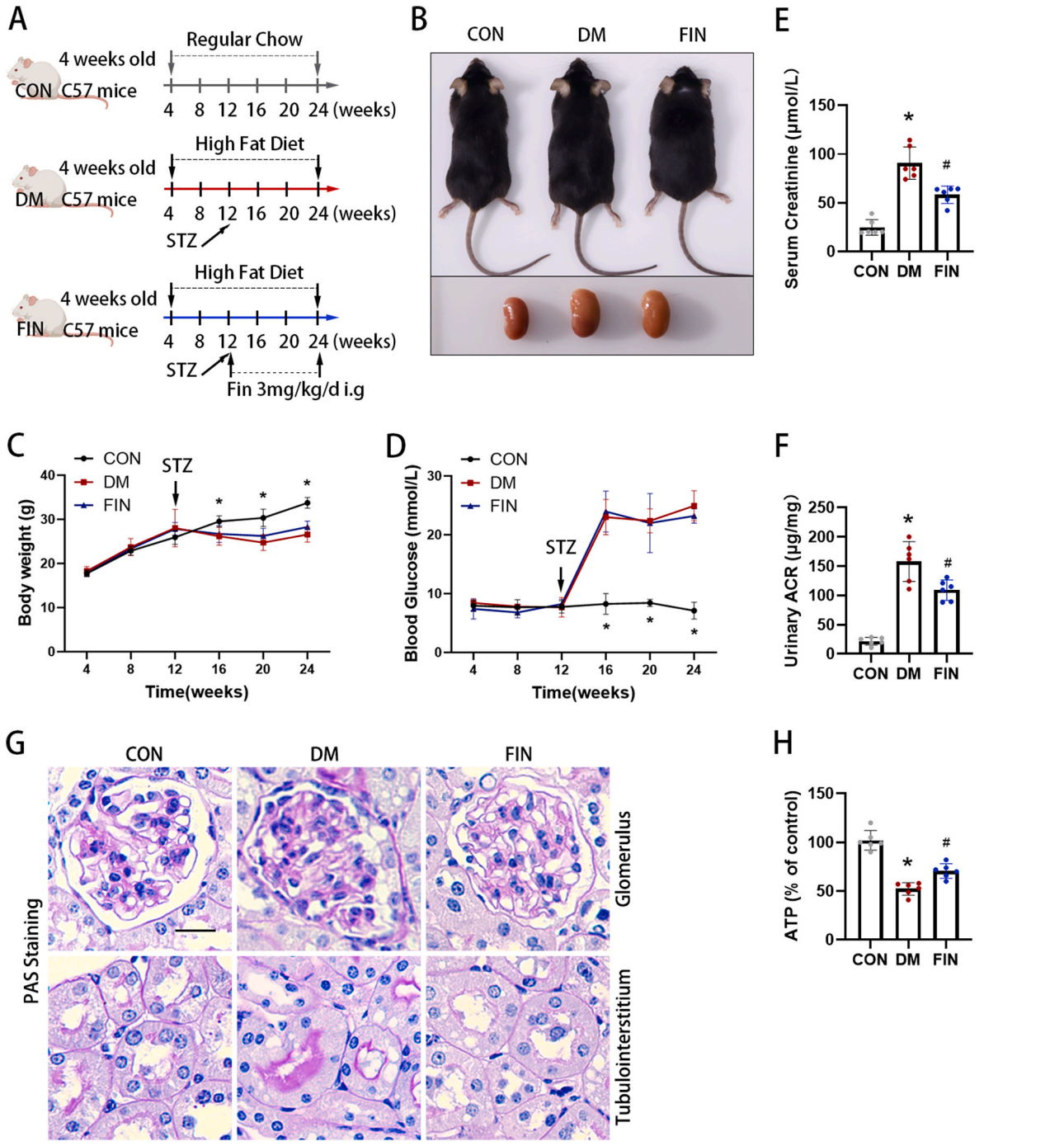

Fig. 1. Effect of finerenone on kidney function and pathologic alterations in HFD/STZ-induced T2DM mice's kidneys. (A) The schematic representation of the study protocol. (B) Representative image of mice and the alterations in kidney morphology. The variations in the body weight (C), blood glucose (D), serum creatinine (E), and urinary ACR (F) are shown (n = 6). (G) PAS staining provides representative histology of the renal cortex. Scale bar: 20 μm. (H) the production of mitochondrial ATP in kidney. All data are shown as means ± SEM.; *P<0.05 *versus* the control group, $^{\#}P < 0.05$ *versus* the T2DM group.

data was presented as means ± SD. The comparison of variables between the two groups was done by the student's t-test. One-way ANOVA was used for comparing variables among multiple groups. Statistical analyses were done using the GraphPad Prism 6 software. A value of $P < 0.05$ was deemed to be statistically significant in all comparisons.

3. Results

3.1. Overexpression of MR accentuates the tubulopathy in HFD/STZ-induced T2DM mice, and FIN treatment attenuated diabetic tubulopathy

HFD and low-dose STZ have been widely used to induce DM in manifesting early stages of renal injury. FIN, a novel non-steroidal MRA, was mixed into the mouse chow diet to inhibit MR hyperactivation. Three groups of mice (n = 6 per group) were allocated for the control (Con), HFD/STZ-induced T2DM, and T2DM with FIN dietary supplementation (FIN) groups (Fig. 1A). Blood glucose, body weight, serum creatinine, and urinary albumin-to-creatinine ratio (UACR) were determined during the study period. As early as 8 weeks on a high-fat diet, the T2DM mice showed higher body weight, increased blood glucose, and elevated serum creatinine and urine ACR levels. Interestingly, the urine ACR and serum creatinine in the T2DM mice that received 12 weeks of finerenone (the FIN group) were decreased remarkably compared to those in the T2DM group (Fig. 1E and F); however, no significant variations in body weight or blood glucose were observed (Fig. 1C and D). PAS staining revealed protein excretion, significant tubular epithelial breaks, hypertrophy of the glomeruli, and a rise in mesangial matrices in the T2DM mice compared to the control group (Fig. 1G). Compared with Con group, the mitochondrial ATP content of the kidney in T2DM group was significantly decreased, and the decline in ATP production could be mitigated after treatment with FIN. As shown by immunohistochemical staining, MR expression in the renal tubule of T2DM mice *versus* the control mice was elevated, which was mostly limited to tubular epithelial cells in tubulointerstitial (Fig. 2A). The morphological features were supported by the intensity of protein bands through the WB experiment (Fig. 2C and D). However, treatment with FIN significantly alleviated the above-mentioned morphological damage in the renal tubule and the overexpression of MR.

