

## Gambogenic acid protects against high glucose-induced damage of renal tubular epithelial cells by inhibiting pyroptosis through regulating the AMPK–TXNIP pathway

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RESEARCH ARTICLE

### Abstract

Diabetic nephropathy, a chronic inflammatory disease, is characterized by hyperglycemia-stimulated pyroptosis of renal tubular epithelial cells. Gambogic acid, a primary component of gamboge resin, exerts detoxification, antioxidant, anticancer, anti-angiogenesis and anti-inflammatory capacities. However, the nephroprotective effect of gambogic acid on diabetic nephropathy remains unknown. Human kidney (renal) epithelial cell line HK-2 was treated with dextrorotatory-glucose (D-glucose) to establish an *in vitro* cell model of diabetic nephropathy, followed by incubation with gambogic acid. CCK-8 was designed to detect cell viability. Enzyme-linked-immunosorbent serologic assay (ELISA) was used to detect the levels of inflammation-related factors. Pyroptosis and underlying mechanism were investigated by Western blot assay. High glucose treatment decreased the viability of HK-2 cell line, while gambogic acid incubation restored the reduced cell viability. High glucose-induced increase in the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin (IL)-6, Monocyte chemoattractant protein-1 (MCP-1) and IL-1 $\beta$  were reduced by gambogic acid. The protein expressions of NLR family pyrin domain containing 3 (NLRP3), N-terminal domain of gasdermin D (GSDMD-N), caspase-1, IL-1 $\beta$  and IL-18 were up-regulated in HK-2 cells after high glucose condition, while down-regulated by incubation of gambogic acid. Gambogic acid attenuated high glucose-induced increase of thioredoxin-interacting protein (TXNIP) and phosphorylated 5' adenosine monophosphate-activated protein kinase (p-AMPK) in HK-2 cell line. Gambogic acid protected renal tubular epithelial cells against high glucose-induced inflammation and pyroptosis through suppression of AMPK–TXNIP pathway, providing a potential strategy for the prevention of diabetic nephropathy.

**Keywords:** AMPK–TXNIP; gambogic acid; high glucose; inflammation; pyroptosis; renal tubular epithelial cells

### Introduction

Diabetic nephropathy, a microvascular complication of diabetes, is characterized by glomerular capillary oxidative stress and inflammation, and persistent reduced

glomerular filtration rate (Rossing *et al.*, 2018). Diabetic nephropathy often results in end-stage renal disease and increases the long-term mortality and morbidity of patients with diabetes mellitus (Satirapoj & Adler, 2015). Although the pathological mechanism of diabetic

nephropathy remains elusive, hyperglycemia is viewed as a key factor to drive the progression of diabetic nephropathy (Hall, 2006). Hyperglycemia induces the activation of distinct cellular signaling involved in oxidative stress and inflammation, promotes glomerular ultrafiltration, renal tubular injury and deterioration of renal function (Rao *et al.*, 2021). It has been reported that suppression of renal tubular injury contributed to the amelioration of diabetic nephropathy (Zhao *et al.*, 2015). Therefore, renal tubular injury is viewed as a therapeutic target for preventing diabetic nephropathy.

Traditional medicinal plants (Keshvari *et al.*, 2020) or their extracts (Rahmati *et al.*, 2021) have been widely used for preventing diabetes. Gambogenic acid is one of the primary components of traditional Chinese medicine Gamboge, which is exuded from *Garcinia hanburyi* tree (Mei *et al.*, 2014). Gambogenic acid exerts antitumor activity against cell apoptosis in breast cancer (He *et al.*, 2015), and induces nasopharyngeal carcinoma cell (Yan *et al.*, 2011) or colorectal cancer (Zhao *et al.*, 2020) through mitochondrial oxidative stress signaling. Moreover, gambogenic acid also protects macrophages against lipopolysaccharide-induced increase in inflammatory factors (Akutsu *et al.*, 2016), and has demonstrated hepatoprotective effect against acetaminophen-induced oxidative stress, apoptosis and inflammation in rats (Ding *et al.*, 2021). Palmitic acid and high glucose-induced inflammatory response during the progression of diabetic retinopathy are repressed by gambogenic acid (Chen *et al.*, 2021). However, the role of gambogenic acid in diabetic nephropathy has not been reported yet.

