

Original Article

Ganoderic acid A attenuates streptozotocin-induced diabetic kidney injury: associations with mitochondrial oxidative stress and TGF- β /Smad signaling

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Abstract: Background: Diabetic kidney disease (DKD) involves renal tubular epithelial injury, mitochondrial oxidative stress, and progressive fibrosis. Ganoderic acid A (GAA) has antioxidant and anti-inflammatory activities, but its potential renoprotective effects have not been fully explored in the context of DKD. This study investigated whether GAA affects high glucose (HG)-induced tubular epithelial injury *in vitro* and streptozotocin-induced diabetic kidney injury (STZ-DKI) *in vivo*, focusing on TGF- β /Smad signaling and mitochondrial impairment. Methods: HK-2 cells were pretreated with GAA before HG exposure. Cell viability, LDH release, apoptosis, oxidative stress, mitochondrial superoxide, membrane potential, ATP levels, mtDNA copy number, complex I activity, and MitoTracker Red CMXRos fluorescence were assessed. Fibrotic responses and TGF- β /Smad signaling were examined by immunoblotting and immunofluorescence, and TGF- β 1 overexpression was used for mechanistic validation. Mice with STZ-DKI received GAA for 8 weeks, followed by assessments of renal function, histopathology, tubular injury, and protein expression. Results: GAA improved HK-2 cell viability and reduced LDH release and apoptosis under HG conditions. It decreased intracellular and mitochondrial ROS, improved mitochondrial-associated parameters, and reduced NOX1/2 while increasing SOD1/2 and COX4 expression. GAA also suppressed EMT and fibrotic markers, including α -SMA, fibronectin, and collagen IV, together with reduced TGF- β 1/Smad2/3 activation. TGF- β 1 overexpression partially reversed these effects. *In vivo*, GAA-treated mice showed improved renal functional parameters and reduced histopathological injury, tubular damage, apoptosis, and fibrosis, along with lower fasting blood glucose levels. Conclusion: GAA attenuates HG-induced renal tubular epithelial injury *in vitro* and ameliorates renal injury in STZ-induced diabetic mice, accompanied by decreased TGF- β /Smad signaling and mitochondrial oxidative stress in both models.

Keywords: Ganoderic acid A, diabetic kidney injury, mitochondrial oxidative stress, renal fibrosis, TGF- β /Smad signaling pathway

Introduction

Diabetic kidney disease (DKD) is a common and disabling microvascular complication of diabetes and a major cause of end-stage kidney disease worldwide [1]. Epidemiological studies estimate that approximately 20-40% of individuals with diabetes develop DKD during their lifetime. Clinically, DKD is generally identified by persistent albuminuria and/or a sustained reduction in estimated glomerular filtration rate (eGFR). In contrast, renal interstitial fibrosis represents an important pathological feature rather than a routine diagnostic criterion [2]. Its pathogenesis involves complex and multifactorial alterations in the glomerular,

tubular, and interstitial compartments. Among these changes, renal tubular epithelial injury, mitochondrial dysfunction, and tubulointerstitial fibrosis are considered key pathological events driving disease progression [3]. Although the use of renin-angiotensin-aldosterone system (RAAS) inhibitors, sodium-glucose cotransporter 2 (SGLT2) inhibitors, and glucagon-like peptide-1 (GLP-1) receptor agonists has improved the clinical management of DKD [4], many patients continue to exhibit residual albuminuria and progressive loss of kidney function [5, 6]. Therefore, identifying novel therapeutic targets and developing multitarget agents remain important priorities.

Aberrant activation of TGF- β /Smad signaling contributes to mitochondrial oxidative stress and plays an important role in the pathogenesis of DKD [7]. Under chronic hyperglycemic and lipotoxic conditions, dysregulated TGF- β /Smad signaling promotes excessive reactive oxygen species (ROS) production from mitochondria in renal tubular epithelial cells [8]. ROS accumulation can disrupt mitochondrial membrane potential, impair ATP production and energy metabolism, and promote tubular epithelial cell apoptosis and tubulointerstitial fibrosis [3]. TGF- β 1/Smad signaling also serves as a central regulatory pathway in renal fibrogenesis [9]. Following TGF- β 1 stimulation, phosphorylated Smad2/3 translocates into the nucleus and regulates the expression of fibrosis-related molecules, including α -smooth muscle actin (α -SMA), fibronectin, and other extracellular matrix components. Meanwhile, reduced expression of the inhibitory Smad protein Smad7 further enhances fibrotic signaling [7, 10]. Moreover, TGF- β 1-induced ROS production can reinforce TGF- β 1/Smad activation, thereby forming a positive feedback loop that amplifies oxidative stress, tubuloglomerular injury, and renal dysfunction [11-13]. Therefore, targeting TGF- β 1/Smad-mediated mitochondrial oxidative stress may represent a potential therapeutic strategy for attenuating DKD progression.

Natural compounds have attracted increasing attention in the prevention and treatment of metabolic and chronic kidney diseases because of their multitarget pharmacological activities and generally favorable safety profiles [14]. *Ganoderma lucidum* has long been used in traditional Chinese medicine, and its major bioactive constituents include polysaccharides and triterpenoids [15]. Among these constituents, ganoderic acids exhibit antioxidant, anti-inflammatory, antifibrotic, and metabolic regulatory activities [16]. Ganoderic acid A (GAA) has been reported to protect against oxidative injury in cardiac and hepatic tissues, potentially by preserving mitochondrial function [17, 18]. GAA may also modulate TGF- β signaling [19], suggesting a potential protective role in diabetes-associated renal injury. However, whether GAA attenuates mitochondrial oxidative stress and renal fibrosis and whether these effects are associated with modulation of TGF- β /Smad signaling remain unknown.

Accordingly, this study aimed to evaluate the effects of GAA against high glucose-induced renal tubular epithelial injury in HK-2 cells and streptozotocin-induced diabetic kidney injury (STZ-DKI) in mice. We further investigated whether these effects were associated with inhibition of TGF- β 1/Smad signaling, attenuation of mitochondrial oxidative stress, and suppression of profibrotic responses. This study may provide experimental evidence regarding the cellular effects of GAA and its association with improved renal injury-related parameters in diabetes-associated renal injury.

Materials and methods

Reagents and chemicals

GAA (purity $\geq 98\%$, MedChemExpress, USA, Cat# HY-N1447) was dissolved in dimethyl sulfoxide (DMSO, Gibco, USA, Cat# D12345) to prepare a stock solution, which was stored at -20°C and protected from light. For *in vitro* experiments, the stock solution was diluted with complete culture medium to the indicated concentrations, with the final DMSO concentration kept consistent across all groups. For *in vivo* experiments, GAA was diluted in sterile normal saline and administered to mice by oral gavage at the designated dose. The vehicle control group received an equivalent volume of the corresponding vehicle.

STZ (MedChemExpress, USA; Cat# HY-13753) was freshly prepared by dissolving it in a citrate buffer solution kept at pH 4.5. Normal glucose, high glucose, and osmotic control culture conditions were set up by using D-glucose (MedChemExpress, USA; Cat# HY-B0389) and D-mannitol (MedChemExpress, USA; Cat# HY-N0378).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Viability Assay Kit, Lactate Dehydrogenase (LDH) Release Assay Kit, Calcein-AM/Propidium Iodide (PI) Live/Dead Cell Staining Kit, Annexin V-APC/PI Apoptosis Detection Kit, Superoxide Dismutase (SOD) Activity Assay Kit, Glutathione Peroxidase (GSH-Px) Activity Assay Kit, Malondialdehyde (MDA) Assay Kit, ATP Assay Kit, DCFH-DA Reactive Oxygen Species Probe, MitoSOX Red Mitochondrial Superoxide Indicator, Mitochondrial Complex I Activity Assay Kit, Mito-Tracker Red CMXRos, Genomic DNA Extraction Kit,

Mitochondrial DNA Copy Number Assay Kit, JC-1 $\Delta\Psi_m$ Assay Kit and TUNEL Apoptosis Detection Kit were obtained from Beyotime Biotechnology (China; Cat# C0048S, C0016, C2013S, AP107-100, S0101S, S0056, S01-31S, S0026, S1105S, S0061S, P0423S, C1035, D0065S, D8033S, C2003S, C1170M).

For TGF- β 1 overexpression, HK-2 cells were transfected with a TGF- β 1 overexpression plasmid or the corresponding empty vector (GeneChem, China) using Lipofectamine 3000 (Invitrogen, USA; Cat# L3000008) according to the manufacturer's instructions. At 48 h after transfection, TGF- β 1 overexpression efficiency was verified by Western blotting before subsequent experiments.

Cell culture and treatments

The human renal proximal tubular epithelial cell line HK-2 was purchased from SUNNCELL (China; Cat# SNL-165). Cells were cultured in DMEM/F-12 medium (Gibco, USA; Cat# 1132-0033) supplemented with 10% fetal bovine serum (FBS; Gibco, USA; Cat# A5256701) and 1% penicillin-streptomycin (Gibco, USA; Cat# 15140122) at 37°C in a humidified incubator containing 5% CO₂. The identity of the cell line was confirmed by short tandem repeat (STR) profiling, and routine mycoplasma testing yielded negative results.

To establish an *in vitro* model of high glucose-induced renal tubular epithelial injury, HK-2 cells were cultured under normal-glucose conditions (NG; 5.5 mM D-glucose), osmotic-control conditions (OC; 5.5 mM D-glucose plus 24.5 mM D-mannitol), or high-glucose conditions (HG; 30 mM D-glucose) for 24 h [20]. Cell injury was evaluated by measuring cell viability and LDH release. The OC group was included in the preliminary cell viability and LDH release experiments to evaluate the effects of increased osmolarity. Based on the absence of significant changes in these two endpoints under OC conditions, the OC group was not carried forward into the subsequent mechanistic experiments. To determine the appropriate concentrations of GAA, cells cultured under NG or HG conditions were treated with GAA at 0, 2.5, 5, 10, 15, or 20 μ M for 24 h, and cell viability was assessed [19]. Based on the preliminary results, 5, 10, and 15 μ M GAA were selected for subsequent experiments and designated

as the HG + GAA-L, HG + GAA-M, and HG + GAA-H groups, respectively. Unless otherwise specified, cells were pretreated with GAA for 2 h before HG exposure.

For the TGF- β 1 overexpression rescue experiments, HK-2 cells were assigned to the following groups: NG, HG, HG + GAA-H, HG + GAA-H + Vector, and HG + GAA-H + TGF- β 1. Cells in the indicated groups were transfected with the TGF- β 1 overexpression plasmid or the corresponding empty vector 24 h before subsequent treatment. The cells were then pretreated with 15 μ M GAA for 2 h and exposed to HG conditions for an additional 36 h in the continued presence of GAA.

STZ-induced diabetic kidney injury model and drug administration

Eight-week-old male C57BL/6 mice were purchased from HuaFukang Bioscience Co., Ltd. (Beijing, China). The mice were housed under specific pathogen-free conditions at 22 $\pm$ 2°C and 50-60% relative humidity with a 12-h light/dark cycle and had ad libitum access to food and water. All animals were acclimatized for at least 7 days before the experiments. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University (Approval No. ZY-20250315).

Diabetes was induced by intraperitoneal injection of STZ at 50 mg/kg once daily for five consecutive days. Control mice received an equivalent volume of citrate buffer. Mice were fasted before STZ administration. After the final injection, fasting blood glucose levels were monitored, and mice with fasting blood glucose levels exceeding 16.7 mmol/L were considered diabetic. Urinary protein levels were subsequently measured to evaluate the development of diabetic kidney injury. Mice exhibiting persistent hyperglycemia and increased urinary protein excretion were considered to have successfully established STZ-DKI and were included in the study [21, 22].