Under normal circumstances, kidney tissue scarcely expresses KIM-1 protein, but the expression level of KIM-1 is significantly increased within a few hours of kidney injury, which is one of the specific biological markers for early renal tubular injury. As shown in WB images, KIM-1 expression has been progressively increased in DM mice kidneys, but not in FIN mice kidneys (Fig. 2C and H), and so do cell models (Fig. 4C and G). This data confirmed that the diabetic state led to tubular

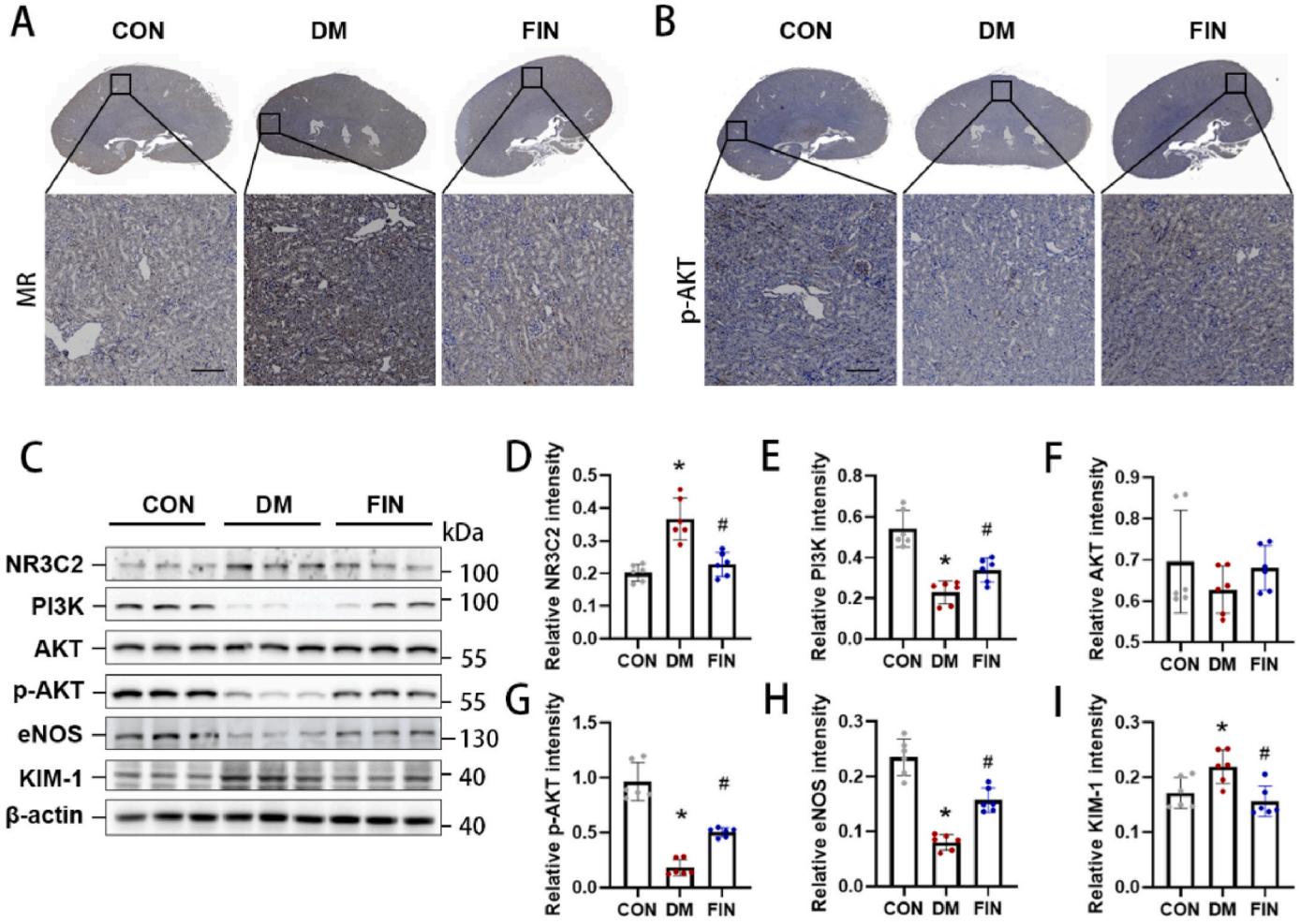

Fig. 2. Finerenone alleviates the overactivation of MR via PI3K/Akt/eNOS signaling pathway in HFD/STZ-induced T2DM mice. (A) Immunohistochemistry staining for MR showed that the MR expression and activity were significantly higher in the T2DM group compared to controls, and significantly reduced in the T2DM mice given finerenone. Scale bar: 200 μm. (B) The immunohistochemistry staining for p-AKT revealed that AKT activity was lowered in the T2DM group and recovered in the finerenone group. Scale bar: 200 μm. (C) The presence of NR3C2, PI3K, AKT, p-AKT, eNOS, and KIM-1 proteins. (D–I) Quantitative analysis of NR3C2, PI3K, AKT, p-AKT, eNOS, and KIM-1 proteins and β-actin served as a loading control (n = 6). Data are shown as means ± SEM; $^{*}P<0.05$ *vs.* the control group, $^{\#}P < 0.05$ *vs.* the T2DM group.

