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Ellagic acid attenuates streptozocin induced diabetic nephropathy via the regulation of oxidative stress and inflammatory signaling

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ABSTRACT

Diabetic nephropathy (DN) is the leading cause of chronic renal disease. Accumulating evidence suggested that oxidative stress and inflammatory processes are involved in the development of DN. In the present study, the DN model was established by injecting mice with STZ (180 mg*kg-1) intraperitoneally, and treated with EA (50, 100 and 150 mg*kg-1) and IRB (positive control) once daily by intragastric gavage. At the same time, rat kidney NRK-52E cells were cultured and incubated with EA and TAK-242 (inhibitor of TLR4) respectively before stimulating with LPS. The mental conditions, body weight, blood glucose, serum albumin (Alb), serum TNF-α, renal function, anti-oxidative enzymes, and protein expression of TLR4, IRAK4, TRAF6, IKKβ, NF-κb P65, HMGB1 in renal tissue were determined. Meanwhile, the proteins expression of TLR4, IRAK1 and NF-κBp65 in cells were further analyzed. The results showed that EA could improve the daily state and body weight; decrease the blood glucose, levels of TNF-α and serum creatinine; elevate the activities of antioxidant enzymes; ameliorate the renal pathology; inhibit the up regulation of expression of proteins TLR4, IRAK4, TRAF6, IKK-β, NF-κBp65 and HMGB1 in DN mice. These results suggested that EA ameliorated STZinduced oxidative renal injury by the inhibition of HMGB1-TLR4-NF-κB pathway.

1. Introduction

Diabetes is a metabolic disease that causes blood glucose to exceed the normal range. Diabetic nephropathy (DN) is the major long term side effects observed due to the diabetes (Shah et al., 2017), about one-third of diabetics suffering from DN (Brosius et al., 2009), which is a chief cause of kidney damage throughout the world (Kowalski et al., 2015). In recent years, the number of patients with DN has increased rapidly and has become a public health disease that endangers human health. Patients often have different degrees of proteinuria, hypertension, edema and other symptoms as the main clinical manifestations. Although there are many drugs available for treatment, at present, diminishing postprandial hyperglycemia is thought to be the best method (Pal et al., 2014).

DN shows chronic inflammatory reaction accompanied by metabolic dysfunction and hemodynamic changes (Kimberly et al., 2014), which suggests that chronic inflammatory systems, innate immunity (including toll like receptors, TLRS) and regulating T cells assume a vital part in its improvement and progression (Odegaard, 2012; Alhaider et al., 2011). But, the mechanism has not been fully elucidated. Recently, more and more studies have demonstrated that DN was closely associated with oxidative stress (Zhang et al., 2012; Keshari et al., 2015; Hakim and Pflueger, 2010), the changes of glomerular hemodynamics and disturbance of glucose metabolism (Qi et al., 2018; Pal et al., 2014). In particular, oxidative stress and pro-inflammatory cytokines are thought to play an important role in the early development of diabetic nephropathy. So DN has been considered as an inflammatory disease (Alhaider et al., 2011).

TLRS is the most important pattern recognition receptor in the surface of innate immune cells, endothelial cells and epithelial cells (Kawai and Akira, 2010; Wada and Makino, 2016). It is also a bridge between nonspecific immunity and specific immunity. At present, there are 13 species of TLR, respectively, TLR1-TLR13, of which TLR12 and TLR13 do not exist in the human body. TLR is a transmembrane protein whose structure is divided into extracellular domain, transmembrane domain and intracellular domain. TLR4 is one of the most widely studied TLR family proteins in the world, and the extracellular domain is a repetitive series of leucine, which mediates the identification of pathogen-related molecular patterns. The intracellular domain is a highly conserved sequence, homologous to the intracellular region of interleukin-1 (IL-1) receptor, so its intracellular domain is called TOLL/IL-1 receptor (TIR). When the corresponding ligands such as

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lipopolysaccharide (LPS) and TLR4 are combined, the signals are transferred to the TIR region, through the medullary differentiation factor 88 (MYD88) dependent or MYD88-independent signaling pathways to further activate the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways and facilitate the release of various cytokines (Lin et al., 2012). Studies have shown that TLR4 could promote inflammation of the renal tubules in DN. TLR4 antagonists could protect DN by inhibiting TGF-β over expression and activating NF-κB (Lin et al., 2013). When NF-κB is activated, it can affect many signaling cascades to maintain redox homeostasis; thereby further modulating the levels of key redox modulators in health and disease (Yerra et al., 2013).

High mobility group protein 1 (HMGB1) belongs to highly migratory protein family (HMG), and it is a highly conserved non-histone protein (Kang et al., 2014a). It is considered that the HMGB1 signaling pathway is mainly mediated by glycation end product receptors and TLRS (including TLR2, TLR4 and TLR9). HMGB1 is mainly located in the nucleus of the cell, which could identify and bind DNA, change the advanced structure of DNA. Thus as a common factor in DNA, HMGB1 plays an important role in cell movement and transcription, replication, repair of DNA. In extracellular domain, HMGB1 and its receptors activate monocytes/macrophages, which eventually leads to NF-κB activation and production of IL-6, IL-1β, TNF-α and other pro-inflammatory factors. NF-κB activation could in turn induce the expression of HMGB1 and its receptors, releasing cytokines that further activate monocytes/macrophages to form positive feedback. In patients suffered from type 2 diabetes and animal models, the content of HMGB1 increased significantly. Studies have shown that metformin significantly reduce the expression of HMGB1 in LPS-stimulated RAW264.7 cells (Tang et al., 2011; Wang et al., 2016). And many studies have shown that HMGB1 were related to adipose tissue inflammation, insulin resistance, islet inflammation and Islet B cell apoptosis (Tsoyi et al., 2011).

STZ is widely employed to induce the experimental model of DN, as it is selectively destroying the pancreatic β-cells by generating reactive oxygen species, which leads to decrease in the insulin secretion (Bellenger et al., 2011; Jin et al., 2009). It is well known that STZ causes cytotoxic action towards β-cells by inhibiting the free radical scavenging enzymes and thereby increasing the oxidative stress which causes cell degeneration (Roy et al., 2013). The method of multiple consecutive injections of the STZ can selectively destroy islets β-cells and induce inflammatory response, which leads to further loss of β-cell activity and causes insulin deficiency and hyperglycemia (Zhu et al., 2017). Therefore, the method of multiple consecutive injections of the STZ is widely used for making experimental diabetic animal models (Bellenger et al., 2011; Wu and Huan, 2008; Zhu et al., 2017).

Natural plants are of importance to the management of many diseases in humans, such as diabetes (Ahmed et al., 2004; Mohamed et al., 2010; Kankana and Mahua, 2017). Numerous herbal medicinal plants are natural sources of antioxidants, which can reduce the oxidative stress generated by STZ in β-cells. World Health Organization (WHO) has recommended the evaluation and application of traditional botanical treatments for diabetes because they are effective, non-toxic, have fewer side effects or have no side effects, and are considered excellent candidates for oral therapy (Day, 1998). Therefore, in recent years, more and more researchers are paying attention to natural plants.

