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P-Coumaric acid alleviates experimental diabetic nephropathy through modulation of Toll like receptor-4 in rats

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ABSTRACT

Aims: Diabetic nephropathy (DN) is responsible for the occurrence of 30–47% of the incident cases of end-stage renal disease (ESRD) worldwide. DN is a chronic inflammatory disorder, which results from hyperglycemia-induced alterations and leads to renal fibrosis and ESRD. Toll like receptor-4 (TLR-4) participates in regulation of inflammatory response through controlling of innate immune system. P-Coumaric Acid (P-CA) is a natural hydroxycinnamic acid derivative and is widely present in vegetables, fruits, mushrooms and cereals. This study aimed to explore the renoprotective effect of P-CA, as anti-inflammatory and antioxidant natural compound, against experimental DN.

Methods: DN was induced by single intraperitoneal injection of streptozotocin (45 mg/kg) in rats. In kidney homogenate, levels of TLR-4, interleukin-6 (IL-6) and transforming growth factor β1 (TGFβ1) were measured using ELISA technique. Also, kidney collagen content was determined colorimetrically.

Key findings: Oral administration of P-CA (100 mg/kg) for 8 weeks significantly alleviated the DN. P-CA significantly reduced serum concentrations of glucose, creatinine, blood urea nitrogen (BUN) and reduced protein content in urine. Also, P-CA significantly increased superoxide dismutase (SOD) activity and significantly reduced kidney contents of malondialdehyde (MDA), TLR-4, IL-6, TGFβ1 and collagen when compared with DN group. Moreover, P-CA significantly improved DN-induced histopathological abnormalities.

Significance: P-CA confers protection against the progression of DN. This renoprotective effect can be attributed to its ability to decrease the generation of inflammatory and fibrotic cytokines in addition to restoring oxidant/antioxidant balance through its ability to down-regulate TLR-4 activation.

1. Introduction

Diabetic nephropathy (DN) is a serious chronic microvascular complication, which causes 30–47% of the incident cases of end-stage renal disease (ESRD) worldwide, posing high healthcare costs of patients with uncontrolled DN [1,2]. DN is characterized by mesangial expansion, interstitial fibrosis, glomerular sclerosis and albuminuria [3]. Pathogenesis of DN is complex, where hyperglycemia induces metabolic and hemodynamic deteriorations, which in their turn result in chronic inflammatory environment and renal fibrosis culminating in renal failure [4,5].

Toll like receptor-4 (TLR-4) is a preserved member of pattern recognition receptors, which plays a central role in innate immune system. TLR-4 responds to several stimuli, including lipopolysaccharide (LPS), hyperglycemia, high-mobility group box1 (HMGB1), advanced glycation end products (AGEs) and oxidative stress. Stimulation of TLR-4 activates mainly nuclear factor kappa B (NF-κB), the master regulatory transcription factor, which controls generation of pro-inflammatory cytokine, such as interleukin-6 (IL-6) [6]. Moreover, TLR-4 mediates renal fibrosis through transforming growth factor β1(TGFβ1) signaling pathway and mediates induction of oxidative stress [7–11]. Wild-type mice treated with STZ showed increased macrophage immunostaining, overexpression of TLR-4, MyD88 and IL-6 and increased deposition of collagen in kidney tissues compared with TLR-4-knockout mice [12]. Thus, TLR-4 functions as a linker, which translates diabetic hemodynamic and metabolic alterations into chronic inflammatory environment leading to renal fibrosis and subsequent ESRD, making it an attractive drug target for prevention of DN.

IL-6 belongs to glycoprotein 130-dependent cytokine family. IL-6 participates in stabilization of chronic inflammation [13,14]. Also, IL-6 mediates angiotensin II-induced endothelial dysfunction leading to increased infiltration of inflammatory cells [15]. IL-6 level was increased

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Table 1
Effect of P-CA on the kidney/body weight index and kidney function parameters.

Groups	Normal control	DN	P-CA100
Kidney/body weight index ($x10^3$)	**3.51 ± 0.14**	**7.12 ± 0.62***	**4.4 ± 0.38$**
Serum Creatinine (mg/dl)	**0.52 ± 0.009**	**1.013 ± 0.016***	**0.72 ± 0.028*'$**
Blood urea nitrogen (mg/dl)	**19.16 ± 1.29**	**60.12 ± 3.16***	**27.48 ± 2.53$**
Creatinine clearance (ml/min)	**0.064 ± 0.003**	**0.017 ± 0.0014***	**0.044 ± 0.0036*'$**
Total proteinuria (mg/day)	**64.17 ± 5.43**	**167.2 ± 4.54***	**92.33 ± 3.22*'$**

Data are mean ± SEM; n = 7. Statistical comparisons were achieved through one-way ANOVA followed by Tukey-Kramer's post hoc test. * = significant at P < 0.05 versus normal control group; $ = significant at P < 0.05 versus DN group. DN, diabetic nephropathy; P-CA100, P-coumaric acid (100 mg/kg, orally).