Following successful establishment of the STZ-DKI model, the mice were randomly assigned to five groups (n = 6 per group): Control, Model, GAA-L, GAA-M, and GAA-H. GAA was administered once daily by oral gavage for 8 consecutive weeks. Mice in the Control and Model

groups received an equivalent volume of normal saline, whereas mice in the GAA-L, GAA-M, and GAA-H groups received GAA at 10, 20, and 30 mg/kg/day, respectively. At the experimental endpoint, 24-h urine samples, blood samples, and kidney tissues were collected. The mice were then deeply anesthetized with 3% isoflurane, and blood samples were collected. Subsequently, the mice were euthanized by cervical dislocation while under deep anesthesia. Death was confirmed by the absence of spontaneous respiration and heartbeat before kidney tissues were harvested. The left kidney was fixed in 4% paraformaldehyde for histopathological examination, immunofluorescence staining, and TUNEL analysis, whereas the right kidney was rapidly frozen in liquid nitrogen and stored at -80°C for subsequent protein and biochemical analyses.

TGF-β1 overexpression plasmid transfection

HK-2 cells were seeded in 6-well plates and cultured to approximately 60-70% confluence. Cells were then transfected with a TGF-β1 overexpression plasmid or the corresponding empty vector using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. At 24 h after transfection, TGF-β1 protein expression was assessed by Western blotting in parallel samples to confirm overexpression efficiency. The transfected cells were subsequently subjected to HG exposure, GAA treatment, and mechanistic analyses.

Molecular docking analysis

Molecular docking was performed to predict the potential interaction between GAA and the human transforming growth factor-β1 (TGF-β1) ligand. The three-dimensional structure of GAA was obtained from the PubChem database (CID: 471002), and the three-dimensional structure of human latent TGF-β1 (PDB ID: 1KLC) was downloaded from the Protein Data Bank. Water molecules were removed from the protein structure, and hydrogen atoms and charges were added before docking. Molecular docking was performed using AutoDock Vina. The docking pose with the lowest predicted binding energy was selected for interaction analysis and visualized using PyMOL.

MTT cell viability assay

HK-2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well and incubated

overnight to allow cell attachment. After the indicated treatments, MTT solution was added to each well, and the cells were incubated at 37°C for 4 h. The culture medium was then carefully removed, and the resulting formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, USA), and cell viability was determined according to the manufacturer's instructions.

LDH release assay

LDH release was assessed as an indicator of cell membrane damage. Following the indicated treatments, culture supernatants were collected and centrifuged at 300×g for 5 min to remove cell debris. The cell-free supernatants were transferred to 96-well plates and mixed with the LDH detection working solution according to the manufacturer's instructions. After incubation at room temperature for 30 min in the dark, absorbance was measured at 490 nm using a microplate reader. Relative LDH release was calculated according to the manufacturer's instructions and expressed as fold change relative to the NG group.

Calcein-AM/PI live-dead staining

Sterile glass coverslips were placed in 24-well plates before HK-2 cells were seeded. Following the indicated treatments for 24 h, the cells were incubated with Calcein-AM and PI at 37°C for 30 min in the dark. Excess staining solution was removed by washing the cells with PBS. Fluorescence images were captured using an Olympus IX73 fluorescence microscope (Olympus, Japan). Live cells exhibited green fluorescence, whereas dead cells exhibited red fluorescence. Five randomly selected fields were analyzed for each sample, and the percentage of live cells was calculated relative to the total number of Calcein-AM-positive and PI-positive cells.

Flow cytometric analysis of apoptosis

Cell apoptosis was assessed using an Annexin V-APC/PI apoptosis detection kit according to the manufacturer's instructions. Briefly, HK-2 cells subjected to the indicated treatments were collected, washed with PBS, and resuspended in binding buffer. The cell suspension was then incubated with Annexin V-APC and propidium iodide (PI) at room temperature for

15 min in the dark. Apoptosis was analyzed using a FACSCanto II flow cytometer (BD Biosciences, USA). Flow cytometric data were analyzed using FlowJo software, and the total apoptotic rate was calculated as the sum of the percentages of early and late apoptotic cells.

Measurement of oxidative stress-related indicators and ATP

After the 36-h treatment, the culture medium was removed, and HK-2 cells were washed twice with ice-cold PBS and lysed in an appropriate lysis buffer. The cell lysates were centrifuged at 4°C to remove cellular debris. SOD and GSH-Px activities and MDA levels were measured using commercial assay kits according to the manufacturers' instructions. Intracellular ATP levels were determined using a luminescence-based ATP assay kit.

For kidney tissue analysis, an appropriate amount of right kidney tissue was weighed and homogenized in ice-cold homogenization buffer on ice. The tissue homogenates were centrifuged at 4°C, and the resulting supernatants were collected for measurement of SOD and GSH-Px activities, MDA levels, and ATP content using the corresponding assay kits according to the manufacturers' instructions.

Measurement of mitochondrial DNA copy number

Total DNA was extracted from HK-2 cells after the indicated treatments using a genomic DNA extraction kit. Following the indicated treatments, total DNA was extracted from HK-2 cells and used as the template for quantitative real-time PCR. Mitochondrial DNA and nuclear DNA were amplified using the primer mixtures supplied with the kit. Relative mitochondrial DNA copy number was calculated as the mtDNA/nDNA ratio according to the manufacturer's instructions and normalized to the NG group.

Measurement of mitochondrial complex I activity

Following the indicated treatments, HK-2 cells were collected and homogenized in the extraction buffer supplied with the kit. After centrifugation, the supernatant was collected, and the protein concentration was determined. Equal amounts of protein from each sample were added to a 96-well plate and mixed with the

reaction reagents according to the manufacturer's instructions. Absorbance changes were monitored using a microplate reader, and mitochondrial complex I activity was calculated according to the kit protocol and normalized to the total protein concentration. The results were expressed relative to the NG group.

Detection of intracellular and mitochondrial ROS

ROS accumulation was detected using the DCFH-DA fluorescent probe according to the manufacturer's instructions. After the indicated treatments for 36 h, HK-2 cells were incubated with DCFH-DA at 37°C for 20 min in the dark. The cells were then washed three times with PBS to remove excess probe. Fluorescence images were captured using a fluorescence microscope, and the fluorescence intensity was quantified using ImageJ software. Intracellular ROS levels were expressed as relative fluorescence intensity.

Mitochondrial superoxide production was assessed using MitoSOX Red according to the manufacturer's instructions. Following the indicated treatments for 36 h, HK-2 cells were incubated with MitoSOX Red at 37°C for 10 min in the dark. After three washes with PBS, fluorescence images were captured using a fluorescence microscope. The red fluorescence intensity was quantified using ImageJ software and expressed as relative fluorescence intensity.

MitoTracker red CMXRos staining

Following the indicated treatments, HK-2 cells were incubated with 100 nM Mito-Tracker Red CMXRos diluted in prewarmed culture medium for 30 min at 37°C in the dark. After staining, the cells were washed twice with prewarmed PBS and replenished with fresh culture medium. Fluorescence images were captured using a fluorescence microscope under identical acquisition settings. Five randomly selected fields were analyzed for each sample using ImageJ software, and the relative MitoTracker Red CMXRos fluorescence intensity was quantified and normalized to the NG group.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was assessed using a commercial JC-1 staining kit according to the manufacturer's

instructions. Following the indicated treatments for 36 h, HK-2 cells were incubated with JC-1 staining solution at 37°C for 20 min in the dark. The cells were then washed twice with JC-1 staining buffer. Fluorescence images of JC-1 aggregates (red fluorescence) and monomers (green fluorescence) were captured using a fluorescence microscope and subsequently merged. Five randomly selected fields were analyzed for each sample using ImageJ software. $\Delta\Psi_m$ was evaluated by calculating the ratio of red to green fluorescence intensity.

Morphological observation

Following the indicated treatments for 36 h, morphological changes in HK-2 cells were observed and photographed using an inverted microscope. Particular attention was paid to HG-induced EMT-like morphological changes, including cell spreading, acquisition of a spindle-shaped morphology, and reduced cell-cell contact, as well as the effects of GAA treatment on these changes.

Immunofluorescence staining

HK-2 cells cultured on sterile coverslips were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 5% BSA at room temperature for 1 h. The cells were then incubated overnight at 4°C with the following primary antibodies: rabbit monoclonal anti- α -SMA antibody (Abcam, UK; Cat# ab124964; 1:200), rabbit polyclonal anti-TGF- β 1 antibody (Abcam, UK; Cat# ab112095; 1:200), and rabbit polyclonal anti-Smad2/3 antibody (CST, USA; Cat# 8685S; 1:200). After washing with PBS, the cells were incubated with the corresponding fluorophore-conjugated secondary antibodies (1:500) at room temperature for 1 h in the dark. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, and fluorescence images were captured using a fluorescence microscope. Five randomly selected fields were analyzed for each sample. The mean fluorescence intensities of α -SMA and TGF- β 1 were quantified over the entire field using ImageJ software, whereas the nuclear fluorescence intensity of Smad2/3 was quantified separately to assess its nuclear accumulation.

For kidney tissue staining, 4- μ m paraffin-embedded sections were deparaffinized in

xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed in citrate buffer, after which the sections were cooled to room temperature and washed with PBS. The sections were then permeabilized with 0.1% Triton X-100 for 10 min, blocked with 5% BSA for 1 h, and incubated overnight at 4°C with anti-TGF- β 1 or anti-Smad2/3 primary antibodies. After washing, the sections were incubated with the corresponding fluorophore-conjugated secondary antibodies for 1 h in the dark, followed by nuclear counterstaining with DAPI. Five randomly selected fields were imaged for each section and analyzed using ImageJ software. TGF- β 1 expression was quantified as the mean fluorescence intensity, whereas Smad2/3 nuclear accumulation was evaluated by measuring its nuclear fluorescence intensity.

To assess renal tubular epithelial injury, kidney sections were incubated overnight at 4°C with a rabbit monoclonal anti-KIM-1 antibody (Abcam, UK; Cat# ab316854; 1:2000). After washing, the sections were incubated with a goat anti-rabbit IgG H&L secondary antibody conjugated to Alexa Fluor 488 (Abcam, UK; Cat# ab150081; 1:1000) at room temperature in the dark. Nuclei were counterstained with DAPI. Fluorescence images were captured under identical imaging conditions. Five randomly selected cortical fields were analyzed for each section, and KIM-1 expression was quantified as the relative fluorescence intensity using ImageJ software.

Western blot analysis

HK-2 cells and kidney tissues were lysed in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors. Kidney tissues were thoroughly homogenized in the lysis buffer on ice. The lysates were centrifuged at 12,000 $\times$ g for 15 min at 4°C, and the resulting supernatants were collected. Protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein (20 μ g) were separated by SDS-PAGE and transferred onto methanol-activated PVDF membranes. The membranes were blocked at room temperature for 1 h with 5% non-fat milk or 5% BSA; 5% BSA was used for the detection of phosphorylated proteins. The membranes were then incubated with the corresponding primary anti-

bodies overnight at 4°C. After three washes with TBST for 5 min each, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies at room temperature for 1 h. Protein bands were visualized using ECL reagents, and band intensities were quantified using ImageJ software. The relative expression levels of total proteins were calculated by normalization to GAPDH. Phosphorylated protein levels were expressed as the ratio of the phosphorylated protein to its corresponding total protein. Caspase-3 activation was evaluated as the ratio of Cleaved Caspase-3 to total Caspase-3.