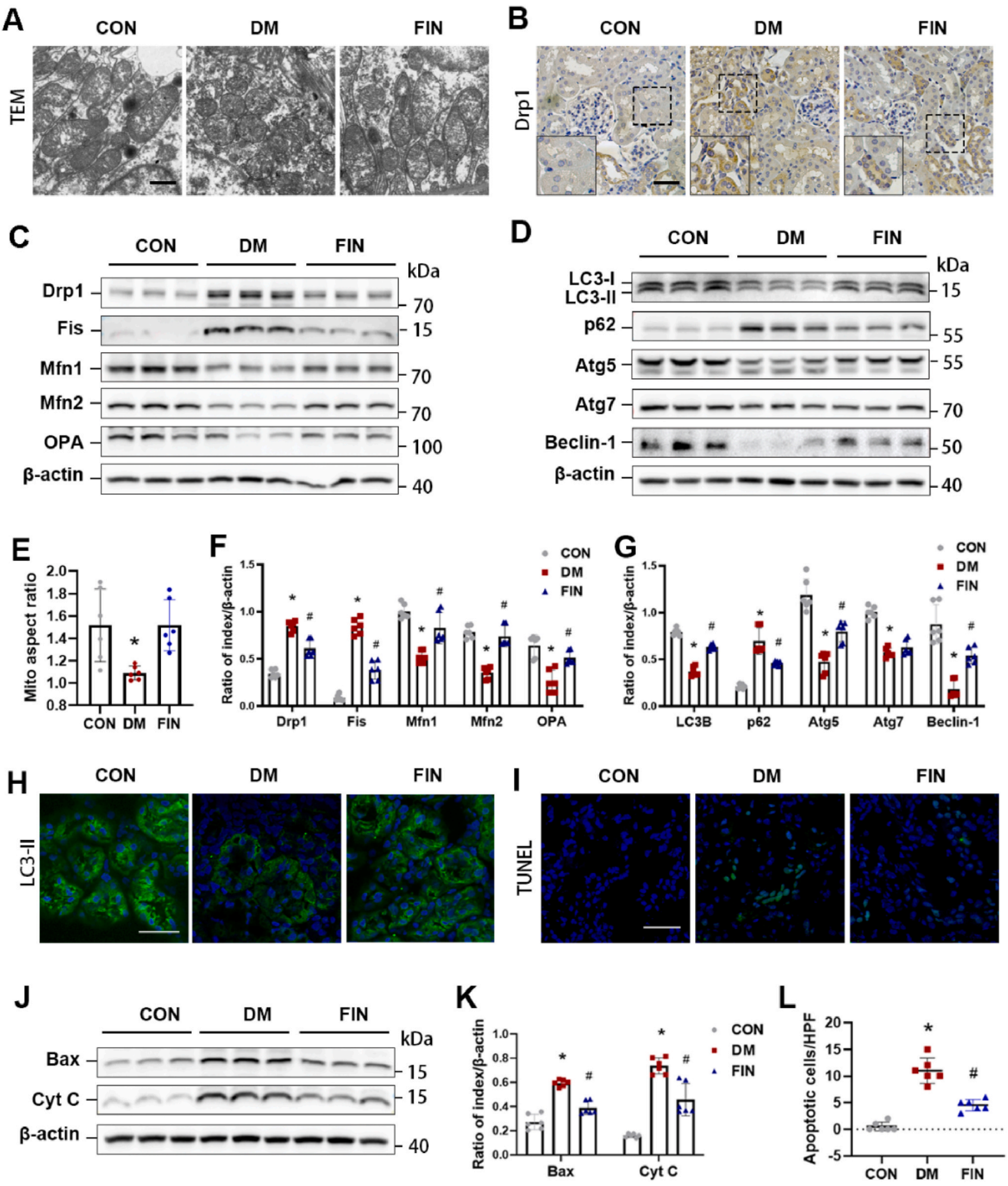

Fig. 3. Alleviation of mitochondrial abnormal dynamics, abnormal mitophagy, and apoptosis in T2DM mice in the finerenone group. (A) Mitochondria in the proximal tubules were viewed using electron microscopy. Scale bar: 1 μm. (B) The mitochondrial pro-fission protein Drp1, mostly found in renal tubules, was increased in the T2DM group, however, finerenone treatment diminished its expression. (C–D) Representative immunoblots and quantitative analysis of the mitochondrial dynamic regulatory proteins, such as Drp1, Fis, Mfn1, Mfn2, and OPA from the control, T2DM, and finerenone groups (n = 6). (E) Quantitative analysis of the aspect ratio of mitochondria (n = 6). (F–G) Representative immunoblots and quantitative analysis of the mitophagy regulating proteins, such as LC3-II, p62, Atg5, Atg7, and Beclin-1 from the above three groups (n = 6). (H) Immunofluorescence staining of LC3-II in the renal tubules from each group. (I–K) Representative immunoblots and quantitative analysis of the apoptosis-related proteins, Bax and Cyt C from the above three groups (n = 6). β-actin served as a loading control. (K–L) Apoptosis was assessed by TUNEL staining (n = 6). The nuclei were counterstained by DAPI. Data are demonstrated as means ± SEM; $^{*}P<0.05$ *vs.* the control group, $^{\#}P < 0.05$ *vs.* the T2DM group.

