Life Sciences 223 (2019) 194–201

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Arctigenin ameliorates renal impairment and inhibits endoplasmic reticulum stress in diabetic *db/db* mice

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ARTICLE INFO

Keywords:
Arctigenin
Diabetic nephropathy
ER stress
Apoptosis

ABSTRACT

Aims: Diabetic nephropathy (DN) is the most common complication of diabetes mellitus. Endoplasmic reticulum (ER) plays an important role in the development and progression of DN. Arctigenin (ATG), a lignan extract from *Fructus Arctii*, exhibits anti-inflammatory, anticarcinogenic, anti-oxidative stress and immunomodulatory properties. The present research aimed to investigate whether ATG could protect against diabetes-related renal injury and inhibit ER stress in *db/db* mice.

Main methods: Male *db/db* mice were randomly divided into two groups: DN group and ATG treatment group (DN + ATG). *db/m* mice were defined as the normal control group (NC). ATG was dissolved in 0.5% carboxymethyl cellulose sodium salt solution and administered orally at a dose of 80 mg/kg to mice in the DN + ATG group once daily for 8 consecutive weeks. HK2 cells were used to determine the effects of ATG on ER stress and cell apoptosis *in vitro*.

Key findings: ATG administration significantly reduced blood glucose, urine albumin excretion, and urine albumin to creatinine ratio, and attenuated renal pathological injury when compared with untreated *db/db* mice. These changes were accompanied by decreased expression of both ER stress-related markers and caspase 12 level in the kidneys of *db/db* mice. *In vitro*, high glucose activated ER stress signal transduction pathway and induced cell apoptosis in HK2 cells, which were blocked by ATG.

Significance: Our results suggest that ATG exerts renoprotective effects on diabetes-related renal injury in *db/db* mice and cytoprotective effects on high glucose induced cell apoptosis and inhibits ER stress.

1. Introduction

Diabetic nephropathy (DN), a serious complication of type 2 diabetes, has become the leading cause of chronic kidney disease (CKD) and end-stage renal disease in the USA [1]. In China, chronic kidney disease related to diabetes has become more common than chronic kidney disease related to glomerulonephritis in both the general population and an urban hospitalized population since 2011 [2]. It is estimated the number of patients with chronic kidney disease related to diabetes is 24.3 million in China [2]. However, the exact pathogenesis of DN has not yet been elucidated so far. It has been generally suggested that oxidative stress, inflammatory response and endoplasmic reticulum (ER) stress predominantly contribute to the pathogenesis of DN [3–5]. Although there are several treatments for diabetic nephropathy (DN), nearly half of DN patients inevitably progress to end stage of renal disease [6]. Therefore, there is an urgency for new, safe and effective agents for DN management.

Arctigenin (ATG) is a natural lignan compound derived mainly from the seeds of *Arctium lappa* (L.). It exerts multiple bioactivities, including anti-inflammatory, anti-oxidative, anti-diabetic, immunoregulation activities [7–10] and anti-tumor metastasis [11]. Previous studies have demonstrated that ATG targeted PI3K/Akt/mTOR, STAT3, RAF-MEK-ERK, wnt/β-catenin and p38 MAPK in cancer [12]. Aside from these molecular mechanism, it has also been reported as an unfolded protein response (UPR) inhibitor to suppress the UPR signaling pathway [13]. It was demonstrated that ATG ameliorated metabolic disorders in ob/ob mice [14] and attenuated myocardial [15] but aggravated renal [16] ischemia/reperfusion injury in rat. However, the effects of ATG on diabetic *db/db* mice are unclear. In the present study, we performed *in vitro* and *in vivo* experiments to explore whether ATG had renoprotective effect on diabetic *db/db* mice and an inhibitory effect on ER stress in the context of DN.

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https://doi.org/10.1016/j.lfs.2019.03.037
Received 18 February 2019; Received in revised form 16 March 2019; Accepted 16 March 2019
Available online 18 March 2019

2. Materials and methods

2.1. Animals

Six-week-old Male *db/db* mice (C57BLKS/J-*LepR^{db}/LepR^{db}*) and their lean non-diabetic littermates *db/m* mice (C57BLKS/J-*LepR^{db}*/+) were purchased from the National Mode Animal Centre of Nanjing University (Nanjing, China). After adaptive feeding for two weeks, *db/db* mice were randomly divided into two groups: DN group (DN) and ATG treatment group (DN + ATG). *db/m* mice were defined as the normal control group (NC). ATG (> 98% purity; molecular weight: 372.41, provided by Aladdin Reagent Co., Ltd. Shanghai, China) was dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC, Sigma) solution and administered orally by a gavage tube at a dose of 80 mg/kg once a day for 8 weeks in DN + ATG group. The NC and DN groups were administered an equal volume of 0.5% CMC. All mice were housed in a specific pathogen-free room and had free access to normal food and water. All animal experiments were approved by the Laboratory Animals Ethical Committee of the First Affiliated Hospital of Wannan Medical College and were carried out in accordance with the National Institutes of Health "Guidelines for Care and Use of the Laboratory Animals".

2.2. Physical and biochemical analysis

Body weight (BW) and blood glucose (BG) were measured. At the end of the study, 24-h urine samples were collected when the mice were placed in metabolic cages. The urinary albumin and urinary creatinine concentrations were assayed using Mouse albumin ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA) and a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions.

