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Nrf2 activator corosolic acid meliorates alloxan induced diabetic nephropathy in mice

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ABSTRACT

Objective: To determine whether corosolic acid (CA) targeting nuclear protein expression of Nrf2 activation can be used to attenuate renal damage and preserve renal function in alloxan diabetic mice.

Methods: A mouse model with diabetic nephropathy was established to examine the Nrf2 expression. Mice were randomly divided into control, diabetic control, and CA groups treated at 0.4 mg/kg, 2 mg/kg and 10 mg/kg *p.o.* for 8 weeks. Diabetes was induced in mice by single intraperitoneal injection of alloxan 200 mg/kg in all groups except the control. The mice with fasting blood glucose level over 200 mg/dL were considered as diabetic and were employed in the study. After 4th and 8th weeks, urine samples were collected (using metabolic cages) to measure protein and urea. Animals were euthanized, and serum samples were collected to estimate the glucose, creatinine, total protein, urea and blood urea nitrogen. Kidney was isolated at the end of experiment for histology to evaluate anti-oxidant parameters. Immunohistochemistry was performed to examine the Nrf2 expression.

Results: CA treatment showed dose dependent reduction in level of biochemical parameters in serum and urine. CA group (10 mg/kg) showed significantly higher body weight and reduced kidney weight. Histopathological examination revealed reduced inflammation, collagen deposition and glomerulosclerosis in renal tissue. CA attenuated renal dysfunction, oxidative stress and inflammatory pro-cytokine levels.

Conclusions: CA treatment exhibited ameliorative effect on kidney in mice with its enhanced Nrf2 expression.

1. Introduction

Diabetic nephropathy (DN) is usually associated with chronic kidney diseases like renal fibrosis, severe tubular necrosis, conservative glomerular dilatation, alteration in mesangial matrix fraction, vascular wall thickening and interstitial inflammation. Herbal medications are preferred as currently available therapies for controlling blood glucose level, which are prone to causing renal damage. Exogenous insulin administration is allied with complications which have effects on longevity and quality of life. But the restriction of most herbal medications is repeated intake of high doses. The available solution is co-administration of Reno protective drugs but they also produce toxicity and limit the efficacy. Henceforth, the urge is to generate efficient treatment alternative to safeguard average renal function and slow down the progression of DN.

DN is a multi-factorial process of extracellular matrix in the mesangium, podocyte injury and loss. Progressive albuminuria is characterized by deregulated lipid and glucose homeostasis, reduction in glomerular filtration rate, excessive deposition, glomerular basement membrane thickening. It is also attended mesengial cytokine stimulation, glomerulosclerosis, elevated oxidative stress, hyperactive polyol and mitogen activated protein kinase pathway [1,2].

Nrf2 protects the body from oxidative stress [3]. In the treatment of diseases like neurodegenerative disease [4], cancer [5], pulmonary fibrosis [6], and diabetes [7], NrF2 is considered as a potential therapeutic target. Nrf2 regulates expression of numerous genes through antioxidant response elements in their promoters to neutralize free radicals and accelerates removal of environmental toxins. It binds to inhibitor kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. An alteration in oxidative or electrophilic stress inducing agents reactive oxygen species (ROS), causes Keap1 to lose its inhibiting ability for Nrf2, which results in the

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disassociation of Nrf2 from Keap1 [8,9]. Oxidative stress is reduced with the simultaneous inactivation of Keap1 and activation of Nrf2 which escort reduction in inflammatory genes interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) [10]. Then Nrf2 can translocate into the nucleus where it combined with the anti-oxidant response element to stimulate the transcription of anti-oxidant genes. It regulates intracellular antioxidants, phase II detoxifying enzymes and many other proteins that detoxify xenobiotics and neutralize ROS to promote cell survival and maintain cellular redox homeostasis [11,12]. NAD(P)H quinone oxidoreductase, glutathione S-transferase, hem oxygenase-1 (HO-1), and-glutamylcysteinesynthetase are among the well-studied Nrf2 target genes that are upregulated through the antioxidant response element regulatory to oxidative stress. Nrf2 conferred protection against high glucose induced oxidative damage.

