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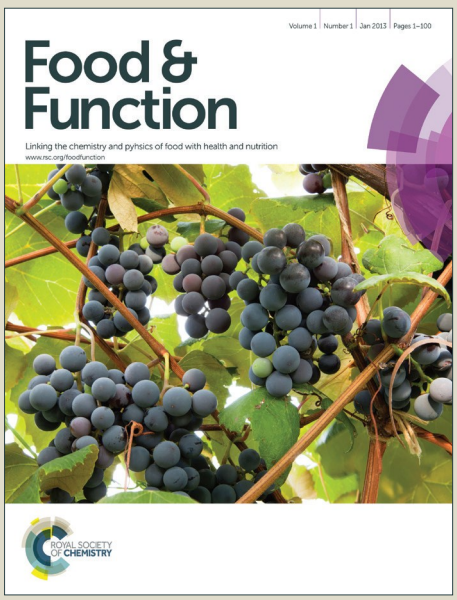

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PAPER

Cordyceps Militaris Fruit Body Extract Ameliorates Membranous Glomerulonephritis by Attenuating Oxidative Stress and Renal Inflammation via NF-κB Pathway

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Membranous glomerulonephritis (MGN) is a common pathogenesis of nephritic syndrome in adult patients. Nuclear factor kappa B (NF-κB) serves as the main transcription factor for the inflammatory response mediated nephropathy. *Cordyceps militaris,* containing various pharmacological components, has been used as a kind of crude drug and folk tonic food for improving immunity and reducing inflammatory. The current study aims to investigate the renoprotective activity of *Cordyceps militaris* aqueous extract (CM) in cationic bovine serum albumin (C-BSA)-induced rat model of membranous glomerulonephritis. Significant renal dysfunction was observed in MGN rats; comparatively, 4-week CM administration strongly decreased the levels of 24-h urine protein, total cholesterol, triglyceride, blood urea nitrogen and serum creatinine, and increased the levels of serum albumin and total serum protein. Strikingly recovering kidney histological architecture was noted in CM-treated MGN rats. Significant improvement in the decreased glutathione peroxidase and superoxide dismutase levels, and enhanced malondialdehvde concentration as observed in serum and kidney was showed in CM-treated rats. Altered levels of inflammatory cytokines including interleukins, monocyte chemoattractant protein-1, intercellular adhesion molecule 1, vascular adhesion molecule 1, tumor necrosis factor-α and 6-keto-prostaglandin F1α, and nuclear transcriptional factor subunit NF-κB p65 reverted to the normal level upon treatment with CM. The present data suggest that CM protects the rats against membranous glomerulonephritis via the normalization of NF-κB activity, thereby inhibits the oxidative damage and reduces inflammatory cytokines levels, which further provide experimental evidence in supporting the clinical use of CM as an effective renoprotective agent.

Introduction

Membranous glomerulonephritis (MGN) is an antibody-mediated disease induced by deposits of immunoglobulins and complement components on the subepithelial layer of the glomerular capillary wall.[1] MGN is considered as one of the leading causes of nephrotic syndrome in adults with no effective therapy.[2] Cationic bovine serum albumin (C-BSA)-induced MGN rat serves as an experimental model due to its similar pathogenesis and immunity with MGN, whose inflammation can be alleviated by Astragalus.[3] Clinically, angiotensin converting enzyme (ACE) inhibition or angiotensin receptor blockade is the most common strategy against membranous nephropathy,[4] but patients have to suffer with serious adverse effects. Although the calcineurin inhibitors (CNIs) cyclosporine and tacrolimus induce complete or partial remission of proteinuria in more than 70% of membranous nephropathy patients, more than 60% of the treated patients suffer from subsequent relapses or treatment dependence.[5, 6] A new treatment strategy is needed to reduce the risk of chronic nephrotoxicity.

Nuclear factor-kappa B (NF-κB), an inflammatory transcription factor, is over-expressed in patients with MGN.[7, 8] NF-κB mediates inflammatory cytokine production in patients with MGN[7] and C-BSA-induced MGN rats,[8] as well as hydroxyl radicals in podocytes to damage the glomerular filtration barrier through targeting glomerular basement membrane (GBM).[9] Down-regulating NF-κB activity by Large Yellow Croaker Swim Bladder and Plumbagin alleviates oxidative stress and glomerular injury in many types of nephritis, but an NF-κB inhibitor in MGN without adverse side effects is not identified.[10,11]

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Natural product has become a promising therapeutic strategy to prevent kidney inflammation due to its high treatment efficiency and less adverse effects. *Cordyceps militaris*, a widely used traditional Chinese medicine, exerts various pharmacological properties, and serves as a kind of crude drug and folk tonic food for improving immunity and reducing inflammation.[12] *Cordyceps sinensis* polysaccharide-enriched extract inhibits platelet-derived growth factor-BB-induced inflammation and ROS production in human mesangial cells to show natural product is beneficial.[13] Ergosterol separated from *Cordyceps militaris* attenuates lipopolysaccharide-induced inflammation in BV-2 microglia cells.[14] We demonstrated that *Cordyceps militaris* mycelium obtained from submerged fermentation alleviates diabetic nephropathy via reducing oxidative stress and improving blood lipid metabolism.[15]

We hypothesized that *Cordyceps militaris* fruit body aqueous extract alleviates glomerulonephritis in C-BSA induced MGN rats, by inhibiting oxidative stress and inflammation. We quantitate urinary protein, albumin, blood lipid, inflammatory cytokine and NF-κB in C-BSA induced rats with/without administration of *Cordyceps militaris* fruit body aqueous extract.

Materials and Methods

Cordyceps militaris extract preparation

The aqueous extract from *Cordyceps militaris* fruit body of the same batch (purchased from Qianxiang co., LTD, Shenyang, China) was extracted with 10 volumes of double distilled (D.D.) water at 45 ℃ for 3 h firstly. After being centrifugated, the residue was extracted at 80 ℃ for another 3.5 h. After the two extracts were merged, the supernatant was sequentially concentrated in an evaporator under reduced pressure, and further freeze-dried to produce the solid aqueous extract (CM). The extraction process was optimized Box-Behnken design according to the content of polysaccharides, total proteins, cordycepic acid, adenosine and cordycepin, which are analysed via phenol-sulfuric acid method,[16] Kjeldahl method[17] and High performance liquid chromatography (HPLC).[18] It was found that CM contained 29.1% polysaccharides, 20.5% total proteins, 6.1% cordycepic acid, 0.2% adenosine, and 0.4% cordycepin. The HPLC chromatogram of CM was shown in Fig.1.