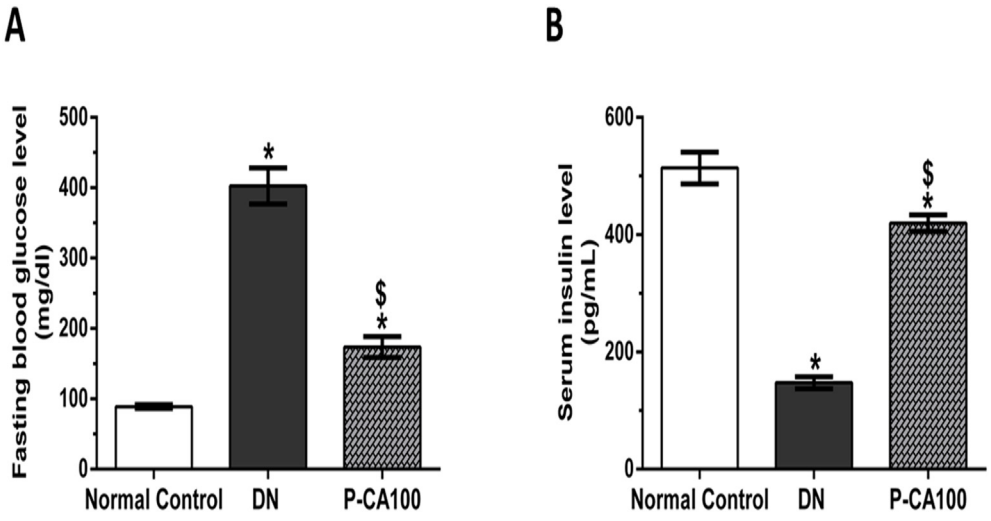

Fig. 1. Effect of p-coumaric acid (P-CA) on fasting blood glucose (A) and serum insulin (B) levels. Statistical comparisons were achieved through one-way ANOVA with Tukey-Kramer's post hoc test. * = significant at P < 0.05 versus normal control group; $ = significant at P < 0.05 versus DN group. Data are mean ± SEM; (n = 7/group). DN, diabetic nephropathy; P-CA100, P-coumaric acid (100 mg/kg, orally).

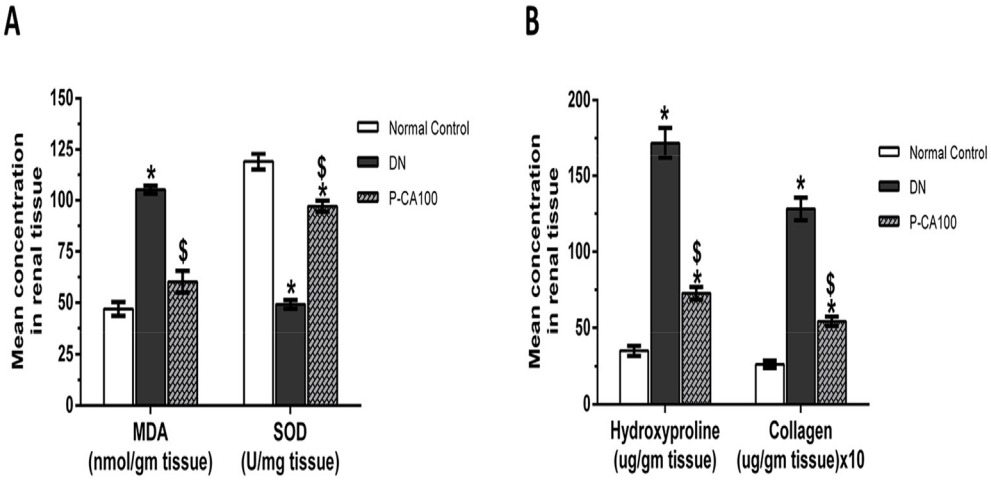

Fig. 2. Effect of p-coumaric acid (P-CA) on superoxide dismutase and malondialdehyde contents (A) and on hydroxyproline and relevant collagen contents (B) in kidney tissue. Statistical comparisons were achieved through one-way ANOVA with Tukey-Kramer's post hoc test. * = significant at P < 0.05 versus normal control group; $ = significant at P < 0.05 versus DN group. Data are mean ± SEM; (n = 7/group). DN, diabetic nephropathy; P-CA100, P-coumaric acid (100 mg/kg, orally).

in parallel to glomerular filtration rate decline and extent of proteinuria in diabetic patients [16]. Moreover, blockade of IL-6 signaling cascades has ameliorative effect on The DN progression [17].

TGFβ1 is the main profibrogenic cytokine, which promotes accumulation of extracellular matrix (ECM) through direct induction of ECM proteins production and through generation of myofibroblasts; mediated by Smad-dependent and non-Smad-dependent cascades [18,19]. Suppression of TGFβ1 is effective strategy to prevent the development and progression of DN [20,21].