Ellagic acid (C14H6O8, EA, the chemical structure shown in Fig. 1A), also named trihydroxy acid, is a product of hydrolyzed tannins, widely contained in nuts, tea, berries, oak wine and other foods (Zhou et al., 2016), it's also the main active ingredient of pomegranate peels, garden burnet and other natural drugs. Human and animal experiments have shown that EA exhibits various biological activities, especially anti-oxidative, anti-inflammatory (Qiu et al., 2013; Larrosa et al., 2010), as well as anti-cancer (Zhao et al., 2013), and has potential preventive or therapeutic effects on chronic diseases such as cardiovascular (Reis Jordao et al., 2017) and neurodegenerative diseases (Ahmed et al., 2016). Nankar have reported that EA can improve insulin sensitizing activity of pioglitazone in L6 myotubes in a synergistic manner (Nankar and Doble, 2015). Therefore, some researchers have begun to further study the role of EA in diabetes. In recent years, more and more studies have revealed that EA can exert an improving effect on anti-diabetic alone (Monia et al., 2017; Simran et al., 2018) or in combination with other drugs (Rakesh and Mukesh, 2017), and there are also more and more about the protective effect of EA on liver toxicity (Abdullah et al., 2018). But so far, little has been reported in the literature on the protective effects of EA on the renal toxicity, especially on the protection of STZ-induced nephrotoxicity in DN mice. Thus, in the present study, we aimed at determining the ameliorative effects of EA against STZ induced DN in mice and LPS induced inflammation in NRK52E cell, and the underlying mechanisms.

2. Materials and methods

2.1. Chemicals

Streptozocin was purchased from Sigma Chemical (St. Louis, MD, USA, No. 14110807, purity≧99%). Ellagic Acid standard (Lot No, C1502031, purity > 98%) was obtained from Aladdin Reagents (Shanghai China). Irbesartan was bought from Hangzhou sanofiaventis minsheng pharmaceutical co. LTD (Shanghai China, No. 20163214, purity > 98%). Total superoxide dismutase (T-SOD, No.20160512), malondialdehyde (MDA, No. 20160426), creatinine (CRE, No.201604320) urea nitrogen (BUN, No.201603421) were supplied by Nanjing Jiancheng Bioengineering Institute. Tumor necrosis factor-α (TNF-α, No. 20160520) ELISA assay kit was obtained from Myhalic biotechnology co. LTD (Wuhan China). Toll-like receptor 4 (TLR4), Interleukin-1 receptor-associated kinase 4 (IRAK4), TNF receptor associated factor 6 (TRAF6), Inhibitor of nuclear factor kappa-B kinase β (IKK β, Nuclear factor-kappa B (NF-κB) p65, High mobility group box-1 protein (HMGB1) antibodies were from Santa Cruz biotechnologies (Santa Cruz, CA). All the other chemicals used were of analytical grade and purchased from Guoyao Chemical Reagent Co., LTD.

2.2. Animals

The male Institute of Cancer Research (ICR) mice (aged 4–6 weeks, body weighted of 18 g–22 g, License number: SCXK (E) 2013–0004) were purchased from Wuhan Institute of Biological Products Co. Ltd. (Wuhan China), which were raised in an environmentally controlled breeding room (temperature: 23 ± 2 °C, humidity: 30%–60%, 12-h light-dark cycle) with enough food and water. All studies were conducted in accordance with the guidelines regarding the care of experimental animals as approved by the Animal Research Central at Wuhan University.

2.3. In vitro model for LPS induced inflammation

The NRK52E cells were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). NRK52E cells were derived from rat renal tubular epithelial cells. NRK52E cells were cultured in DMEM medium supplemented with 10% FBS and 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C and in 5% CO_2. When cells were cultured at a density of $1*10^5$ cells·ml^{-1}, cells were seeded into 6-well plates with glass slides. They were randomly divided into four different groups (blank, LPS, LPS + TAK-242, LPS + 100 μg mL^{-1} EA). Groups of TAK-242 and 100 μg L^{-1} EA were given 0.1 mg mL^{-1} LPS stimulation for 24-h after drug intervention.

2.4. In vivo animal experimental protocol

After two weeks of adaptive feeding, 48 mice were randomly divided into six groups(each group with 8 mice), as follow: normal

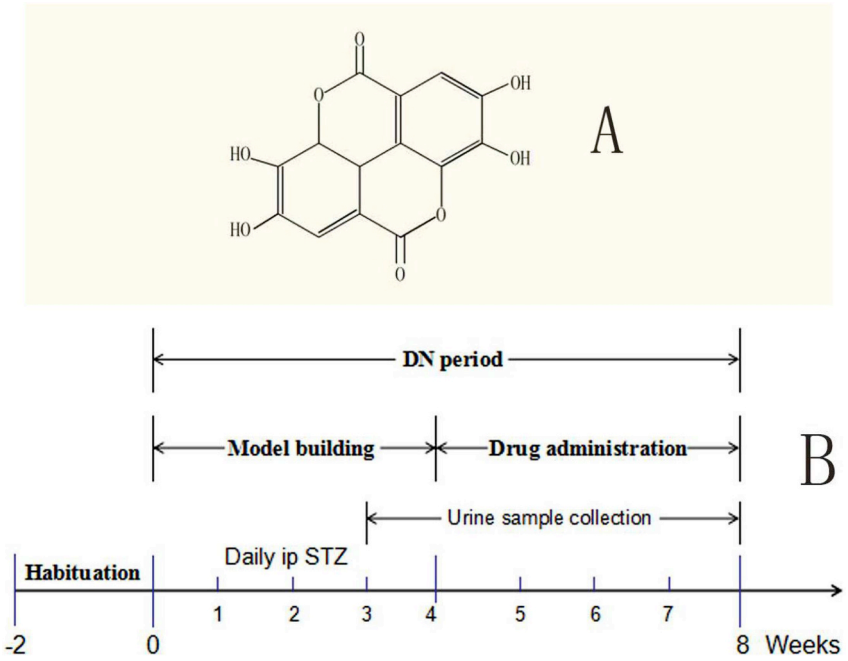


Fig. 1. (A) Molecular structure of EA. (B) Schematic representation of treatment schedule followed for the modulation of DN using STZ and EA.

control group (SHAM), STZ model group (MODEL), irbesartan positive control group (IRB, 180 mg kg^{-1}), high dose of EA group (HEA, 150 mg kg^{-1}), medium dose of EA group (MEA, 100 mg kg^{-1}), low dose of EA group (LEA, 50 mg kg^{-1}) respectively. All mice fasted for 8 h, except for the normal control group, the remaining mice were injected intraperitoneally with STZ (180 mg kg^{-1}·d^{-1} for 4 weeks), which was dissolved in the pH4.5 citric acid buffer in ice bath to establish DN model (Kang et al., 2014b). To single out the successful model, blood glucose in mice was measured for 3 times by blood glucose meter (ONE TOUCH Ultra Easy), and it indicated the DN model was successfully established if the blood glucose > 16.7 mmol L^{-1} every time on the 3rd day, 10th day and 17th day. After the discrimination of model, the successful modeling mice were administered orally with EA or normal saline. The mice in IRB group were administrated with 20 mg kg^{-1} IRB. The mice in EA group were administered with EA (The EA was prepared with 0.01 mol L^{-1} NaOH solution) once a day for four consecutive weeks. In the meantime, the SHAM and model groups were given by gavage of normal saline with the same volume. During the whole study, all mice were fed normally except for necessary fasting without insulin and hypoglycemic drugs. Body weights and blood glucose were monitored every two weeks throughout the treatment period. The animal experiments were carried out in accordance with Institutional Animal Ethical Committee (IAEC) guidelines. All efforts were made to minimize animal suffering.

From the twenty-first day after the STZ injection, the mice were placed in metabolic cages for collection of urine samples. Than 100 μl aliquot of urine sample was thawed at 4 °C and diluted at a ratio of a three-fold volume of methanol to precipitate proteins. The mixture was centrifuged at 12000 rpm for 10 min in the high-speed refrigerated centrifuge at 4 °C, and the clear supernatant was immediately stored at −80 °C until tested for biochemical indicators. At the end of the treatment protocol, all mice were euthanized, and blood samples were collected from the tail vein and centrifuged at 3000 rpm for 10 min to obtain clear serum. The bilateral kidney tissues from all groups were excised and weighed, one side of the kidney was cut longitudinally, with 4% of poly-formaldehyde fixed, used for pathological studies by HE staining and PAS staining, The rest of the kidney was collected for assessing the other biochemical and molecular aspect.