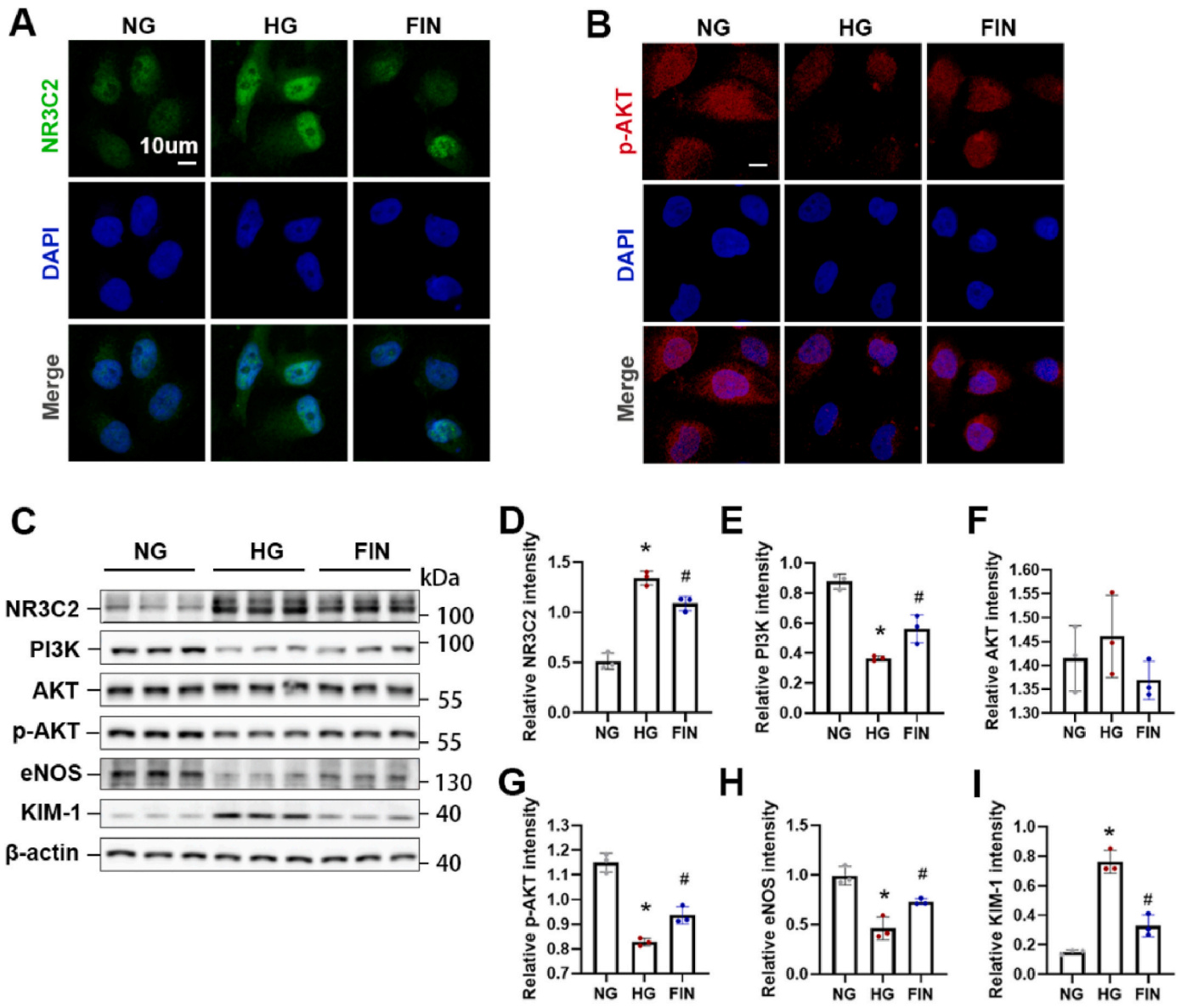

Fig. 4. Finerenone alleviates the overactivation of MR via PI3K/Akt/eNOS signaling pathway in HK-2 cells treated with HG. (A) HK-2 cells were immunostained to show the localization of NR3C2 (green) and p-AKT (red). Scale bar: 10 μm. (C–I) By immunoblotting, the presence of NR3C2 and KIM-1 were increased under HG ambience, but AKT was unaffected. Expressions of PI3K, p-AKT, and eNOS were decreased. Concurrently, the expression and activity of the PI3K/Akt/eNOS pathway and KIM-1, a marker of renal tubular injury, were markedly increased in the HK-2 cells treated with finerenone. Interestingly, the overactivation of MR was also reversed (n = 3). Data are shown as means ± SEM; $^{*}P<0.05$ *vs.* the NG group, $^{\#}P < 0.05$ *vs.* the HG group.

injury and interstitial fibrosis, thereby accelerating the progression of DT, which was restored by blocking MR activation.

3.2. FIN treatment on mitochondrial homeostasis disorder of mitochondrial morphology, dynamics, mitophagy, and apoptosis in the renal tubules of HFD/STZ-induced T2DM mice

The regulatory mechanisms of mitochondrial homeostasis, including mitochondrial dynamics (fission and fusion), mitophagy, and biogenesis of mitochondria are multifaceted. Consistent with the outcomes of recent literature, our *in vivo* studies found that mitochondrial fission proteins were increased, whereas fusion proteins and mitophagy proteins were reduced in T2DM mice compared to the control mice on immunoblot analysis (Fig. 3B, C, and 3D). RAAS activation in the kidney played a significant role in the advancement of DKD by enhancing the non-classical aldosterone-MR effect, the MR hyperactivation [13]. To determine whether activation of MR was an essential step in diabetes-induced renal tubular mitochondrial dysfunction, mitochondrial morphology, dynamics-associated proteins, and mitophagy-associated proteins were examined in the kidneys of FIN-treated mice (Fig. 3A–F).

On electron microscopic analysis, the renal tubular cells from the control mice displayed many elongated mitochondria organized along the membrane. However, the mitochondria in the tubules of T2DM mice were short and round shaped or fragmented and scattered. On the other hand, treatment with FIN significantly reduced the mitochondrial fission in the renal tubules of DM kidneys (Fig. 3A and E). Additionally, the expression of mitochondrial-shaping proteins was elucidated. The Drp1-mitochondrial pro-fission protein, expressed mostly in the renal tubules by immunohistochemistry, was increased in the DM mice; however, its in-situ expression was decreased to some extent in the FIN-treated mice (Fig. 3B). Conversely, the Mfn2-mitochondrial pro-fusion protein was significantly reduced in the tubules of the DM mice, but in the proximal tubules, it was largely recovered after the FIN therapy (Fig. 3C).

Concurrently, immunoblot analysis found the up-regulation of pro-fission proteins (Drp1 and Fis1) and mitophagy protein (p62), and down-regulation of pro-fusion and mitophagy proteins (Mfn1, Mfn2, OPA, LC3-II, Atg5, Atg7, and Beclin-1), while repaired levels of these proteins in the kidneys of DM mice were seen after treating with FIN (Fig. 3C, D, 3F, and 3G). Notably, expression of Atg7, a pivotal autophagy effector enzyme that in concert with other Atg proteins, was not upregulated after the FIN treatment. The LC3-II immunofluorescence staining was significantly decreased in the tubules of the T2DM group, and the autophagosome development was largely recovered following