The primary antibodies used in this study were as follows: Bcl-2 (Abcam, UK, Cat# ab182858; 1:1000), Bax (Abcam, UK, Cat# ab32503; 1:1000), Cleaved Caspase-3 (Abcam, UK, Cat# ab32042; 1:1000), Caspase-3 (Abcam, UK, Cat# ab32351; 1:1000), NOX1 (Abcam, UK, Cat# ab131088; 1:1000), NOX2 (Abcam, UK, Cat# ab310337; 1:1000), SOD1 (Abcam, UK, Cat# ab308181; 1:1000), SOD2 (Abcam, UK, Cat# ab68155; 1:1000), α -SMA (Abcam, UK, Cat# ab124964; 1:1000), Fibronectin (Abcam, UK, Cat# ab45688; 1:1000), Collagen IV (Abcam, UK, Cat# ab6586; 1:1000), TGF- β 1 (Abcam, UK, Cat# ab215715; 1:1000), Smad2 (Abcam, UK, Cat# ab40855; 1:1000), p-Smad2 (Abcam, UK, Cat# ab280888; 1:1000), Smad3 (Abcam, UK, Cat# ab40854; 1:1000), p-Smad3 (Abcam, UK, Cat# ab63403; 1:1000), as well as GAPDH (Abcam, UK, Cat# ab181602, 1:10000) as the internal loading control.

Detection of ROS in renal tissues

Fresh kidney tissues were embedded in optimal cutting temperature (OCT) compound, rapidly frozen, and sectioned at a thickness of 8 μ m using a cryostat. The frozen sections were mounted on glass slides, air-dried at room temperature for approximately 15 min, and gently washed three times with PBS.

To assess renal ROS accumulation and mitochondrial superoxide production, the sections were incubated with DCFH-DA working solution for 30 min or MitoSOX Red working solution for 10 min at 37°C in the dark, respectively. The sections were then washed three times with PBS for 5 min each to remove excess fluorescent probes. Fluorescence images were captured using a fluorescence microscope under

identical imaging conditions. Five randomly selected fields were analyzed for each sample, and the mean fluorescence intensity was quantified using ImageJ software.

Biochemical analysis of serum and urine samples

At the experimental endpoint, 24-h urine samples were collected from individual mice using metabolic cages. Blood samples were subsequently collected from the retro-orbital venous plexus and allowed to clot at room temperature, followed by centrifugation to obtain serum. Urinary albumin and creatinine concentrations were measured using commercial assay kits, and the urinary albumin-to-creatinine ratio (UACR) was calculated. Serum creatinine (CRE), blood urea nitrogen (BUN), and insulin levels were also measured using the corresponding commercial kits. As an exploratory metabolic index, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following formula: $\text{HOMA-IR} = \text{fasting blood glucose (mmol/L)} \times \text{fasting insulin (mU/L)} / 22.5$ [23]. Body and kidney weights were recorded, and the kidney-to-body weight ratio was calculated. All biochemical assays were performed according to the manufacturers' instructions.

Histopathological staining

Kidney tissues were fixed overnight in 4% paraformaldehyde, dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin. Paraffin-embedded tissues were cut into 4- μ m sections, deparaffinized in xylene twice for 10 min each, and rehydrated through a descending ethanol series from 100% to 75% for 5 min at each concentration. The sections were then subjected to hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome staining. For H&E staining, the sections were stained with hematoxylin for 5 min, differentiated, blued, and counterstained with eosin for 3 min. For PAS staining, the sections were oxidized with periodic acid for 5 min, incubated with Schiff reagent for 15 min, and counterstained with hematoxylin. Masson's trichrome staining was performed to visualize collagen deposition. After staining, the sections were dehydrated, cleared, mounted with neutral mounting medium, and examined under a light microscope.

Images were acquired under identical settings. Five non-overlapping cortical fields per section and at least 10 intact glomeruli per mouse were analyzed.

H&E staining was used to evaluate renal histopathological changes, including glomerular hypertrophy, tubular epithelial injury, tubular lumen dilation, and interstitial inflammatory cell infiltration. Renal histological injury was graded using a semi-quantitative scale from 0 to 4: 0, normal; 1, mild injury involving < 25% of the examined area; 2, moderate injury involving 25-50%; 3, severe injury involving 50-75%; and 4, extensive injury involving > 75% [24].

PAS staining was used to assess glomerular matrix deposition and mesangial expansion. Mesangial matrix expansion was scored as follows: 0, normal; 1, mild expansion involving < 25% of the mesangial area; 2, moderate expansion involving 25-50%; 3, severe expansion involving 50-75%; and 4, extensive expansion involving > 75% [25].

Masson's trichrome staining was used to evaluate renal interstitial fibrosis. The fibrotic area was quantified using ImageJ software and expressed as the percentage of blue-stained collagen area relative to the total tissue area [26].

TUNEL fluorescence assay

Apoptotic cells in kidney tissues were detected using a TUNEL assay kit according to the manufacturer's instructions. Briefly, paraffin-embedded kidney sections were deparaffinized, rehydrated, and treated with proteinase K. The sections were then incubated with the TUNEL reaction mixture at 37°C for 1 h in the dark, followed by nuclear counterstaining with DAPI. Fluorescence images were captured using a fluorescence microscope under identical imaging conditions. Five randomly selected non-overlapping fields per section were analyzed using ImageJ software, and the percentage of TUNEL-positive cells was calculated relative to the total number of DAPI-positive cells.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 software. Continuous data are presented as the mean $\pm$ standard deviation (SD).

Cell viability data involving both treatment group and exposure time, as shown in **Figure 1A**, were analyzed using two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. The comparison between the Vector and TGF- β 1 overexpression groups in the transfection-efficiency experiment was performed using an unpaired two-tailed Student's t-test. Other comparisons among three or more independent groups were performed using one-way ANOVA followed by Tukey's multiple-comparison test. Renal histological injury scores and mesangial expansion scores were analyzed using the Kruskal-Wallis test followed by Dunn's multiple-comparison test. *In vitro* experiments were independently repeated at least three times, and six mice were included in each animal group. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

GAA attenuates high glucose-induced injury and apoptosis in HK-2 cells

To evaluate whether GAA protects renal tubular epithelial cells against HG-induced injury, an *in vitro* model of high glucose-induced renal tubular epithelial injury was established using HK-2 cells. MTT assay results showed that HG treatment significantly reduced HK-2 cell viability in a time-dependent manner at 24 and 48 h compared with the NG group; OC treatment showed no significant effect on cell viability or LDH release at either time point (**Figure 1A**). LDH release into the culture supernatant was subsequently measured, as increased LDH release reflects plasma membrane damage and cytotoxicity [27]. The OC group showed no significant change in LDH release compared with the NG group, whereas HG treatment significantly increased LDH release (**Figure 1B**). These findings suggest that the HG-induced decrease in cell viability and increase in LDH release were not caused by increased osmolarity alone.

We next evaluated the cytotoxicity and protective effects of GAA. GAA at concentrations up to 20 μ M showed no significant cytotoxicity toward HK-2 cells, whereas it significantly attenuated the HG-induced reduction in cell viability (**Figure 1C, 1D**). Further experiments showed that low, medium, and high concentrations of GAA significantly increased cell viability under HG con-

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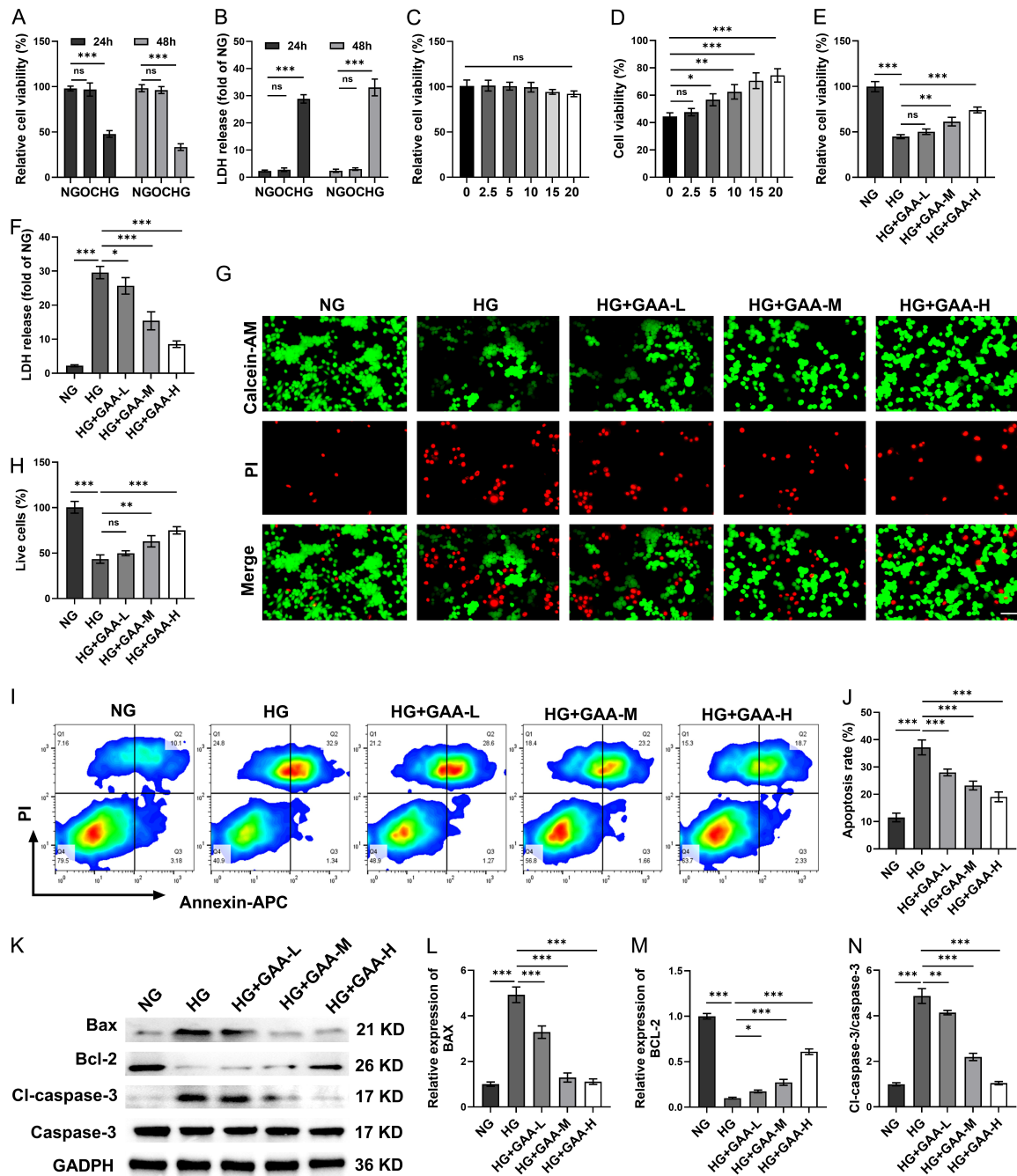


Figure 1. Ganoderic acid A (GAA) attenuates high glucose-induced cellular injury and apoptosis. (A, B) Cell viability (A) and lactate dehydrogenase (LDH) release (B) were measured after HK-2 cells were cultured under normal-glucose (NG), osmotic-control (OC), or high-glucose (HG) conditions for 24 or 48 h. (C, D) The effects of different concentrations of GAA (0, 2.5, 5, 10, 15, and 20 μM) on HK-2 cell viability under NG (C) and HG (D) conditions. (E, F) Cell viability (E) and LDH release (F) were measured in the NG, HG, HG + GAA-L, HG + GAA-M, and HG + GAA-H groups using MTT and LDH assays, respectively. GAA-L, GAA-M, and GAA-H represent 5, 10, and 15 μM GAA, respectively. (G, H) Representative images of Calcein-AM/PI live/dead staining (G; scale bar = 50 μm) and quantification of the percentage of viable cells (H). (I, J) Cell apoptosis was assessed by Annexin V-APC/PI flow cytometry (I), and the total apoptotic rate was quantified (J). (K) Western blot analysis of Bax, Bcl-2, total caspase-3, and cleaved caspase-3 protein expression. (L-N) Densitometric quantification of Bax (L), Bcl-2 (M), and the ratio of cleaved caspase-3 to total caspase-3 (N). ns, not statistically significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

ditions (**Figure 1E**) and reduced HG-induced LDH release (**Figure 1F**). Calcein-AM/PI staining, which distinguishes live cells from dead cells [28], further showed that HG exposure markedly decreased the proportion of viable cells, whereas GAA pretreatment increased the proportion of viable cells (**Figure 1G, 1H**). Consistently, Annexin V-APC/PI flow cytometry demonstrated that GAA reduced the HG-induced total apoptotic rate in a concentration-dependent manner (**Figure 1I, 1J**).