2.3. Histology analysis

10% formalin-fixed, paraffin-embedded renal tissues were sectioned (4-μm thickness) and stained with hematoxylin-eosin (HE) and Periodic acid-Schiff reagent (PAS). For immunohistochemistry, paraffin-embedded renal sections (4-μm thickness) were dewaxed, hydrated, blocked and stained with monoclonal antibodies against GRP78 and CHOP (both from Cell Signaling Technology, Danvers, MA). All the measurements were calculated by ImagePro Plus Systems.

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) staining

TUNEL staining using the DeadEnd™ Colometric TUNEL System (Promega, Madison, WI, USA) was carried out according to the manufacturer's protocols. Briefly, paraffin-embedded renal sections were dewaxed and reacted with protease K at 37 °C for 10 min. Then, the slices were further-developed and mounted. TUNEL-positive cells were counted under a light microscope and expressed as the rate of apoptosis (%).

2.5. Cell culture

HK2, the human proximal tubular epithelial cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Minimum Essential Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY, USA) and 1% Penicillin-Streptomycin at 37 °C in a 5% CO_2 incubator. To evaluate the effect of ATG on HG-induced ER stress in HK2 cells, the cells were synchronized with FBS-free medium for 24 h when they reached 80% confluence, and then cultured with DMSO or ATG (1 μM) 6 h before treatment with NG (normal glucose, 6 mM D-glucose), HG (high glucose, 30 mM D-glucose) and HM (high mannitol, normal glucose with 24 mM of D-mannitol). All assays were performed at least in triplicate and repeated three times.

2.6. Western blot analysis

The protein concentrations of renal tissues or cell lysates were determined with a Bio-Rad protein assay kit (Bio-Rad; bovine serum albumin was used as a standard). Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk, the membranes were incubated with one of the following primary antibodies: anti-GRP78 (1:1000), anti-CHOP (1:1000), caspase 12 (1:1000), and β-actin (1:1000) antibodies. The membranes were washed with TBST (150 mM NaCl, 20 mM Tris–HCl, and 0.1% Tween 20) and incubated for 1 h at room temperature with secondary antibody (1:4000). Imaging was performed using the Bio-Rad Imaging System. The densitometry of the bands was performed on image-scanning analysis software (Labworks) and reported as optical density per square millimeter.

2.7. Cell apoptosis assay

Cell apoptosis was examined by flow cytometry using the Annexin V-FITC Kit (BD Biosciences, San Diego, CA, USA), according to the manufacturer's protocols. In brief, the cells were harvested and adjusted with 1× binding buffer at a concentration of 1×10^6 cells/mL. Then, 100 μL of binding buffer, 5 μL of Annexin V-FITC, and 5 μL of propidium iodide (PI) solution were added to the cells and incubated at room temperature for 15 min in the dark. The stained cells were subsequently analyzed using a flow cytometer.

2.8. Statistical analysis

Statistical analyses were performed using SPSS 17.0. All results are presented as mean ± SEM, and comparison between multiple groups were analyzed using one-way ANOVA, followed by the Dunnett's multiple range test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. ATG attenuated renal injury in diabetic db/db mice

To examine whether ATG could exert beneficial effects on *db/db* mice, we examined the changes in blood glucose (BG), body weight (BW), urine albumin and creatinine ratio (UACR), and 24-h urine albumin excretion ratio (UAER). Compared with normal control mice, *db/db* mice exhibited significant renal injury characterized by increased levels of UACR, and 24-h UAER ($P < 0.05$). Meanwhile, ATG treatment significantly ameliorated the increase in BG, BW, UACR, and 24-h UAER (Fig. 1). These results show that ATG is an effective agent for diabetic *db/db* mice.

3.2. Alterations of renal morphology in different groups

HE and PAS staining showed typical renal pathological changes in *db/db* mice at 16 weeks, including mesangial cell proliferation and focal mesangial matrix accumulation and expansion. Consistent with the changes in biochemical markers, these changes were significantly ameliorated in the ATG-administered group compared with those in the untreated *db/db* mice (Fig. 2). These results show that ATG has beneficial effects in preventing renal injury.

3.3. ATG suppresses ER stress induced in the kidneys of db/db mice

To explore whether ER stress is induced in the kidneys of *db/db* mice, we examined the expression of ER stress markers in the kidneys of