CA is a multifunctional pentacyclic triterpenoid presenting in some edible plants including *Lagerstroemia speciosa* and *Eriobotrta japonica* with multiple pharmacological activities. CA has exhibited potent free radical scavenging, anti-hyperglycemic, anti-hyperlipidemic, antihypertensive, antioxidant, anti-inflammatory, antifungal, antiviral activities and anticancer effects [13] in their potential clinical value and application projection as remedial drug for diabetic comorbidities. The previous studies demonstrated the anti-diabetic effect of CA in KK-Ay mice, an animal model of type 2 diabetes [14,15]. CA was reported to have effective hypotensive, diuretic/Saluretic, anti-hyperlipidemic, antioxidant and hypoglycemic effects [14]. Some pentacyclic triterpenoids distinctly confined PC12 cells against subsequent H_2O_2- and MPP^+-induced restoration of GSH, diminution of catalase and SOD, increase of lipid peroxidation, and release of IL-6 and TNF-α, which definitely contributed to the diminishing oxidative and inflammatory injury [16,17]. As diabetic nephropathy is associated with inflammatory responses in which NF-κB pathway plays a crucial role, it was a sincere attempt to discover the action of Corosolic acid on the activation of NF-κB and TNF-α in the kidney of alloxan-induced diabetic mice with renal injury.

2. Material and method

2.1. Chemicals and reagents

Alloxan was procured from Sigma–Aldrich Corporation St Louis, MO, USA. Corosolic acid was purchased from Aktin Chemicals Inc. China batch number CA-160106 HPLC). NBT (CAS No. 298-83-9) was obtained from Hi-media Laboratories Ltd., India. Nrf2 (C-20): sc-722 primary antibody (Lot number B1215), goat anti-rabbit IgG-FITC: sc-2012 secondary antibody (Lot numberG2314) and ImmunoCruz™ rabbit ABC Staining System: sc-2018 (Lot number L0114) were obtained from Santa Cruz Biotechnology, Inc., USA.

2.2. Animal model and experimental protocol

The study protocol was well designed and approved (IAEC/CPCSEA/RCPIPER/2015-25) by Institutional Animal Ethics Committee (Registration Number- Reg. No. 651/02/c/CPCSEA) of R.C Patel Institute of Pharmaceutical Education and Research, Shirpur, India.

Seventy male Swiss albino mice (20–30) g were procured from the central animal house facility of the institute. The animals were systematically placed in a controlled condition of 12 h light and 12 h dark cycle at room temperature (25 ± 2) °C with 50% ± 10% humidity in polypropylene cages. Then animals were supplemented with Standard palletized feed (Amrut Feed, Pune, India) and tap water *ad libitum*, and given around a week time to get acclimatized to the experimental conditions before the initiation of experiment. All animals were maintained as per the standards prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (Government of India). After fed with regular diet for 1 week, they were randomly assigned to a normal control group (n = 6) and an alloxan-induced group (n = 65), which were given a single intraperitoneal injection with 200 mg/kg alloxan diluted with sterile saline just before use. A single dose of sterile saline was given to the control group. On the third day after the alloxan injection, the mice with fasting blood glucose level over 200 mg/dL were considered diabetic and were employed in the study [18,19]. Sixteen diabetic mice were selected and randomly divided into two groups: diabetic group (n = 8) and corosolic acid-treated diabetic group (n = 8). A total of 24 diabetic mice were selected and randomly allocated to 4 treatment groups containing 8 mice each. The groups were as follows: Diabetic group (n = 8), corosolic acid 0.4 mg/kg group (n = 8), corosolic acid 2 mg/kg group (n = 8), corosolic acid 10 mg/kg (n = 8). Corosolic acid was administered orally as a solution in distilled water and the diabetic group received equal volume of distilled water orally. Equal volume of distilled water was given to mice in both the control group and the diabetic group and the oral administration was performed between 8:30 and 9:30 every morning. Urine was collected from the mice housed in metabolic cages for 24 h in the 4th and 8th week before the end of the experiment. At the end of 8th week, the animals were sacrificed and blood samples were collected by drainage from the retro orbital venous plexus. Serum was obtained by centrifuge at 3000 ×g for 15 min and stored at −80 °C until it was used for biochemical analysis. Meanwhile, kidney samples were rapidly excised, weighed and stored at −80 °C or fixed in 10% neutral-buffered formalin.

2.3. Assessment of renal function

At the end of the 8th week, blood samples and 24 h urine samples were collected from each mouse, under light ether anesthesia through retro orbital puncture. The plasma and urine levels of glucose, blood urea nitrogen, creatinine and total protein were examined to assess the renal function. The glomerular filtration rate was further identified by measuring creatinine clearance and urea clearance.