Increasing evidence has demonstrated that high glucose stimulates the injury of renal tubular epithelial cells by promoting apoptosis, inflammation and oxidative stress (Zhang *et al.*, 2019). Therefore, high glucose-induced human kidney (renal) epithelial cell line HK-2 has been widely used as an *in vitro* cell model of diabetic nephropathy (Zhang *et al.*, 2019). The effects of gambogenic acid on the viability, inflammation and pyroptosis of high glucose-induced HK-2 cells are investigated in the present study. In addition, the underlying mechanism has been assessed to provide novel insight into the therapeutic intervention of diabetic nephropathy patients.

## Materials and methods

### Cell culture and treatment

Human renal epithelial cell line (HK-2) was provided by the Procell Life Science and Technology Co. Ltd. (Wuhan, China). Cells were cultured in low-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA,

USA) containing 1% penicillin–streptomycin (Gibco) and 10% fetal bovine serum (FBS; Gibco) at 37°C. Dextrorotatory-glucose (D-glucose; 20, 40, 60 or 80  $\mu$ M) (Sigma-Aldrich, St. Louis, MO, USA) was used to incubate HK-2 cells for 72 h. HK-2 cell line was also incubated with 0.5-, 1-, 2-, 4-, 8- or 16- $\mu$ M gambogenic acid (Shanghai Aladdin Biochemical Technology Co. Ltd.; Shanghai, China) for 24 h. HK-2 cell line after incubation with 40- $\mu$ M D-glucose was incubated with 0.5-, 1- or 2- $\mu$ M gambogenic acid, followed by functional assays.

### Cell viability and enzyme-linked-immunosorbent serologic assay (ELISA)

HK-2 cell line was seeded into 96-well plates and incubated with D-glucose or gambogenic acid. CCK-8 solution (Beyotime, Beijing, China) was added into each well, and the absorbance at 450 nm was measured by microplate reader (BioTek, Winooski, VT, USA). HK-2 cell line was incubated with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Beijing, China), and centrifuged at 12,000 g for 1 h for extracting supernatant. Protein concentration in the supernatant was determined by Pierce™ BCA Protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), and the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin (IL)-6, Monocyte chemoattractant protein-1 (MCP)-1 and IL-1 $\beta$  were measured using commercial kits (MultiSciences, Beijing, China).

### Western blot test

Proteins isolated from HK-2 cells were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membrane. The membrane was blocked and probed with specific antibodies: anti-NLR family pyrin domain containing 3 (NLRP3) and anti-N-terminal domain of gasdermin D (GSDMD-N) (1:2,000; Abcam, Cambridge, UK); anti-caspase-1 and anti-IL-1 $\beta$  (1:2,500; Abcam); anti-IL-18 and anti-thioredoxin-interacting protein (TXNIP, 1:3,000; Abcam); and anti-5' adenosine monophosphate-activated protein kinase (anti-AMPK), anti-phosphorylated AMPK (p-AMPK) and anti- $\beta$ -actin (1:3,500; Abcam). Following incubation with horseradish peroxidase-conjugated secondary antibody (1:4,000; Abcam) and tetramethylbenzidine, the protein bands were visualized using chemiluminescence (Sigma-Aldrich).

### Statistical analysis

Data with at least triple replicates were expressed as mean  $\pm$  standard error of mean (SEM), and analyzed by Student's *t*-test or one-way analysis of variance (ANOVA)

using the SPSS software;  $P < 0.05$  was considered as statistically significant.