P-Coumaric Acid (P-CA) is a member of hydroxycinnamic acid family. P-CA is widely present in free or conjugated forms in vegetables (tomatoes and potatoes), fruits (pears and apples), mushrooms and cereals [22]. P-CA exhibited antidiabetic and potent antioxidant effects [23,24]. Moreover, P-CA showed significant anti-inflammatory action in rat model of rheumatoid arthritis [25,26]. P-CA also exhibited several beneficial anti-cancer effects [27,28].

The renoprotective ability of P-CA to halt and delay the DN progression and the its underlying mechanisms of actions have yet to be elucidated. Moreover, the antagonistic activity of P-CA against TLR-4 has to be studied yet. Therefore, this study was undertaken to explore renoprotective effect of P-CA against the progression of DN in rats.

2. Methods and materials

2.1. Experimental animals

Thirty-six adult (14 weeks) male Sprague Dawley albino rats (200 ± 20 g) were obtained from Egyptian Organization for Biological Products and Vaccines (Giza, Egypt). Rats were kept under controlled conditions of temperature (25 °C ± 2) with a systematic constant 12 h

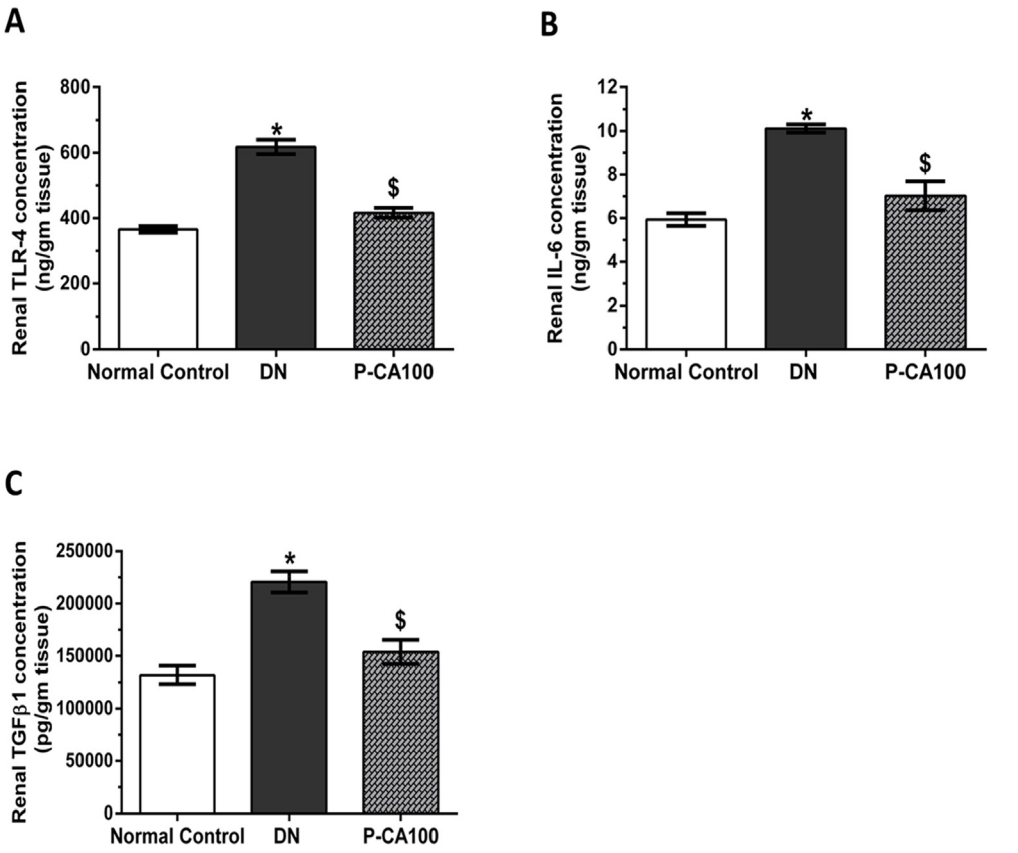

Fig. 3. Effect of p-coumaric acid (P-CA) on kidney contents of toll like receptor-4 (A), interleukin-6 (B) and Transforming growth factor β1 (C). Statistical comparisons were achieved through one-way ANOVA with Tukey-Kramer's post hoc test* = significant at $P < 0.05$ versus normal control group; $ = significant at $P < 0.05$ versus DN group. Data are mean ± SEM; (n = 7/group). DN, diabetic nephropathy; P-CA100, P-coumaric acid (100 mg/kg, orally).

on/off light cycle and were allowed free access to food and water ad libitum. This study was conducted according to the protocol approved by "Research Ethics Committee, Faculty of Pharmacy, Mansoura University" which complies with the guidelines of Laboratory Animal Care (NIH publication no. 85–23, revised 1985).

2.2. Chemicals and drugs

P-CA (≥98% purity) and streptozotocin (STZ) were bought from Sigma-Aldrich Chemicals (St. Louis, Mo, USA). All other chemicals and reagents were of great analytical grades and obtained from standard commercial suppliers.