2.5. Measurement of body weight, blood glucose and renal index

Body weights of mice were measured at 2-week intervals. Blood glucose was detected in two week intervals by One Touch Ultra blood glucose monitoring system (Life Scan Inc., Milpitas, CA, USA) by blood sampling from the tail vein. Renal index (RI) was ratio of kidney weight (KW) and body weight (BW), and was calculated by following formula: RI (KW/BW) = [left kidney weight (mg) + right kidney weight (mg)]/(2*body weight) (g) (Pal et al., 2014).

2.6. Biochemical index analysis

The levels of blood urea nitrogen (BUN) and creatinine (CR) in urine and serum samples were measured spectrophotometrically by means of urease and enzymatic method for assaying respectively. Total superoxide dismutase activity (T-SOD) and malondialdehyde (MDA) in kidney tissues and serum were estimated using commercially available assay kits. The concentration of microalbuminurea (m-ALB) protein in 24 h urine and TNF-α in serum were measured by ELISA assay kit (RD system). The 24 hm-ALB was calculated according to the following equation: 24 hm-ALB (ug) = m-ALB c (ug·ml^{-1})*VU (ml). All biochemical index analysis followed the manufacturer's protocols.

2.7. Histopathologic examination

The kidneys were fixed with paraformaldehyde overnight. The fixed tissue specimens were dehydrated in graded alcohol solutions, cleared in toluene, and embedded in paraffin (JB-P5). To assess histopathologic changes in the kidneys, Sections (3 μm) were stained with the hematoxylin and eosin (H&E) and trichromeperiodic acidschiff stain (PAS) according to standard procedure and examined under a microscope. Ultra-thin sections were observed with the electron microscope (OLYMPUS BX51).

2.8. Western blot analysis

To invest the effect of EA and IRB on the TLR4-NF-κB signaling pathway, several proteins in mice kidney tissue homogenate was determined by Western Blot. Kidney tissues were lysed by 1 ml RIPA

(radioimmunopre cipitation assay) lysis buffer to extract total protein samples. The tissue homogenates were collected by centrifugation at 12,000 rpm for 30 min at 4 °C, and the supernatant was saved and used for the concentration determination by BCA protein Assay kit. Then each sample containing same amounts of protein (40 μg) was subjected to sodium dodecyl sulfate-polyacryl amide gel electro-phoresis (SDS-PAGE) gels and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. 5% fat-free milk (in PBST) was used to block membranes for 1 h at room temperature. After incubating 1-antibo overnight at 4 °C, the membranes were washed three times with TBST and incubated with 2-antibodies contain TLR4, IRAK4, TRAF6, IKKβ, NF-κbp65 and HMGB1 proteins for 2 h at room temperature. Finally, an enhanced chemiluminescence (ELC) was adopted to color the brands. Pictures of proteins were taken and processed by Image-Pro-Plus 6.0 software.

Western Bolt also was applied for vitro experiment to assess the influence of EA to the relevant proteins. Cells from each group (control group, LPS group, LPS + TAK-242 positive control group, LPS + 100 μg·ml^{-1} EA group) were harvested and lysised in total protein extraction reagent with protease inhibitors. Then the lysate was centrifuged at 12,000 rpm for 30 min and supernatant was collected. The rest step is identical to describe above.

2.9. Statistical analysis

Analysis was carried out by the Statistical Package Social Sciences 19.0 system (SPSS Inc., Chicago, Illinois, USA), and the results were represented as the means ± standard deviation (SD) of at least three independent experiments. The data was compared by two-way analysis of variance (ANOVA) followed by t tests. Differences were considered statistically significant when P value was less than 0.05.

3. Results

3.1. Psychosis status, blood glucose and renal index

After 17 days establishment of DN model, the mice in normal group were active, sensitive to sound and light, possessed white and shiny hair and their feces were granular, while the mice in control group were gaunt, listless, unresponsive and their hair were lusterless, their feces were much and thin. As shown in Table 1, the mice in EA groups and IRB group appear better on physiological status than model group. Comparing with model group, the weights and blood glucose of mice in IRB group and EA groups were subtle improving, but there was no statistical significance compare with sham group ($p > 0.05$). Table 2 presented the renal index of mice in each group, there was significant difference between sham group and the other groups ($p < 0.01$), however, no significant difference among model group, IRB group and EA groups. IRB and EA could not reverse kidney hypertrophy caused by diabetes.

3.2. Effect of EA on m-ALB, BUN and Cr in DN mice

m-ALB is an early manifestation of renal impairment in diabetic patients, Cr is a product of muscle energy metabolism while urea is that of protein metabolism. All the products are excreted by kidneys and they are well known markers which indicate the normal functioning of the kidney (Bagshaw and Gibney, 2008). As seen in Fig. 2 and Fig. 3, results showed that STZ-treatment significantly ($p < 0.01$) enhanced m-ALB, the BUN and Cr in serum, and the BUN and Cr were significantly ($p < 0.01$) decreased in urine, suggesting that STZ-treatment (180 mg ml^{-1}) could develop DN in ICR mice. In contrast, IRB, MEA and HEA significantly decreased the m-ALB containing in 24 h urine ($p < 0.01$). LEA also lowered the 24 h urine albumin, but there was no significant difference comparing with the model group ($p > 0.05$). After molding, the Cr and BUN in serum were increased significantly and they were decreased in urine. IRB could reverse this trend significantly ($p < 0.01$), EA could also change the trend dose-dependently. In addition, HEA changed the trend of Cr significantly compared with the model group ($p < 0.01$), but there was no significant difference compared with the sham group ($p > 0.05$). The results were seen in Fig. 3.

3.3. Histopathology analysis

Histological evaluation was conducted by using H&E and PAS to evaluate the histopathological features of mice kidney issue. By HE means, cell nucleus was stained blue, cytoplasm was stained red, and after PAS staining, neutral mucus substance appeared red while cell nucleus appeared blue. As shown in Fig. 4, compared to the sham group, the mesangial cells were moderately hyperplasia, Bowman's capsule expanded, mesangial thickened, the glomerular basement membrane thickened, matrix hyperplasia accumulated, some inflammatory cells infiltrated, and staining showed slight nodular glomerular sclerosis in model group. On the contrary, with the pretreatment of IRB, the expansion of the Bowman's capsule was relieved, the glomerular size was almost no different from the normal group, the mesangial cells were not obvious hyperplasia, and the mesangial was not obviously thickened. LEA group still showed thickening of the mesangial, slight hyperplasia of stroma of the mesangial, thickening of the glomerular basement membrane, but the expansion of the Bowman's capsule was relieved. In MEA group and HEA group, the glomerular size was similar to the normal group, and the expansion of Bowman's capsule and the thickening of the basement membrane of the renal tubular were relieved obviously, and the mesangial thickening and mesangial cells hyperplasia were also relieved apparently. No inflammatory cells infiltration was found when treated with IRB, LEA, MEA and HEA. It could be known that IRB and MEA have a certain effect on the pathology of renal injury caused by DN.

3.4. Effects of EA on renal anti-oxidation status

T-SOD is a kind of metal enzyme that could clear free radicals in the

Table 1
Effect of EA on body weight and blood glucose (n = 8, means ± sd).