## Results

### Gambogenic acid enhanced the viability of high glucose-treated HK-2 cell line

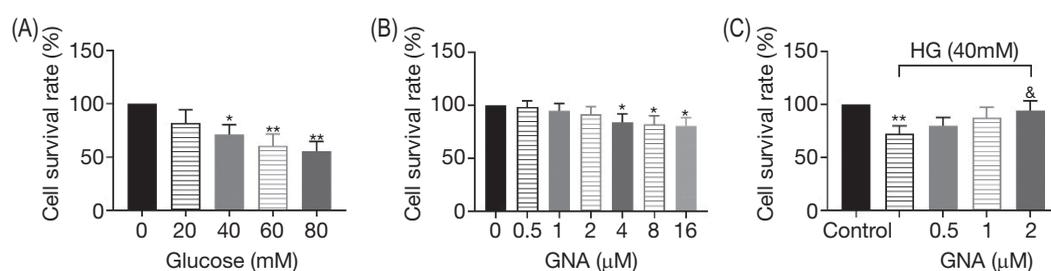
High concentrations of D-glucose (40, 60 or 80  $\mu\text{M}$ ) reduced the viability of HK-2 cells in a dose-dependent manner (Figure 1A), suggesting the cytotoxicity of high glucose on renal tubular epithelial cells. Administration of gambogenic acid below 2- $\mu\text{M}$  concentration had no significant effect on the viability of HK-2 cells (Figure 1B), while 4- $\mu\text{M}$  concentration or more (4, 8 or 16  $\mu\text{M}$ ) decreased the viability of HK-2 cell line (Figure 1B). HK-2 cell line was co-incubated with 40- $\mu\text{M}$  glucose and 0.5-, 1- or 2- $\mu\text{M}$  gambogenic acid to investigate the nephroprotective effect of gambogenic acid against high glucose-caused decrease in cell viability. Indeed, the decreased viability of HK-2 cells caused by high glucose was increased by gambogenic acid in a dose-dependent manner (Figure 1C).

### Gambogenic acid suppressed inflammation in high glucose-treated HK-2 cell line

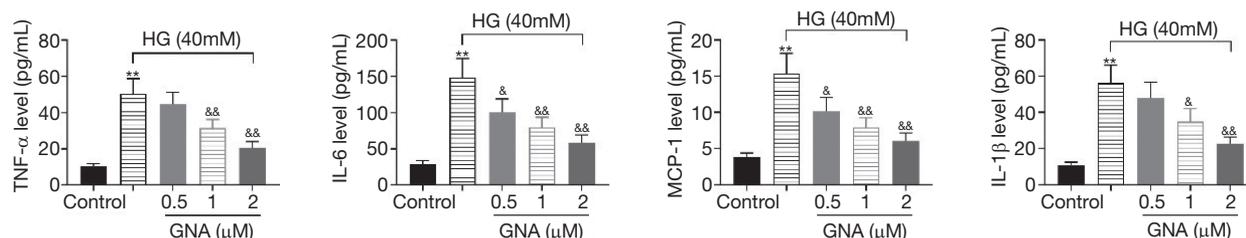
The levels of proinflammatory cytokines, TNF- $\alpha$ , IL-6, MCP-1 and IL-1 $\beta$ , were up-regulated in HK-2 cells after exposed to high glucose (40  $\mu\text{M}$ ) (Figure 2). However, gambogenic acid down-regulated the levels of TNF- $\alpha$ , IL-6, MCP-1 and IL-1 $\beta$  of high glucose-treated HK-2 cells (Figure 2) in a dose-dependent manner, indicating the anti-inflammatory effect of gambogenic acid on high glucose-treated HK-2 cells.