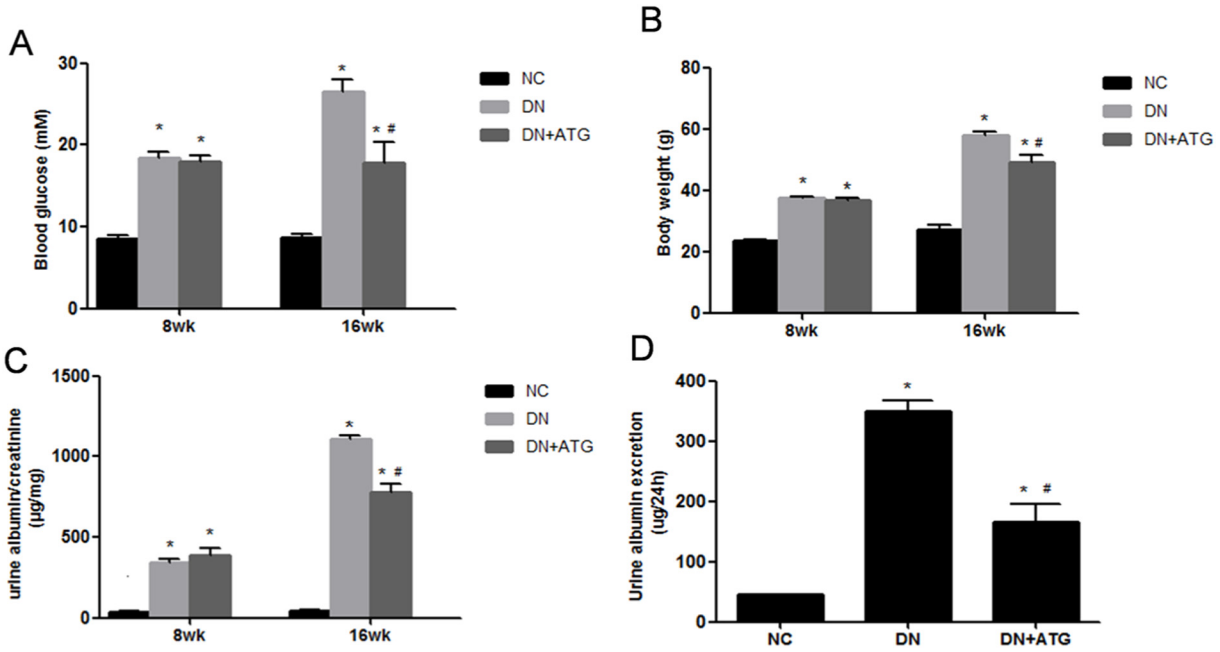

Fig. 1. ATG attenuates renal injury in diabetic mice. The ATG-treated group (DN + ATG, n = 10) was administered 80 mg/kg ATG dissolved in 0.5% carboxymethyl cellulose sodium salt (CMC) solution daily by gavage for 8 weeks. Mice in the control group (NC, n = 10) or diabetes group (DN, n = 10) received the same amount of 0.5% CMC. Body glucose (A), blood weight (B), urine albumin and creatinine ratio (C), and urine albumin excretion (D) of *db/db* mice decreased after ATG treatment. Values are expressed as means ± SEM. $^{*}P < 0.05$ *versus* NC group. $^{\#}P < 0.05$ *versus* DN group.

db/db mice. Western blot analysis showed that the expression of GRP78 and CHOP, two representative molecules involved in ER stress, in the renal cortex were markedly higher than that in non-diabetic *db/m* mice. ATG administration significantly downregulated the expression of these two molecules (Fig. 3). In addition, consistent changes were found in immunohistochemistry analysis. As shown in Fig. 4, GRP78 was predominantly expressed in the renal tubules and few stained regions were observed in the glomeruli. Increased CHOP staining was detected mainly in the glomeruli of diabetic *db/db* mice. Taken together, these results indicate that ER stress is induced in the kidneys of *db/db* mice, and ATG effectively inhibits the activation of ER stress mediated by diabetes.

3.4. ATG inhibits apoptosis in the kidneys of diabetic db/db mice

To determine the role of apoptosis in DN and further assess whether the renoprotective effects of ATG in DN involved inhibition of apoptosis, *in situ* TUNEL assay was performed on kidney sections, and the expression levels of apoptosis-related proteins were determined by western blot. We observed that at 16 weeks, diabetic *db/db* mice