The establishment of rats model with membranous glomerulonephritis

C-BSA preparation

Crystallized unmodified BSA was chemically cationized to prepare C-BSA according to Border's method.[8] Briefly, an anhydrous ethylenediamine (EDA, Sigma-Aldrich, Germany) solution was prepared by mixing 67 ml EDA and 500 ml D.D. water. The pH was adjusted to 4.75 with 350 ml of 6 M HCl at 25 □. After addition of 1.8 g 1-ethyl-[(3-dimethylaminopropyl)-carbodiimide hydrochloride] (EDC, Sigma-Aldrich, Germany), 5 g native BSA (Amresco, Solon, USA) dissolved in 25 ml D.D. water was added to the EDA solution. With continuous stirring, the reaction lasted for 120 min, and then 30 ml 4 M acetate buffer was added before being stopped. The product was dialyzed against distilled water at 4

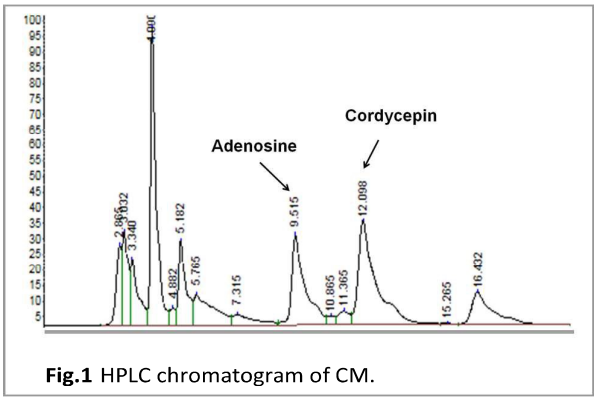

Fig.1 HPLC chromatogram of CM.

℃ for 48 h, lyophilized, and stored at – 80 ℃.

Animal care

The Institution Animal Ethics Committee of Jilin University reviewed and approved the entire animal protocol prior to conducting the experiments. Wistar male rats weighing 120 g-140 g (SCXK(JI)-2011-0003) (Purchased from Norman Bethune University of Medical Science Jilin University, Jilin, China) were maintained on a 12-h light/dark cycle (lights on 07:00-19:00) at 23±1 ℃ with water and food available *ad libitum*.

MGN rats model preparation and drug administration

50 male Wistar rats were injected subcutaneously with 0.5 mg incomplete Freund's adjuvant at day 1 to prevent autoimmunity, following with C-BSA (40 mg/kg) injection via tail vein every day for three weeks to induce membranous glomerulonephritis. All successfully established MGN rats were separated randomly into 5 groups. Another 10 male Wistar rats injected with normal saline were served as control group (CTRL).

Control group: Normal rats were orally treated with 5 ml/kg normal saline everyday;

Model group: MGN rats were orally treated with 5 ml/kg normal saline everyday;

Positive control group: MGN rats were orally treated with 10 mg/kg Tripterygium wilfordii glycosides (TG) everyday;

Test drug group I: MGN rats were orally treated with 0.5 g/kg CM everyday;

Test drug group II: MGN rats were orally treated with 1.0 g/kg CM everyday;

Test drug group III: MGN rats were orally treated with 2.0 g/kg CM everyday.

Drug administration was lasted for 4 weeks, and the bodyweight and 24-h urine protein of each rat were monitored for two weeks during the whole experimental period. At the end of the experiment, blood and kidney samples were collected.

Biochemical criterions determination

Samples collection

24-hour urine collections were performed in each animal after placement in metabolic cage every week. Before sacrifice, blood was sampled from rat's tail under anesthesia with 200 mg/kg pentobarbital. After sacrifice, kidneys were collected, and one part was homogenized in D.D. water (or RIPA buffer) with three washes in ice-cold physiological saline, and the other part was placed in 4% paraformaldehyde for histopathological examination.

Renal function assessment

Renal function was measured by detecting the levels serum creatinine (Scr), blood urea nitrogen (BUN), serum albumin and total protein, and 24-h urine protein concentration. All factors were examined by related assay kits obtained from NanJing Biotechnology Co. Ltd. (NanJing, China) according to operating manual.

Blood lipid assessment

It has been reported that blood lipid, like fatty acids, reduces the production of inflammatory cytokines.[19] The concentration of total cholesterol (TC) and triglyceride in serum were determined via related assay kits obtained from NanJing Biotechnology Co. Ltd. (NanJing, China).

Oxidative stress assessment

After 4-week drug administration, the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), and the levels of malondialdehvde (MDA) in serum and kidney of MGN rats and normal rats were analyzed using commercial assay kits obtained from NanJing Biotechnology Co. Ltd. (NanJing, China).

Renal cytokines detection

The levels of inflammatory cytokines including interleukin-2 (IL-2), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), vascular adhesion molecule 1 (VCAM-1), and 6-keto-prostaglandin F1α (6-keto-PGF1α) in serum of experimental rats were measured by specific enzyme-linked immuno sorbent assay (ELISA) kits (R&D systems, USA) according to the manufacturer's instructions. The concentrations of test samples were analyzed from the related standard plot.

NF-κB p65 subunit detection

Since the levels of p65 correlates positively with NF-κB pathway, the activation of NF-κB p65 was analyzed using NF-κB active ELISA kit (R&D systems, USA) according to the manufacturer's instructions.

Histopathological examination

Collected kidney tissues were immerged in 4% paraformaldehyde for 48 h, and then dehydrated in gradient ethanol (50%, 70%, 80%, 90%, 95% and 100%) step-by-step. Samples were immerged in xylene for 30 min and incubated with first paraffin at 65 °C overnight. After being embedded in wax, tissues were cut into serial sections at 5 μm thickness using microtome (Leica, Germany) and spread over microscopy slides. Sections were deparraffiniaed with fresh xylene for 10 min, hydrated with gradient ethanol (100%, 90%, 80% and 70%), and then washed with D.D. water for three times. The sections were analyzed via haematoxylin and eosin staining (H&E staining) and examined by a light microscope digital camera (Nikon Instruments, Tokyo, Japan). The severity of glomerulonephritis (GN) was graded on a 0–4 scale as follows: 0, normal; 1, mild increase in mesangial cellularity and matrix; 2, moderate increase in mesangial cellularity and matrix, with thickening of the GBM; 3, serious increase in mesangial cellularity and matrix, with thickening of the GBM; 4, serious increase in mesangial cellularity and matrix, with thickening of the GBM, diffuse endocapillary hypercellularity and segmental necrosis.[20]

Western blot analysis

Kidney tissues were homogenized with 10 volumes of lysis buffer (Sigma-Aldrich, USA) containing 1X protease inhibitor cocktail (Sigma-Aldrich, USA) and 1 mM Phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich, USA). The homogenate was centrifuged at 10836×g for 10 min and supernatants were used as the whole protein extract. Total protein was estimated using Bradford method. About 30 μg protein was separated with 10% SDS-PAGE and then transferred onto a nitrocellulose membrane (0.45 μm, Bio Basic, Inc., USA) with mini-protein two-gel electrophoresis system (Bio-Rad, USA). The transferred membrane was blocked through incubation with 5% bull serum albumin (BSA) for 3 h at room temperature. The membranes were then blotted 4 °C overnight with primary antibodies at phosphor (P)-AKT, total (T)-AKT, P-NF-κB p65, T-NF-κB p65, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA) at dilution of 1 : 1000, following by treatment with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 (Santa Cruz, USA). Immunoreactive bands were visualized with an ECL detection system (GE Healthcare, UK). The intensity of the bands was quantified by scanning densitometry using Image J.