Bcl-2, Bax, and cleaved caspase-3 are important regulators of mitochondria-mediated apoptosis [29]. Western blot analysis showed that HG exposure increased the expression of the pro-apoptotic proteins Bax and cleaved caspase-3 while decreasing the expression of the anti-apoptotic protein Bcl-2 (**Figure 1K-N**). These HG-induced alterations in apoptosis-related proteins were significantly reversed by GAA treatment. Collectively, these findings indicate that GAA attenuates HG-induced injury and apoptosis in HK-2 cells.

GAA attenuates HG-induced mitochondrial oxidative stress and mitochondrial impairment

Mitochondrial oxidative stress and dysfunction contribute importantly to renal tubular injury during DKD progression [30]. To investigate whether GAA regulates oxidative stress and cellular energy metabolism, we first measured oxidative stress-related biochemical indicators and ATP levels in HK-2 cells. SOD and GSH-Px are important antioxidant enzymes, whereas MDA is commonly used as an indicator of lipid peroxidation [31]. Compared with the NG group, HG exposure significantly decreased SOD and GSH-Px activities, increased MDA levels, and reduced ATP levels, indicating enhanced oxidative stress and impaired cellular energy metabolism. In contrast, pretreatment with low, medium, and high concentrations of GAA increased SOD and GSH-Px activities, decreased MDA levels, and increased ATP levels under HG conditions (**Figure 2A-D**).

To further assess mitochondrial-associated functional changes, mtDNA copy number and mitochondrial complex I activity were measured. HG exposure significantly decreased the mtDNA/nDNA ratio and complex I activity compared with the NG group, whereas GAA pretreatment attenuated these reductions in a concentration-dependent manner (**Figure 2E, 2F**).

We next evaluated intracellular ROS accumulation and mitochondrial superoxide production using fluorescent probes. DCFH-DA staining, which is commonly used to assess intracellular ROS levels [32], showed that HG exposure markedly increased green fluorescence intensity, whereas GAA pretreatment reduced intracellular ROS accumulation in a concentration-dependent manner (**Figure 2G, 2H**). Consistently, MitoSOX Red staining showed that HG increased mitochondrial superoxide production, which was significantly attenuated by GAA pretreatment (**Figure 2I, 2J**). MitoTracker Red CMXRos staining further showed that HG exposure markedly reduced the MitoTracker Red CMXRos fluorescence intensity, whereas GAA pretreatment progressively increased this signal, suggesting attenuation of the HG-induced loss of mitochondrial-associated features (**Figure 2K, 2L**).

Because $\Delta\Psi_m$ is an important indicator of mitochondrial functional status [33], JC-1 staining was further performed. HG exposure markedly decreased the red-to-green fluorescence intensity ratio, indicating a reduction in $\Delta\Psi_m$ and mitochondrial dysfunction. GAA pretreatment significantly attenuated the HG-induced reduction in $\Delta\Psi_m$ (**Figure 2M, 2N**).

NOX1 and NOX2 are important ROS-generating enzymes [34], whereas SOD1 and SOD2 contribute to antioxidant defense, and COX4 is closely associated with mitochondrial respiratory function [35]. Western blot analysis showed that HG exposure increased NOX1 and NOX2 protein expression while decreasing the expression of SOD1, SOD2, and COX4 compared with the NG group. These changes were significantly reversed by GAA treatment, as reflected by reduced NOX1 and NOX2 expression and increased SOD1, SOD2, and COX4 levels (**Figure 2O-T**). Collectively, these findings indicate that GAA attenuates HG-induced oxidative stress and ameliorates mitochondrial impairment, as evidenced by improvements in mtDNA copy number, complex I activity, ATP levels, MitoTracker Red CMXRos fluorescence intensity, $\Delta\Psi_m$, and oxidative stress-related markers.

GAA attenuates HG-induced EMT-like and pro-fibrotic changes in HK-2 cells

EMT-like changes and ECM accumulation are important features associated with renal inter-

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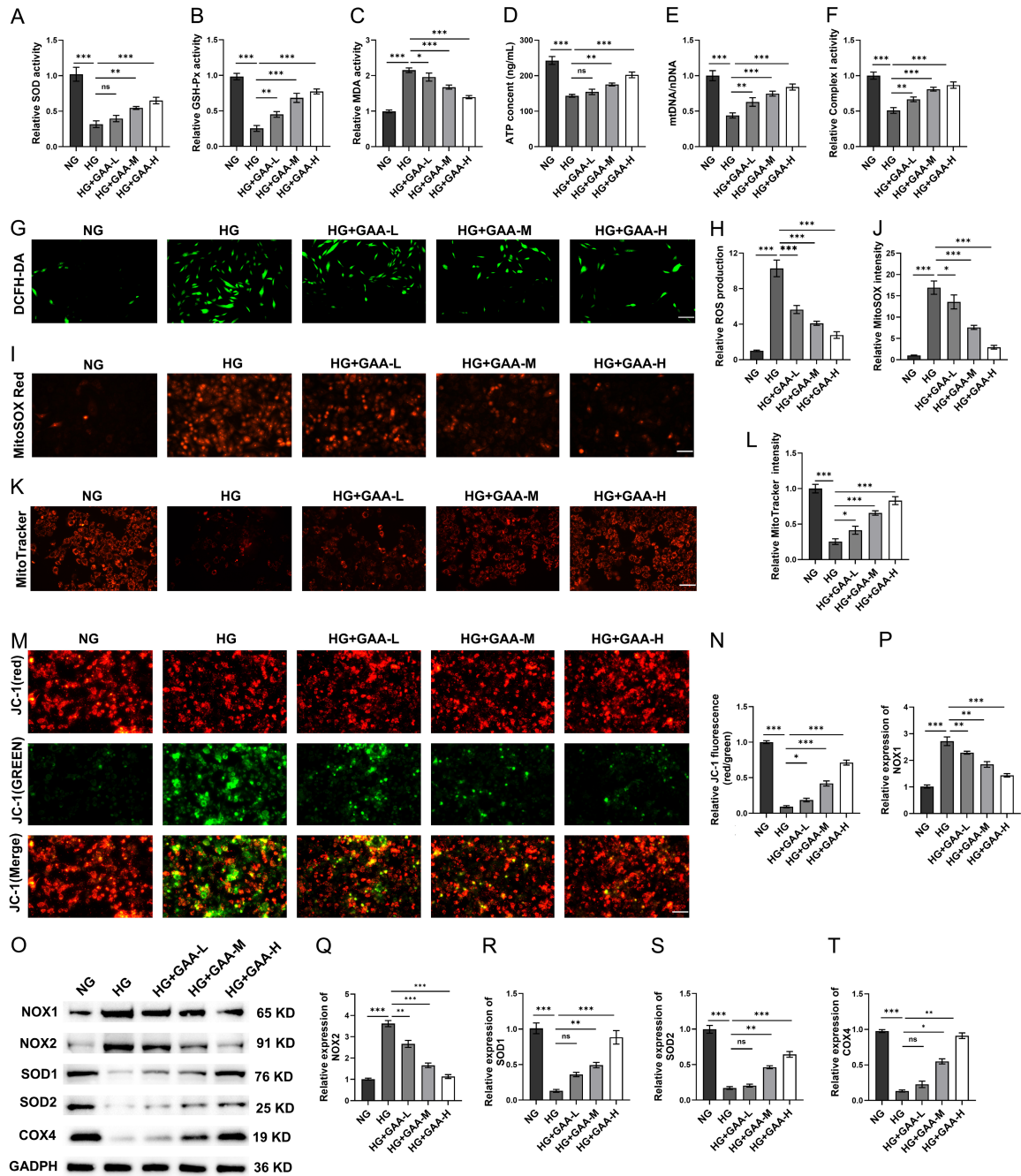


Figure 2. GAA alleviates high glucose-induced oxidative stress and mitochondrial dysfunction. (A-D) Superoxide dismutase (SOD) activity (A), glutathione peroxidase (GSH-Px) activity (B), malondialdehyde (MDA) levels (C), and ATP levels (D) were measured in the indicated groups (NG, HG, HG + GAA-L, HG + GAA-M, and HG + GAA-H) using commercial assay kits. (E) Mitochondrial DNA copy number was evaluated based on the mtDNA/nDNA ratio. (F) Mitochondrial complex I activity was measured using a commercial assay kit. (G, H) Intracellular ROS accumulation was detected using the DCFH-DA fluorescent probe (G; scale bar = 50 μ m), and the relative fluorescence intensity was quantified (H). (I, J) Mitochondrial superoxide production was assessed by MitoSOX Red staining (I; scale bar = 50 μ m), and the mean fluorescence intensity was quantified (J). (K, L) MitoTracker Red CMXRos staining images are shown (K; scale bar = 50 μ m), and the relative fluorescence intensity was quantified (L). (M, N) Mitochondrial membrane potential ($\Delta\Psi$ m) was assessed by JC-1 staining (M; scale bar = 50 μ m), and the red-to-green fluorescence intensity ratio was quantified (N). (O-T) Representative Western blot images of NOX1, NOX2, SOD1, SOD2, and COX4 (O), with the corresponding protein levels normalized to GAPDH (P-T). ns, not statistically significant; * P < 0.05, ** P < 0.01, *** P < 0.001.