2.3. Experimental design

Diabetes mellitus (DM) was induced in rats after a week of acclimatization. DM was generated by single intraperitoneal (i.p.) injection of STZ (45 mg/kg) dissolved in a 0.1 M chilled citrate buffer (pH 4.5) after fasting for 12 h [29]. Normal control group were injected with equal volume of the vehicle. 4 h after injection of STZ, the injected rats were supplied with 10% glucose solution for 24 h to avoid fatal hypoglycemia which was induced by the released insulin from the destructed pancreatic β cells. 48 h after induction of DM, blood glucose level of the rats was checked and measured from the tail vein utilizing OneTouch glucometer (LifeScan, USA). All rats with blood glucose level above 250 mg/dl were considered diabetic rats and were subsequently divided into DN and P-CA100 groups in random manner.

All of rats in this study were allocated into 3 different groups (n = 12/group) as follows: normal control group, non-diabetic rats were administered with 0.5% carboxymethyl cellulose (CMC) solution once daily using orogastric gavage tube; DN group, diabetic rats were administered with 0.5% CMC solution once daily using orogastric gavage tube; P-CA100 group, diabetic rats received P-CA (100 mg/kg) once daily suspended in 0.5% CMC solution using orogastric gavage tube [25]. We started administration of P-CA 48 h post STZ injection and continued for 8 weeks.

2.4. Sample collection and processing

After eight weeks of treatment with P-CA, 24-h urine samples were obtained from rats utilizing metabolic cages. Urine samples were then centrifuged at 340 g and 4 °C for 2 min and were utilized immediately for biochemical analysis. Blood samples were withdrawn from rats through retro-orbital puncture under light ether anesthesia. Then, we centrifuged these blood samples at 765 g and 4 °C for 5 min for separation of serum, which was stored at −20 °C until its biochemical analysis. After animal sacrification by decapitation, kidneys were rapidly excised, rinsed with ice-cold saline and weighed for calculation of kidney/body weight index. The right kidney was cut lengthwise and fixed in 10% neutral formalin for histopathological examination. The left kidney was homogenized in chilled 0.5% potassium chloride and centrifuged at 765 g and 4 °C for 10 min. The supernatants were preserved at −80 °C for biochemical analysis [30].

2.5. Biochemical analysis of serum

Insulin level was measured using commercial enzyme linked immune sorbent assay (ELISA) kit from Cloud-clone Company (Houston, USA) in accordance to the given instructions. Also, level of fasting blood glucose and kidney function tests as blood urea nitrogen (BUN) and serum creatinine were estimated using kits from Biodiagnostic Company (Dokki, Giza, Egypt).

2.6. Biochemical analysis of urine

24-h urine total protein level and 24-h urine creatinine level were estimated using kits from Spinreact Company (Spain) and Biodiagnostic Company (Dokki, Giza, Egypt), respectively.