Statistical analysis

All data were expressed as mean ± S.D.. A one-way analysis of variance (ANOVA) was used to detect statistical significance followed by post-hoc multiple comparisons (Dunn's test) using SPSS 16.0 software (IBM corporation, Armonk, USA). A P-value<0.05 was considered to be statistically significant. Graphs were plotted with the OriginPro 8.5 software.

Results

The effect of CM on renal function in MGN rats

Compared to normal control rats, C-BSA injection resulted in nearly three-fold reduction of 24-h urine protein level (P<0.01; Fig.2A). Similar as TG treatment, CM at doses of 1.0 mg/kg and 2.0 mg/kg strongly suppressed the hyper-level of 24-h urine protein (P<0.01 Fig.2A). Interestingly, during the 8 weeks, no significant changes on bodyweight among experimental rats were observed (Not shown in data). In non-treated MGN rats,

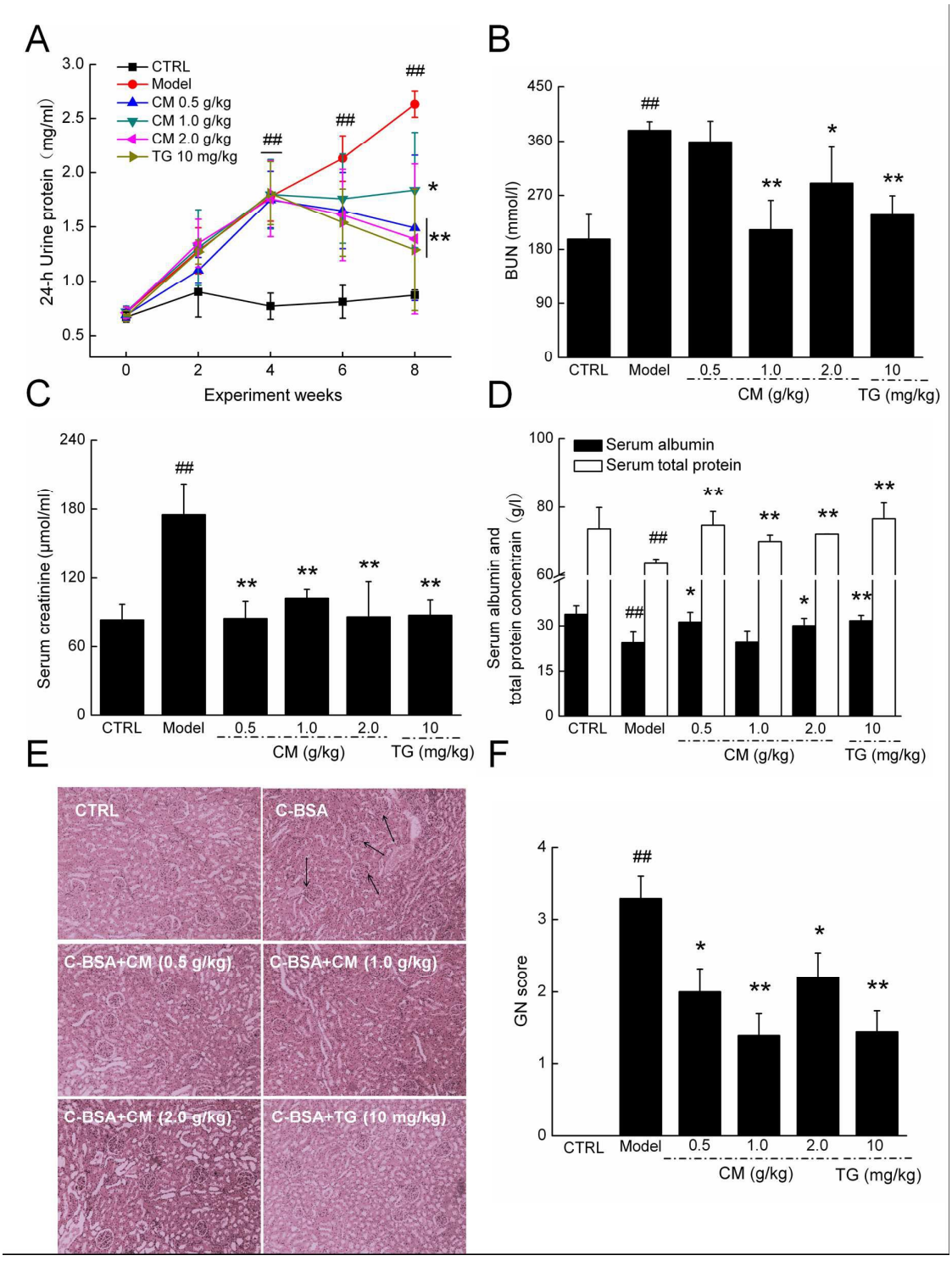

Fig.2 24-h urine protein (A) in control and MGN rats was monitored every two weeks during the whole experiment, and the levels of BUN (B), Scr (C), and albumin and total protein (D) in serum were detected at the end of the experiment. (E) Histopathological analysis in kidney collected from all experimental rats through H&E staining (n=10; x200). (F) Gomerulonephritis disease was scored from (0) to (4) as described in materials and methods. Data were expressed as mean ± S.D. (n = 10) and analyzed using a one-way ANOVA followed by Dunn's test. ## $P < 0.01$ versus the control rats, * $P < 0.05$ and ** $P < 0.01$ versus the MGN rats.

Table 1 The regulatory effects of CM and TG on the antioxidant status in experimental rats

		CTRL	Model	CM (g/kg)			TG (mg/kg)
				0.5	1.0	2.0	10
Serum	SOD (U/mL)	318±51	228±47$^{\#}$	251±52	258±51	273±41	273±37
	MDA (nmol/mL)	7.81±0.88	14.90±2.22$^{\#\#}$	13.40±2.75	10.05±2.42**	7.95±1.22**	7.66±3.42**
	GSH-Px (μmol/L)	458±116	267±43$^{\#\#}$	307±69	363±44**	365±53**	394±62**
Kidney	SOD (U/mgprot)	121±34	75±13$^{\#}$	122±48*	93±33	100±16*	121±21**
	MDA (nmol/mgprot)	7.00±1.36	11.33±2.56$^{\#\#}$	11.22±2.69	8.19±1.23*	7.31±2.03**	7.48±1.54**
	GSH-Px (μmol/gprot)	5879±589	3793±420$^{\#\#}$	4015±662	4054±606	5143±733**	5371±623**

The levels of MDA and the activities of SOD and GSH-Px in serum and kidney of MGN and control rats were detected after 4-week administration of CM (0.5 mg/kg, 1.0 mg/kg and 2.0 mg/kg) or TG (10 mg/kg). Values were expressed as mean ± S.D. (n = 10). # P < 0.05 and ## P < 0.01 versus control rats. * P < 0.05 and ** P < 0.01 versus MGN rats.