Groups	Week 2		Week 4		Week 6		Week 8	
	BW (g)	BG (mM)	BW (g)	BG (mM)	BW (g)	BG (mM)	BW (g)	BG (mM)
SHAM	27.12 ± 2.1	4.7 ± 1.3	31.12 ± 1.7	4.9 ± 1.3	34.12 ± 1.7	5.1 ± 1.3	40.12 ± 1.9	6.3 ± 1.5
Model	26.52 ± 1.8	14.7 ± 1.2$^{\triangle}$	23.52 ± 1.3$^{\triangle}$	19.7 ± 1.2$^{\triangle\triangle}$	25.52 ± 1.3$^{\triangle}$	20.7 ± 1.4$^{\triangle\triangle}$	28.52 ± 2.1$^{\triangle\triangle}$	22.2 ± 0.8$^{\triangle\triangle}$
IRB	25.35 ± 1.9	14.2 ± 0.9$^{\triangle}$	22.35 ± 1.6$^{\triangle}$	19.2 ± 0.9$^{\triangle\triangle}$	27.35 ± 1.6$^{\triangle}$	14.4 ± 1.2$^{\triangle\#}$	33.35 ± 1.8$^{\triangle\#}$	8.5 ± 1.6$^{\#\#}$
HEA	26.62 ± 1.2	13.8 ± 1.3$^{\triangle}$	22.62 ± 1.5$^{\triangle}$	20.8 ± 1.3$^{\triangle\triangle}$	25.62 ± 1.5$^{\triangle}$	14.8 ± 0.9$^{\triangle\#}$	32.62 ± 1.6$^{\triangle\#}$	8.7 ± 1.3$^{\#\#}$
MEA	25.24 ± 1.4	14.0 ± 1.1$^{\triangle}$	21.24 ± 2.0$^{\triangle}$	20.0 ± 1.1$^{\triangle\triangle}$	24.24 ± 2.0$^{\triangle}$	15.0 ± 0.7$^{\triangle\#}$	30.24 ± 2.2$^{\triangle}$	11.2 ± 1.5$^{\triangle\#\#}$
LEA	25.06 ± 1.6	13.9 ± 0.8$^{\triangle}$	22.06 ± 1.4$^{\triangle}$	19.9 ± 0.8$^{\triangle\triangle}$	25.06 ± 1.4$^{\triangle}$	15.6 ± 1.1$^{\triangle\#}$	30.06 ± 2.4$^{\triangle}$	12.1 ± 0.9$^{\triangle\#\#}$

Notes: n = 8; $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ VS. SHAM group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ VS. model group.

Table 2
Kidney index of each group. (means ± sd).

	SHAM (n = 8)	MODEL (n = 8)	IRB (n = 8)	LEA (n = 8)	MEA (n = 8)	HE (n = 8)
KW/BW(mg/g)	7.11 ± 0.57	13.95 ± 1.52**	13.32 ± 0.97**	14.47 ± 1.13**	14.76 ± 2.98**	14.25 ± 1.37**

Mean values were significantly different compared with the SHAM group: *$p < 0.05$, **$p < 0.01$ VS. SHAM group.

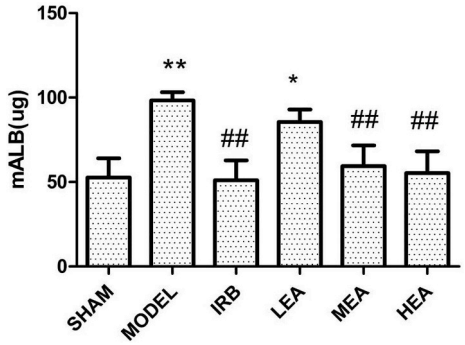


Fig. 2. 24 h m-ALB of each group. Values are means, with their standard deviation represented by vertical bars. Mean values were significantly different compared with the SHAM group: *$p < 0.05$, **$p < 0.01$. Mean values were significantly different compared with the model group: $^{\#}{}^{\#}p < 0.01$.

body, and its vigor reflects the ability of the organism to remove free radicals. The content of MAD reveals the degree of the free radical attack. Therefore, to evaluate the radical scavenging activity of EA on STZ-induced generation of ROS, T-SOD and MDA were carried out. As shown in Fig. 5, the levels of T-SOD in kidney of model group were subtle decreasing, but there was no obvious change on the contents of T-SOD and MDA in kidney tissue, while the contents of T-SOD and MAD in serum changed significantly ($p < 0.01$). In model group, the content of T-SOD in serum was significantly lower than that in SHAM group ($p < 0.01$), and IRB significantly increased the T-SOD level, which was obviously different from the model group ($p < 0.01$). EA improved T-SOD activity of DN mice in some degree, but there was statistical differences between the model group and the SHAM group ($p < 0.05$). MDA in serum increased significantly($p < 0.01$) after modeling, the content of MDA was reduced to normal level basically treated with IRB, no difference comparing with SHAM group and lower than model group ($p < 0.01$). EA also decreased the content of MDA in serum of the DN mice comparing with the model group ($p < 0.01$) and there was no statistical difference ($p > 0.05$) with the sham group.

3.5. EA reduced STZ-induced elevation of pro-inflammatory cytokines

The level of TNF-α in serum were shown in Fig. 6, Levels of pro-inflammatory cytokines TNF-α were significantly ($p < 0.01$) increased in the model group in comparison with the SHAM group. Both IRB and EA decreased TNF-α content in serum obviously ($p < 0.01$) and dose-dependently. By the way, EA was more effective than IRB in decreasing the level of serum TNF-α.

3.6. Western blot analysis

TLR4/NF-κB is important signaling pathways involved in the protection against inflammation. As shown in Fig. 7, STZ-treatment significantly($p < 0.01$) increased the level of TLR4, IRAK4, TRAF6, IKKβ, NF-κbp65 and HMGB1 as compared to the SHAM group, suggesting that the TLR4/NF-κB signaling pathway was activated in the STZ-induced DN group. And protein levels of TLR4, IRAK4, TRAF6, IKKβ, NF-κbp65 and HMGB1 were significantly down-regulated after 4 weeks of treatment with EA ($p < 0.05$), indicating that EA can inhibit the renal TLR4/NF-κB signaling pathway in DN mice.

In Fig. 8, LPS-treatment significantly increased the expression of TLR4, IRAK1 and NF-κbp65 proteins as compared to the control group ($p < 0.01$). However, pretreatment with 100 μg ml^{-1} EA inhibited the increase of LPS-induced TLR4 protein expression obviously ($p < 0.01$)