2.4. Histological examination

The histological examination of kidney specimen was conducted using hematoxylin and eosin staining. The kidney was excised from each of the sacrificed mouse, rinsed in saline, blotted on tissue paper for weighing and half of the kidney section were immediately stored in 10% formalin solution. The specimen was split longitudinally and processed for histological examination.

2.5. Estimation of Nrf-2 expression by immunohistochemistry

The sections of kidney were embedded in paraffin and placed on poly-L-lysine coated slides, sequentially treated with 100%

xylene, 100% ethanol, 90% ethanol, 70% ethanol and 50% ethanol in a graded manner. Each treatment lasted for 3 min. The slides were then place in distilled water for 10 min. The sections were incubated with citrate buffer (pH 6.0) at 95 °C for 5 min in water bath for antigen retrieval. Then, it was cooled in citrate buffer for 20 min and washed in deionized water 3 times, washed two times in TBS for 5 min each, with excess liquid aspirated. Specimens were incubated for 1 h in 1.5% normal blocking serum in TBS. The blocking buffer was drained and Nrf2 primary antibody was added (Santa Cruz Biotechnology, Inc., USA) at 4 °C overnight. Slides were rinsed for 2 times in TBS with 0.025% triton X-100 with gentle agitation, and incubated in 3% H_2O_2 in TBS for 15 min. Biotinylated secondary antibody was added and incubated for 30 min, rinsed 3 times for 5 min each with TBS. AB enzyme was added and incubated for 30 min, rinsed 3 times for 5 min each with TBS. The slides were developed with DAB, rinsed with distilled water for 5 min, dehydrated and observed in inverted microscope (Motic AE31, MXH-100 camera) and images were taken using Motic Images plus 2.0 software, Asia.

2.6. Estimation of release of cytokines

Ice-chilled phosphate buffer (50 mM pH 7.4) was used for preparing kidney homogenate (10%). One hundred (100) µL of the supernatant was taken for the estimation of TNF-α, IL-1β and IL-6 using ELISA kits by centrifugation at 2 000 rpm for 20 min at 4 °C.

2.7. Estimation of oxidative stress

Kidney was taken out from liquid nitrogen and transferred to ice-cold phosphate buffer saline (pH 7.4). Fine slices of the tissue were made by cross-chopping with a surgical scalpel, suspended in chilled 0.25 M sucrose solution and quickly blotted on a filter paper. Tissues were minced and homogenized finally to release soluble proteins in ice chilled phosphate buffer (50 mM pH 7.4). The supernatant was used for estimation of tissue parameters, in which the homogenate was produced by centrifugation at 7 000 rpm for 20 min using high speed centrifuge.

2.8. Assessment of protein content

Protein content was measured using total protein kit (span Diagnostic laboratories Pvt. Ltd., Delhi, India). Thirty (30) µL supernatant of renal tissue homogenate was mixed up with 3.0 mL of biuret test reagent, kept for 5 min and absorbance was read at 578 nm.

$$\text{Total protein concentration (mg/dL)} = \frac{\text{Absorbance of test} \times 6.5}{\text{Absorbance of standard}}$$

2.9. Estimation of lipid per oxidation

According to the method of Ohkawa *et al.* [20], lipid peroxides in the kidney were measured as malondialdehyde (MDA), an index of lipid per oxidation. Briefly, 0.2 mL of homogenate was mixed with 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 30% acetic acid (pH 3.5) and 1.5 mL of 0.8% thiobarbituric acid. The reaction mixture was cooled on ice bath after heating for 60 min at 95 °C. After cooling, 1.0 mL distilled water and 5.0 mL *n*-butanol: pyridine (15:1 v/v) solution were added and centrifuged at 5 000 rpm for 20 min. The absorbance of the pink colour in organic layer was measured at 532 nm. The levels were expressed as µg/mg of protein using 1,1,3,3-tetraethoxypropane (Sigma Chemicals, USA) as the standard MDA.

2.10. Estimation of superoxide dismutase (SOD)

Slight modifications were made in the method of Kono *et al.* [21] for determining SOD activity. One hundred (100) µL tissue supernatant, 2.85 mL of 0.1 M phosphate buffer (pH 8.4) and 50 µL of 7.5 mM pyrogallol were added and the absorbance was measured at 420 nm for 3 min at 30 s intervals. Enzyme levels were expressed as U/mg protein.