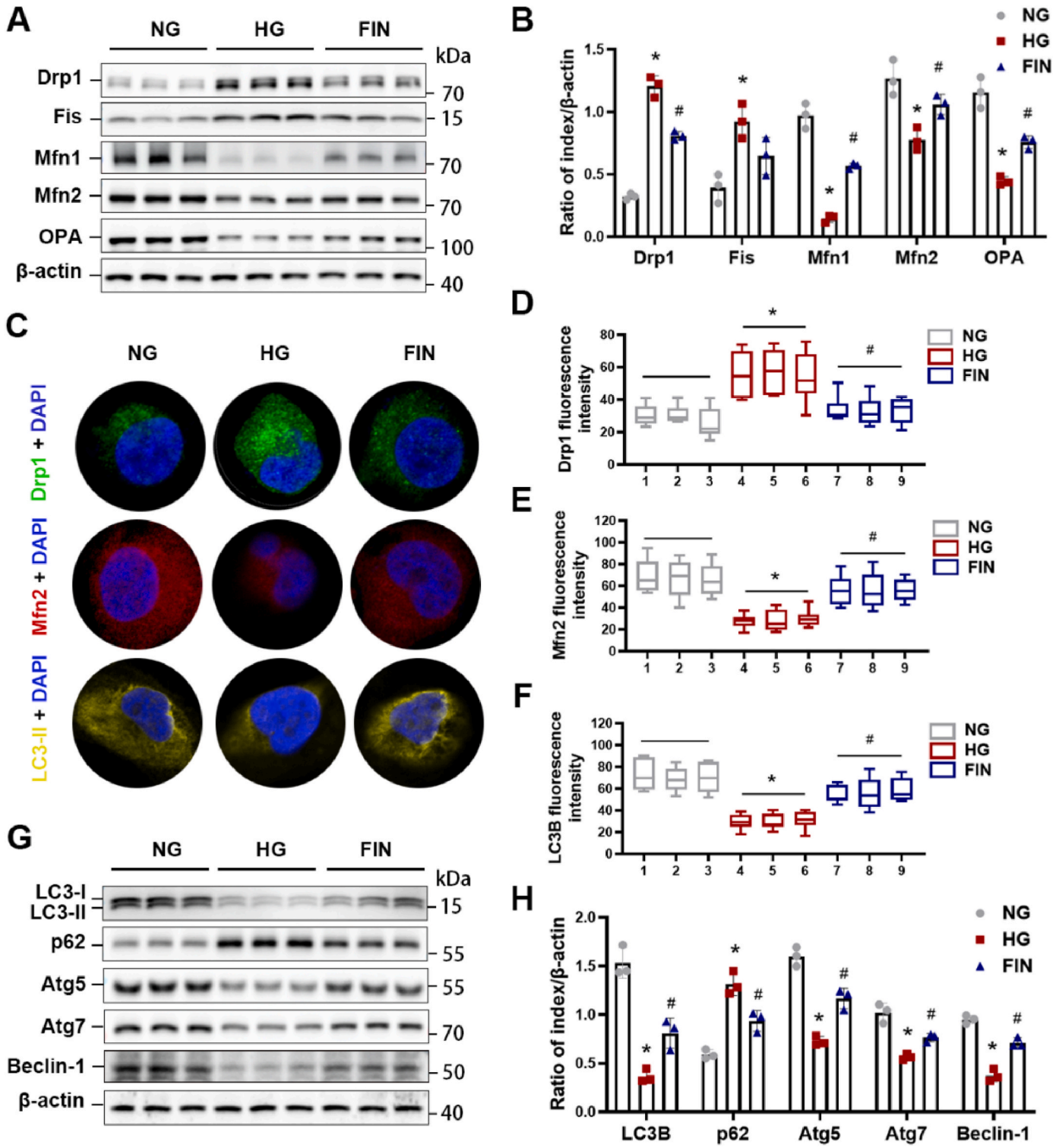

Fig. 5. Finerenone attenuates HG-induced mitochondrial dynamics and abnormal mitophagy in HK-2 cells. (A–B) Quantitative analysis of mitochondrial dynamic regulatory proteins and representative immunoblots containing Drp1, Fis, Mfn1, Mfn2, and OPA from the NG, HG, and FIN groups (n = 3). (C) Confocal images of Drp1 (green), Mfn2 (red), and LC3-II (yellow) in HK-2 cells. The nuclei were counterstained with DAPI (blue). (D–F) Quantification of the degree of Drp1, Mfn2, and LC3-II by ImageJ (n-3). (G–H) Representative immunoblots and quantitative analysis of the mitophagy regulating proteins, such as LC3-II, p62, Atg5, Atg7, and Beclin-1 from the above three groups (n = 3). Data are shown as means ± SEM; $^{*}P<0.05$ *vs.* the NG group, $^{\#}P < 0.05$ *vs.* the HG group.

the FIN therapy (Fig. 3H). Moreover, the appearance of apoptosis-related proteins (Bax and Cyt C) was reduced in the FIN-treated group (Fig. 3J and K). The kidney tissues from the T2DM group that were labeled with TUNEL staining exhibited increased apoptosis, which was significantly lowered by the FIN therapy, particularly in the renal tubules (Fig. 3I and L). As demonstrated by these outcomes, FIN ameliorated tubular mitochondrial dysfunction and apoptosis in the kidneys of HFD/STZ-induced T2DM mice *in vivo*.

3.3. Reduction of PI3K, Akt phosphorylation, and eNOS in DM state and recovery of T2DM-induced inactivation of the PI3K/Akt/eNOS signaling pathway by the blockade of MR

The role of PI3K in regulating mitochondrial function draws attention to diabetes [22]. The PI3K/Akt/eNOS signaling pathway controls and is controlled by multiple regulators that are implicated in mitochondrial function. Given the profound effects of PI3K on mitochondrial intrinsic apoptosis, we tested whether blockade of MR (FIN treatment) could activate PI3K, which then could mediate mitochondrial energy homeostasis through Akt phosphorylation. In our *in vivo* studies, the presence of PI3K, Akt, the phosphorylation of Akt and eNOS were elucidated in the WB experiment. On immunoblot analysis, down-regulation of PI3K, and the phosphorylation of Akt in HFD/STZ-induced T2DM mice were observed, while no remarkable differences in the Akt were found among the CON, DM, and FIN groups. However, restoration of the PI3K/Akt/eNOS signaling pathway in the kidneys of T2DM mice was seen after FIN treatment (Fig. 2C, E, 2G and 2H). Similar to the animal experiments, exposure of HK2 cells to HG ambiance resulted in a significant decrease of PI3K, eNOS and the phosphorylation of Akt, and the FIN treatment reversed these changes significantly (Fig. 4B, C, 4E, 4G, and 4H). Meanwhile, no notable alteration of Akt was noticed among the three groups *in vitro*. The inactivated PI3K/Akt/eNOS signaling pathway by the diabetic state was restored by FIN treatment, which correlated well with the mitochondrial function, suggesting that the activation of MR as a key factor within the PI3K/Akt/eNOS signaling pathway, specific to mitochondrial dysfunction induced by the renal tubular injury in diabetes mellitus.