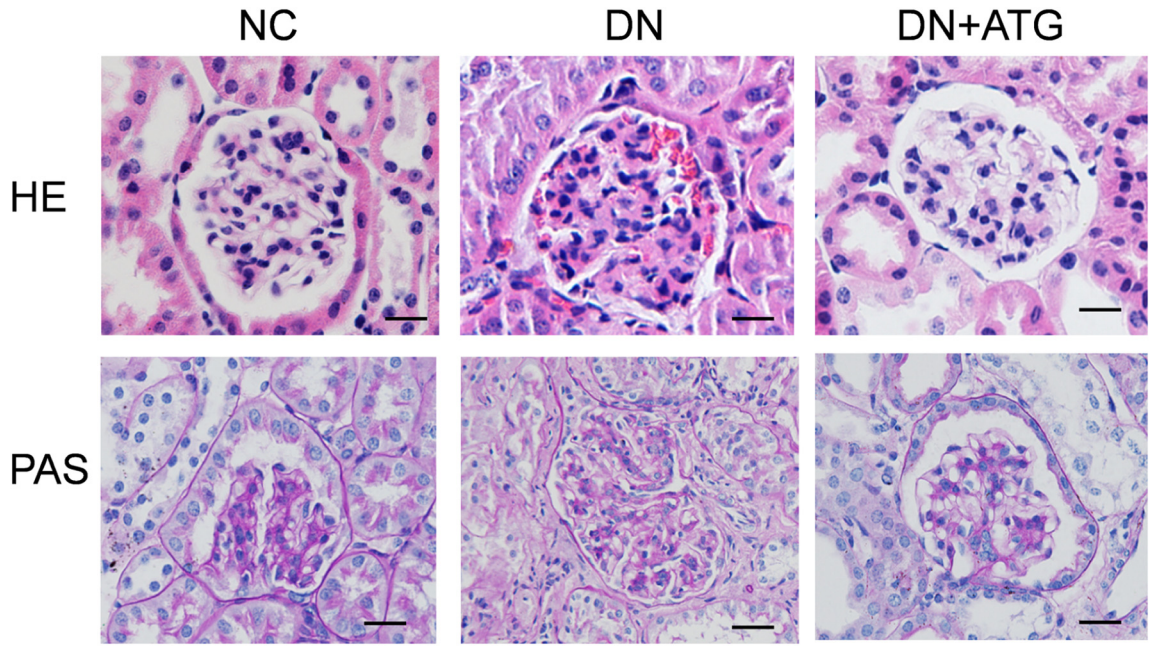

Fig. 2. Alterations of renal morphology in different groups. Representative images of hematoxylin-eosin (HE) and Periodic acid Schiff (PAS)-stained kidney sections from different groups. Bar = 50 μm.

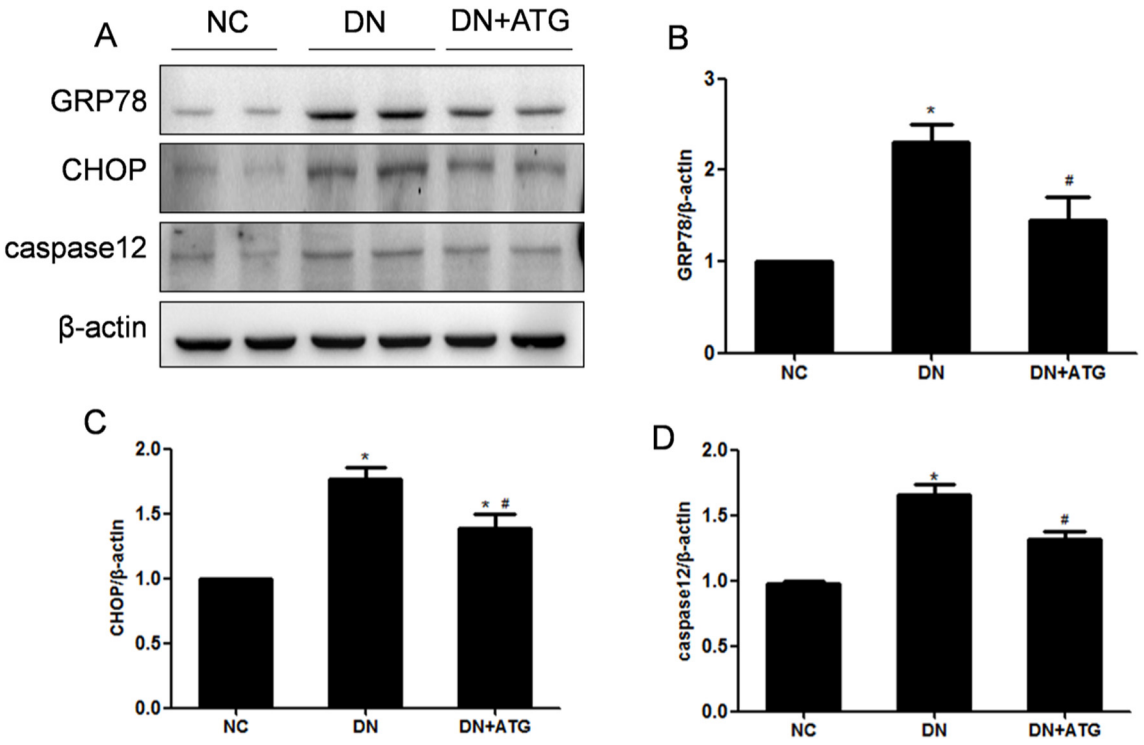

Fig. 3. ATG suppresses ER stress induced in the kidneys of *db/db* mice. The expression levels of GRP78, CHOP, caspase12 and β-actin were analyzed by Western blot (A). Quantification of the protein levels of GRP78 (B), CHOP (C) and caspase12 (D), and corrected by reference to the value of β-actin. Values are expressed as means ± SEM. *$P < 0.05$ *versus* NC group. #$P < 0.05$ *versus* DN group. All experiments were performed in triplicate.

displayed increased number of apoptotic cells compared with the normal control mice ($P < 0.05$), whereas ATG treatment significantly reduced the number of apoptotic cells compared with the DN group (Fig. 5). As shown in Fig. 3, the expression of caspase 12 was markedly increased in *db/db* mice compared with that in *db/m* mice. ATG treatment significantly suppressed the increase of caspase 12 in *db/db* mice, indicating that ATG could alleviate diabetes-induced apoptosis.