2.11. Estimation of reduced glutathione (GSH)

GSH was estimated by the method of Owens and Belcher *et al.* [22]. Briefly, 100 µL of tissue homogenate was mixed with 100 µL of 10% trichloro acetic acid and vortexed. The contents were then centrifuged at 5 000 rpm for 10 min. Subsequently 0.05 mL of supernatant was mixed with a reaction mixture containing 3.0 mL of 0.3 M phosphate buffer (pH 8.4) and 0.5 mL 5,5′ dithiobis-(2-nitrobenzoic acid). The absorbance was measured at 412 nm using a spectrophotometer within 10 min. GSH was determined from a standard curve using commercially available standard GSH (Sigma Chemicals, USA). The amount was expressed as µg/mg of protein.

2.12. Estimation of catalase

Catalase activity was estimated by the method described by Abraham *et al.* [23]. A total of 50 µL of tissue supernatant, 1.0 mL of 50 mM phosphate buffer (pH 7) and 0.1 mL of 30 mM hydrogen peroxide were added and a decrease in absorbance at 240 nm was measured every 5 s for 30 s. Catalase levels were expressed as U/mg protein and the total protein was estimated according to the method of Lowry *et al.* [24].

2.13. Assessment of food and water intake

The food and water intake were recorded before shifting the mice to metabolic cages. The average food and water intake were measured by giving weighed amount of food and water in each cage, and determining the weight of remaining chow in each cage and amount of water in each bottle the next day.

2.14. Statistical analysis

Graph Pad Prism version 5.0 software, USA was used for statistical analysis. The results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Bonferroni multiple comparison test was used to analyze the data from sets of experimental parameters for statistical comparison between groups and $P < 0.05$ was considered as significant within group test.

3. Results

3.1. Influences of kidney hypertrophy in alloxan induced diabetic mice by CA

The mean kidney weight and ratio of kidney weight to body weight in vehicle treated alloxan diabetic mice were significantly

Table 1

Effect of CA on renal function related parameters in alloxan induced diabetic mice.

Group	Body weight (g)		Urine output (mL/24 h)		Kidney weight (mg) after 8 week	KW/BW mg/g after 8 week
	Before study	After study	Week 4	Week 8		
Normal	28.3 ± 1.0	29.1 ± 0.8	2.00 ± 0.10	2.10 ± 0.07	225.00 ± 12.47	7.60 ± 0.20
Alloxan	23.5 ± 1.1	21.5 ± 0.8$^{\#\#}$	3.80 ± 0.20$^{\#\#}$	4.30 ± 0.49$^{\#\#}$	328.00 ± 17.02$^{\#\#}$	15.20 ± 0.40$^{\#\#}$
CA (0.4)	25.5 ± 1.5	24.1 ± 1.4	2.60 ± 0.15*	2.40 ± 0.17**	270.00 ± 11.57*	11.10 ± 0.07*
CA (5)	25.1 ± 1.9	24.1 ± 1.3	2.50 ± 0.19**	2.30 ± 0.17**	251.00 ± 15.83*	10.30 ± 0.10**
CA (10)	24.1 ± 1.7	24.3 ± 1.2	2.40 ± 0.06**	2.20 ± 0.06**	234.00 ± 11.81**	9.60 ± 0.14**

Values are expressed as means ± SD, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with alloxan.