92.0% and 110.8% increment of BUN and Scr levels, and 28.1% and 13.8% decrement of albumin and total protein concentrations in serum were observed, which were strikingly reversed to the normal level by 4-week TG and CM orally administration (P<0.01, Fig. 2B, C and D). Additionally, the incidences of glomerular basement membrane thickening or mesangial proliferation, and inflammatory infiltrate injuries noted in kidney tissue of MGN rats were significantly ameliorated by both CM and TG treatment (Fig. 2E). All these results suggest that CM possesses renoprotective activities against renal injury in rats caused by chronic C-BSA injection.

The regulatory effect of CM on blood lipid

As reported, due to the metabolic disorders, hyper-level of TC and triglyceride in serum were found in nephritis patients.[21] 121.9% and 26.7% enhancement of serum TC and triglyceride concentration were noted in MGN rats (P<0.01; Fig. 3). For serum TC level, 2.0 g/kg CM and 10 mg/kg TG treatment resulted in 31.3% and 29.6% reduction compared with non-treated MGN rats (P<0.01; Fig. 3A). Only CM other than TG at dose of 2.0 g/kg suppressed high serum concentration of triglyceride up to 19.7% (P<0.01; Fig. 3B).

The anti-oxidative effect of CM in MGN rats

Oxidative stress has been implicated in inflammation which was directly related to the level of MDA and the activities of SOD and GSH.[22] Overproduction of MDA and hypo-activities of SOD and GSH-Px were observed in serum and kidney of

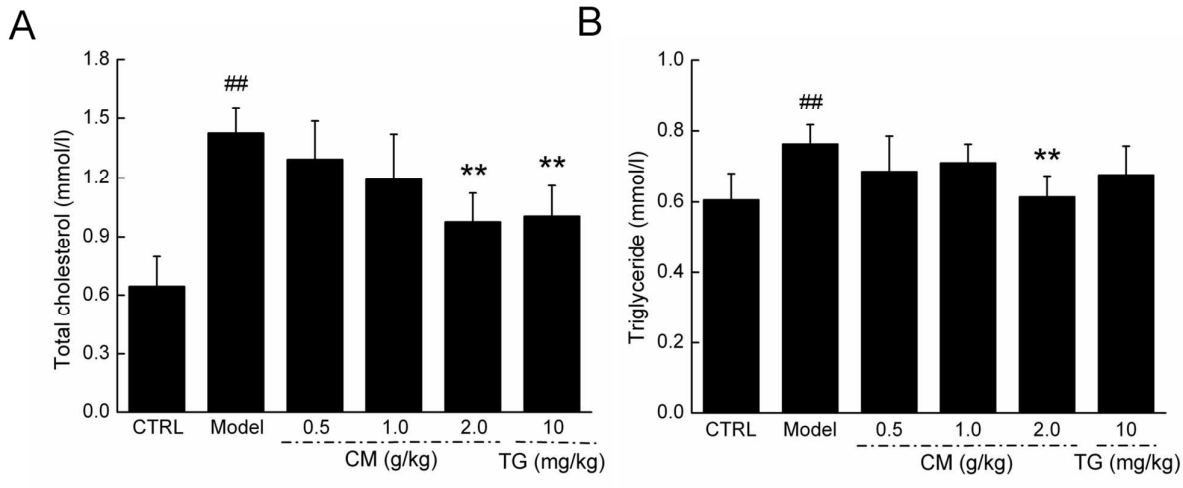

Fig.3 C-BSA-induced MGN rats were treated with or without CM at doses ranged from 0.5 g/kg to 2 g/kg and TG at 10 mg/kg for 4 weeks. CM successfully normalized the pathological changes on serum concentration of total cholesterol (A) and triglycerides (B) in MGN rats compared with control rats. Data were expressed as mean ± S.D. (n = 10) and analyzed using a one-way ANOVA followed by Dunn's test. ## P < 0.01 versus the control rats, ** P < 0.01 versus the MGN rats.