3.5. ATG prevents high glucose-induced ER stress in HK2 cells

To determine the effects of high glucose (HG) on the activation of ER stress *in vitro*, HK2 cells were incubated with high concentrations of glucose for 24 h. As shown in Fig. 6, HG stimulation led to the upregulation of GRP78, CHOP, and caspase 12. As an osmotic control, mannitol had no significant effect on the activation of ER stress. Our data suggested that ER stress was induced in HG-stimulated cultured HK2 cells. To determine whether the inhibition of ER stress by ATG depended on its ability to lower BG *in vivo*, we pretreated HK2 cells with ATG at a concentration of 1 μM for 6 h. As shown in Fig. 6, ATG significantly inhibited the upregulation of GRP78, CHOP and caspase 12 in HK2 cells exposed to HG. Taken together, these data suggest that ATG exerts inhibitory effects on ER stress and ER-associated apoptosis pathways independently of glucose reduction.

3.6. ATG inhibits high glucose-induced apoptosis in HK2 cells

We performed an in-depth study to determine the role of HK2 cell apoptosis under HG conditions. Flow cytometry analysis showed that HG induced HK2 cell apoptosis, which was attenuated by ATG treatment (Fig. 7). These data suggest that ATG protects against HG-induced apoptosis in HK2 cells.

4. Discussion

ATG, a natural lignan compound extracted from *Arctium lappa*, was reported to

exhibit numerous pharmacological actions, including antidiabetes, antitumor, antioxidant, and neuron protective activities [10,14,17]. Previous studies have reported that ATG protect against TGF-β1-promoted tubulointerstitial fibrosis, which may provide a basis for developing therapeutic strategies to treat chronic renal inflammation and tubular EMT [18]. ATG attenuates ischemic stroke and exerts anti-arrhythmia role in ischemia/reperfusion injury [15,19]. However, pretreatment of ATG aggravates I/R induced acute kidney injury [16]. Till now, little is known about the effects and mechanisms of ATG on diabetes induced renal injury. Malhotra et al. reported the effect of antioxidants on ER stress [20]. ER stress is intimately connected with oxidative stress. Some studies have shown that antioxidants can reduce levels of ER stress [21,22]. In our previous study, inhibiting ER stress could attenuate renal injury in *db/db* mice [23]. Thus, we explored whether ATG as an antioxidant may exert renoprotective effects on diabetic *db/db* mice and inhibitory effects on ER stress in DN.

Herein, we discovered that ATG administration lowered BG and BW level, reduced UACR and UAE and attenuated renal histopathology damage in diabetic *db/db* mice, which suggested that ATG is an effective agent for the treatment of DN. Meanwhile, we found ATG could downregulate the expression of GRP78 and CHOP, which indicated that ATG could effectively inhibit ER stress signaling pathway. *In vitro* experiment showed that ATG effectively protected HK2 cells against HG-induced apoptosis, indicating that the cytoprotection of ATG is independent of glucose decline.

ER stress has been found to be important in the development of diabetic nephrology and ER stress induced apoptosis plays a key role in the pathways to cell death [24]. Accumulated evidence shows that a number of insults, including hypoxia, oxidative stress, hyperglycaemia can lead to the accumulation of unfolded or misfolded proteins in the ER lumen [25,26]. When protein overloading in ER lumen overwhelms its capability of protein processing, ER stress is initiated and the unfolded protein response (UPR) is activated, which induces an adaptive response to restore normal ER function [27–31]. If the stress is too