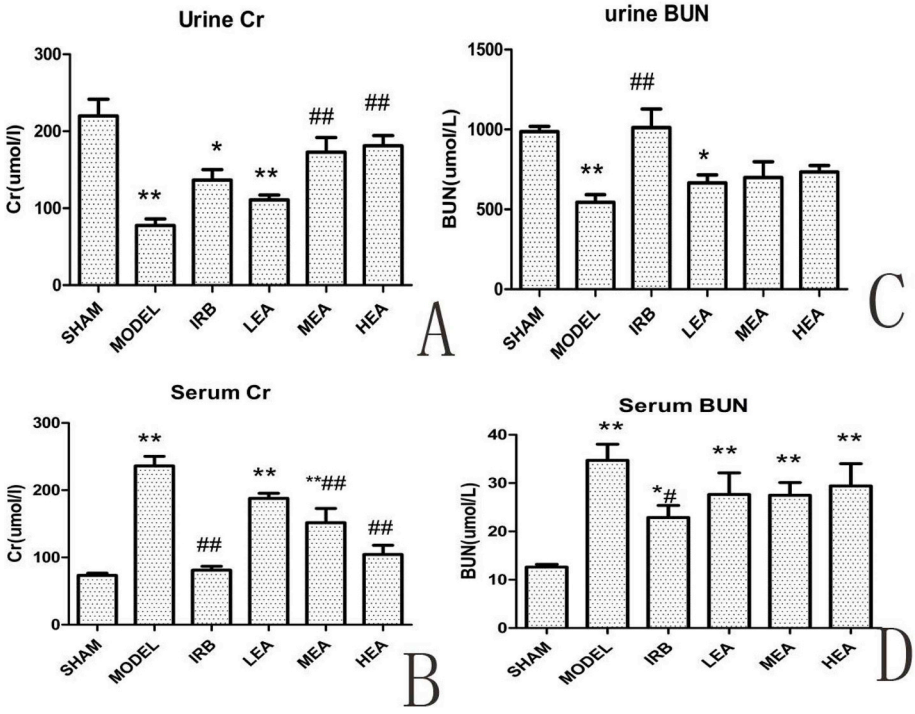
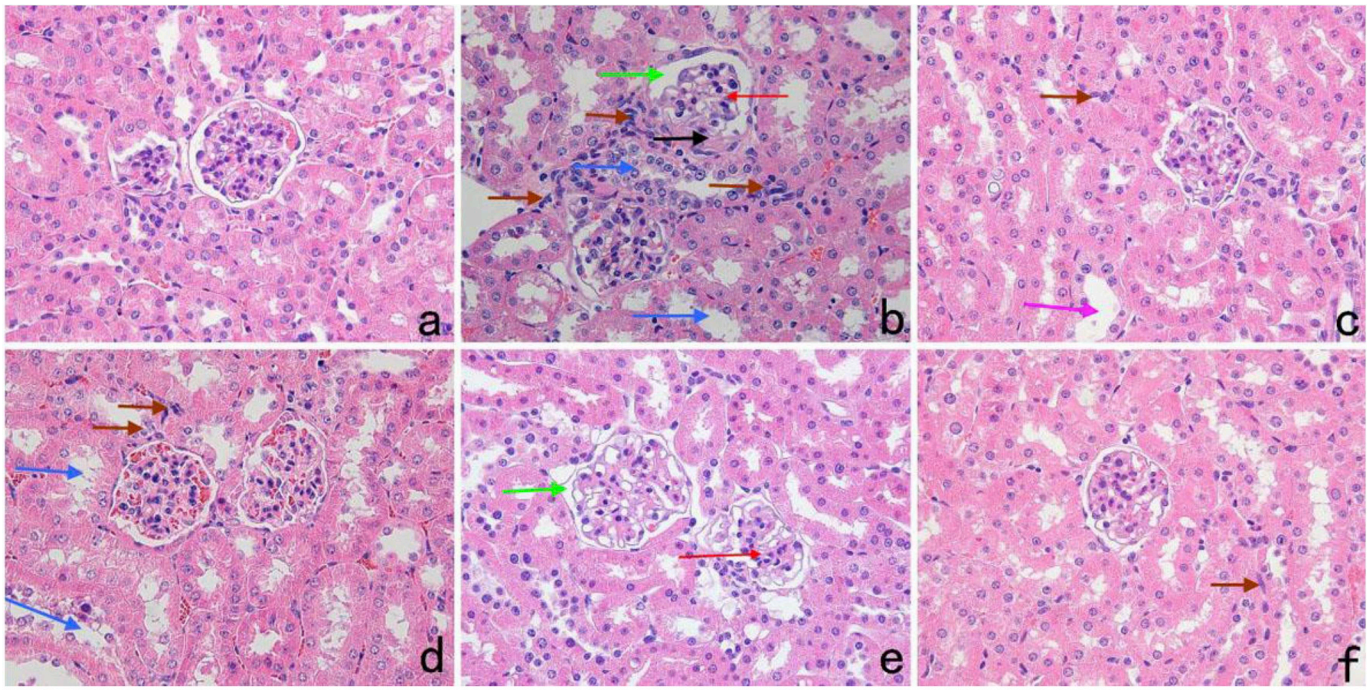


Fig. 3. Assessment of the levels of BUN and Cr in serum and urine. (A) The level of Cr in urine. (B) The level of Cr in serum. (C) The level of BUN in urine. (D) The level of BUN in serum. Values are means, with their standard deviation represented by vertical bars. Mean values were significantly different compared with the SHAM group: *$p < 0.05$, **$p < 0.01$. Mean values were significantly different compared with the Model group: $^{\#}{}^{\#}p < 0.01$.



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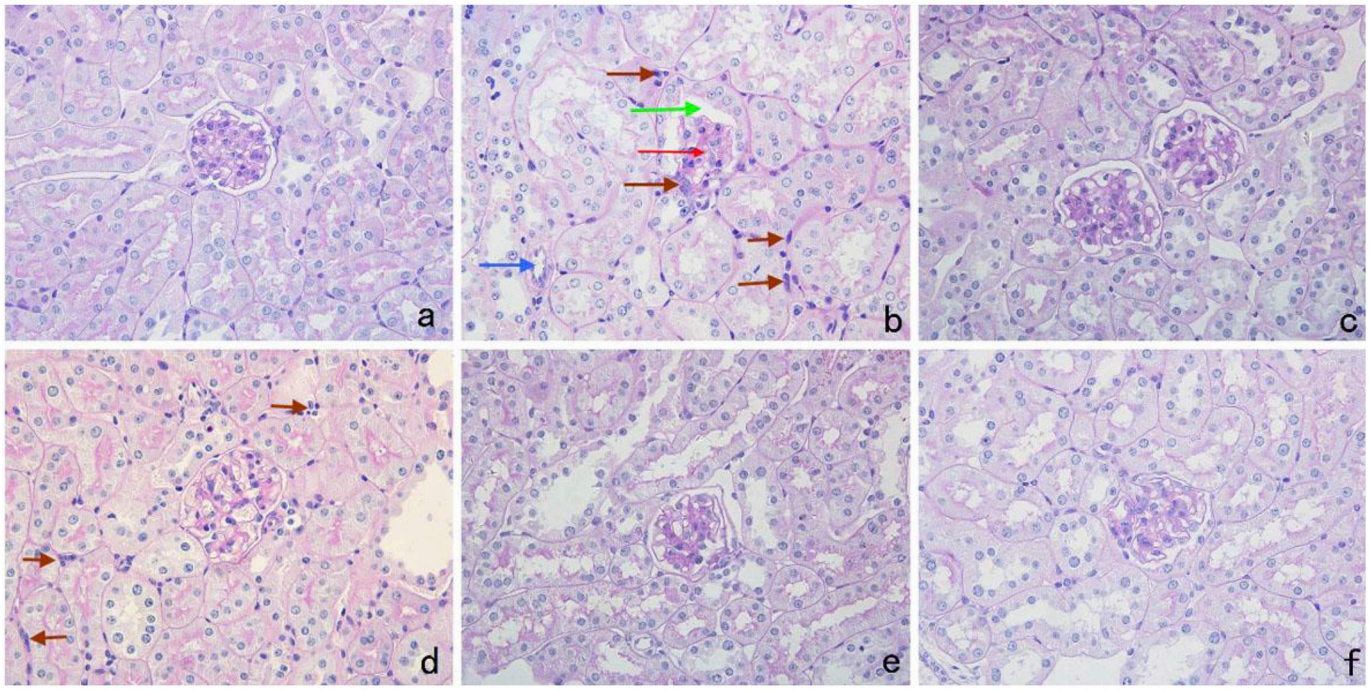

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Fig. 4. H&E (A) and PAS (B) staining of kidney vertical section (200 ×). (a) SHAM group. (b) Model group. (c) IRB group. (d) LEA group. (e) MEA group. (f) HEA group.

and decreased the relevant downstream proteins expression, such as IRAK1 ($P < 0.01$) protein and NF-κBp65 ($P < 0.01$) protein. IRAK1 was activated by P-IRAK4, phosphorylated to P-IRAK1 and activated NF-KB further. The results suggested that EA could significantly inhibit the expression of TLR4, IRAK1, P-IRAK1 and p65 proteins.