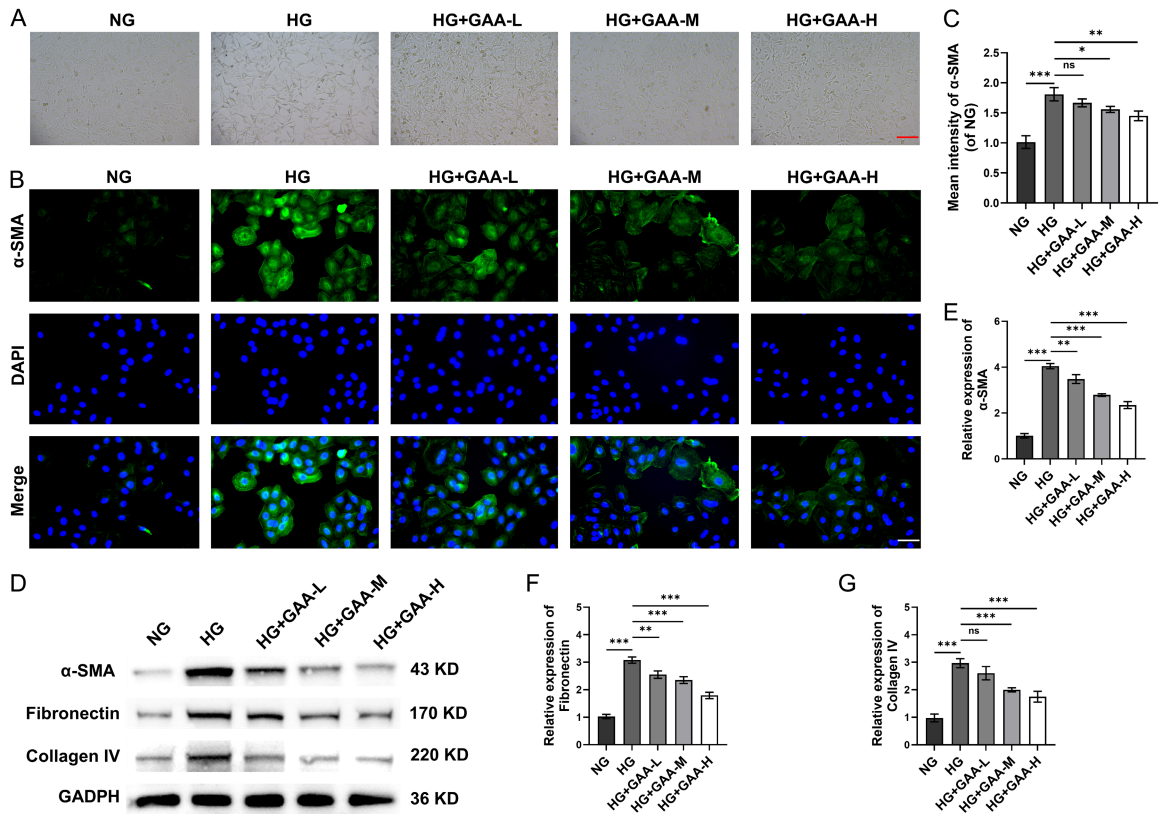


Figure 3. GAA attenuates high glucose-induced fibrotic phenotype in HK-2 cells. (A) Representative phase-contrast images showing morphological changes in HK-2 cells in the indicated groups (NG, HG, HG + GAA-L, HG + GAA-M, and HG + GAA-H) (scale bar = 200 μm). (B, C) Representative immunofluorescence images of α-SMA expression (B; scale bar = 50 μm) and quantification of the relative fluorescence intensity (C). (D-G) Representative Western blot images of α-SMA, fibronectin, and collagen IV expression (D), with densitometric quantification of α-SMA (E), fibronectin (F), and collagen IV (G) normalized to GAPDH. ****P* < 0.001.

stitial fibrosis [36]. Under phase-contrast microscopy, HK-2 cells in the NG group exhibited a typical cobblestone-like epithelial morphology. After 36 h of HG exposure, the cells became elongated, showed reduced cell-cell contact, and acquired a spindle-shaped, fibroblast-like morphology, indicating EMT-like phenotypic changes. These morphological alterations were markedly attenuated by GAA pretreatment, with more cells retaining an epithelial-like appearance (Figure 3A).

α-SMA, a marker associated with acquisition of a myofibroblast-like phenotype [37], was further examined by immunofluorescence staining. HG exposure significantly increased α-SMA fluorescence intensity, whereas GAA pretreatment markedly reduced this increase (Figure 3B, 3C). Western blot analysis further showed that HG exposure increased the protein levels of α-SMA, fibronectin, and collagen IV com-

pared with the NG group. Among these proteins, fibronectin and collagen IV are major ECM components, whereas α-SMA reflects a profibrotic phenotypic transition. GAA treatment significantly reduced the HG-induced expression of these fibrosis-related proteins (Figure 3D-G). Collectively, these findings indicate that GAA attenuates HG-induced EMT-like changes and profibrotic responses in HK-2 cells.

GAA suppresses HG-induced activation of TGF-β/Smad signaling

To investigate the mechanism underlying the antifibrotic effects of GAA, molecular docking, immunofluorescence staining, and Western blotting were performed to evaluate the potential relationship between GAA and TGF-β/Smad signaling. Molecular docking analysis suggested a potential interaction between GAA and

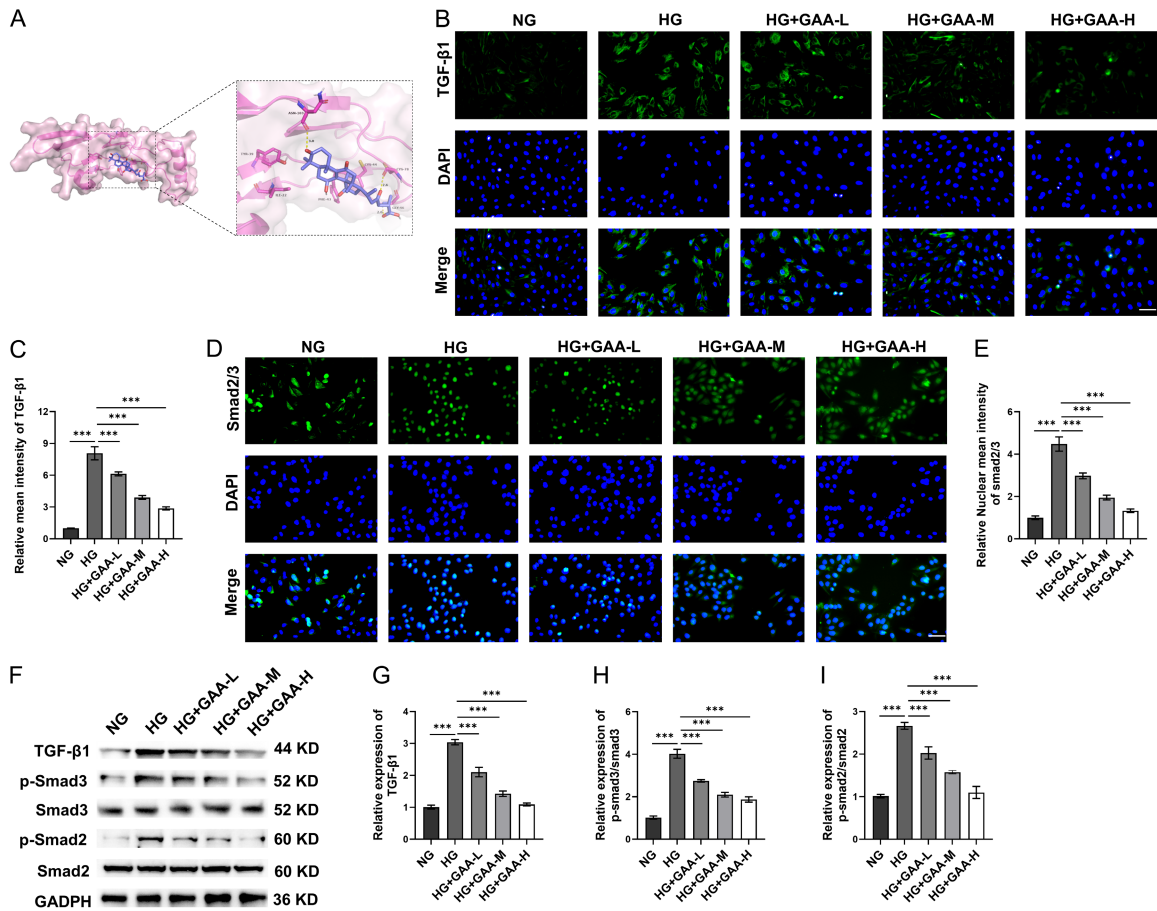


Figure 4. GAA attenuates high glucose-induced activation of TGF-β/Smad signaling in HK-2 cells. (A) Predicted binding mode of GAA within a binding pocket on the TGF-β1 ligand obtained by molecular docking analysis. (B, C) Representative immunofluorescence images of TGF-β1 expression in HK-2 cells from the indicated groups (B; scale bar = 50 μm) and quantification of the relative fluorescence intensity (C). (D, E) Representative immunofluorescence images showing Smad2/3 nuclear accumulation (D; scale bar = 50 μm) and quantification of the mean nuclear fluorescence intensity (E). (F-I) Representative Western blot images of TGF-β1, p-Smad3, Smad3, p-Smad2, and Smad2 expression (F). TGF-β1 protein expression was normalized to GAPDH (G), whereas Smad3 and Smad2 phosphorylation levels were expressed as the ratios of p-Smad3/Smad3 (H) and p-Smad2/Smad2 (I), respectively. ****P* < 0.001.

TGF-β1, with GAA fitting into a predicted surface pocket on TGF-β1 and forming multiple noncovalent contacts (Figure 4A).

TGF-β1 is a major profibrotic cytokine that promotes renal fibrosis by inducing Smad2/3 phosphorylation and nuclear translocation, thereby regulating the transcription of fibrosis-related genes [38]. Immunofluorescence staining showed that HG exposure significantly increased TGF-β1 fluorescence intensity in HK-2 cells, whereas GAA pretreatment attenuated this increase (Figure 4B, 4C). HG exposure also promoted Smad2/3 nuclear accumulation, as indicated by increased nuclear fluorescence intensity, which was markedly reduced by GAA

pretreatment (Figure 4D, 4E). Consistently, Western blot analysis showed that HG exposure increased TGF-β1 protein expression and elevated the ratios of p-Smad3/Smad3 and p-Smad2/Smad2, whereas GAA pretreatment significantly reversed these changes (Figure 4F-I), indicating activation of TGF-β/Smad signaling. GAA treatment significantly reduced TGF-β1 expression and decreased the phosphorylation levels of Smad2 and Smad3 under HG conditions.

Collectively, molecular docking suggested a potential interaction between GAA and the TGF-β ligand, while the cellular experiments demonstrated that GAA attenuated HG-induced

activation of TGF- β /Smad signaling by reducing TGF- β 1 expression, Smad2/3 phosphorylation, and Smad2/3 nuclear accumulation.

TGF- β 1 overexpression partially reverses the protective effects of GAA against HG-induced injury

To assess whether TGF- β 1/Smad signaling contributes to the protective effects of GAA, TGF- β 1 was transiently overexpressed in HK-2 cells. Western blot analysis confirmed that TGF- β 1 protein expression was significantly increased in cells transfected with the TGF- β 1 overexpression plasmid compared with those transfected with the empty vector (**Figure 5A, 5B**). TGF- β 1 overexpression also increased the phosphorylation levels of Smad2 and Smad3 and partially reversed the inhibitory effects of GAA on TGF- β /Smad signaling under HG conditions (**Figure 5C-F**).

Functional analyses showed that GAA markedly reduced HG-induced apoptosis, whereas TGF- β 1 overexpression partially reversed this protective effect (**Figure 5G, 5H**). TGF- β 1 overexpression also attenuated the protective effects of GAA on mitochondrial function, as evidenced by increased intracellular ROS accumulation (**Figure 5I, 5J**) and mitochondrial superoxide production (**Figure 5K, 5L**), as well as decreased ATP levels (**Figure 5M**). TGF- β 1 overexpression also partially reversed the GAA-induced increases in mtDNA copy number and mitochondrial complex I activity (**Figure 5N, 5O**) and reduced MitoTracker fluorescence intensity (**Figure 5P, 5Q**). JC-1 staining further showed that TGF- β 1 overexpression attenuated the GAA-mediated improvement in mitochondrial membrane potential (**Figure 5R, 5S**). Moreover, TGF- β 1 overexpression partially restored the expression of the fibrosis-related proteins α -SMA, fibronectin, and collagen IV, which had been reduced by GAA, while attenuating the GAA-induced increase in COX4 expression (**Figure 5T-X**).

Collectively, these findings indicate that the protective effects of GAA against HG-induced mitochondrial oxidative stress, mitochondrial impairment, apoptosis, and profibrotic responses are at least partially mediated through inhibition of TGF- β 1/Smad signaling.

GAA improves renal functional parameters and attenuates histopathological injury in mice with STZ-DKI

To evaluate the protective effects of GAA *in vivo*, renal functional parameters and histopathological changes were examined in mice with STZ-DKI. UACR, serum creatinine (CRE), and blood urea nitrogen (BUN) are commonly used indicators of renal dysfunction [39]. Compared with the Control group, the Model group exhibited significantly increased UACR, CRE, and BUN levels. GAA treatment, particularly at the medium and high doses, significantly reduced these renal functional parameters (**Figure 6A-C**). In addition, the Model group showed lower serum insulin levels, higher HOMA-IR values, and an increased kidney-to-body weight ratio compared with the Control group. GAA treatment increased serum insulin levels and reduced HOMA-IR values and the kidney-to-body weight ratio to varying degrees (**Figure 6D-F**). Serum insulin and HOMA-IR are presented as descriptive metabolic parameters only, as the repeated low-dose STZ model primarily reflects β -cell injury rather than insulin resistance.