### Gambogenic acid suppressed pyroptosis in high glucose-treated HK-2 cell line

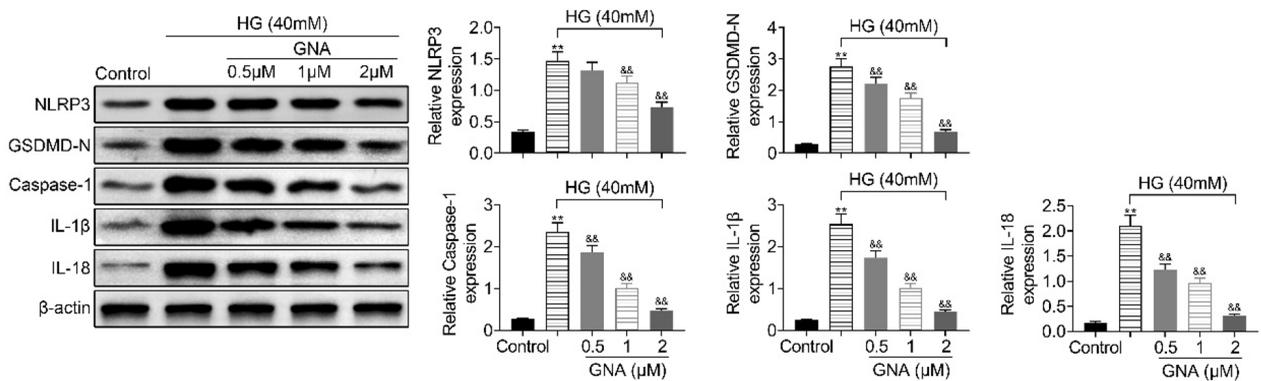
High glucose induces the up-regulation of NLRP3, GSDMD-N, caspase-1, IL-1 $\beta$  and IL-18 in HK-2 cells (Figure 3). However, the levels of NLRP3, GSDMD-N, caspase-1, IL-1 $\beta$  and IL-18 in high glucose-treated HK-2 cells are reduced by gambogenic acid in a dose-dependent manner (Figure 3). These results demonstrated that gambogenic acid suppresses pyroptosis in high glucose-treated HK-2 cell line.



**Figure 1.** Gambogenic acid enhanced the viability of high glucose (HG)-treated HK-2 cells. (A) D-glucose reduced the viability of HK-2 cells in a dose-dependent manner. (B) Gambogenic acid at concentrations of 0, 0.5, 1 and 2  $\mu\text{M}$  had no significant effect on the viability of HK-2 cells, while at concentrations of more than 4  $\mu\text{M}$  (8 and 16  $\mu\text{M}$ ) decreased the viability of HK-2 cells. (C) Gambogenic acid enhanced the viability of high glucose-treated HK-2 cells in a dose-dependent manner. \*, \*\*vs. control,  $P < 0.05$ ,  $P < 0.01$ . &vs. HG group,  $P < 0.05$ .



**Figure 2.** Gambogenic acid suppressed inflammation in high glucose (HG)-treated HK-2 cells. Gambogenic acid down-regulated the levels of TNF- $\alpha$ , IL-6, MCP-1, and IL-1 $\beta$  in high glucose-treated HK-2 cells. \*\*vs. control,  $P < 0.01$ . & $P < 0.05$ . &&vs. HG group,  $P < 0.01$ .



**Figure 3.** Gambogic acid suppressed pyroptosis in high glucose (HG)-treated HK-2 cells. Gambogic acid down-regulated the levels of NLRP3, GSDMD-N, caspase-1, IL-1 $\beta$  and IL-18 in high glucose-treated HK-2 cells. \*\*vs. control,  $P < 0.01$ . &&vs. HG group,  $P < 0.01$ .