4. Discussion

Diabetic nephropathy (DN) is the major source of end-stage renal sickness and its occurrence is growing due to the worldwide pandemic of diabetes (Tesch and Allen, 2007). However, the underlying molecular mechanisms have not been understood in most cases. Ellagic acid

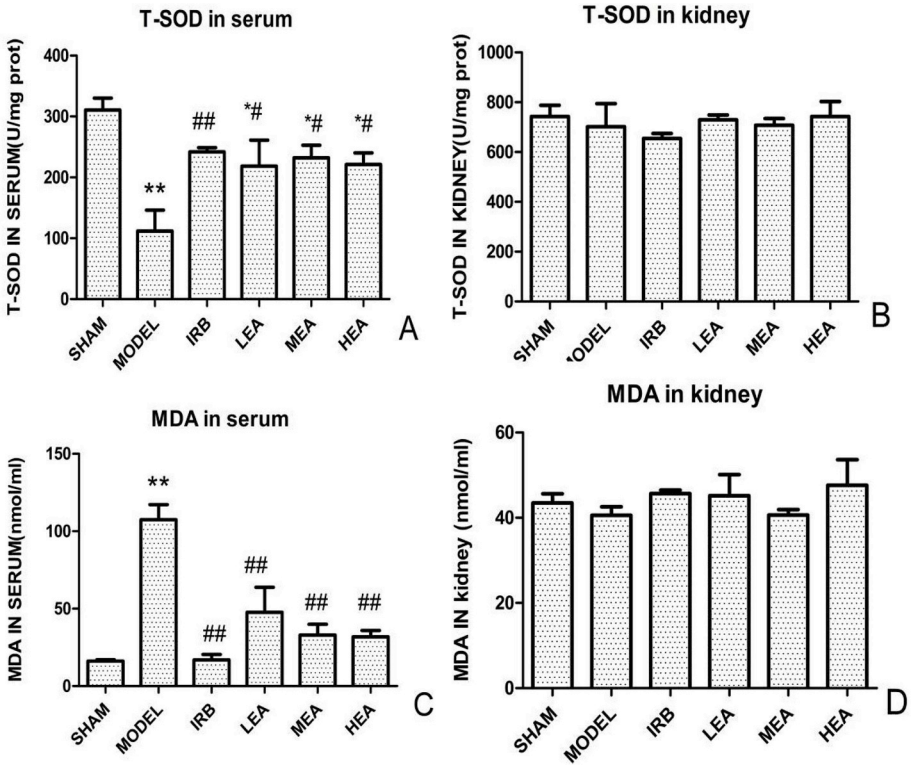

Fig. 5. Effect of EA on oxidative stress associated parameters: (A) Level of T-SOD in serum. (B) Level of T-SOD in kidney tissue. (C) Level of MDA in serum. (D) Level of MDA in kidney tissue. Values are means, with their standard deviation represented by vertical bars (n = 6). Mean values were significantly different compared with those of the SHAM group: $^{*}p < 0.05$, $^{**}p < 0.01$. Mean values were significantly different compared with the Model group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

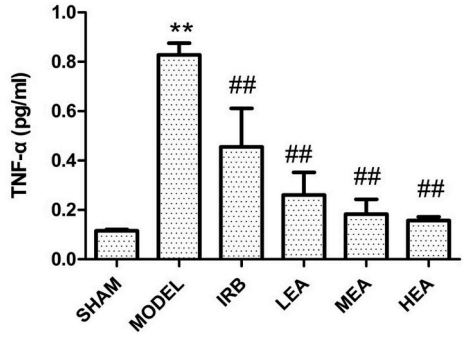

Fig. 6. Effect of EA on TNF-α in serum. Values are means, with their standard deviation represented by vertical bars (n = 6). Mean values were significantly different compared with those of the SHAM group: $^{**}p < 0.01$. Mean values were significantly different.

(EA) is a natural phenolic compound with a strong antioxidant activity found in many fruits and vegetables. In some other studies, it has been proved that EA has a protective effect on kidney damage caused by diabetes (Ahad et al., 2014). In this study, we investigated the protective effect and potential mechanism of EA on DN mice.

In vivo experiment, the diabetic model was established on ICR mice by intraperitoneal injection of STZ, and the effect of EA on DN was studied after kidney injury and IRB was employed as a positive drug. It is known that in the urine of the normal organism contains only a very small amount of m-ALB, and in the urine of diabetic nephropathy patients, the m-ALB is more than 20 mg L^{-1} and it has appeared before the kidneys show pathological changes. The progression of DN is closely related to the severity of proteinuria, and m-ALB is often used as a marker for early DN (Park et al., 2015). The contents of Cr and BUN in blood and urine reflect the glomerular filtration rate indirectly, and they were taken as direct in vivo index for nephropathy in STZ-treated mice (Odetti et al., 2003). In the present study, the effect of EA treatment on DN mice was investigated. Our results showed that oral treatment of EA can significantly alleviate kidney damage as evidenced by the decrease in serum parameters such as 24 h m-ALB, blood Cr and BUN content, the increase in urine parameters such as Cr and BUN content. In addition, Ahad (Ahad et al., 2014) also reported that EA can change these parameters in serum and urine of DN rats. These are consistent with previous reports (Mehrzadi et al., 2018; Aldawsari and Hosny, 2018). Studies have shown that kidney damage is mitigated with improvement in clearance of blood urea and decreased protein excretion (Sugimoto et al., 2001). The effects of MEA and HEA were stronger than LEA. MEA and HEA significantly improved the kidney damage caused by diabetes mellitus. Cheyi Chao reported that 2.5% or 5% EA mixed with food could protect kidney damage caused by diabetes (Chao et al., 2010), in agreement with our results.

Several investigations illustrated that renal injuries have a connection to the accumulation of reactive oxygen species (ROS) under hyperglycemic conditions (Zhang et al., 2017; Jeong et al., 2009). When organism is exposed to a variety of harmful stimuli, high-active molecules such as reactive oxygen species (ROS, including O_2-, H_2O_2, HO_2 ·and · OH) produce too much, it called oxidative stress, and the accumulation of excess ROS in vivo can attack the unsaturated fatty acid of bio-membrane, triggering lipid peroxidation, degrading polyunsaturated fatty acids and formatting lipid peroxide (such as MDA). Therefore, MDA is an important index to reveal the degree of lipid peroxidation and oxidative stress in the body. SOD is one of the vital antioxidant enzymes that can remove ROS, if the activity of SOD was increased, oxidative stress would be alleviated (Xu et al., 2016). So its activity is often used to evaluate the antioxidant ability of organisms. Diabetes is closely associated with kidney damage and oxidative stress (Aghadavod et al., 2016). High glucose as a harmful stimulation, induce the body to produce oxidative stress, the increase of ROS, and through the chain reaction, the damage further aggravated. Early studies reported that pomegranate peel tannins improved DN by alleviating oxidative stress of kidney in diabetic model rats (Mestry et al., 2017), and pomegranate peel tannins had obvious effect to eliminate ·OH and superoxide anion free radical. EA is one of the main components of pomegranate peel tannins, and it has been proved that EA has good antioxidant activity (Galano et al., 2014). A recent study has provided