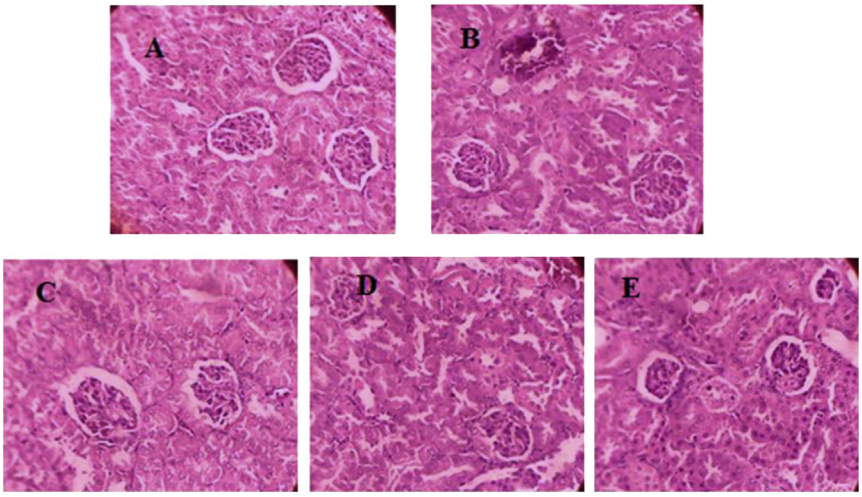

Figure 1. Effect of CA on alloxan induced histopathological changes in renal tissues.
Mice were intraperitoneally injected with Alloxan (200 mg/kg). Mice were intragastrically administered with CA at 0.4 mg/kg, 2 mg/kg and 10 mg/kg for 8 weeks. (A) Renal section from normal mice, (B) the renal section from the mice administered with alloxan 200 mg/kg, (C) the renal section from mice administered with alloxan 200 mg/kg and CA 0.4 mg/kg, (D) the renal section from mice administered with alloxan 200 mg/kg and CA 2 mg/kg, (E) the renal section from mice administered with alloxan 200 mg/kg and CA 10 mg/kg.

Table 2

Effect of different treatments of CA on antioxidant renal parameter in alloxan induced diabetic mice in week 8.

Group	Total protein (g/dL)	Catalase (U/mg of protein)	SOD (U/mg of protein)	GSH (U/L)	MDA (μg/mg of protein)
Normal	151.5 ± 5.8	0.49 ± 0.05	0.53 ± 0.04	0.45 ± 0.02	23.7 ± 2.7
Alloxan	246.8 ± 6.4$^{\#\#}$	0.07 ± 0.01$^{\#\#}$	0.03 ± 0.09$^{\#\#}$	0.04 ± 0.02$^{\#\#}$	101.1 ± 7.2$^{\#\#}$
CA (0.4)	209.6 ± 4.2*	0.17 ± 0.06*	0.29 ± 0.05*	0.22 ± 0.04*	69.0 ± 8.5
CA (5)	194.5 ± 5.6**	0.21 ± 0.08*	0.33 ± 0.04**	0.26 ± 0.03**	63.6 ± 8.6*
CA (10)	183.6 ± 4.3**	0.24 ± 0.08**	0.38 ± 0.04**	0.31 ± 0.02**	51.9 ± 7.5**

Values are expressed as means ± SD, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with alloxan.

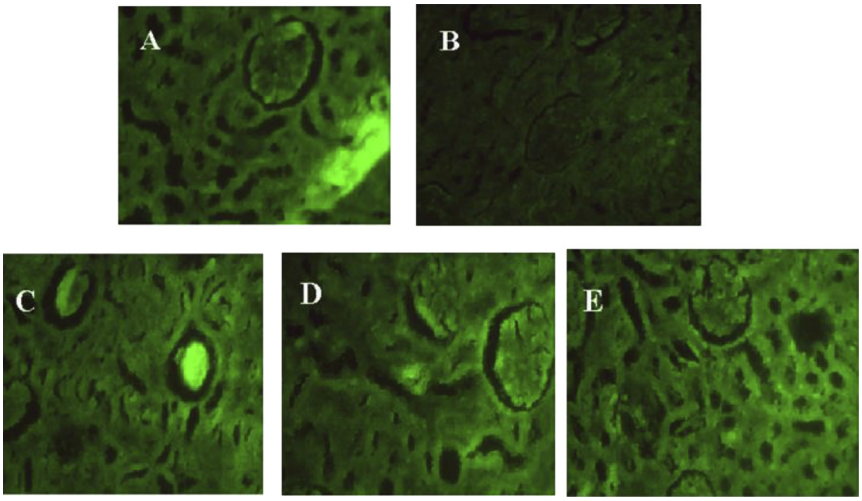

Figure 2. Immunohistochemical study of Nrf2 in mice kidney tissue section obtained from (A) Renal section from normal mice, (B) the renal section from the mice administered with alloxan 200 mg/kg, (C) the renal section from mice administered with alloxan 200 mg/kg and CA 0.4 mg/kg, (D) the renal section from mice administered with alloxan 200 mg/kg and CA 2 mg/kg, (E) the renal section from mice administered with alloxan 200 mg/kg and CA 10 mg/kg.

increased as compared to those in control group ($P < 0.01$). As shown in Table 1, diabetic mice with 10 mg/kg, 2 mg/kg, and 0.4 mg/kg CA treatment had significantly higher body weight and lower kidney weight respectively. Kidney hypertrophy was obviously ameliorated in CA treated group at week 4 and 8 ($P < 0.01$) .