### Gambogic acid suppressed the activation of AMPK–TXNIP signaling in high glucose-treated HK-2 cell line

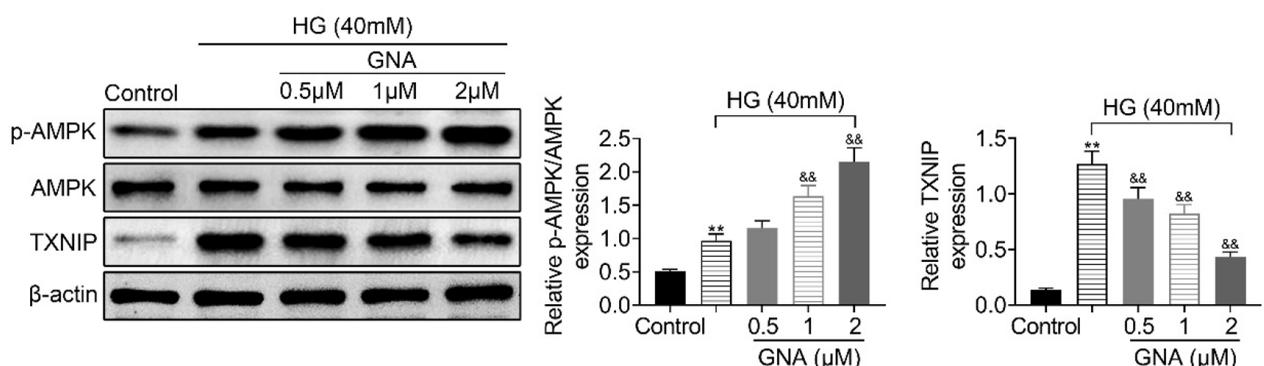
High glucose induces the up-regulation of TXNIP (Figure 4) and down-regulates the protein expression of p-AMPK in high glucose-treated HK-2 cells (Figure 4). Administration of gambogic acid reduces the protein expression of TXNIP and promotes the protein expression of p-AMPK in high glucose-treated HK-2 cell line (Figure 4). Gambogic acid suppresses the activation of AMPK–TXNIP signaling to protect HK-2 cell line against high glucose condition.

## Discussion

Chinese herbal medicines demonstrate long-term beneficial effects in the patients with diabetic nephropathy (Guo *et al.*, 2021). For example, kolaviron, derived from *Garcinia kola* seeds, suppressed inflammation and oxidative stress in rats with diabetic nephropathy (Ayepola *et al.*, 2014). Prenylated caged xanthenes, derived from

*Garcinia hanburyi*, are established to function as an inhibitor of protein tyrosine phosphatase 1B and thus involved in the progression and development of diabetes (Tan *et al.*, 2017). Gambogic acid is a xanthonoid derived from *Garcinia hanburyi* that protects against high glucose-induced inflammatory response in diabetic retinopathy (Chen *et al.*, 2021). The present study established that gambogic acid exerted protective effect against diabetic nephropathy by regulating cell viability, inflammation and pyroptosis.

Gambogic acid is regarded as a tumor suppressor through inhibiting the proliferation of tumor cells (Xu & Wantao, 2012). High concentration of gambogic acid ( $\geq 4 \mu\text{M}$ ) in this study reduced the viability of HK-2 cells. However, its lower concentration ( $\leq 2 \mu\text{M}$ ) increased the viability of high glucose-treated HK-2 cells. Hyperglycemia induces the inflammatory response of renal tubular epithelial cells, and contributes to the pathogenesis of diabetic nephropathy (Salti *et al.*, 2020). Suppression of high glucose-induced inflammation relieves renal tubular injury in diabetic



**Figure 4.** Gambogic acid suppressed activation of AMPK–TXNIP signaling in high glucose (HG)-induced HK-2 cells. Gambogic acid reduced the protein expression of TXNIP and promoted the protein expression of p-AMPK in high glucose-treated HK-2 cell line. \*\*vs. control,  $P < 0.01$ . &&vs. HG group,  $P < 0.01$ .