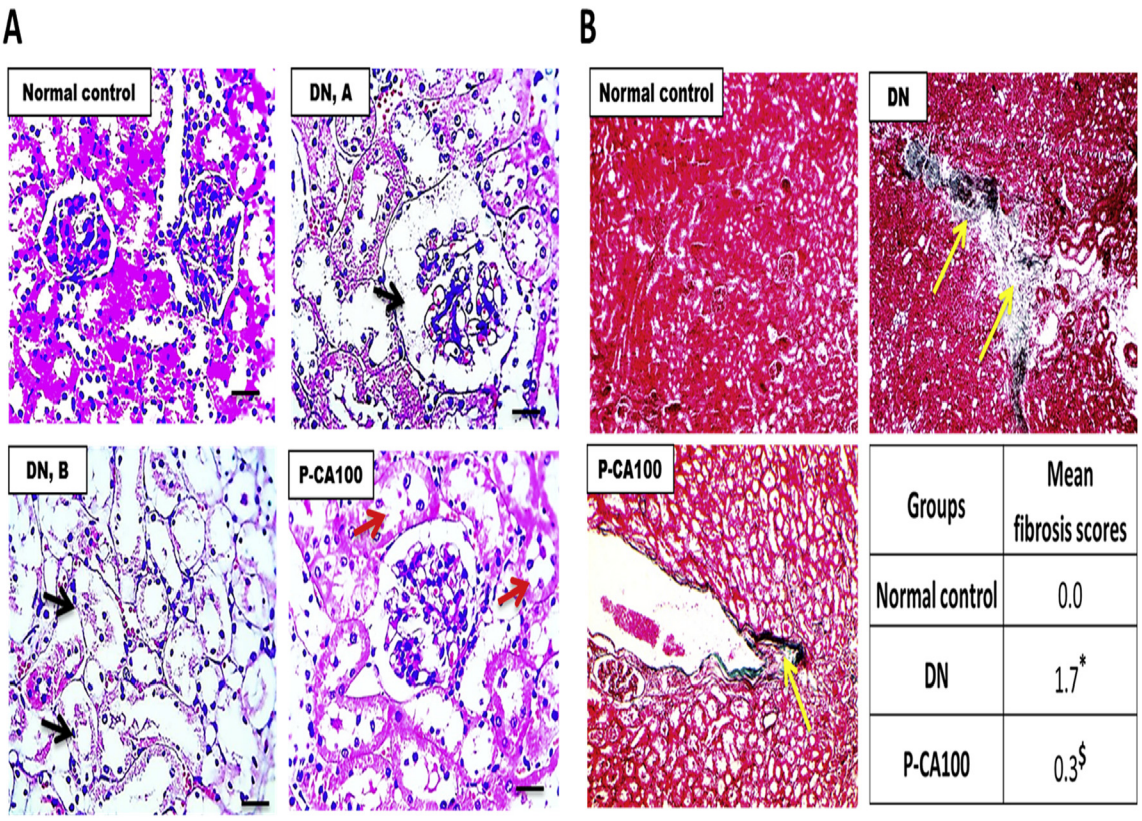

Fig. 4. Effect of p-coumaric acid (P-CA) on histopathological examination. (A) Histopathological investigation of hematoxylin and eosin (H&E) stained kidney sections (x400, bar = 50), (n = 6/group). Normal control exhibited normal kidney histology. DN group exhibited swollen Bowman's capsule with presence of eosinophilic proteinaceous material (black arrow) (A) and separation of epithelium from basement membrane of renal tubules with Marked tubular dilation (black arrows) (B). P-CA100 treated group exhibited moderate tubular dilation and presence of few desquamated cells in tubular lumen (red arrows). DN, diabetic nephropathy; P-CA100, P-coumaric acid (100 mg/kg, orally). **(B)** Histopathological investigation of Masson's trichome stained kidney sections and semi-quantitative fibrosis scoring (x100, bar = 100), (n = 6/group). Normal control group showed no fibrosis. DN group showed moderate perivascular fibrosis. P-CA100 treated group showed mild perivascular fibrosis. Statistical comparisons were achieved through Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. * = significant at $P < 0.05$ versus normal control group; $ = significant at $P < 0.05$ versus DN group. DN, diabetic nephropathy; P-CA100, P-coumaric acid (100 mg/kg, orally). . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Creatinine clearance was calculated utilizing the equation "creatinine clearance (mL/min) = [urinary creatinine level (mg/dl) × urine flow (mL/min)]/serum creatinine level (mg/dl)". Urine flow per minute was deduced by dividing 24-h urine volume by 1440 (number of minutes in a day).

2.7. Evaluation of oxidative stress parameters

Kidney SOD activity and MDA content were estimated utilizing kits from Biodiagnostic Company (Dokki, Giza, Egypt).

2.8. Assessment of kidney hydroxyproline and collagen contents

100 mg kidney tissue was utilized for measurement of renal hydroxyproline content [31]. The procedure depends on base hydrolysis instead of acid hydrolysis for tissue dissolution. Absorbance of the reddish purple complex was read at 550 nm. Renal collagen content was obtained by multiplying kidney hydroxyproline content by 7.46, where hydroxyproline accounts for 13.5% of collagen structure [32,33].

2.9. Evaluation of renal contents of toll like receptor-4, interleukin-6 and transforming growth factor β1

Kidney homogenate was utilized for measurement of TLR-4 level via commercially available ELISA kit from Cloud-clone Company (Houston, USA). Also, IL-6 and TGFβ1 levels were measured utilizing ELISA kits from Affymetrix ebioscience (San Diego, USA).

2.10. Histopathological examination

Fixed longitudinal halves of right kidneys were dehydrated using ethanol, cleared in xylene, and finally fixed in paraffin wax. Two sets of 5-μm thick sections were sliced; the first set of slides was stained with hematoxylin and eosin (H&E) to determine histopathological abnormalities in the kidneys resulted from DN progression and the effect of treatment with P-CA on these abnormalities. The second set of slides was stained with Masson's trichrome stain to estimate the severity of fibrosis semi-quantitatively, which was scored from 0 to 3 in accordance to method suggested by Ref. [34],"0, normal; 1, mild fibrosis; 2, moderate fibrosis; and 3, severe fibrosis".

2.11. Statistical analysis

Results were showed as mean ± standard error of the mean (SEM). For statistical comparisons, one way analysis of variance (ANOVA) and Tukey's post hoc test were applied to parametric data. Analysis of histopathology scores (non-parametric data) was carried out by Kruskal-Wallis test and Dunn's post-hoc test. Statistical computations and graphing were done utilizing GraphPad Prism V 6.01 (GraphPad Software Inc., San Diego, CA, USA). In all tests, statistical significance was deemed at P values of less than 0.05.