3.2. Effect of CA on histopathological changes in renal tissues

The light microscopic evaluation of kidney sections revealed normal cell architecture of normal group tissue in Figure 1. The kidney of diabetic alloxan mice revealed severe tubular degeneration or necrosis, moderate degrees of glomerular dilatation, vascular wall thickening and interstitial inflammation. The severity of renal injury was attenuated by CA at 10 mg/kg. The result indicated that CA obviously ameliorated the histopathological condition as compared to alloxan mice.

3.3. Effect of CA oxidative stress in renal tissues

Oxidative stress eminence was evaluated by the measurement of MDA level as a marker of lipid peroxidation. Lipid peroxidation was determined by measuring the level of MDA, SOD, and CAT. Alloxan induced diabetes is a state of marked oxidative stress as demonstrated by a significant increase ($P < 0.01$) in the MDA level and a significant decrease in the GSH level ($P < 0.01$), SOD activity ($P < 0.01$) and catalase ($P < 0.01$) activities in kidney tissue homogenates. Alloxan induced diabetic mice significantly declined the activities of SOD and CA at 10 mg/kg, restored the level of SOD and CAT ($P < 0.01$). Our result stated that CA at 10 mg/kg attenuated the oxidative stress in alloxan stimulated mice (Table 2).

3.4. Effect of CA on level of inflammatory cytokines in renal tissues

The effect of CA on generation of pro-inflammatory cytokines expression of TNF-α, IL-1β and IL-6 in renal tissue homogenate was evaluated. The alloxan mice showed a marked increase in level of cytokines as compared to normal group, whereas treatment with CA, UA and OA 10 mg/kg pronouncedly decreased ($P < 0.01$) the content of TNF-α, IL-1β and IL-6 in kidney tissue. The TNF-α content, IL-1β and IL-6 remarkably decreased in CA ($P < 0.01$), which was more efficient than alloxan mice. The data indicated that CA 10 mg/kg might reduce the synthesis and release of inflammatory cytokines in alloxan diabetic nephropathy.

3.5. Effect of CA, UA and OA on protein expression of Nrf2 in renal tissues

To explore the potential mechanism of CA on alloxan induced diabetic nephropathy, Nrf2 expression in kidney tissue were detected. The protein of Nrf2 was markedly decreased in alloxan mice; however, treatment with CA at 10 mg/kg effectively increased the level of Nrf2 compared to alloxan mice. The subsequent intensity was measured using ImageJ1.44 software as shown in Figure 2.

Table 3
Effect of different treatments of CA on serum biochemical parameter in alloxan induced diabetic mice.

Group	Blood glucose (mg/dL)		Serum creatinine (mg/dL)		Serum total protein (g/dL)		Serum urea (mg/dL)		Serum BUN (mg/dL)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Normal	94.8 ± 1.5	95.80 ± 1.90	0.30 ± 0.05	0.31 ± 0.05	4.70 ± 0.10	4.70 ± 0.10	36.6 ± 0.7	39.8 ± 1.6	17.0 ± 0.3	19.0 ± 0.7
Alloxan	467.8 ± 2.7$^{\#\#}$	495.00 ± 7.50$^{\#\#}$	1.40 ± 0.09$^{\#\#}$	1.80 ± 0.10$^{\#\#}$	6.60 ± 0.20$^{\#\#}$	6.40 ± 0.20$^{\#\#}$	101.1 ± 1.3$^{\#\#}$	122.2 ± 3.6$^{\#\#}$	47.0 ± 0.6$^{\#\#}$	57.0 ± 1.7$^{\#\#}$
CA (0.4)	259.5 ± 1.9*	232.60 ± 6.07*	1.10 ± 0.05	0.90 ± 0.11**	5.70 ± 0.10*	5.60 ± 0.10**	85.3 ± 1.4	84.7 ± 3.0*	40.0 ± 0.6	40.0 ± 1.4*
CA (5)	231.9 ± 4.4**	216.60 ± 5.09**	1.00 ± 0.09*	0.70 ± 0.05**	5.50 ± 0.10**	5.20 ± 0.09**	78.1 ± 2.5**	77.7 ± 3.0**	36.0 ± 1.2**	36.0 ± 1.4**
CA (10)	198.1 ± 3.1**	198.10 ± 3.40**	0.60 ± 0.05**	0.40 ± 0.05**	5.20 ± 0.09**	4.90 ± 0.10**	70.1 ± 2.9**	69.3 ± 3.1**	33.0 ± 1.3**	32.0 ± 1.4**

Values are expressed as means ± SD, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with alloxan.