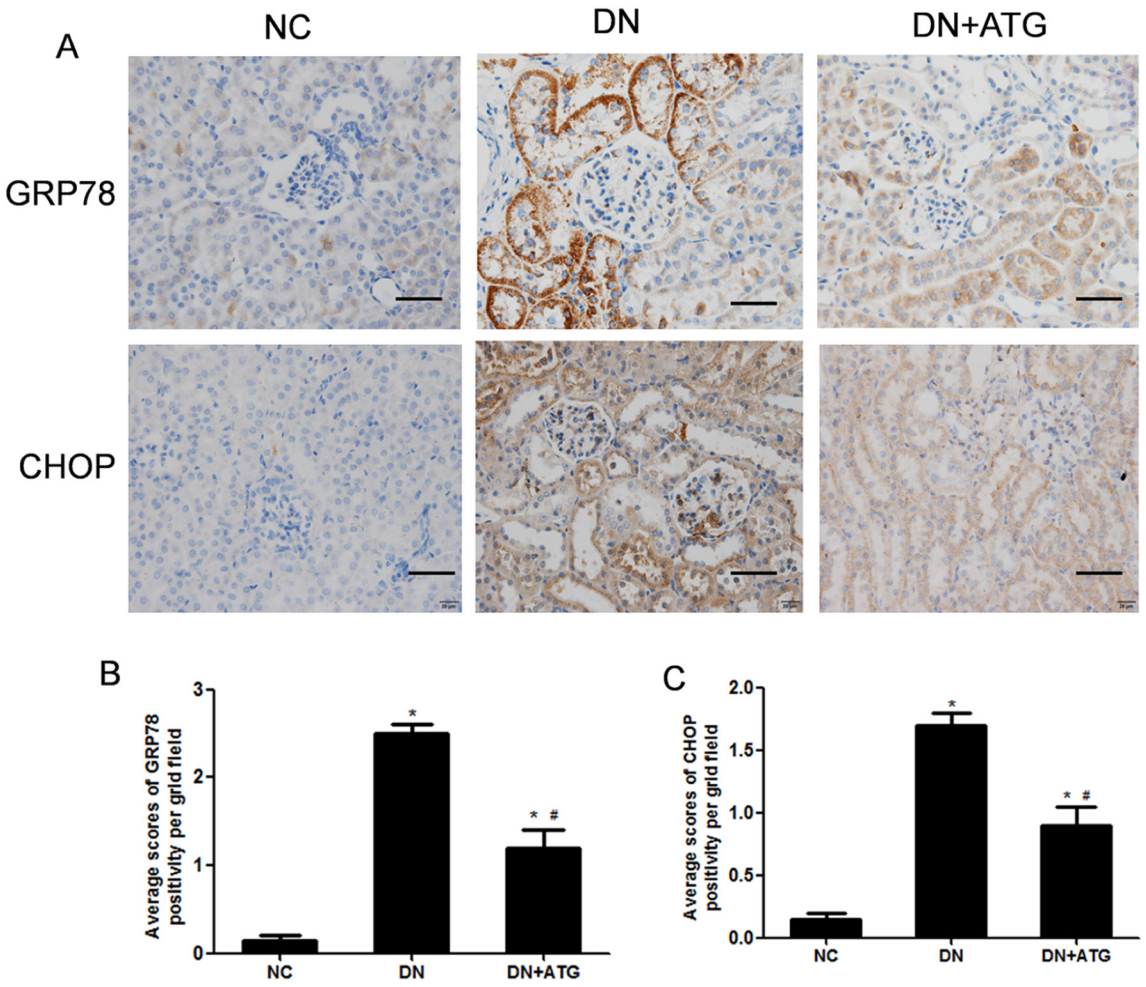

Fig. 4. ATG suppresses GRP78 and CHOP expression in the renal tissues of mice. Immunohistochemical staining analysis of GRP78 and CHOP expression in the renal tissues of mice. Bar = 50 μm (A). Histograms represent semi-quantitative of GRP78 (B) and CHOP (C) expression in the renal tissues. Values are expressed as means ± SEM. $^{*}P < 0.05$ *versus* NC group. $^{\#}P < 0.05$ *versus* DN group.

prolonged or severe and ER homeostasis cannot be restored, an apoptotic signaling pathway is triggered. Therefore, inhibiting ER stress mediated cell apoptosis may provide new therapeutic approaches for DN.

GRP78 is a central molecular chaperon of ER stress, as it binds to three transmembrane proteins (PERK, IRE1, and ATF6) to maintain the inactivated state [3]. In agreement with previous study that has demonstrated that ATG also suppressed the induction of GRP78 in PANC-1 cells under glucose starvation [13]. Aside this, ATG blocked the phosphorylation level of phosphorylated PERK in HT-29 cells [32]. Inhibiting PERK/eIF2α pathway by ATG in either BV2 or primary neuronal cells ameliorates memory impairment in Alzheimer's Disease Model Mice [33]. Based on above research, it is indicated ATG could inhibit ER stress signaling pathway in multiple experiment model.

However, ER stress is a two-edged sword. If an adaptive UPR could not counterbalance the stress, then the ER would initiate apoptotic pathway. CHOP acts as a transcriptional factor, which is involved in ER stress-induced apoptotic cell death, and has been characterized as a proapoptotic protein [34,35]. The expression of CHOP was increased during ER stress and correlated with the onset of ER stress-associated apoptosis [36]. CHOP deletion protects cells from ER stress [34]. We found that the downregulation of CHOP in ATG-treated group was accompanied by a decline in apoptotic renal cells by using TUNEL assay. Caspase 12 is specifically localized on the cytoplasmic side of the ER in rodents, which is considered as a specific marker of ER stress-induced apoptosis [37–39]. During ER stress-induced apoptosis cascade, caspase 12 is cleaved and activated, which subsequently triggers the activation of cytoplasmic caspase 3 and leads to the induction of cell apoptosis [40]. However, inconsistent with previous study, it was reported that ATG couldn't decrease apoptotic tubular cells despite of reduced infiltrating inflammatory cells and proinflammatory cytokine in ischemia/reperfusion induced acute kidney injury [16]. This discrepancy remains unclear so far, but it may be explained by differences in the animal models and internal environment. Thus, ATG suppressed ER stress signaling pathway, which was correlated with a beneficial effect on the physiological and pathophysiological processes of DN. High concentrations of glucose could induce several biological processes, including NADPH oxidase activation and reactive oxygen species production, which could also induce ER stress [41]. Our data demonstrated that ATG inhibited HG-induced ER stress *in vivo* and *in vitro*. As a potent antioxidant agent, the inhibition of ER stress by ATG may be the indirect effects of its inhibition on oxidative stress. Hence, we would like to investigate the inhibitory effect of ATG on ER stress with signaling pathway inhibitor and gene knockout mouse in the near future.

In summary, our findings showed that the protective effects of ATG on diabetes-related renal injury and the inhibitory effects of ATG on ER stress *in vivo* and *in vitro*. These data also provide further evidence for the clinical application of ATG in the prevention of DN.