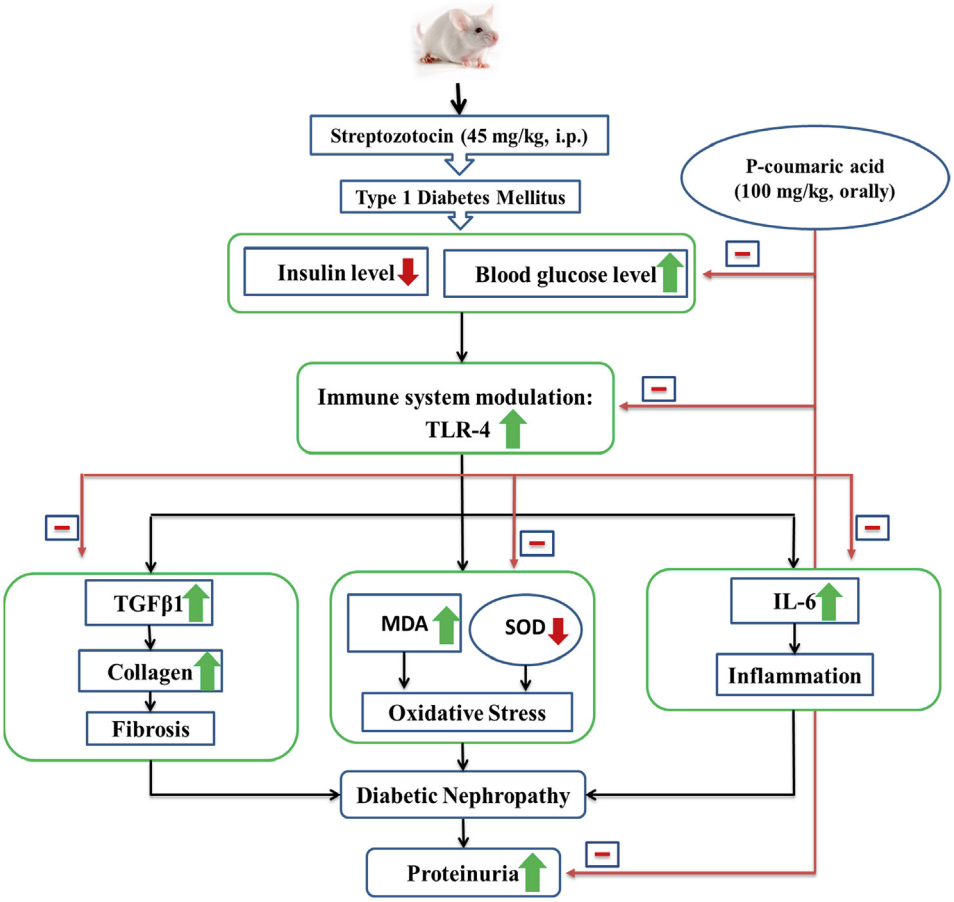

Fig. 5. The presumed renoprotective effects of p-coumaric acid against diabetic nephropathy progression through blockade of toll like receptor-4 signaling cascade. TLR-4, toll like receptor-4; IL-6, interleukin-6; TGFβ1, transforming growth factor β1; SOD, superoxide dismutase; MDA, malondialdehyde;, decrease.

3. Results

3.1. Effect of p-coumaric acid on the kidney/body weight index

DN group exhibited significant elevation in the kidney/body weight index by about 2 folds when compared with normal rats. Treatment with P-CA(100 mg/kg/24hrs, orally) for 8 weeks led to significant reduction in the kidney/body weight index by about 38% in comparison to DN group, (Table 1).

3.2. Effect of p-coumaric acid on kidney function parameters

DN group exhibited significant increase in BUN and serum creatinine levels by about 3.1 folds and 1.9 folds, respectively, when compared with normal rats. P-CA (100 mg/kg) significantly reduced BUN and serum creatinine levels by about 55% and 25%, respectively, when compared with DN group. But, P-CA treated group showed significant increase in serum creatinine level in comparison to normal rats, (Table 1).

Total proteinuria was significantly increased and creatinine clearance was significantly decreased in DN group by about 2.6 folds and 74%, respectively, in comparison to normal rats. P-CA(100 mg/kg) significantly lowered total proteinuria and increased creatinine clearance by about 55% and 2.6 folds, respectively, when compared with DN group. They still exhibited significant difference from normal control, (Table 1).

3.3. Effect of p-coumaric acid on serum glucose and insulin levels

Fasting blood glucose level was significantly increased in DN group with significant decrease in insulin level by about 4.5 folds and 71%, respectively, in comparison to normal rats. P-CA (100 mg/kg) led to significant decrease in fasting blood glucose level and significant increase in insulin secretion by about 57% and 2.9 folds, respectively, when compared with DN group. They still exhibited significant difference from that of normal rats, (Fig. 1, A&B,respectively).

3.4. Effect of p-coumaric acid on oxidative stress

DN group exhibited significant elevation in MDA content and significant decline in SOD activity in renal homogenate by about 2.25 folds and 59%, respectively, in comparison to normal rats. P-CA (100 mg/kg) significantly lowered content of MDA and increased SOD activity by approximately 43% and 1.9 folds, respectively, when compared with DN group, but SOD activity in P-CA treated rats was still significantly different from that of normal rats, (Fig. 2,A).