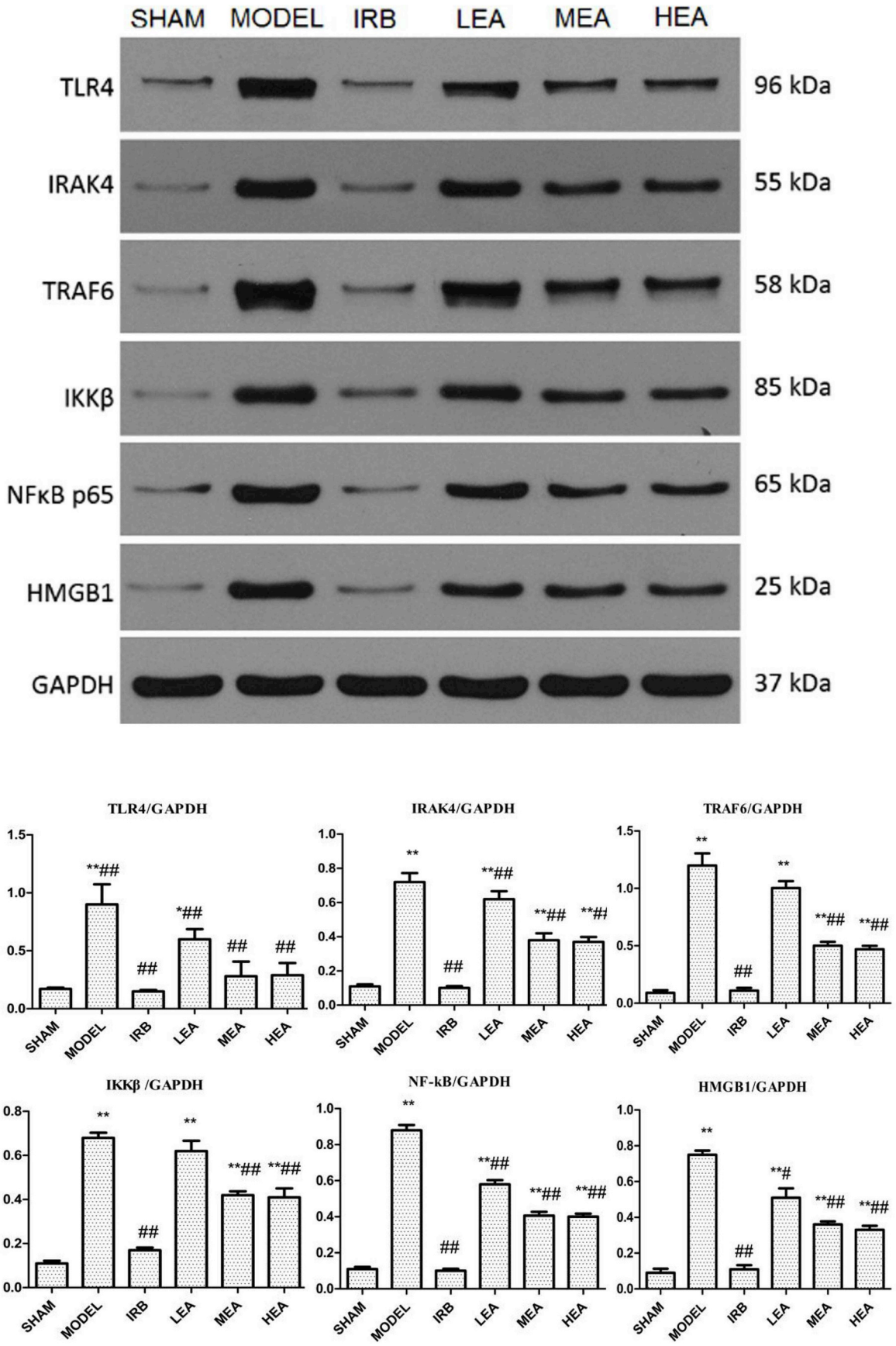

Fig. 7. Western blot results of TLR4, IRAK4, IKKβ, NF-κbp65, HMGB1. Values are means, with their standard deviation represented by vertical bars (n = 6). Mean values were significantly different compared with those of the SHAM group: $^{**}p < 0.01$. Mean values were significantly different compared with those of the Model group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

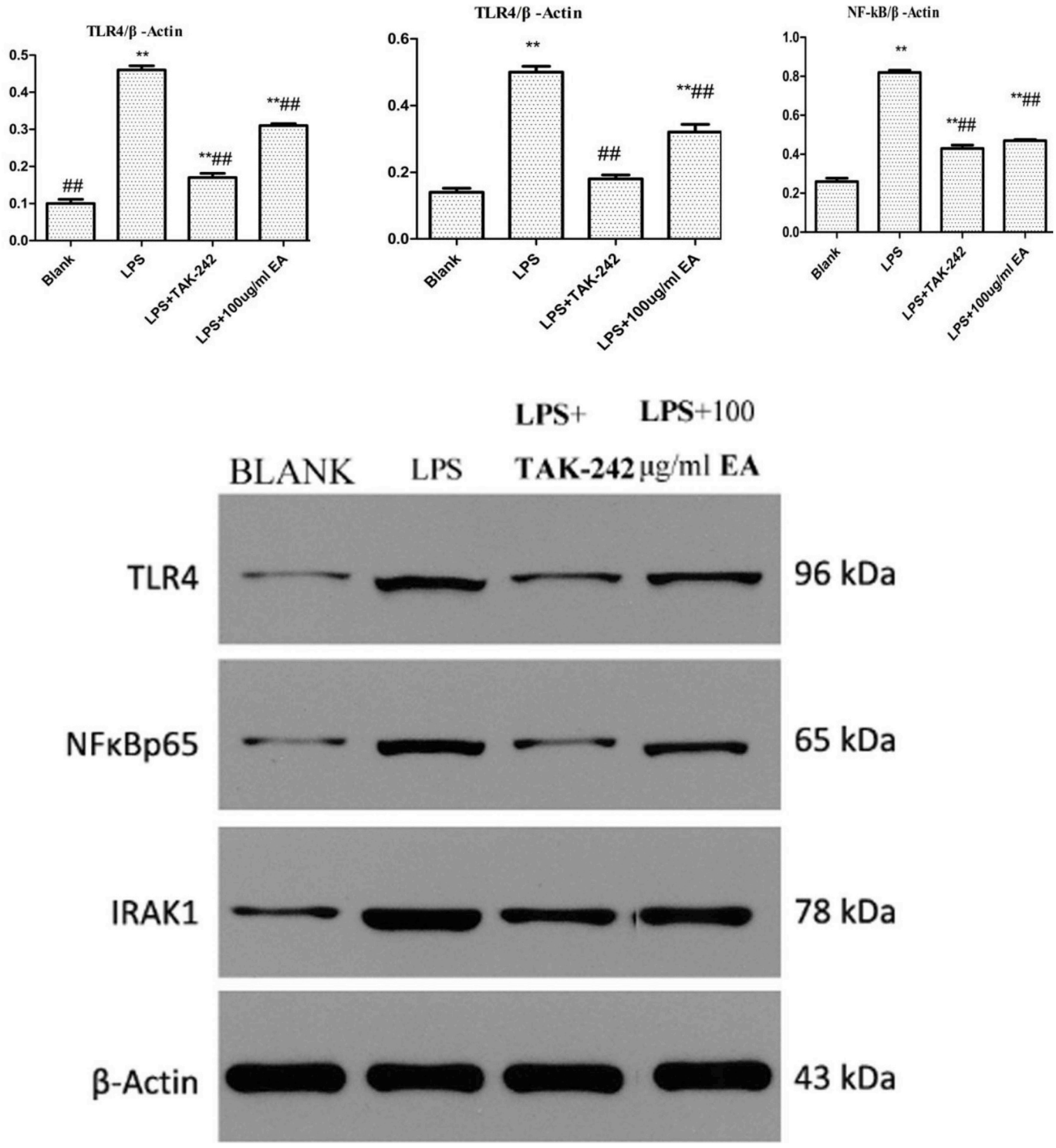

Fig. 8. Western blot result of TLR4, IRAK1 and p65. Values are means, with their standard deviation represented by vertical bars (n = 6). Mean values were significantly different compared with those of the Blank group: $^{**}p < 0.01$. Mean values were significantly different compared with those of the LPS group: $^{\#}$ $^{\#}p < 0.01$.

evidence that EA improves vascular function in blood vessels exposed to hyperglycemic conditions through the reduction of oxidative stress (Rozentsvit et al., 2017). The current animal study proved that EA increased the T-SOD activity of DN mice serum and decreased MDA in serum content significantly, which showed that EA alleviated the symptoms of DN by reducing oxidative stress in DN mice, the results were consistent (Simran et al., 2018).