Acknowledgements

This work was supported by the Foundation of Yijishan Hospital of

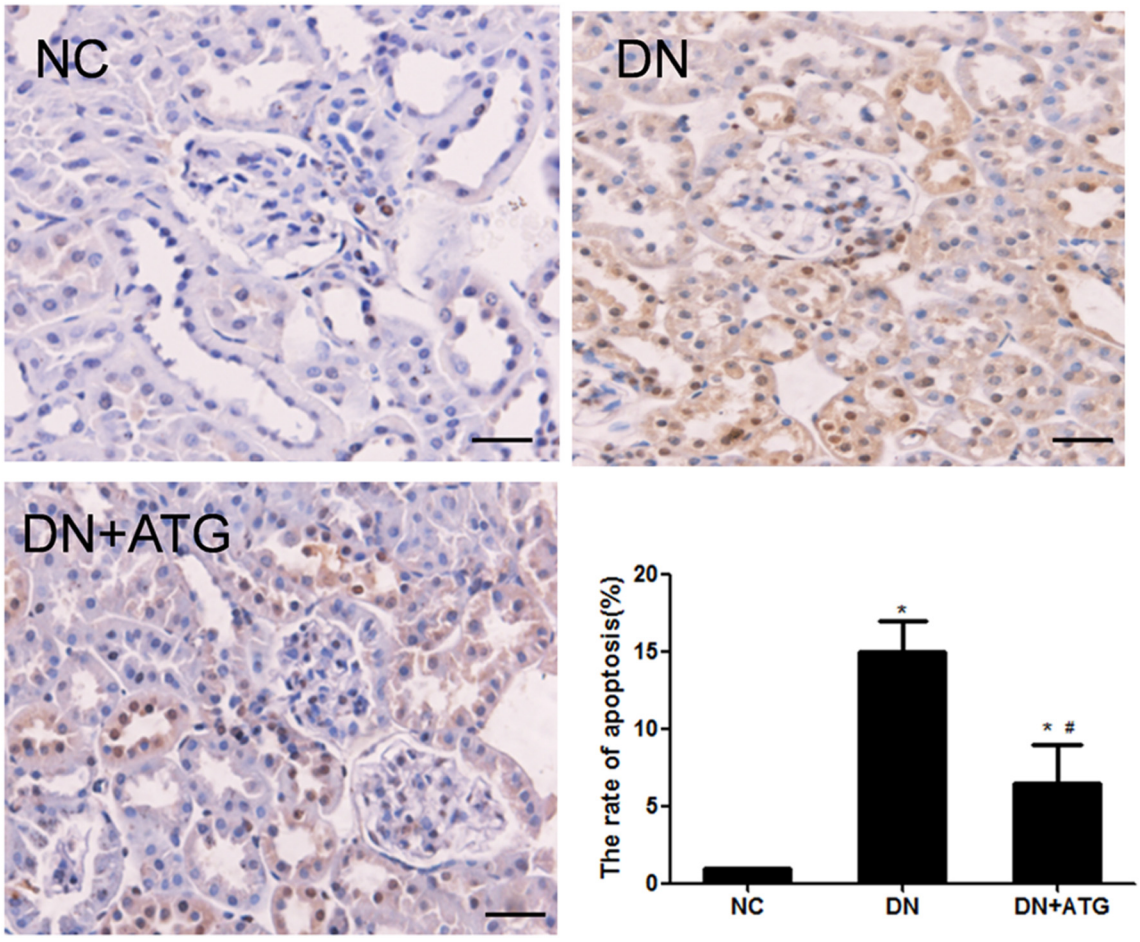

Fig. 5. ATG inhibits apoptosis in kidneys of diabetic *db/db* mice. Apoptosis of renal cells in kidneys of each group were determined by TUNEL assay. Values are expressed as means ± SEM. $^{*}P < 0.05$ compared with NC group, $^{\#}P < 0.05$, compared with DN group.

Wannan Medical College (KY24560348), the Doctoral Research Fund of Wannan Medical College (WYRCQD201709), Anhui Provincial Natural Science Foundation (1908085MH248), and the Projects of Anhui Province Key Laboratory of Biological Macro-molecules Research (LAB201801).