3.4. Alterations in mitochondrial dynamics and autophagy by HG ambiance and attenuation of the mitochondrial dynamics and autophagy disruption by inhibition of MR

Mitochondria are dynamic organelles that allow eukaryotic cells to adapt to changes in their environment to survive via dynamic changes, such as undergoing continuous fission and fusion [23]. Besides, mitophagy is the selective elimination of injured mitochondria by autophagy. Given these dynamics, the influence of HG on both mitochondrial dynamics and mitophagy was evaluated. The HK-2 cells exposed to HG displayed higher percentages of fragmented mitochondria (Fig. 6A). Specifically, the presence of Drp1 and Fis1 proteins increased, while the protein levels of Mfn1, Mfn2, and OPA decreased compared to the control cells (Fig. 5A and B). Additionally, Drp1 and Mfn2 fluorescence intensities were comparable to the quantitative evaluations of immunoblot (Fig. 5C, D, and 5E). During the process of mitophagy, LC3-I transformed to LC3-II [24]. Immunofluorescence staining of LC3-II labeled the autophagosome, whereas immunoblot analysis of mitophagy-associated proteins quantified mitophagy. Reduction of mitophagy was regarded after counting cells with punctate LC3-II appearance (Fig. 5C and F). WB also showed a reduced protein level of mitophagy-related proteins (LC3-II, Beclin-1, Atg5, Atg7, and others), and these proteins were reduced in cells subjected to HG conditions (Fig. 5G and H).

To determine whether inhibition of MR hyperactivation had any protective effect on mitochondrial dynamics (fusion/fission) and the mitophagy process, non-steroidal MRA-FIN was treated in the HK-2 cells. Based on the therapeutic effect of the drug (Supplemental Fig. 2), 5 μM was selected as the testing concentration of FIN in the *in vitro* cultures of HK-2 cells. Notably, activated Drp1 was attenuated, and inactivated Mfn2 was alleviated in the FIN group, and this process was accompanied by an antagonistic effect on MR. Similar to the results of the mice experiments, restoration of mitophagy was observed in the FIN-treated cells. Whether it was immunofluorescence or a Western blot, an upsurge of mitophagy was noted in the HK-2 cells treated with FIN compared to the cells under HG ambiance.

3.5. Exposure of HK-2 cells to HG ambiance causes morphological changes in mitochondria, oxidative stress, and apoptosis, but treatment with FIN reduces mitoROS production and apoptosis in tubular cells

Disruption of mitochondrial function and abnormally fragmented mitochondria may invariably result in mitochondrial oxidative stress and cell apoptosis. Mitochondrial morphological changes were observed in HK2 cells stained with MitoTracker red using laser scanning confocal microscopy. As shown in Fig. 6A and C, in the NG (5 mM) group, mitochondrial fluorescence was uniformly present, and they transformed into local punctate over burst fluorescence signal of varying strength after the HG exposure for 24 h. In the FIN group, the non-steroidal MRA-FIN protected the mitochondria and ameliorated the mitochondrial morphological abnormalities (Fig. 6A and C).

Increased mitoROS production was strongly associated with mitochondrial dysfunction, impaired OXPHOS, and an imbalance of energy homeostasis in DT [3]. We also detected oxidative stress and apoptosis in the *in vitro* experiment. Under HG ambiance, both the mitochondrial and intracellular levels of ROS were increased. In contrast, in the FIN-treated HK-2 cells, mitochondrial and intracellular levels of ROS were comparatively lower in intensity of staining with MitoSOX and H_2-DCFDA probes, respectively (Fig. 6B and D).

Immunoblotting revealed a substantial rise in pro-apoptotic proteins Bax and Cyt C in the HG group. Surprisingly, all of these impacts were at least partially mitigated by the FIN treatment (Fig. 6E and F). The HK-2 cells in the HG group subjected to TUNEL staining indicated concomitant upsurges of mitoROS and apoptosis, and they were significantly lowered by the FIN treatment (Fig. 6H). Therefore, FIN, by inhibiting the activation of MR, exerted the antiapoptotic and antioxidant properties in the renal tubular epithelial cells.

4. Discussion

Proteinuria and ultrastructural glomerular lesions have always been focused when exploring the pathogenesis of DKD [25]. However, the main emphasis on the glomerular changes in DKD has partially shifted to renal tubular changes since clinical manifestations of renal disorder were highly linked to tubular interstitial fibrosis and tubular atrophy [3,26]. Treatments that particularly target pathophysiological disruptions in the renal tubules have been challenged [27]. In this study, significant lesions in the renal tubule were found in HFD/STZ-induced T2DM mice (Fig. 1G). A critical question, “Can relieving tubular injury reverse DKD progression?” needed to be addressed. The current study (Fig. 1E, F, and 1G) found that FIN, a selective non-steroidal MRA, not only ameliorated DT but also, improved renal function by lowering serum creatinine and urinary ACR. FIN also alleviated glomerular irregularities to a certain degree (Fig. 1G), indicating that renal glomeruli and tubules were inextricably linked to DKD. Chen [28] and Lee [7] et al. found that inhibition of SGLT2 by empagliflozin in diabetic renal tubular injury decreased albuminuria, BUN, and body and kidney weight in addition to reducing tubulointerstitial damage. Based on this finding, the strategy focusing on the renal tubules, specific to mitochondrial fragmentation, mitochondrial fission, and mitophagy may help understand the pathogenesis and improve treatment for DKD [7,29].

The renal tubules actively reabsorb filtered initial urine, which requires abundant energy, and the renal tubules are therefore rich in mitochondria [30]. Mitochondria maintain their homeostasis by