Furthermore, it is commonly understood that the high level of oxidative stress is not only related to the functional changes, including the increased concentration of Cr and BUN, but also lead to a structural abnormalities of kidneys such as the increased glomerular filtrationrate and glomerulosclerosis (Kitada et al., 2003). As we observed in the present work, the DN mice exhibited the severe symptoms, such as the glomerular basement membrane thickened, glomerulosclerosis and the moderately hyperplasia of mesangial cells. However, four weeks treatment of diabetic mice with EA effectively prevented the above

functional and morphologic alterations.

Oxidative stress increases the production of cytokines, and oxygen-containing derivatives act as a second messenger to activate NF- κB, leading transcription of cytokines, growth factors and extracellular matrix proteins encoded genes (Ha et al., 2002). Tumor necrosis factor (TNF-α) is a cytokine secreted by macrophages/monocytes, which could activate neutrophils and lymphocytes, increase the permeability of vascular endothelial cells and induce the synthesis and release of other cytokines. It could be combined with receptor TNFR1 and TNFR2 to participate in inflammatory and immune reaction. TNFR1 involves in activating NF, inducing apoptosis and IL-6 to mediate inflammation and promoting fibroblast proliferation. TNFR2 involves in cell migration, regeneration, proliferation and regulation of TNF1 mediated apoptosis. TNF-α could activate the transcription and expression of inflammatory mediators mediated by the NF-κB pathway and mediate the expression of various inflammatory mediators in mesangial cells through the NF-κB pathway. This is a vicious circle, which aggravates the inflammatory reactors.

The vivo experiments presented that EA decreased the level of TNF-α in DN kidney injury mice significantly and showed dose-dependent, and the effect was better than IRB, which may be related to the antioxidant activity of EA. In line with previous data, our western blot analysis results revealed that the mechanism of EA on DN may be associated with the activation of TLR4 to NF-κB pathway, which revealed that EA is a potent inhibitor of NF-κB activation (Anitha et al., 2013; Edderkaoui et al., 2008). By inducing type 2 diabetic rats and then the rats were administering EA. The results showed that EA significantly improved renal dysfunction and oxidative stress in diabetic rats, and verified that EA can alleviate inflammatory process via inhibiting the NF-κB pathway in diabetic rats (Ahad et al., 2014), consistent with our results. What's more, EA relieved oxidative stress by raising T-SOD activity and lowering MDA level, reduced the production of inflammatory factors (such as TNF-α) by decreasing excessive ROS in organism, and decreased the HMGB1 and TLR4 in the kidney tissue of DN mice at the same time, and cut down TLR4 downstream protein such as IRAK4, TRAF6, IKKβ, so that NF-κB activation further reduced and cytokines like TNF-α also decreased as a result.

HMGB1 is secreted by immunocytes (such as macrophages, monocytes and dedritic cells) and acts as a cytokine medium after secretion. TLR4 is one of HMGB1's receptors, and HMGB1 could up-regulate NF-kB through TLR4, causing macrophages and neutrophils to produce and release more cytokines, stimulating the release of more ROS, and NADPH oxidative activation, further leading to tissue damage and inflammatory response. Our experiments showed that the effect of EA in DN maybe through down-regulating HMGB1-TLRS and downstream proteins expression in DN mice.

TLRs are a core element in the innate immune system, which plays an essential role in regulating inflammatory responses, especially TLR4 (Banerjee and Gerondakis, 2007). It has been well documented that TLR4 is involved in progressive renal fibrosis by triggering multiple inflammatory pathways (Pulskens et al., 2010). In this process, some key transcription factors of TLRs activate downstream transcriptional regulators (such as NF-κB) to initiate expression of ROS and inflammation-related factors (Liu et al., 2014). So in our current vitro experiment, the inhibitory effect of EA on LPS induced TLR4 elevation was studied. The results revealed that EA could reduce the increase of LPS-induced TLR4 and inhibit the expression of downstream proteins such as IRAK1, P-IRAK1 and p65 proteins, which indicated that EA could through inhibit TLR4 and TLR4/NF-κB pathways to alleviate a range of inflammatory responses. Our finding provided supportive evidence that the mechanism of action of EA on DN mice is likely to be mediated by the TLR4/NF-κB signaling pathway.

In addition, urolithin-A (UA) and urolithin-B (UB) are the metabolic products of EA, widely distributing in the blood, urine, bile, feces and colon tissues of human body or other mammals in the form of sulfuric acid, glycosylation, methylation, etc (González-Barrio et al., 2011). As a natural product, UA exhibits various biological activities, especially anti-oxidative, anti-tumor and anti-inflammatory (Piwowarski et al., 2015). Previous studies demonstrated that UA effectively attenuated cisplatin-induced kidney damage and showed significantly greater effect than its precursor EA on preserving the normal kidney architecture by down-regulating the proinflammatory cytokines (Melissa et al., 2017). And UA treatment diminished the LPS-evoked activation of NADPH oxidase (NOX), the results indicate that UA treatment attenuates pro-inflammatory mediator production by suppressing NOX-derived reactive oxygen species-mediated PI3-K/Akt/NF-κB and JNK/AP-1 signaling pathways in LPS-stimulated macrophages (Komatsu et al., 2018). Moreover, UA and UB could prevent the occurrence of cardiac dysfunction in streptozotocin-induced diabetic rats by preventing the initial inflammatory response of myocardial tissue to hyperglycaemia and the negative impact of the altered diabetic milieu on cardiac performance, as measured at cellular and organ levels (Monia et al., 2017). After EA enters the body, it is converted into urolithins by intestinal metabolism. UA and UB are the main components of EA metabolites. Therefore, we conclude that urolithins may be one of the mechanisms of EA. At present, we are studying the effect of UA on LPS-induced NRK-52E cell inflammation. Pre-experimental results also show that UA can significantly reduce LPS-induced increase in TLR4 and reduce inflammation. In line with previous results, Piwowarski reported that urolithins could inhibit LPS-induced inflammation, the inhibitory effect of urolithins on iNOS and proinflammatory cytokines expression in RAW 264.7 macrophages depending on TLR4/NF-κB pathway inhibition indicates a potential mechanism of action (Piwowarski et al., 2015). So our finding laid the foundation for the further study of the related activities of urolithins.

5. Conclusion

In summary, the present study for the first time uncovers that EA can significantly ameliorate the renal function and pathological changes in DN mice induced by STZ. Therefore, EA might be a new therapeutic approach in preventing STZ-induced toxicity in humans. Moreover, EA treatment to STZ-induced diabetic mice exhibited a significant ameliorative potential probably through the inhibition of TLR4/NF-κB signaling system (Fig. 9). Further detailed studies are in progress to elucidate the precise mechanism.

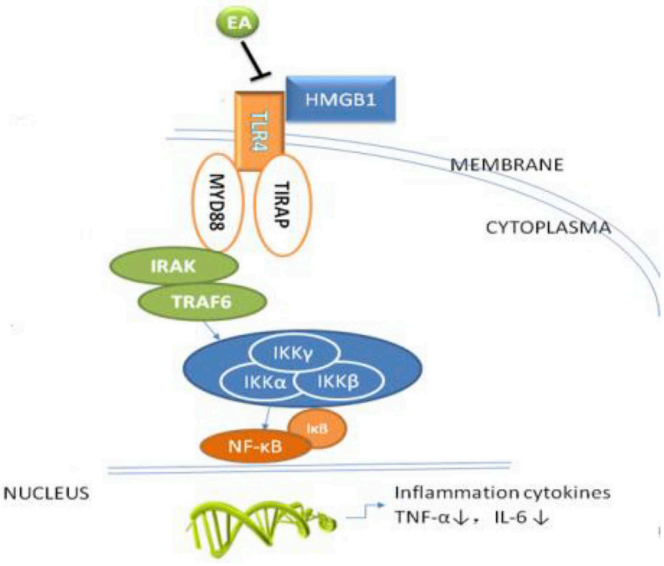

Fig. 9. Schematic diagram for protective effects of EA against STZ induced DN.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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