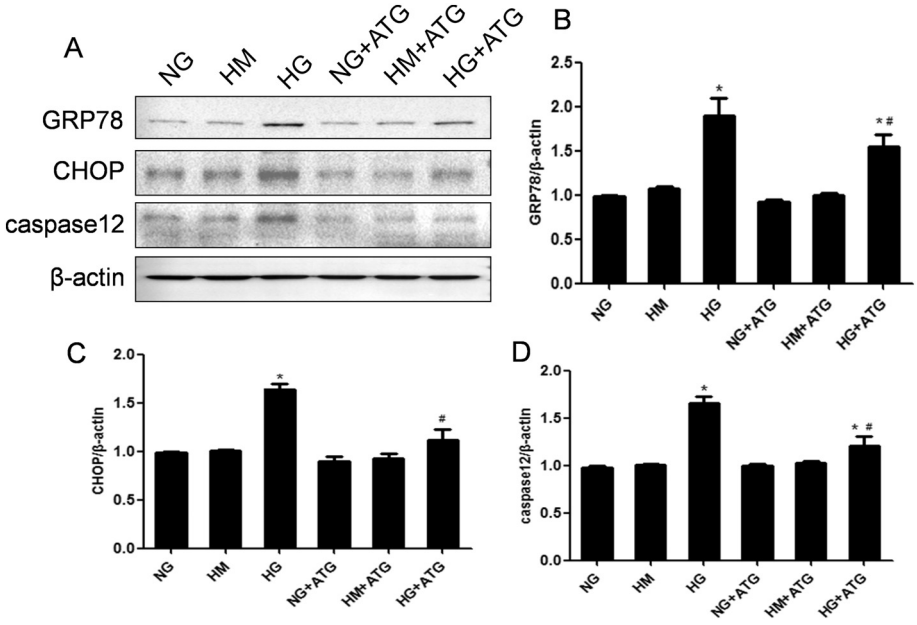

Fig. 6. ATG prevents high glucose-induced ER stress in HK2 cells. HK2 cells were pretreated with 1 μM ATG for 6 h followed by incubation with normal glucose (6 mM, NG) or normal glucose with equal osmolarity of mannitol (HM) or high glucose (30 mM, HG) at 30 mM for another 24 h. The expression levels of GRP78, CHOP, caspase12 and β-actin in HK2 cells were detected by western blot analysis (A). B–D. Quantification of the protein levels of GRP78 (B), CHOP (C) and caspase12 (D) are normalized by reference to the value of β-actin. Values are expressed as means ± SEM. $^{*}P < 0.05$ compared with NG group, $^{\#}P < 0.05$ compared with DN group. All experiments were performed in triplicate.

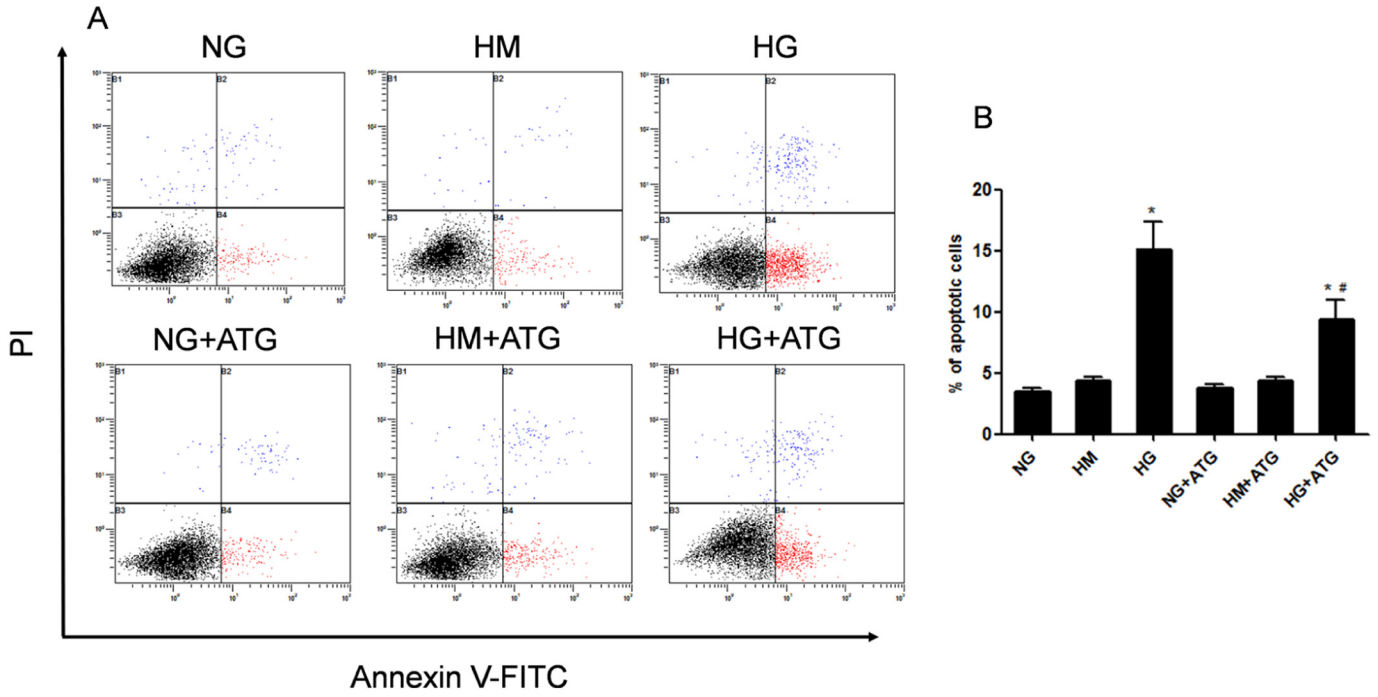

Fig. 7. ATG inhibits high glucose-induced apoptosis in HK2 cells. HK2 cells were pretreated with 1 μM ATG for 6 h followed by incubation with normal glucose (6 mM, NG) or normal glucose with equal osmolarity of mannitol (HM) or high glucose (30 mM, HG) at 30 mM for another 24 h. A. Representative images of apoptotic HK2 cells detected by flow cytometry. B. quantification of apoptotic HK2 cells. Values are expressed as means ± SEM. $^{*}P < 0.05$ compared with NG group, $^{\#}P < 0.05$ compared with DN group. All experiments were performed in triplicate.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Jing Zhang and Guodong Wang conceived and designed the study and drafted the manuscript. Jing Zhang, Peng Cao and Jingjing Gui performed the experiments. Jun Han and Yuwei Wang supported part materials and analyzed the data. All authors read and approved the final manuscript.

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