Histopathological examination further showed that GAA attenuated STZ-induced renal pathological changes. H&E staining revealed marked renal structural abnormalities in the Model group, including glomerular hypertrophy, tubular epithelial injury, tubular lumen dilation, and interstitial inflammatory cell infiltration. These pathological changes and the corresponding histological injury scores were significantly alleviated by GAA treatment (**Figure 6G, 6H**). TUNEL staining showed a marked increase in apoptotic cells in the kidneys of Model mice, whereas GAA treatment significantly reduced the proportion of TUNEL-positive cells (**Figure 6I, 6J**). PAS staining revealed increased mesangial matrix deposition and mesangial expansion in the Model group, whereas Masson's trichrome staining showed extensive collagen deposition in the renal tubulointerstitial region. GAA treatment significantly reduced the mesangial expansion scores and fibrotic areas (**Figure 6K-N**). To further assess renal tubular epithelial injury, immunofluorescence staining was performed for KIM-1, a marker of tubular injury. KIM-1 fluorescence intensity was markedly increased in the kidneys of Model mice and

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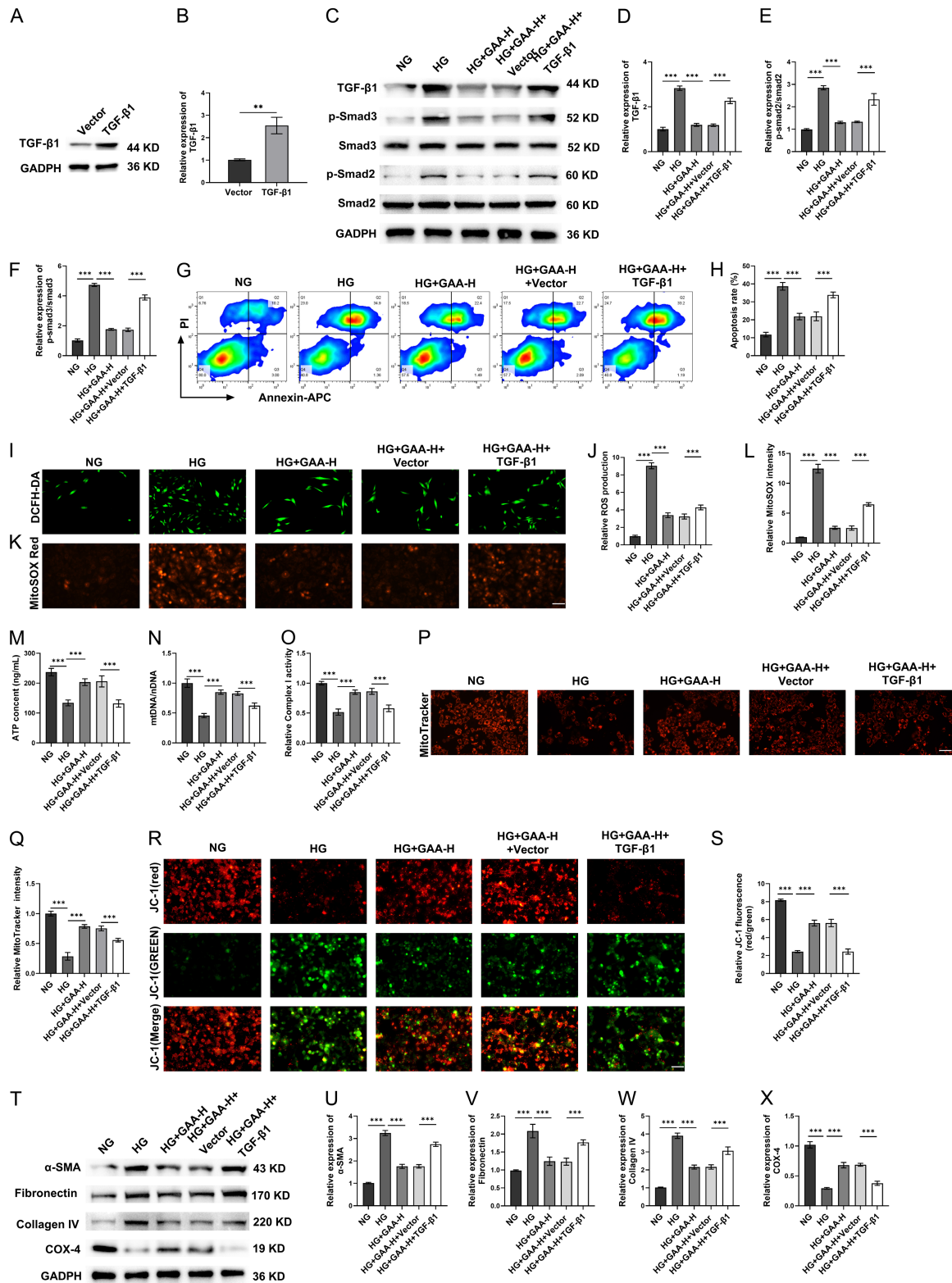


Figure 5. TGF-β1 overexpression reverses the protective effects of GAA against high glucose-induced injury in HK-2 cells. (A, B) Western blot verification of TGF-β1 overexpression in HK-2 cells (A) and the corresponding densitometric quantification (B). (C-F) Representative Western blot images of TGF-β1, p-Smad2, Smad2, p-Smad3, and Smad3 in the indicated groups (C), with corresponding quantification of TGF-β1 expression and the p-Smad2/Smad2 and p-Smad3/Smad3 ratios (D-F). (G, H) Representative Annexin V-APC/PI flow cytometry plots (G) and quantification of

the total apoptotic rate (H). (I, J) Representative DCFH-DA fluorescence images showing intracellular ROS accumulation (I; scale bar = 50 μ m) and quantification of the relative fluorescence intensity (J). (K, L) Representative MitoSOX Red fluorescence images showing mitochondrial superoxide production (K; scale bar = 50 μ m) and quantification of the relative fluorescence intensity (L). (M) Intracellular ATP levels were measured using a luciferase-based luminescence assay. (N) Mitochondrial DNA copy number was evaluated based on the mtDNA/nDNA ratio. (O) Mitochondrial complex I activity was measured using a commercial assay kit. (P, Q) Representative MitoTracker Red CMXRos staining images (P; scale bar = 50 μ m) and quantification of the relative fluorescence intensity (Q). (R, S) Representative JC-1 staining images showing changes in mitochondrial membrane potential ($\Delta\Psi$ m) (R) and quantification of the red-to-green fluorescence intensity ratio (S). (T-X) Representative Western blot images of α -SMA, fibronectin, collagen IV, and COX4 (T), with the corresponding densitometric quantification presented in (U-X). *** $P < 0.001$.

was significantly reduced following GAA treatment (**Figure 6O, 6P**).

Western blot analysis showed that Bax and cleaved caspase-3 expression were increased, whereas Bcl-2 expression was decreased, in the Model group compared with the Control group. These changes were significantly reversed by GAA treatment (**Figure 6Q-T**). Moreover, the protein levels of α -SMA, fibronectin, and collagen IV were markedly increased in the Model group and significantly reduced following GAA treatment (**Figure 6U-X**).

Taken together, these findings indicate that GAA improves renal functional parameters and attenuates apoptosis, renal tubular epithelial injury, histopathological injury, and fibrotic responses in mice with STZ-DKI.

GAA attenuates mitochondrial oxidative stress and improves mitochondrial-associated parameters in mice with STZ-DKI

To assess the effects of GAA on renal oxidative stress and mitochondrial-associated parameters, oxidative stress-related indicators and ATP levels were measured. Compared with the Control group, the Model group exhibited significantly decreased SOD and GSH-Px activities and increased MDA levels. These alterations were significantly ameliorated by GAA treatment, particularly at the medium and high doses (**Figure 7A-C**). In addition, ATP levels were markedly reduced in the Model group but were partially restored following GAA administration (**Figure 7D**).

Renal ROS accumulation and mitochondrial superoxide production were further assessed using fluorescent probes. DCFH-DA staining showed markedly increased ROS fluorescence intensity in the Model group, which was significantly reduced by GAA treatment (**Figure 7E, 7F**). Similarly, MitoSOX Red staining revealed

increased mitochondrial superoxide production in the kidneys of Model mice, whereas GAA administration markedly attenuated this increase (**Figure 7G, 7H**). Western blot analysis further showed that COX4 expression was significantly decreased in the Model group and partially restored by GAA treatment (**Figure 7I, 7J**). Collectively, these findings indicate that GAA attenuates renal oxidative stress and mitochondrial dysfunction in mice with STZ-DKI, as evidenced by enhanced antioxidant capacity, reduced ROS accumulation, and improved ATP and COX4 levels.

GAA attenuates activation of TGF- β /Smad signaling in kidney tissues of mice with STZ-DKI

To further determine whether GAA regulates profibrotic signaling *in vivo*, TGF- β /Smad signaling was examined in kidney tissues. Immunofluorescence staining showed weak TGF- β 1 fluorescence in the renal tubules and glomeruli of the Control group. In contrast, TGF- β 1 fluorescence intensity was markedly increased in the Model group and was significantly reduced by GAA treatment (**Figure 8A, 8B**).

Smad2/3 nuclear accumulation was also evaluated by immunofluorescence staining. Compared with the Control group, the Model group exhibited significantly increased nuclear Smad2/3 fluorescence intensity, whereas GAA treatment markedly attenuated this increase (**Figure 8C, 8D**).

Interestingly, GAA treatment decreased TGF- β 1 and Smad2/3 fluorescence intensity in a dose-dependent manner, with the high-dose group reaching levels comparable to those in the Sham group.