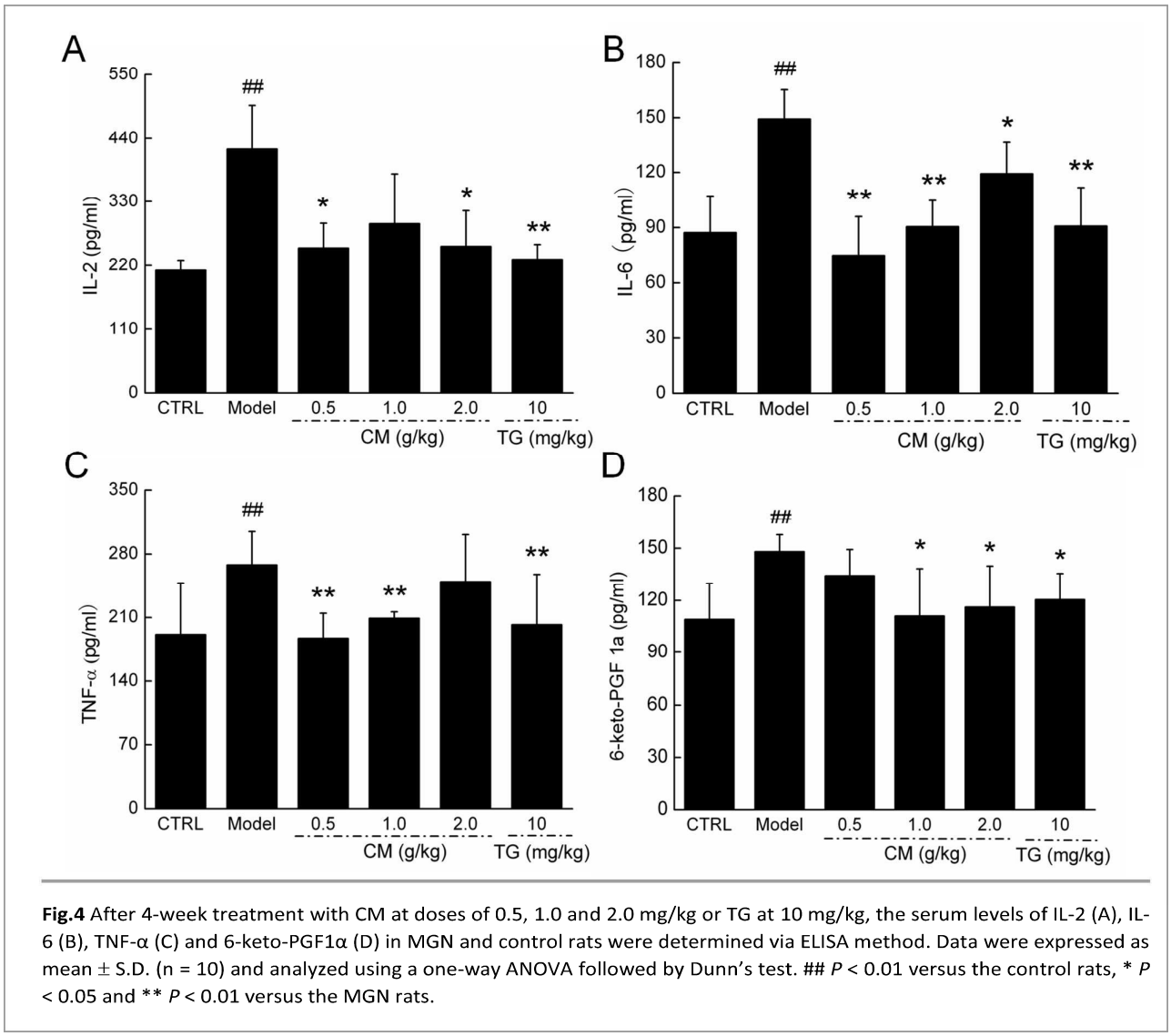

Fig.4 After 4-week treatment with CM at doses of 0.5, 1.0 and 2.0 mg/kg or TG at 10 mg/kg, the serum levels of IL-2 (A), IL-6 (B), TNF-α (C) and 6-keto-PGF1α (D) in MGN and control rats were determined via ELISA method. Data were expressed as mean ± S.D. (n = 10) and analyzed using a one-way ANOVA followed by Dunn's test. ## $P < 0.01$ versus the control rats, * $P < 0.05$ and ** $P < 0.01$ versus the MGN rats.

MGN rats compared with normal control group ($P<0.05$; Tab.1). In serum, 4-week TG and CM treatment restored MDA and GSH-Px concentration to normal level ($P<0.01$; Tab.1); however, no significant influence on SOD activity was noted. Similar as TG of anti-oxidative activities in kidney, CM at dose of 2.0 g/kg resulted in a 35.5% reduction on MDA level, and 33.3% and 35.6% increment on SOD and GSH-Px activities compared with MGN rats ($P<0.05$; Tab.1).

The effect of CM on IL-2, IL-6, TNF-α and 6-keto-PGF levels

The effect of CM treatment on inflammatory cytokines levels were represented in Fig. 3. As higher level of 6-keto-PGF1α was observed during renal injury,[23] 6-keto-PGF1α was also analyzed in our study. MGN rats exhibited significant increase in serum levels of IL-2 and IL-6 compared with normal control rats ($P<0.01$; Fig. 4A and B). CM oral administration strongly reduced the hyper-levels of IL-2 and IL-6 up to 40.6% and 50.1 % ($P<0.01$; Fig. 4A and B). A significant enhancement of TNF-α and 6-keto-PGF in serum levels were noted in non-treated MGN rats; contrastively, 4-week CM treatment reduced nearly 39.9% TNF-α and 24.5% 6-keto-PGF levels ($P<0.01$; Fig. 4C and D). The similar regulatory effects of TG on inflammatory cytokines were found.

The effect of CM on MCP-1, ICAM-1, VCAM-1 and NF-κB p65 levels

In MGN rats, the serum levels of MCP-1 (649 pg/ml), ICAM-1 (82 ng/ml), VCAM-1 (98 ng/ml) and NF-κB p65 (761 pg/ml) were significantly augmented compared with normal control rats ($P<0.01$; Fig. 5). Except for ICAM-1, 4-week TG treatment at 10 mg/kg attenuated the hyper-level of MCP-1, VCAM-1 and NF-κB p65 ($P<0.05$; Fig. 5). Treatment with CM at indicated doses for 4 weeks caused a significant fall in the serum levels of MCP-1 (190 pg/ml), ICAM-1 (20 ng/ml), VCAM-1 (17 ng/ml) and NF-κB p65 (436 pg/ml) compared with MGN rats ($P<0.05$; Fig. 5).