3.5. Effect of p-coumaric acid on kidney hydroxyproline and relative collagen contents

DN group exhibited significant increase in kidney contents of hydroxyproline and collagen by about 4.8 folds when compared with normal rats. P-CA (100 mg/kg) led to significant decrease in kidney hydroxyproline and relevant collagen contents by approximately 57% in comparison to DN group. They still exhibited significant difference from that of normal rats, (Fig. 2,B).

3.6. Effect of P-CA on renal contents of toll like receptor-4, interleukin-6 and transforming growth factor β1

DN group exhibited significant increase in levels of TLR-4, IL-6 and TGFβ1 by about 1.7 folds, 1.7 folds and 1.9 folds, respectively, when

compared with normal rat. P-CA (100 mg/kg) significantly reduced levels of TLR-4, IL-6 and TGFβ1 by about 40%, 31% and 35%, respectively, when compared with DN group (Fig. 3A and B, C, respectively).

3.7. Effect of P-CA on histopathological examination

H&E stained kidney sections of normal control exhibited normal kidney architecture. Kidneys of DN group exhibited swollen Bowman's capsule with presence of eosinophilic proteinaceous material (black arrow) and Marked tubular dilation with separation of epithelium from basement membrane of renal tubules (black arrows). Rats treated with P-CA exhibited improved renal histology evidenced by moderate tubular dilation with presence of few desquamated cells in lumen of tubules (red arrows), (x400, bar = 50), (Fig,4,A).

As shown in (Fig. 4,B), Masson's trichrome stained kidney sections of normal control exhibited absence of fibrosis. DN group exhibited moderate degree of perivascular fibrosis (yellow arrow). Rats treated with P-CA exhibited mild degree of perivascular fibrosis (yellow arrow), (x100,bar = 100).

4. Discussion

Chronic inflammation is practically a key contributor in pathogenesis of DN because it functions as a link between hyperglycemia-induced metabolic and hemodynamic alterations and renal fibrosis leading to renal failure [35,36]. In the current study, we investigated the renoprotecive effect of P-CA in rat model of STZ-induced DN. DM was induced by single injection of STZ (45 mg/kg, i.p.), which induced death of pancreatic β cells mediated by STZ-induced intracellular oxidative stress and DNA fragmentation [37]. Results demonstrated that P-CA functionally, histopathologically and biochemically protected against the progression of DN.

Hyperglycemia is responsible for the initiation of diabetic pathogenic conditions including metabolic alterations, such as AGEs formation, and hemodynamic alterations, such as renin-angiotensin system activation. P-CA exerted significant hypoglycemic effect mediated by increased secretion of insulin. These observations are in agreement with ameliorative effect of P-CA on rat model of DM [23]. In addition, previous studies demonstrated that the hypoglycemic effect of P-CA is also attributed to P-CA-induced mitigating actions, including modulation of glucose metabolism enzymes, enhancement of glucose homeostasis, reduction of absorption of dietary carbohydrate from intestine and its anti-inflammatory and anti-oxidant effects [22]. But, the hypoglycemic effect is insufficient for protection against DN. Previous studies reported that diabetic patients with normoglycemia ultimately developed DN due to metabolic memory [38]. The metabolic memory is attributed to chronic inflammation, oxidative stress and AGEs formation [39]. Therefore, there is a necessary need to develop new remedies, which have capability to prevent mediators of metabolic memory and participate in achievement of glycemic control.

P-CA significantly attenuated functional markers of the progression of DN. Indeed, P-CA significantly decreased BUN, serum creatinine and total proteinuria and increased creatinine clearance compared to DN group. Also, findings of histopathological examination support our biochemical results. Indeed, P-CA mitigated degeneration of glomerular and tubular architecture and it exhibited decreased accumulation of collagen in kidney sections stained with Masson's trichome stain when compared with DN group. Moreover, P-CA exhibited anti-hypertrophic effect evidenced by significant reduction in kidney/body weight index. The previous observations indicate that P-CA is effective compound for halting the progression of DN. These observations are in agreement with renoprotective effect of P-CA on cisplatin-induced kidney injury and are in agreement with ameliorative effect of P-CA on hypoxic cerebral edema [40,41].