nephropathy (Su *et al.*, 2020). Consistent with a previous study establishing that gambogic acid inhibits lipopolysaccharide-induced up-regulation of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\beta$ , IL-12 $\beta$  and IL-23 $\alpha$  in macrophages (Akutsu *et al.*, 2016), incubation with gambogic acid down-regulates the levels of TNF- $\alpha$ , IL-6, MCP-1 and IL-1 $\beta$  in high glucose-treated HK-2 cells. Therefore, gambogic acid protects renal tubular epithelial cells against high glucose condition through proliferative and anti-inflammatory effects. High glucose also induces the apoptosis of renal tubular epithelial cells through Rho kinase signal (Li *et al.*, 2018), and hyperglycemia-induced apoptosis of renal tubular epithelial cells is the characteristic of diabetic nephropathy (Zhang *et al.*, 2019). Gambogic acid exerts pro-apoptotic effect on tumor cells (Chen *et al.*, 2015). The effect of gambogic acid on the apoptosis of high glucose-treated HK-2 cells must be investigated in the future research.

Pyroptosis, characterized by release of inflammatory factors (IL-1 $\beta$  and IL-18) through activation of caspase-1, is a pattern of programmed cell death and conceived as a recommendation for clinical diagnosis, targeted treatment and prognosis of kidney diseases (Zhang *et al.*, 2021). Pyroptosis facilitates local inflammation and promotes the progression of diabetic complications (Mamun *et al.*, 2021). Moreover, activation of NLRP3 inflammasome contributes to caspase-1-mediated pyroptosis and progression of diabetic nephropathy (Li *et al.*, 2020). High glucose also induces the expression of protein levels of NLRP3, GSDMD, caspase-1, IL-1 $\beta$  and IL-18 in renal tubular epithelial cells, and suppression of NLRP3-mediated pyroptosis ameliorates high glucose-induced cell injury (Ke *et al.*, 2020). A previous study has proved the suppressive effect of gambogic acid on NLRP3-mediated pyroptosis signaling in diabetic retinopathy (Chen *et al.*, 2021). Results in this study demonstrated that gambogic acid reduced the levels of NLRP3, GSDMD, caspase-1, IL-1 $\beta$  and IL-18, thus exerting anti-diabetic effect against high glucose.

AMPK signaling regulates cell growth and energy metabolism, thus implicating in the pathogenesis of diabetic retinopathy (Kubota *et al.*, 2011). The level of p-AMPK was down-regulated in a mouse model with diabetic nephropathy, and activation of AMPK pathway promoted autophagy and reduced renal cell apoptosis, thus alleviating renal injury induced by high glucose (Song *et al.*, 2021). AMPK promoted the activation of NLRP3 inflammasome to suppress hyperglycemia-induced pyroptosis in cardiomyocytes through degeneration of TXNIP (Wei *et al.*, 2019). AMPK–TXNIP–NLRP3 signaling was involved in the pathogenesis of diabetic nephropathy. (An *et al.*, 2020; Yang *et al.*, 2021). Gambogic acid promoted the activation of AMPK pathway (Kim *et al.*,

2014). Here, gambogic acid promoted the protein expression of p-AMPK and reduced the protein expression of TXNIP in high glucose-induced HK-2 cells, suggesting that AMPK–TXNIP was associated with gambogic acid-mediated diabetic retinopathy.

## Conclusion

Gambogic acid exerts renal protective effects against high glucose condition by inhibiting inflammation and pyroptosis. Gambogic acid suppresses the activation of AMPK–TXNIP-mediated NLRP3 inflammasome to attenuate high glucose-induced injury in HK-2 cells. Therefore, gambogic acid could be a novel therapeutic avenue for diabetic nephropathy. However, the effect of gambogic acid on animal model with diabetic nephropathy must be investigated in the future research.

## Competing interests

The authors state that there were no conflicts of interest to disclose.

## Contribution of authors

Ningxu Li and Xiuying Wen designed and Mingjuan Tang conducted the experiments. Xiangmei Peng analyzed and interpreted the data. Qizhi Sheng and Ping Liu prepared the manuscript with contributions of all coauthors.

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