Table 4
Effect of different treatments of CA on urine parameter in alloxan induced diabetic mice.

Group	Urine glucose (mg/dL)		Urine creatinine (mg/dL)		Urine total protein (g/dL)		Urine urea (mg/dL)		Urine BUN (mg/dL)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Normal	1.1 ± 0.2	3.0 ± 0.6	1.7 ± 0.3	1.7 ± 0.2	5.00 ± 0.30	4.7 ± 0.1	62.3 ± 3.1	65.7 ± 3.5	29.1 ± 1.4	30.6 ± 1.6
Alloxan	612.0 ± 92.2$^{\#\#}$	675.2 ± 20.5$^{\#\#}$	4.9 ± 0.3$^{\#\#}$	0.5 ± 0.4$^{\#\#}$	7.50 ± 2.10$^{\#\#}$	6.6 ± 0.2$^{\#\#}$	116.1 ± 7.7$^{\#\#}$	134.7 ± 6.7$^{\#\#}$	54.2 ± 3.6$^{\#\#}$	62.9 ± 3.1$^{\#\#}$
CA (0.4)	333.8 ± 70.1	270.5 ± 39.7*	2.7 ± 0.1**	2.9 ± 0.8**	5.80 ± 0.20*	5.7 ± 0.1*	82.1 ± 5.0*	86.1 ± 4.1**	38.3 ± 2.3*	40.2 ± 1.9**
CA (5)	280.4 ± 47.0*	232.4 ± 19.6**	2.4 ± 0.1**	2.8 ± 0.7**	6.20 ± 0.10**	5.5 ± 0.1**	72.9 ± 3.9**	79.9 ± 6.3**	34.0 ± 1.8**	37.3 ± 2.9**
CA (10)	170.9 ± 5.8**	170.9 ± 17.2**	2.1 ± 0.1**	2.5 ± 0.4**	5.20 ± 0.09**	4.9 ± 0.1**	70.1 ± 2.9**	69.3 ± 3.1**	33 ± 1.3**	32 ± 1.4**

Effect of different treatments of CA on urine parameter in alloxan induced diabetic mice.
Values are expressed as means ± SD, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with alloxan.

Table 5
Effect of different treatments of CA on food and water intake in alloxan induced diabetic mice.

Group	Food intake (g)		Water intake (mL)	
	Week 4	Week 8	Week 4	Week 8
Normal	2.60 ± 0.02	2.4 ± 0.4	1.50 ± 0.08	1.70 ± 0.07
Alloxan	4.60 ± 0.40$^{\#\#}$	5.7 ± 0.1$^{\#\#}$	4.10 ± 0.10$^{\#\#}$	4.80 ± 0.20$^{\#\#}$
CA (0.4)	3.50 ± 0.20*	4.2 ± 0.1**	2.30 ± 0.10*	3.40 ± 0.20**
CA (5)	3.20 ± 0.10**	3.6 ± 0.1**	2.40 ± 0.10**	3.00 ± 0.10**
CA (10)	3.40 ± 0.08**	3.3 ± 0.1**	2.00 ± 0.10**	2.20 ± 0.10**

Values are expressed as means ± SD, $n = 6$. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with normal, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with alloxan.

3.6. General parameter of each group

In alloxan induced mice, blood glucose, blood urea nitrogen, serum creatinine, urea, total protein, urine glucose, urine blood urea nitrogen, urine creatinine, urine urea and urine total protein over 24 h were significantly enhanced compared with control group; While these indicators were significantly reduced over 24 h in diabetic mice of CA treated group (Tables 3 and 4).

3.7. Food and water intake

The food and water intake at week 4 and 8th week are presented in Table 5. Treatment with CA at 10 mg/kg significantly decreases the food and water intake at 4th and 8th week.

4. Discussion

As expected, our current study demonstrated that alloxan treated mice manifested heavy infiltrations of inflammatory cells, interstitial fibrosis, tubular atrophy, dilatation and glomerulosclerosis. In previous study, it has been noted that marked influence of hyperglycemia that further induced AGE production, protein kinase C pathway, the hexosamine pathway, stress signalling and mitochondrial dysfunction, leads to produce of ROS and favors the development of diabetic complications [25,26]. The current research has further explored that hyperglycemia induces not only oxidative stress but also ER stress. Nrf2 is a transcriptional factor that activates antioxidant genes which manifest in many tissues including kidney. Furthermore, the Nrf2/Keap1 is a major cellular defense system against cytotoxic, responding to oxidative stress and being researched as a therapeutic target for diabetic complications [27,28]. Moreover, our research is in agreement with the anti-glycative effect of CA lowered glucose in blood and kidney contributing to lowering AGEs formation in blood and kidney [29–31]. Thus aforementioned data suggests that CA ameliorated protective effect on renal injury of alloxan mice by its antioxidant and anti-inflammatory activity via Nrf2 pathway. These results are consistent with the study of Jimenez-Osorio *et al.* [32], which researched the protective effect via down regulation of Nrf2 as an important target during chronic hyperglycaemias.