TLR-4 is a conserved member of pattern recognition receptors, which are essential players in regulation of innate immune system. TLR-4 is widely expressed by antigen-presenting cells, mesangial cells, endothelial cells, podocytes and myofibroblasts. TLR-4 facilitates foundation of chronic inflammatory milieu in response to hyperglycemia, AGEs, LPS and oxidative stress. Activation of TLR-4 signaling cascade is initiated by formation of TLR-4/co-receptor myeloid differentiation factor 2 (MD-2) complexes followed by dimerization of these complexes in response to the stimuli with the aid of cell-surface cluster of differentiation 14 (CD14) leading to conformational changes and recruitment of adaptor proteins including myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adaptor inducing interferon (TRIF). Activation of TLR-4 cascade initiates NF-κB signaling cascade via both MyD88-dependent pathway and TRIF-dependent pathway leading to establishment of oxidative stress and production of cytokines, including IL-6 and TGFβ1, and chemokines, such as CC Ligand 2 (CCL2). TLR-4 will first stimulate resident cells to produce of CCL2, which in its turn recruits and promotes infiltration and accumulation of activated inflammatory cells, mainly macrophages, leading to chronic sterile inflammation and subsequent fibrosis in diabetic kidneys [6–11]. In our experiment, P-CA significantly reduced kidney TLR-4 level when compared with DN group. This observation suggests that P-CA is a potent ameliorative agent against the progression of DN through suppression of TLR-4.

Generation of ROS and activation of TLR-4 depend on each other. Oxidative stress is a key feature of DN and initiates several harmful signaling pathways including TLR-4 signaling pathways. Oxidative stress overexpresses TLR-4 by induction of translocation of TLR-4 to cell surface via ceramide generation [42]. Oxidant/antioxidant balance is indicated by two common parameters, which are SOD (antioxidant enzyme) level and lipid peroxidation in term of MDA content. In our experiment, P-CA significantly reduced MDA content and significantly increased SOD level in renal homogenate when compared with DN group. These observations are in agreement with antioxidant effect of P-CA on oxidative stress-induced apoptosis of human lens epithelial cells [24].

IL-6 belongs to glycoprotein 130-dependent family of cytokines. IL-6 plays critical roles in the DN progression [43]. Hyperglycemia induces IL-6 production from kidney resident cells, including podocytes, endothelial cells and mesangial cells. IL-6 is essential for stabilization of the established chronic inflammation through its downstream STAT3/IL-17 signaling cascade [13]. IL-6 promotes infiltration of inflammatory cell mediated by angiotensin II-induced endothelial dysfunction [15]. Also, IL-6 induces differentiation of CD4 T cells and monocytes into inflammatory T helper17 (Th17) cells and macrophages, respectively, both of which play critical roles in the pathogenesis and progression of DN. Moreover, IL-6 improves proliferation of Th17 cells and prevents their apoptosis [13,44,45]. Macrophages are a main source of proinflammatory and profibrogenic mediators, such as tumor necrosis factor α and TGFβ1 [46]. In addition, Th17 cells promote the DN progression through production of several proinflamatory and profibrogenic cytokines, such as interleukin-17 [47,48]. In our experiment, P-CA significantly decreased IL-6 levels in renal homogenate when compared with DN group. This result is in agreement with anti-inflammatory effect of P-CA in rat model of rheumatoid arthritis [25].

Renal fibrosis is defined as deposition of abnormal amount of ECM proteins in kidney tissues leading to dysregulation of cellular transduction and degeneration of kidney construction [49]. TGFβ1 is the master profibrogenic cytokine that belongs to TGFβ superfamily [49]. TGFβ1 induces renal damage via activation of Smad-dependent and non-Smad-dependent cascades. TGFβ1 increases renal deposition of ECM proteins, such as collagen and fibronection, through direct activation of ECM genes expression and indirectly by stimulation of local fibroblasts and initiation of trans-differentiation of resident cells into myofibroblasts, both of which are the key source of ECM proteins [18,50]. TGFβ1 participates in generation of myofibroblasts through promotion of both epithelial-mesenchymal and endothelial-

mesenchymal transitions [18,19,51,52]. Also, TGFβ1 stabilizes the structure of collagen via promotion of both collagen cross-linking by over expression of pro-collagen lysyl hydroxylase 2, which is essential for hydroxylation of lysyl residues of collagen, and formation of cross-links between elastin and collagen fibers mediated by increasing transcription of lysyl oxidase [18,53,54]. Moreover, TGFβ1 suppresses degradation of the ECM by stimulation of transcription of tissue inhibitor of metalloproteinases-1 and plasminogen activator inhibitor-1, which prevent the action of matrix metalloproteinases leading to interstitial fibrosis and glomerulosclerosis [18,55]. In our experiment, P-CA significantly reduced TGFβ1 level in renal homogenate when compared with DN group. This result is in agreement with the beneficial impact of P-CA on the renal hydroxyproline and relevant collagen contents. In our experiment, P-CA significantly decreased the kidney hydroxyproline and relevant collagen contents when compared with DN group.

In conclusion, our data demonstrated that P-CA halted the progression of DN by not only its hypoglycemic effect, but also P-CA attenuated oxidative stress, via regulation of SOD and MDA levels; inflammatory response, mediated by TLR-4 and IL-6; and renal collagen deposition and fibrosis, mediated by TGFβ1, (Fig.5). The current study participated in a better understanding of the underlying protective mechanisms of P-CA included in prevention of DN. Renoprotective effect of P-CA in the early stages of DN should be investigated clinically.

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Declaration of competing interest

None.

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