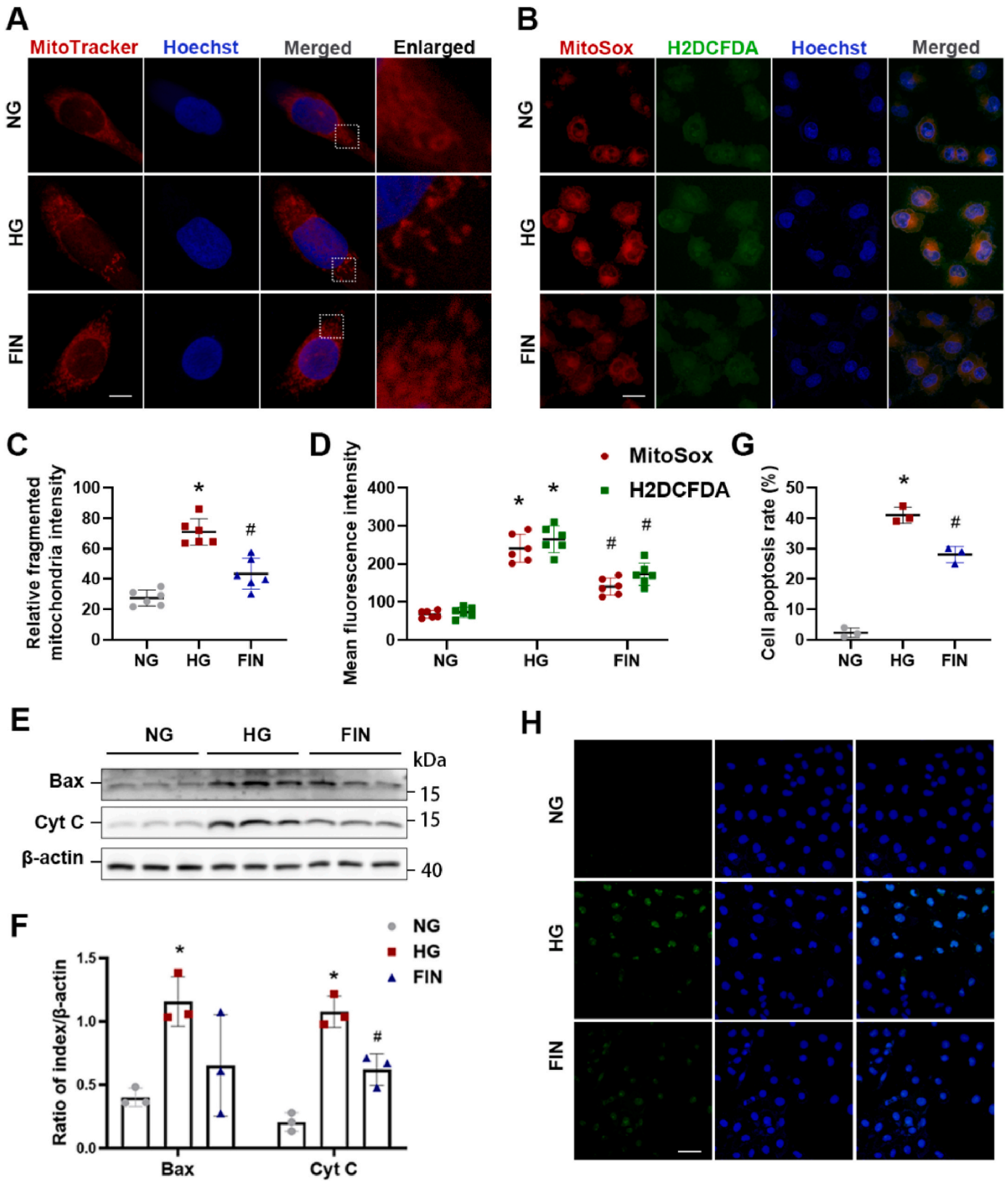

Fig. 6. Effect of finerenone on dysfunctional mitochondria, mitoROS generation, and HK-2 cell apoptosis. (A) MitoTracker (red) staining evaluated the mitochondrial morphology. Scale bar: 5 μm. (B) Mitochondrial and intracellular ROS production was assessed by H_2-DCFDA (green) or MitoSOX (red) staining. Scale bar: 20 μm. (C–D) The degree of mitochondrial fragmentation was quantified, and mitochondrial oxidative stress in the NG, HG, and FIN groups was evaluated by ImageJ (n = 6). (E–F) Quantitative analysis and representative immunoblots of the apoptosis-related proteins, Bax and Cyt C (n = 3). (G–H) TUNEL staining was used to determine the apoptosis. Scale bar: 50 μm. Quantitative analysis of the TUNEL-positive tubular cells in the above groups as evaluated by ImageJ (n = 3). Data are indicated as means ± SEM; $^{*}P<0.05$ *vs.* the CON group, $^{\#}P < 0.05$ *vs.* the HG group.

mitochondrial dynamics and kinetics, mitophagy, and mitoROS regulation, and it has been evaluated in numerous diseases [31–34], but studies on mitochondrial dysfunction in DKD, are limited. Based on some existing studies, mitochondrial homeostasis played bidirectional roles in several renal illnesses, such as DKD [35] and AKI [36]. Mitophagy and mitochondrial fusion provide cytoprotective effects during kidney damage by DKD, while mitochondrial fission precipitates the renal damage [37,38].

Our study revealed that most of mitochondria in the injured renal tubules of T2DM mice were fragmented and distributed in a disorganized manner in the cells (Fig. 3A). Furthermore, mitophagy was decreased in the tubules and the wounded mitochondria were removed less efficiently. Enhanced fragmentation of mitochondria, decreased mitophagy, and fission and fusion under the HG atmosphere were identified in the tubular cells in *in vitro* and *in vivo* experiments (Figs. 3, 5 and 6). The mitochondrial function was perturbed and resulted in an increase in mitoROS production and activation of the mitochondrial apoptotic cascade (Fig. 7).

The MR (encoded by NR3C2) is a transcription factor that binds to appropriate ligands to activate a signal cascade. MR activation has distinct negative impacts on different cell types, including cardiomyocytes, vascular smooth muscle cells, endothelial cells, adipocytes, and inflammatory cells in addition to its renal function, resulting in severe renal and CVS consequences, and thus seeking curative abilities of pharmacological MRAs [39]. A single-cell sequencing study revealed that the expression of mineralocorticoid receptors was increased in the proximal tubule in diabetic patients compared to the control kidney. In addition to inducing changes in gene expression, non-genomic effects of aldosterone have been documented in epithelial cells. This study found that the non-genomic effects of aldosterone could be crucial in the context of diabetes-induced organ injury and it was the significance and value of this research, as shown in Fig. 7, that the red pentagon represented the aldosterone, which binds to the non-classical MR pathway (expressed in the proximal tubular epithelial cells).