Moreover, the elevated level of blood glucose in alloxan induced mice model was well established, while the treatment of CA at 10 mg/kg significantly declines the blood glucose level, indicating significant protective effect of CA against diabetes. Meanwhile, the histopathological examination of alloxan treated kidney sections revealed severe tubular necrosis, intense degrees

of glomerular dilatation, vascular wall thickening and heavy interstitial inflammation, whereas CA attenuated these alterations respectively. The analytical results indicated that CA effectively ameliorated the histopathological condition in alloxan induced diabetic nephropathy.

Inflammatory cytokines such as IL-1β, IL-6 and TNF-α accelerate development and progression of diabetic nephropathy. Former studies showed that IL-1β is incriminated in the progression of abnormalities in intraglomerular hemodynamics related to prostaglandin synthesis by mesangial cells [33–35]. In addition, IL-6 is also involved in intensifying the fibronectin level, hastening mesangial cell proliferation, disturbing extracellular matrix dynamics and increasing endothelial permeability [36]. Furthermore, it has been estimated in previous study that TNF-α is cytotoxic to glomerular, mesangial and epithelial cells and is able to induce direct renal damage through the generation of reactive free radicals [37,38].

The findings suggested that the accumulated ROS interact with polyunsaturated fatty acid to form lipid per oxidation resulted damage in kidney tissue, which have been proposed to be underlying mechanism for diabetic nephropathy [39,40]. The subsequent oxidative stress and ROS degraded membrane polyunsaturated fatty acids and produced 4-hydrosylnonenal and malondialdehyde [41,42]. MDA level is often used as an index of lipid per oxidation and increased MDA is a hallmark of severe oxidative stress in tissue injury [43,44]. Numerous evidence showed that SOD and MDA are mainly associated with diabetic nephropathy [45,46]. Alloxan induced diabetic mice markedly increased the MDA level in kidney homogenate, indicating enhanced lipid per oxidation. In the present study, we documented that CA effectively increased the activities of SOD and CAT, and reduced the content of MDA in kidney tissues in alloxan mice. Hence, it is reasonable to suppose that the use of antioxidant defense of renal tissue by exogenous antioxidants possesses additional properties such as anti-inflammatory, anti-diabetic and cytoprotective effect, should be a strategy to conserve the kidney from the oxidative damage.

Furthermore, Nrf2 mediated the protective defense mechanism to cope with inflammation, excessive ROS and oxidative stress, resulting in renal damage [47,48]. Illuminating the Nrf2 mediated protective mechanism against diabetic nephropathy will develop novel therapeutic intervention to prevent or slow the progression of diabetic nephropathy. HO-1 is the major antioxidant and cytoprotective enzyme mediated by Nrf2 activation. Nrf2/HO-1 signalling could modulate the oxidative mediators including MDA, CAT, and SOD in renal damage of diabetic mice [48–50].

In current study, we found that Nrf2 expression was manifestly increased in alloxan mice with nephritic injury, implying that the contents of TNF-α, IL-1β and IL-6 were also concerned in the progression of renal inflammation. CA treatment efficaciously increased the level of Nrf2 expression by attenuating renal dysfunction, oxidative stress and inflammatory pro-cytokine levels. These findings recommended that CA is a promising therapeutic agent for the treatment of DN with its enhanced Nrf2 expression.

Our experimental result divulges that CA effectively attenuated the expression of Nrf2 and provided protective effect. Moreover, CA treatment in mice induced by alloxan exhibited an ameliorative potential by attenuating the hyperglycemia-mediated oxidative stress, hereby alleviating the ultrastructure alteration which included tubulonecrosis, glomerulosclerosis and glomerular basement thickening in renal tubules. Further detailed studies are in progress to explicate the precise mechanism by which CA ameliorates its potential in other diabetic complication.

Conflict of interest statement

The authors declare no conflict of interest.

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