Western blotting was subsequently performed to further assess TGF- β /Smad signaling in kidney tissues (**Figure 8E**). TGF- β 1 protein expres-

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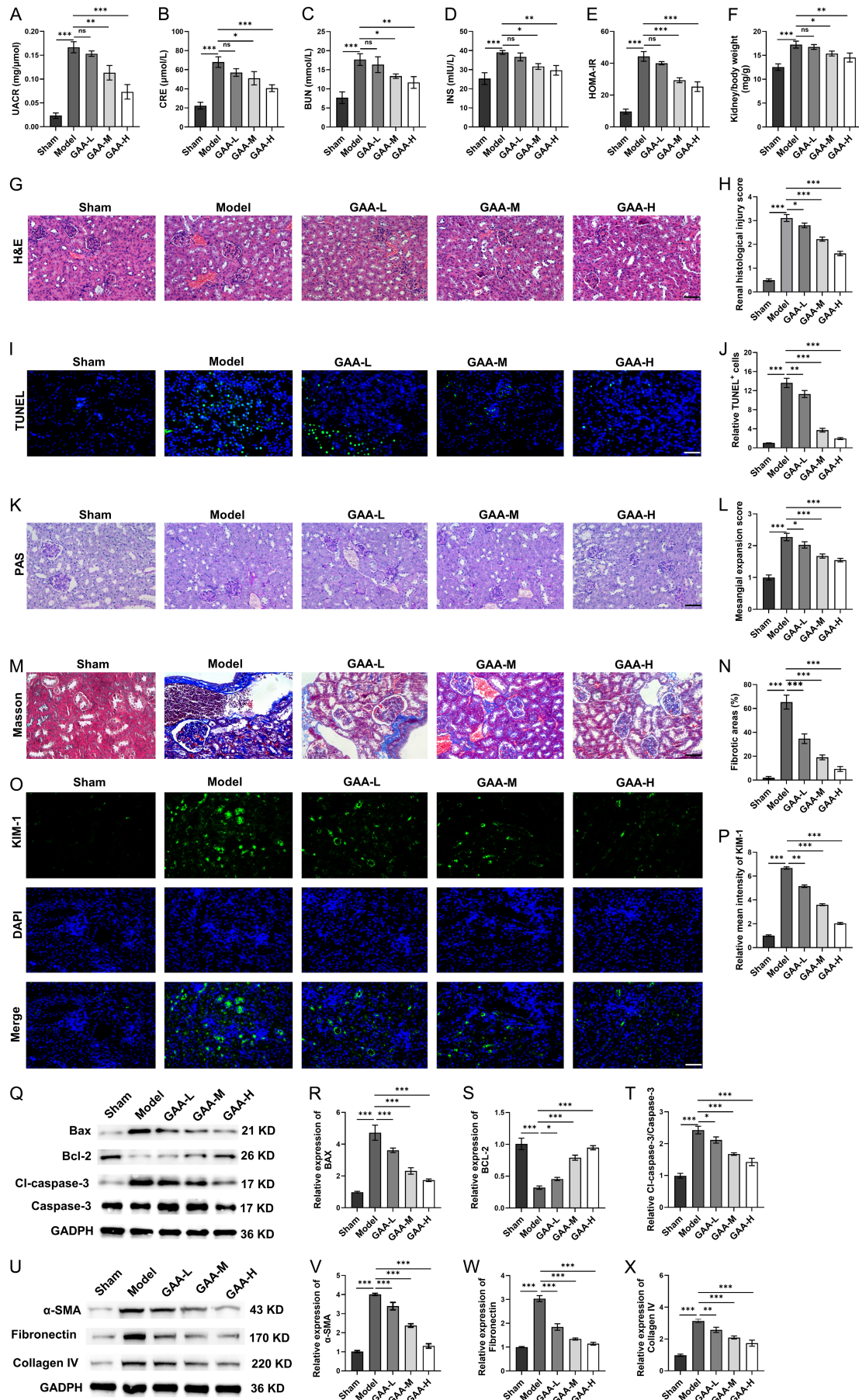


Figure 6. GAA improves renal functional parameters and attenuates histopathological injury in mice with Streptozotocin-induced Diabetic Kidney Injury (STZ-DKI). (A-F) Urinary albumin-to-creatinine ratio (UACR; A), serum creatinine (CRE; B), blood urea nitrogen (BUN; C), serum insulin levels (INS; D), HOMA-IR values (E), and the kidney-to-body weight ratio (F) were assessed in the Sham, Model, GAA-L, GAA-M, and GAA-H groups. (G, H) Representative images of hematoxylin and eosin (H&E)-stained kidney sections (G; scale bar = 50 μ m) and the corresponding renal histological injury scores (H). (I, J) Representative images of TUNEL staining in kidney tissues (I; scale bar = 50 μ m) and quantification of TUNEL-positive cells (J). (K, L) Representative images of periodic acid-Schiff (PAS) staining (K; scale bar = 50 μ m) and the corresponding mesangial matrix expansion scores (L). (M, N) Representative images of Masson's trichrome staining (M; scale bar = 50 μ m) and quantification of the percentage of fibrotic area (N). (O, P) Representative immunofluorescence images of kidney injury molecule-1 (KIM-1) expression in kidney tissues (O; scale bar = 50 μ m) and quantification of the relative fluorescence intensity (P). (Q-T) Representative Western blot images of Bax, Bcl-2, total caspase-3, and cleaved caspase-3 in kidney tissues (Q), with densitometric quantification of Bax (R), Bcl-2 (S), and the ratio of cleaved caspase-3 to total caspase-3 (T). (U-X) Representative Western blot images of α -SMA, fibronectin, and collagen IV in kidney tissues (U), with densitometric quantification of α -SMA (V), fibronectin (W), and collagen IV (X), normalized to GAPDH. ns, not statistically significant; * P < 0.05, ** P < 0.01, *** P < 0.001.

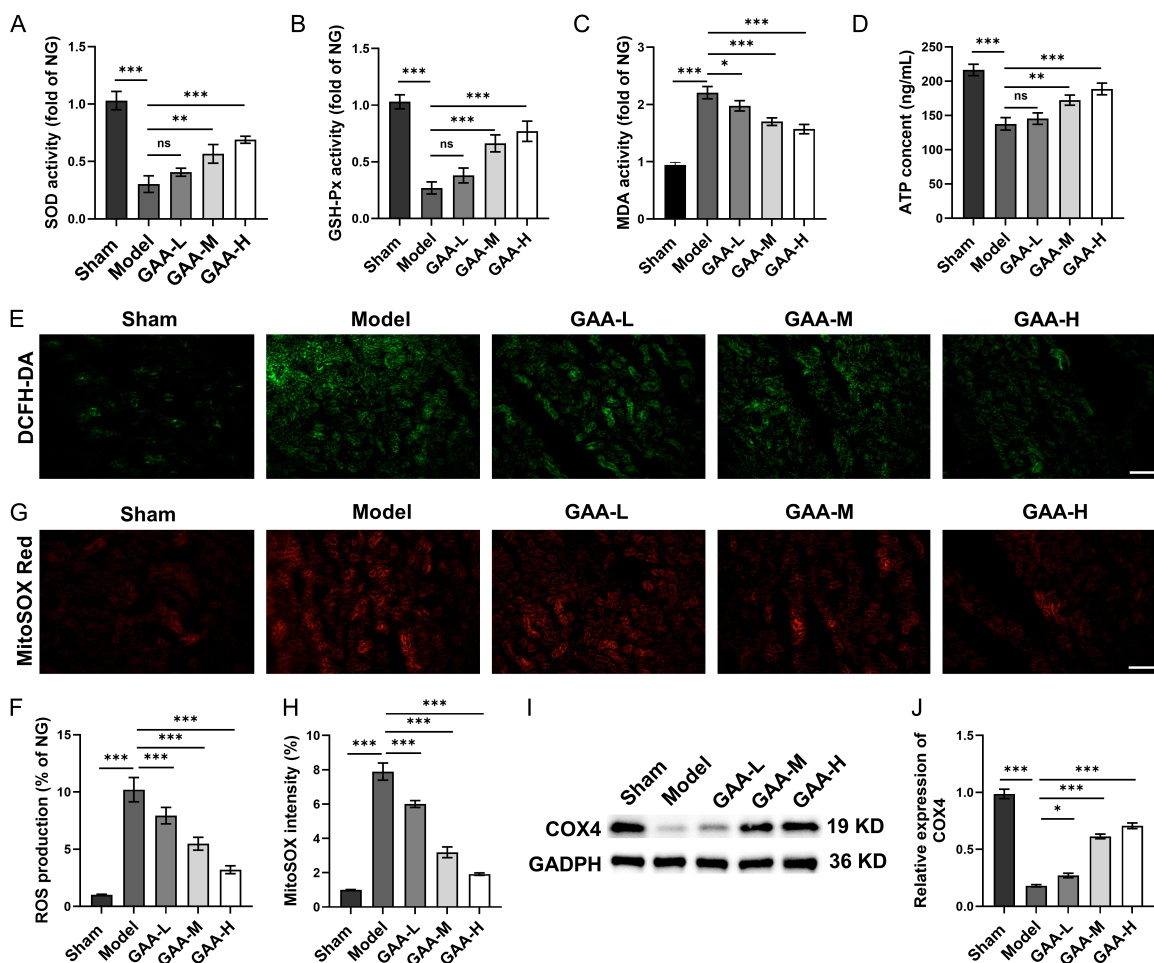


Figure 7. GAA attenuates mitochondrial dysfunction and oxidative stress in kidney tissues of mice with STZ-DKI. (A-C) Superoxide dismutase (SOD) activity (A), glutathione peroxidase (GSH-Px) activity (B), and malondialdehyde (MDA) levels (C) were measured in kidney tissues from the Sham, Model, GAA-L, GAA-M, and GAA-H groups using commercial assay kits. (D) ATP levels in kidney homogenates were measured using a luciferase-based luminescence assay. (E, F) Representative images of DCFH-DA staining in kidney sections (E; scale bar = 50 μ m) and quantification of the relative ROS fluorescence intensity (F). (G, H) Representative images of MitoSOX Red staining in kidney sections (G; scale bar = 50 μ m) and quantification of mitochondrial superoxide fluorescence intensity (H). (I, J) Representative Western blot images of COX4 expression in kidney tissues (I) and the corresponding densitometric quantification normalized to GAPDH (J). ns, not statistically significant; * P < 0.05, ** P < 0.01, *** P < 0.001.

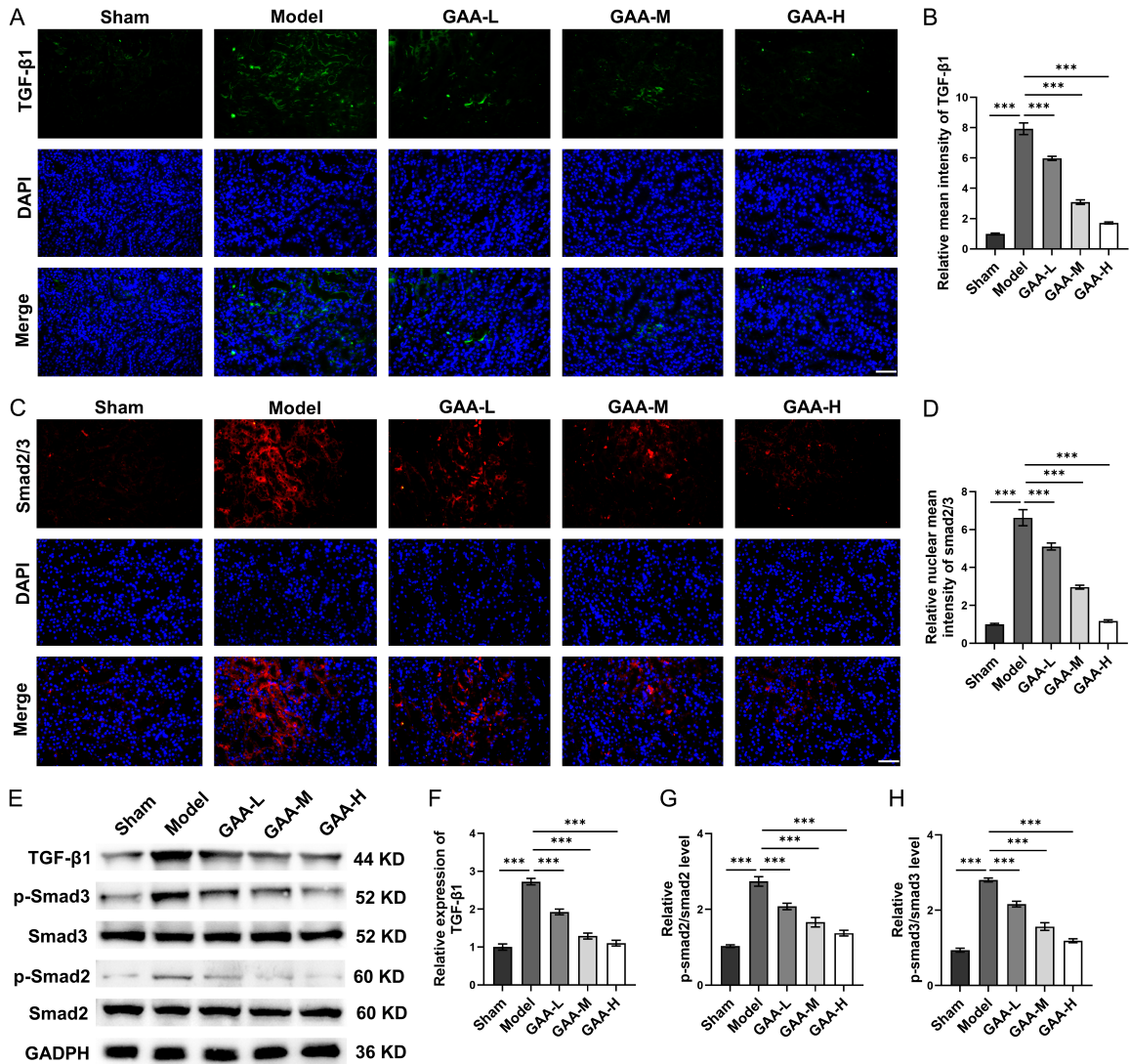


Figure 8. GAA suppresses activation of the TGF- β /Smad signaling pathway in kidney tissues of mice with STZ-DKI. (A, B) Representative immunofluorescence images of TGF- β 1 expression in kidney sections (A; scale bar = 50 μ m) and quantification of the relative fluorescence intensity (B). (C, D) Representative immunofluorescence images showing Smad2/3 localization in kidney tissues (C; scale bar = 50 μ m) and quantification of the nuclear fluorescence intensity (D). (E) Representative Western blot images of TGF- β 1, p-Smad2, Smad2, p-Smad3, and Smad3 in kidney tissues from the Sham, Model, GAA-L, GAA-M, and GAA-H groups. (F-H) Densitometric quantification of TGF- β 1 expression normalized to GAPDH (F), the p-Smad2/Smad2 ratio (G), and the p-Smad3/Smad3 ratio (H). *** P < 0.001.