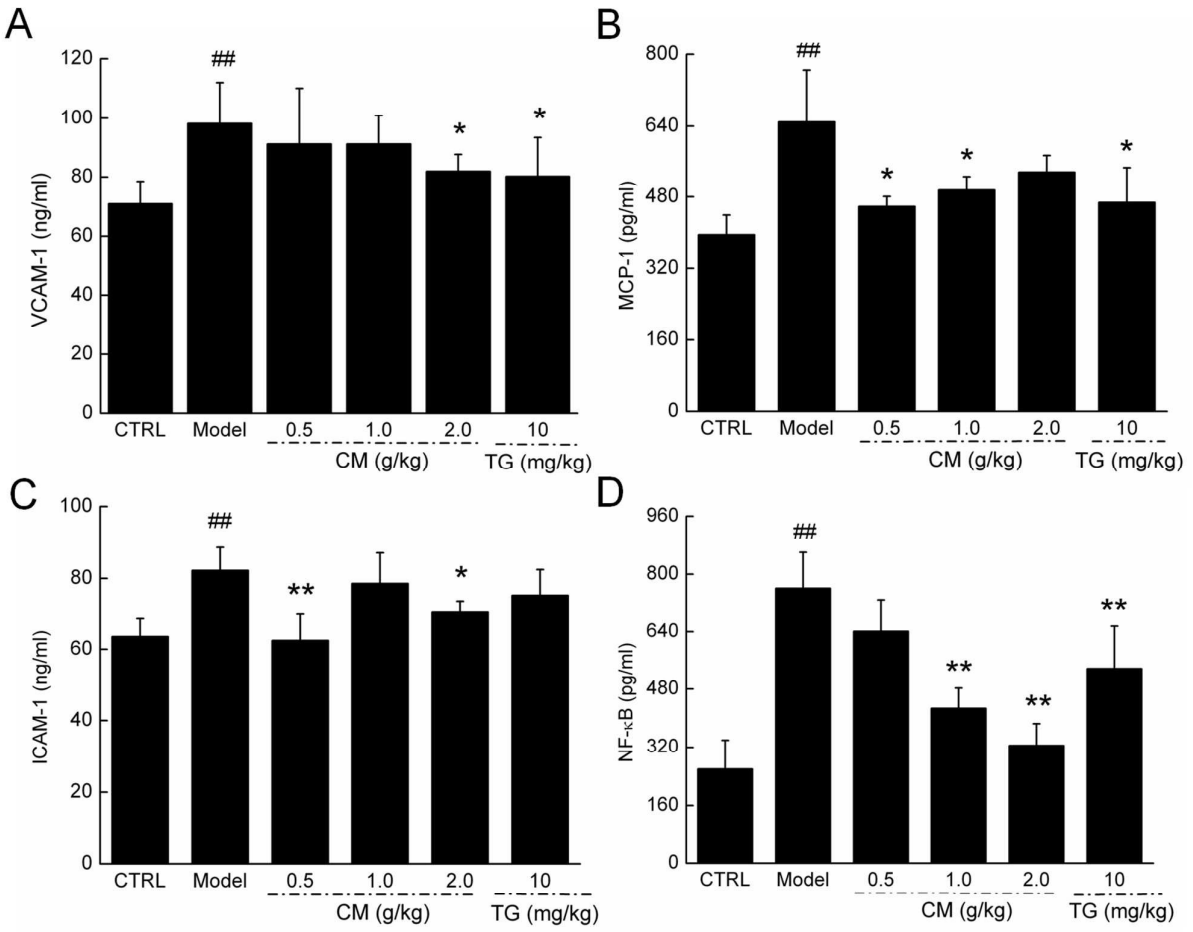

Fig.5 C-BSA-induced MGN rats were treated with or without CM and TG at indicated doses for 4 weeks. At the end of the experiment, the levels of VCAM-1 (A), MCP-1 (B) and ICAM-1 (C), and the activities of NF-κB p65 (D) in serum were analyzed. Data were expressed as mean ± S.D. (n = 10) and analyzed using a one-way ANOVA followed by Dunn's test. ## P < 0.01 versus the control rats, * P < 0.05 and ** P < 0.01 versus the MGN rats.

The regulation effect of CM on protein expressions in kidney

The expressions of phosphor-AKT and phosphor-NF-κB p65 in kidney of experimental rats were detected via western blot to investigate the preliminary signal molecular pathway during CM-mediated anti-membranous glomerulonephritic activities. The enhanced activities of AKT and NF-κB p65 were found in kidney of MGN rats compared with control rats (P<0.01; Fig. 6), which were significantly restored to the normal level after 4-week TG and CM treatment (P<0.05; Fig. 6).

Discussion

In the present study, we prove that CM is a promising Chinese herb medicine, which can alleviate kidney injury in MGN, without any adverse side effects. Hyperlipidemia, proteinuria, Scr, BUN, blood albumin and protein are endpoints of chronic kidney disease.[21, 24-26] Albumin, the most prevalent serum protein, contributes to the cure of edema caused by nephropathy.[26] Low serum level of albumin is the manifestation of poor renal function.[24] Our data show that CM restores all of the above parameters in a dose dependent manner, suggesting CM protects kidney from immune related damage. Specifically, CM targets lipid metabolism and damages the integrity of the glomerular filtration barrier. In histological studies on C-BSA treated rats, CM administration alleviates the kidney structural changes, suggesting a restoration of kidney injury.

Oxidative stress and chronic inflammation are two major causes of kidney injury.[27, 28] Therefore, the anti-oxidation and anti-inflammatory methods and drugs are intensely studied.[29-31] The problem is that targeting single or several molecules in the complex signaling pathways generate many adverse side effects. For example, the bardoxolone methyl, a promising anti-oxidation medicine, generates adverse side effects such as weight loss, muscle spasm, proteinuria, and abnormal high level of serum megnesium and alanine aminotransferase (ALT), and death. Therefore, its phase III clinical trial is terminated prematurely.[32, 33] *Tripterygium wilfordii* is a widely used herbal

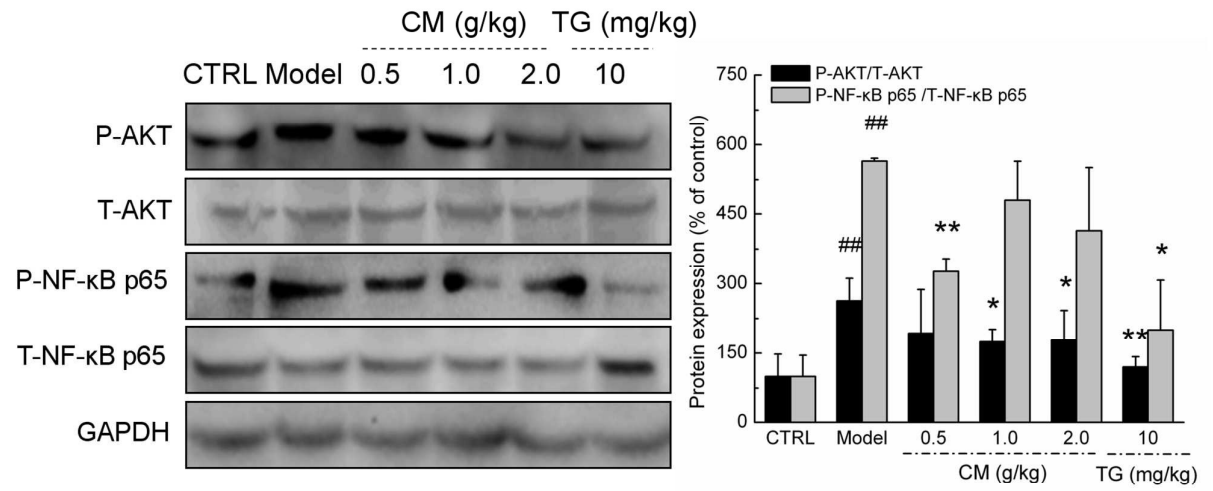

Fig.6 After 4-week treatment with CM or TG, the phosphorylation of AKT and NF-κB p65 in kidney tissue of experimental rats were determined via western blot. Quantification data of the expression of P-AKT and P-NF-κB p65 were normalized by corresponding T-AKT, T-NF-κB p65 and GAPDH, respectively. Data were expressed as mean ± S.D. (n = 10) and analyzed using a one-way ANOVA followed by Dunn's test. ## $P < 0.01$ versus the control rats, * $P < 0.05$ and ** $P < 0.01$ versus the MGN rats.

medicine for rheumatoid arthritis and other autoimmune diseases treatment; however, its adverse reactions including hepatotoxicity and nephrotoxicity have been frequently reported in clinic.[34] CM is a traditional Chinese herb medicine. Its nature of crude drug suggests multi-effective component, which might target many molecules in the signaling of inflammation and oxidative stress. This "systemic targeting" will completely eliminate the inflammation and oxidative stress in a much natural way, so that less adverse side effect is expected. Furthermore, CM, as a folk tonic food, has been practiced by Chinese people for thousands of years, further emphasizes its safety with less adverse side effects. In the acute toxic test performed in our preliminary study provides experimental basis for its safety, and CM showed no influences on animal behaviours and body organs.

Oxidative factors production is physiologically relevant as an important step in inflammation.[35] The alteration of glomerulus structure and function caused by oxidative injury is related to the damage of ROS on mesangial and endothelial cells.[34] Antioxidant enzymes including GSH-Px and SOD prevent oxidative stress injury in animals.[37, 38] Thymoquinone, an antioxidant, presents renoprotective effect related to its anti-oxidant property.[39] We confirmed that *Cordyceps militaris* mycelium displayed anti-nephropathic activity via reducing oxidative stress response.[15] The modulation of oxidative system contributes to CM-mediated anti-membranous glomerulonephritic property.