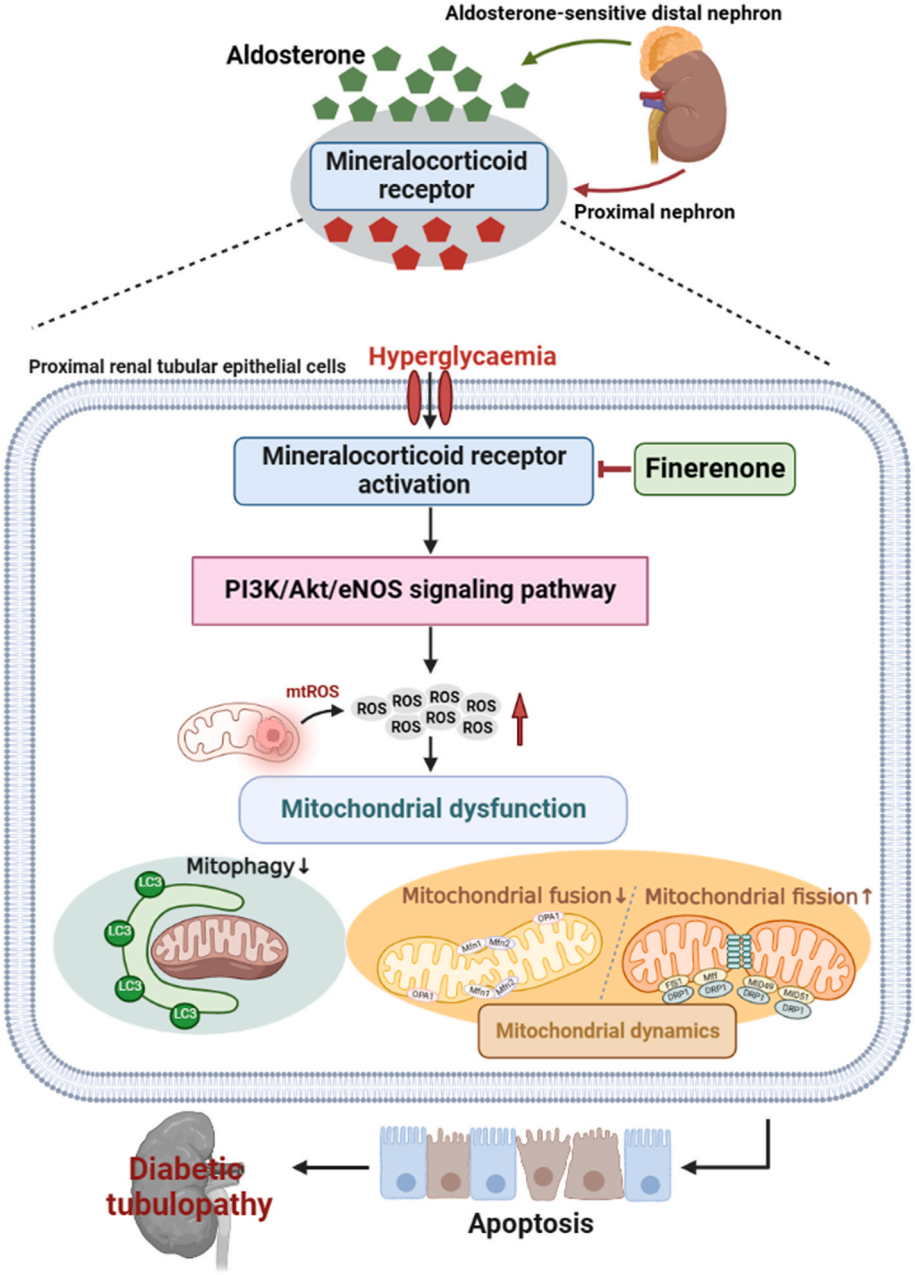

Fig. 7. Proposed model for the signaling pathway by which MR participates in the diabetic tubulopathy via PI3K/Akt/eNOS pathway.

Kolkhof et al. [40] demonstrated that FIN, but not eplerenone, enhanced the systolic and diastolic left ventricular function and decreased the brain natriuretic peptide prohormone plasma levels in rats that suffered from chronic heart failure [40]. Likewise, in a rat model of acute ischemia leading to CKD renal damage, FIN inhibited ROS generation during reperfusion, thus alleviating proteinuria and renal structural and functional injuries [41]. FIN, a selective non-steroidal MRA, is a viable drug for the treatment of DKD due to its claimed effect of MR stimulation in inflammation, oxidative stress, and fibrosis [42]. However, the mechanism via which MRAs exert the renal protective effect in DKD is unknown and poorly studied. According to the major FIDELIO-DKD clinical study, FIN delayed CKD progression and lowered the risk of adverse cardiovascular events in T2DM patients. The fundamental mechanisms through which MR activation leads to kidney damage need to be explored. As demonstrated in Figs. 2 and 4, under HG ambience, MR was increased in tubular cells, where it influenced mitochondrial dynamics, including mitophagy via PI3K/Akt/eNOS pathway. PI3K/Akt/eNOS is a traditional signaling pathway that is involved in cell development and survival as a result of extracellular signals [19,43]. PI3K phosphorylation stimulates its downstream protein Akt, phosphorylates Akt (p-Akt) and eNOS, regulating a variety of physiological functions, such as cell differentiation, propagation, migration, and apoptosis [19,44]. In rat kidney fibroblast cells, ligation of aldosterone to MR led to fast stimulation of the growth factor receptors in kidney fibroblasts and the induction of PI3K/Akt/eNOS signaling, which activated the cell proliferation [18]. In this model of T2DM, a key curative function of FIN was elucidated, which was achieved by the suppression of MR via PI3K/Akt/eNOS signaling pathway, hence normalizing the mitochondrial dysfunction and decreasing the mitoROS production and tubular cell apoptosis with the improvement in kidney functions (Figs. 1–3). Despite this investigation efforts, some difficulties remained unresolved. For example, the mechanism by which MR activation modulated the PI3K and downstream Akt/eNOS still has to be explored. What's more, whether MR activation in epithelial cells directly controls mitochondrial homeostasis is worthy of further investigation.

In conclusion, the current study found that DM activated the MR, triggering mitochondrial dysfunction. FIN prevented mitochondrial homeostasis imbalance in the DM mice or the HG-induced HK-2 cells through the PI3K/Akt/eNOS pathway, which positively contributed to an overall improvement of renal tubular function in this abnormal condition. Hence, the experimental results supported the clinical indication of FIN, exploring its therapeutic effect in alleviating tubular injury and improving overall renal function in patients whit CKD and T2DM.

Ethics approval and consent to participate

The research study was authorized by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University and adhered to the principles of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

All data will be made available upon request.

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Author contributions

Pei Wang and Zhangsuo Liu designed the study. Lan Yao, Mei Hong and Bingyu Li carried out the cell culture experiments. Lan Yao, Xin Wang, Bohan Chen and Yamin Liu performed the animal experiments. Lan Yao drafted the manuscript, and prepared the figures and tables. Pei Wang and Xianhui Liang revised the manuscript. All authors approved the final manuscript.

Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102946.

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