sion was significantly increased in the Model group compared with the Sham group, whereas GAA treatment reduced its expression in a dose-dependent manner (Figure 8F). Similarly, the ratios of p-Smad2/Smad2 and p-Smad3/Smad3 were markedly elevated in the Model group, indicating activation of TGF- β /Smad signaling. GAA treatment significantly decreased both phosphorylation ratios, while the total protein levels of Smad2 and Smad3 remained largely unchanged (Figure 8G, 8H).

Taken together, these findings indicate that GAA suppresses pathological activation of TGF- β /Smad signaling in mice with STZ-DKI by reducing TGF- β 1 expression, Smad2/3 phosphorylation, and Smad2/3 nuclear accumulation.

Discussion

DKD is a serious microvascular complication of diabetes and may ultimately progress to ESRD

[40]. Chronic hyperglycemia and lipid metabolic disturbances contribute to multiple renal pathological changes, including glomerulosclerosis, tubular atrophy, and progressive tubulointerstitial fibrosis. Within this complex pathological process, injury and phenotypic alterations of renal tubular epithelial cells (RTECs) are increasingly recognized as important drivers of DKD progression [41]. Under hyperglycemic and lipotoxic conditions, RTECs may undergo excessive apoptosis, oxidative stress, and EMT-like phenotypic changes [42]. In the present study, GAA markedly attenuated HG-induced apoptosis and improved cell viability in HK-2 cells. GAA also reduced the HG-induced expression of α -SMA, fibronectin, and collagen IV. Among these proteins, α -SMA reflects acquisition of a myofibroblast-like phenotype, whereas fibronectin and collagen IV are major extracellular matrix components. These findings suggest that GAA attenuates profibrotic phenotypic changes and ECM accumulation in renal tubular epithelial cells, suggesting a protective effect against HG-induced fibrotic responses.

Mitochondrial dysfunction and oxidative stress can reinforce each other, forming a self-perpetuating cycle that contributes to the progression of DKD [43]. Under chronic hyperglycemic conditions, excessive mitochondrial reactive oxygen species (ROS) production in renal tubular epithelial cells can lead to loss of mitochondrial membrane potential, impaired ATP production, and reduced antioxidant enzyme activities, including those of SOD and GSH-Px [44]. In the present study, GAA attenuated HG-induced mitochondrial impairment, accompanied by reduced intracellular and mitochondrial ROS accumulation, increased ATP levels, and attenuation of the HG-induced loss of $\Delta\Psi_m$. These mitochondrial protective effects were accompanied by an increased Bcl-2/Bax balance and reduced cleaved caspase-3 expression, indicating attenuation of mitochondria-associated apoptotic signaling. Given that renal tubular epithelial cell apoptosis contributes to tubular atrophy and progressive renal dysfunction, these improvements in mitochondrial-associated parameters may contribute to the protective effects of GAA against HG-induced renal tubular epithelial injury.

Multiple signaling pathways contribute to renal fibrosis in DKD, among which aberrant activa-

tion of TGF- β 1/Smad signaling is recognized as a central profibrotic mechanism [9]. Upon activation, TGF- β 1 induces the phosphorylation of Smad2/3, followed by their nuclear translocation and regulation of profibrotic gene transcription. Concurrent downregulation of the inhibitory Smad protein Smad7 can further amplify this signaling cascade [45]. Recent studies have shown that therapeutic agents, including SGLT2 inhibitors and other triterpenoid compounds, can modulate TGF- β signaling, supporting the relevance of targeting this pathway in DKD [46, 47]. Consistent with these findings, our results showed that GAA reduced HG-induced TGF- β 1 expression, inhibited Smad2/3 phosphorylation, and attenuated Smad2/3 nuclear accumulation in HK-2 cells.

To further investigate the mechanism underlying the mitochondrial protective effects of GAA, a gain-of-function rescue experiment was performed by overexpressing TGF- β 1. TGF- β 1 overexpression partially reversed the inhibitory effects of GAA on intracellular ROS accumulation and mitochondrial superoxide production and attenuated its protective effects on mitochondrial membrane potential and ATP levels. These findings indicate that inhibition of TGF- β 1/Smad signaling contributes, at least in part, to GAA-mediated alleviation of HG-induced mitochondrial oxidative stress and dysfunction. The rescue experiment further supports a functional association between TGF- β 1/Smad activation and mitochondrial impairment in renal tubular epithelial cells. However, additional mechanisms, including the potential direct antioxidant effects of GAA, cannot be excluded.

Finally, the *in vivo* effects of GAA were evaluated in mice with STZ-DKI. GAA administration was associated with improvements in renal functional parameters, as evidenced by reduced UACR, serum creatinine, and blood urea nitrogen levels, and attenuated histopathological changes, including mesangial matrix expansion and collagen deposition. Consistent with the *in vitro* findings, immunofluorescence and Western blot analyses showed that GAA treatment was accompanied by reduced activation of renal TGF- β /Smad signaling, reduced oxidative stress, and partially restored COX4 expression in kidney tissues. The consistency between the *in vitro* mechanistic findings and the *in vivo* observations is consistent with a

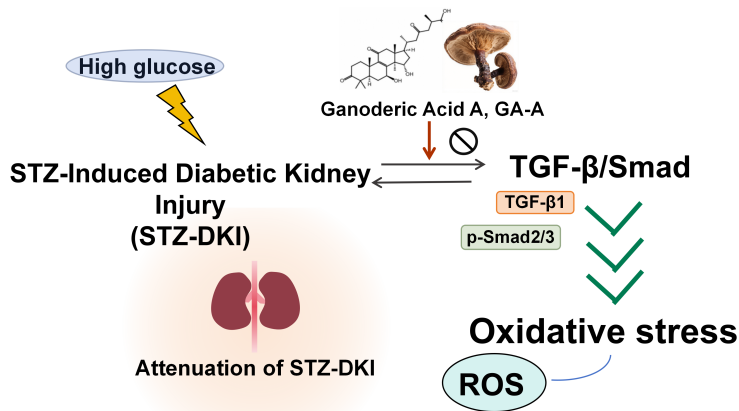


Figure 9. Ganoderic acid A (GAA) attenuates HG-induced renal tubular epithelial injury *in vitro* and streptozotocin-induced diabetic kidney injury (STZ-DKI) *in vivo*, accompanied by reduced TGF-β1/Smad2/3 signaling, mitochondrial oxidative stress, and fibrotic responses.

renoprotective potential of GAA. Collectively, these *in vivo* findings suggest that reduced TGF-β/Smad signaling and attenuated mitochondrial oxidative stress and dysfunction were associated with the renal improvements observed after GAA treatment, while improved systemic glycemic status may also have contributed to these effects.

Taken together, the *in vitro* and *in vivo* findings indicate that the effects of GAA are associated with reduced TGF-β1/Smad2/3 signaling, mitochondrial oxidative stress, and fibrotic responses, as summarized in **Figure 9**.

Several limitations of the present study should be acknowledged. Because a glycemia-matched control group was not included, the contribution of the glucose-lowering effect of GAA to its *in vivo* renal improvements cannot be fully dissociated from its potential direct renal effects. However, under fixed HG conditions *in vitro*, GAA attenuated HK-2 cell injury, mitochondrial oxidative stress, apoptosis, and profibrotic responses, and these effects were partially reversed by TGF-β1 overexpression, supporting a direct cellular protective effect under controlled glucose conditions. Future studies using insulin-treated or other glycemia-matched control groups will help clarify the relative contributions of systemic glycemic improvement and direct renal effects.

The *in vitro* experimental design also has certain limitations. The osmotic-control group was included in the initial cell viability and LDH

release experiments but was not carried out through all mechanistic assays, whereas the 2-h GAA pretreatment primarily models a preventive rather than a therapeutic intervention. Future studies should therefore extend osmotic controls to selected mechanistic endpoints and compare GAA pretreatment with administration after HG-induced injury. In addition, although histopathological assessment and KIM-1 staining strengthened the evidence that GAA attenuates tubular and tubulointerstitial injury, further tubular injury scoring, assessment of additional injury markers such as NGAL,

and co-localization with tubular markers would help clarify the spatial relationship among tubular injury, TGF-β/Smad activation, oxidative stress, and fibrosis.

Conclusion

GAA attenuates HG-induced renal tubular injury *in vitro* and ameliorates renal injury in STZ-induced diabetic mice, in association with reduced mitochondrial oxidative stress, apoptosis, and fibrotic responses. TGF-β1 overexpression rescue experiments support the involvement of TGF-β1/Smad signaling in the cellular effects of GAA under fixed HG conditions. These findings support further investigation of the therapeutic potential of GAA in diabetes-associated renal injury.

Disclosure of conflict of interest

None.

Abbreviations

ATP, adenosine triphosphate; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; BUN, blood urea nitrogen; BSA, Bovine serum albumin; Calcein-AM, calcein acetoxymethyl ester; COX4, cytochrome c oxidase subunit 4; CRE, creatinine; DKD, diabetic kidney disease; DAPI, 4',6-diamidino-2-phenylindole; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; DMSO, Dimethyl sulfoxide; ECM, extracellular matrix; ECL, enhanced chemiluminescence; EMT, epi-

thelial-mesenchymal transition; GAA, ganoderic acid A; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GSH-Px, glutathione peroxidase; H&E, Hematoxylin and eosin; HG, high glucose; HK-2, human kidney-2 cells; HOMA-IR, homeostasis model assessment of insulin resistance; HRP, horseradish peroxidase; INS, insulin; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; KW/BW, kidney weight/body weight; LDH, lactate dehydrogenase; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NG, normal glucose; NOX1, NADPH oxidase 1; NOX2, NADPH oxidase 2; OCT, optimal cutting temperature compound; OC, osmotic control; OD, optical density; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinylidene fluoride; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SGLT2, sodium-glucose cotransporter 2; Sham, sham-operated/control group; SOD, superoxide dismutase; SPF, specific pathogen free; STZ, streptozotocin; STR, short tandem repeat; TGF- β 1, transforming growth factor- β 1; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; UACR, urinary albumin-to-creatinine ratio; WB, Western blot; α -SMA, alpha-smooth muscle actin.

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