Due to the participation of inflammatory mediators, MGN is considered as an immune-mediated inflammatory disease.[40] The overexpression of IL-2 activates pro-inflammatory CD4+ T cells, which exacerbates the glomerular damage by recruiting the ratio of macrophages and neutrophils.[41] IL-6, secreted by the glomerular membrane system, is responsible for the proliferation of mesangial cells and the release of inflammatory mediators including superoxide anion.[42] Skimmin slows down the progression of membranous glomerulonephritis by inhibiting IL-1β and IL-6 expression and immune complex deposition.[43] CM reverses the hyper-levels of IL-2, IL-6 and TNF-α caused by chronic C-BSA injection indicating that interleukins participate in CM-mediated renoprotection. CM also restores the hyper-levels of VCAM-1, ICAM-1 and MCP-1 in MGN rats. Exposure to inflammatory stimuli, the expression of especial adhesion molecules is disordered in MGN patients.[44] The deregulated adhesion molecules such as VCAM-1 and ICAM-1 fail to capture flowing leukocytes, which would persistently infiltrate into inflamed tissues.[45]

Our data support the idea that reducing kidney inflammation, especially NF-κB activity, potentially restores the integrity of glomerular filtration barrier. The regulatory effects of CM on NF-κB p65 and AKT activation were observed in MGN rats. Inhibiting AKT, one of physiological activators of NF-κB,[46] suppresses the adhesion molecules expressions,[47] and further prevents high glucose-induced glomerulosclerosis.[48] The NF-κB p65 transcriptional activation promotes inflammatory cytokines expressions and oxidative stress responses, which are involved in the progression of kidney tissue injury.[8, 43] BAY 11-7082, an inhibitor of IκB, downregulating NF-κB activation, successfully inhibits the oxidative damage from diabetic nephropathy.[49] Another study shows that cobalt chloride attenuates oxidative stress and inflammation via suppressing NF-κB activity in human renal proximal tubular epithelial cells.[50] We found that combining with NF-κB deactivation, the IκBα expression was reduced (Data not shown), which was consistent with several previous studies.[51, 52] IκBα sequesters

NF-κB in an inactivate form in the cytoplasm; however, the non-canonical NF-κB pathway is independent of IκBα regulation.[52, 53] It is suggested that a non-canonical NF-κB pathway which not directly dependent on IκBα may contribute to CM-mediated renoprotection in C-BSA-induced MGN rats. Further study related to the roles of IκBα during CM's renoprotection will be performed.

Conclusions

In conclusion, CM protects the rats against membranous glomerulonephritis induced by C-BSA injection via reducing NF-κB activity, thereby inhibits the oxidative damage and reduces inflammatory cytokines levels. Further investigations are ongoing for getting purified polysaccharide from *Cordyceps militaris* fruit body with anti-MGN effects. Based on acute and chronic toxicity tests performed in our separated experiments, CM is a safe agent with the maximal oral tolerance dose of 120 g/kg in rats. Our data provide experimental evidence in supporting the clinical use of CM as an effective and safe renoprotective agent.

Conflict of interest

None

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References

1. D. Kerjaschki and M. G. Farquhar, *The Journal of experimental medicine*, 1983, **157**, 667-686.
2. I. Horvatic and K. Galesic, *Lijecnicki vjesnik*, 2012, **134**, 328-339.
3. S. G. Li, Y. Chen and Y. Q. Zhang, *Zhong yao cai = Zhongyaocai = Journal of Chinese medicinal materials*, 2010, **33**, 1913-1916.
4. A. V. Kshirsagar, P. H. Nachman and R. J. Falk, *Seminars in nephrology*, 2003, **23**, 362-372.
5. D. C. Cattran, C. Greenwood, S. Ritchie, K. Bernstein, D. N. Churchill, W. F. Clark, P. A. Morrin and S. Lavoie, *Kidney international*, 1995, **47**, 1130-1135.
6. M. Praga, V. Barrio, G. F. Juarez, J. Luno and M. Grupo Espanol de Estudio de la Nefropatia, *Kidney international*, 2007, **71**, 924-930.
7. S. A. Mezzano, M. Barria, M. A. Droguett, M. E. Burgos, L. G. Ardiles, C. Flores and J. Egido, *Kidney international*, 2001, **60**, 1366-1377.
8. P. Pan, Y. J. Wang, L. Han, X. Liu, M. Zhao and Y. F. Yuan, *Journal of ethnopharmacology*, 2010, **131**, 203-209.
9. S. J. Mudge, K. Paizis, R. B. Auwardt, V. Levidiotis, S. A. Fraser and D. A. Power, *Nephron. Experimental nephrology*, 2010, **116**, e23-31.
10. X. Jiang, X. Zhao, H. Luo and K. Zhu, *Nutrients*, 2014, 6, 1223-1235.
11. T. Wang, F. Wu, Z. Jin, Z. Zhai, Y. Wang, B. Tu, W. Yan and T. Tang, *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 2014, 64, 177-183.
12. S. K. Das, M. Masuda, A. Sakurai and M. Sakakibara, *Fitoterapia*, 2010, **81**, 961-968.
13. Y. Wang, Y. Wang, D. Liu, W. Wang, H. Zhao, M. Wang and H. Yin, *Carbohydrate polymers*, 2015, **125**, 135-145.
14. N. Nallathamby, L. Guan-Serm, S. Vidyadaran, S. N. Abd Malek, J. Raman and V. Sabaratnam, *Natural product communications*, 2015, **10**, 885-886.
15. Y. Dong, T. Jing, Q. Meng, C. Liu, S. Hu, Y. Ma, Y. Liu, J. Lu, Y. Cheng, D. Wang and L. Teng, *BioMed research international*, 2014, **2014**, 160980.
16. J. Baharara and E. Amini, *Avicenna journal of medical biotechnology*, 2015, **7**, 151-158.
17. M. Kato, T. Yamazaki, H. Kato, S. Eyama, M. Goto, M. Yoshioka and A. Takatsu, *Analytical sciences : the international journal of the Japan Society for Analytical Chemistry*, 2015, **31**, 805-814.
18. S. P. Li, F. Q. Yang and K. W. Tsim, *Journal of pharmaceutical and biomedical analysis*, 2006, **41**, 1571-1584.
19. S. Khalatbari Soltani, R. Jamaluddin, H. Tabibi, B. N. Mohd Yusof, S. Atabak, S. P. Loh and L. Rahmani, *Hemodialysis international. International Symposium on Home Hemodialysis*, 2013, **17**, 275-281.
20. Y. Li, I. Raman, Y. Du, M. Yan, S. Min, J. Yang, X. Fang, W. Li, J. Lu, X. J. Zhou, C. Mohan and Q. Z. Li, *PloS one*, 2013, **8**, e67790.
21. Y. B. Chong, D. Y. Yap, C. S. Tang and T. M. Chan, *Nephrology*, 2011, **16**, 511-517.
22. P. Codoner-Franch, V. Valls-Belles, A. Arilla-Codoner and E. Alonso-Iglesias, *Translational research : the journal of laboratory and clinical medicine*, 2011, **158**, 369-384.
23. Y. Wang, J. Tong, R. Tang, H. Dong and J. Xu, *Nephron. Physiology*, 2004, **98**, p80-88.
24. X. Jiang, X. Zhao, H. Luo and K. Zhu, *Nutrients*, 2014, **6**, 1223-1235.
25. Z. Zhang, P. Wu, J. Zhang, S. Wang and G. Zhang, *Pharmacological research*, 2016, **105**, 74-83.
26. Z. J. Chou An, Zhou Yuan, Hua Jing, Wu Junbiao, *Traditional Chinese Drug Research & Clinical Pharmacology*, 2012, **23**, 626-630.
27. G. C. Liu, F. Fang, J. Zhou, K. Koulajian, S. Yang, L. Lam, H. N. Reich, R. John, A. M. Herzenberg, A. Giacca, G. Y. Oudit and J. W. Scholey, *Diabetologia*, 2012, **55**, 2522-2532.
28. 26. J. Y. Moon, K. H. Jeong, T. W. Lee, C. G. Ihm, S. J. Lim and S. H. Lee, *American journal of nephrology*, 2012, **35**, 164-174.
29. S. M. Yang, S. M. Ka, H. L. Wu, Y. C. Yeh, C. H. Kuo, K. F. Hua, G. Y. Shi, Y. J. Hung, F. C. Hsiao, S. S. Yang, Y. S. Shieh, S. H. Lin, C. W. Wei, J. S. Lee, C. Y. Yang and A. Chen, *Diabetologia*, 2014, **57**, 424-434.
30. M. Patel, X. X. Wang, L. Magomedova, R. John, A. Rasheed, H. Santamaria, W. Wang, R. Tsai, L. Qiu, A. Orellana, A. Advani, M. Levi and C. L. Cummins, *Diabetologia*, 2014, **57**, 435-446.
31. Y. Ishikawa, T. Gohda, M. Tanimoto, K. Omote, M. Furukawa, S. Yamaguchi, M. Murakoshi, S. Hagiwara, S. Horikoshi, K. Funabiki and Y. Tomino, *Experimental diabetes research*, 2012, **2012**, 702948.
32. C. Zoja, A. Benigni and G. Remuzzi, *Nephrology, dialysis, transplantation : official publication of the European Dialysis*

and Transplant Association - European Renal Association, 2014, **29 Suppl 1**, i19-i24.
33. D. K. Singh, P. Winocour and K. Farrington, *Nature reviews. Endocrinology*, 2011, **7**, 176-184.
34. Y. He, S. Shi, R. Zhang, W. Shen, J. Tu, Z. Ding and Y. Fan, *Drug and chemical toxicology*, 2015, **38**, 145-151.
35. M. T. Sultan, M. S. Butt, R. Karim, W. Ahmed, U. Kaka, S. Ahmad, S. Dewanjee, H. Z. Jaafar and M. Zia-Ul-Haq, *BMC complementary and alternative medicine*, 2015, **15**, 330.
36. A. Ragheb, A. Attia, W. S. Eldin, F. Elbarbry, S. Gazarin and A. Shoker, *Saudi journal of kidney diseases and transplantation : an official publication of the Saudi Center for Organ Transplantation, Saudi Arabia*, 2009, **20**, 741-752.
37. T. O. Barreto, L. S. Cleto, C. R. Gioda, R. S. Silva, A. C. Campi-Azevedo, J. de Sousa-Franco, J. C. de Magalhaes, C. L. Penaforte, K. M. Pinto, S. Cruz Jdos and E. Rocha-Vieira, *European journal of applied physiology*, 2012, **112**, 2523-2530.
38. S. K. Powers, K. J. Sollanek, M. P. Wiggs, H. A. Demirel and A. J. Smuder, *Free radical research*, 2014, **48**, 43-51.
39. M. Erboga, M. Kanter, C. Aktas, U. Sener, Z. Fidanol Erboga, Y. Bozdemir Donmez and A. Gurel, *Biological trace element research*, 2015, DOI: 10.1007/s12011-015-0453-x.
40. F. R. Santos, *Jornal brasileiro de nefrologia : 'orgao oficial de Sociedades Brasileira e Latino-Americana de Nefrologia*, 2014, **36**, 59-62.
41. R. Bertelli, A. Di Donato, M. Cioni, F. Grassi, M. Ikehata, A. Bonanni, M. P. Rastaldi and G. M. Ghiggeri, *PloS one*, 2014, **9**, e111285.
42. L. Ma, Y. Gao, G. Chen, J. Gong, D. Yang, Y. Xie, M. Wang, H. Chen and M. Song, *Medical science monitor : international medical journal of experimental and clinical research*, 2015, **21**, 356-362.
43. S. Zhang, H. Xin, Y. Li, D. Zhang, J. Shi, J. Yang and X. Chen, *Evidence-based complementary and alternative medicine : eCAM*, 2013, **2013**, 819296.
44. E. Honkanen, E. von Willebrand, A. M. Teppo, T. Tornroth and C. Gronhagen-Riska, *Kidney international*, 1998, **53**, 909-917.
45. R. S. Cotran and J. S. Pober, *Kidney international*, 1989, **35**, 969-975.
46. J. Zdychova and R. Komers, *Physiological research / Academia Scientiarum Bohemoslovaca*, 2005, **54**, 1-16.
47. W. Huang, X. Mo, X. Wu, W. Luo and Y. Chen, *Molecular and cellular biochemistry*, 2015, **407**, 173-179.
48. Shemesh, II, B. Rozen-Zvi, Y. Kalechman, U. Gafter and B. Sredni, *PloS one*, 2014, **9**, e114287.
49. S. R. Kolati, E. R. Kasala, L. N. Bodduluru, J. R. Mahareddy, S. K. Uppulapu, R. Gogoi, C. C. Barua and M. Lahkar, *Environmental toxicology and pharmacology*, 2015, **39**, 690-699.
50. S. W. Oh, Y. M. Lee, S. Kim, H. J. Chin, D. W. Chae and K. Y. Na, *Journal of Korean medical science*, 2014, **29 Suppl 2**, S139-145.
51. R. Min, Z. Zun, Y. Min, D. Wenhu, Y. Wenjun and Z. Chenping, *Oral diseases*, 2011, **17**, 362-369.
52. V. Camara-Clayette, Y. Lecluse, C. Schrader, W. Klapper, W. Vainchenker, O. Hermine and V. Ribrag, *European journal of cancer*, 2014, **50**, 159-169.
53. C. Y. Sasaki, T. J. Barberi, P. Ghosh and D. L. Longo, *The Journal of biological chemistry*, 2005, **280**, 34538-34547.