Diabetic Renal Injury Induced in Experimental Rats

Role of Curcuminoids as Probable Therapeutic Agent

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***Abstract*- Background: Renal injury may develop in uncontrolled diabetic manifestations, mostly attributed to increased oxidative stress and release of pro-inflammatory mediators and finally leading to diabetic complications. Methods: Curcumenoids which have anti-oxidant and anti-inflammatory properties were tested in alloxan-induced hyperglycemia in rats on oxidative stress, gene expression of tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1), in relation to microalbuminuria and renal function. Results: We found that the onset of microalbuminuria preceded the increase in serum glucose after alloxan administration. Gene expression of TNF-α and TGF-β1 showed a gradual increase after one and two weeks of alloxan administration as compared to the normal group. Curcumenoids administration decreased gene expression of TNF-α and TGF-β1 in kidneys, serum-glucose, fructosamine, urea, creatinine, C-reactive protein, malondialdehyde, urinary microalbumin and total protein. Histological examination of kidney tissues showed significant improvement in Curcumenoids-treated rats as compared to untreated diabetic rats. Conclusions: Curcumenoids modulated renal injury of alloxan-induced diabetic rats as revealed by observed biochemical data. This may refer to it as therapeutic candidate for treatment of diabetic renal injury and clinical trials are mostly requested.**

Keywords*- Nephropathy; Curcumenoids; Malondialdehyde; Tumor Necrosis Factor-alpha; Transforming Growth Factor-beta1; Microalbuminuria

I. INTRODUCTION

Long standing diabetes usually leads to structural and functional abnormalities in the vasculature which characterize diabetic related micro and macro-vascular complications [1]. Diabetic vascular complications are currently the principal causes of morbidity and mortality in diabetic patients (type I and II), leading to a mortality rate 3-4 times more than that of healthy populations [2]. Renal microangiopathy usually contributes to nephropathy which is the leading cause of end-stage renal failure (ESRF) [3]. Despite that DN has been traditionally considered as a non-immune disease, however, accumulating evidences now indicate that immunologic and inflammatory mechanisms may play a significant role in its development and progression [4]. TNF-α is a multifunctional regulating cytokine involved in the inflammatory response in diabetes [5, 6]. It inhibits insulin signaling pathways [7], impairs peripheral glucose uptake and alters the expression of major genes that control glucose and lipid metabolism [8, 9]. Transforming growth factor-β1 (TGF-β1) represents an important mediator in the pathogenesis of diabetic nephropathy [10, 11] and may inhibit matrix degradation, upregulate adhesion molecules and enhance chemoattraction [12]. Flavonoids are phenolic compounds widely distributed in a wide variety of edible plants including leafy vegetables, fruits and beverages (tea, red wine). They have been reported to exert multiple biological effects, including antiviral, anti thrombotic, anti-ischemic, anti-inflammatory, anti histaminic, anti oxidant and free radical scavenging abilities [13]. The phenolic compounds widely distributed in plants are the major compounds associated to human health and beneficial effects in inflammatory diseases additionally cancer.

Curcumenoids as a natural product are characterized by a variety of pharmacological actions through inhibition of inducible nitric oxide synthase (iNOS) additionally its potential as radical scavenger [14]. The latter effect is mediated through multiple mechanisms involving inhibition of the activation of various transcription factors such as nuclear factor kappa B (NFκB), activated protein-1 (AP-1) and peroxisome proliferator activated receptor-ϒ (PPAR-ϒ) [15]. Additional mechanism includes down regulation of the production of proinflammatory cytokines like interleukine-1β (IL-1β) [16].

Present study aimed mainly to investigate the effect of curcuminoids administration on the expression of certain cytokines, onset of microalbuminuria, oxidative stress and specific biomarkers in alloxan diabetic rats. This may be of value for treatment of diabetic renal injury.

II. MATERIALS AND METHODS

A. Animals

48 adult male albino rats weighing 170 ± 20 g were housed under environmentally-controlled conditions and allowed one week for acclimatization at room temperature with a 12 hours dark/light cycle before beginning the experimental work. Rats were fed rodent chow and allowed free access of drinking water. The animals were maintained and used in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (The University of Zagazig, Zagazig, Egypt).

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B. Experimental Protocol

Diabetes was induced by administration of a single dose of alloxan intraperitoneally (90 mg/kg body weight), serum glucose and urinary microalbumin were checked at 2, 4, 6 days and at 3 and 6 weeks after alloxan-treatment. Rats which achieved serum glucose level more than 200 mg/dl were expressed as diabetic and enrolled in the study (n=8). Three experimental groups (n=8) were used: normal rats (NC), alloxan-induced diabetic rats which received no treatment and served as diabetic control (DC) and the last group received daily oral dose of Curcumenoids (50 mg/kg), isolated and purified from Curcuma Longa family Zingebracae according to piper et al, [17], for 6 weeks.

C. Blood and Urine Sampling

At the end of the experiment (6 weeks), rats were fasted overnight, blood samples were collected and centrifuged directly for serum separation. Samples were processed instantly for determination of glucose, creatinine, urea, CRP and fructosamine. Urine samples were collected and processed for determination of urinary microalbumin and total protein.

D. Tissue Collection

Following blood collection, rats were killed by decapitation, kidneys were removed instantly. One kidney was quickly frozen in liquid nitrogen (-170 ℃) and stored at -20 ℃ for determination of malondialdehyde (MDA) and gene expression of TNF-α and TGF-β1.

E. Immunohistological Analysis in Kidney Tissue of Paraffin Blocks

The image of each reaction was captured using a 40X objective (Bar = 50 μm) with a numerical aperture of a high resolution of 16-bit digital camera (1280X1024 pixel). Images were viewed and recorded using Olympus microscope, equipped with a Spot digital camera, using computer program MAtLAB software (Image J, The Mathworks Inc, USA).

F. Analytical Methods

Serum glucose was determined using commercial kits provided by Spinreact Kits, Barcelona, Spain. Fructosamine was determined using QCA Kits, Barcelona, Spain. Creatinine and urea were determined using Diamond Kits, Cairo, Egypt. Urinary microalbumin content was determined using Organtec ELISA Kits, Munich, Germany. CRP was determined using BD Biosciences ELISA kits, NJ, USA. Protein in urine was determined using Linear kits, Barcelona, Spain.

G. Kidney MDA Level

Malondialdehyde was determined as marker of lipid peroxidation [18] using Bio-Diagnostic Kits, Cairo, Egypt.

H. RNA Isolation and RT-PCR Assay for TGF-β1 and TNF-α Genes

For the detection of TGF-β1 and TNF-α by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), RNA was extracted using SV Total RNA isolation system (Promega, Madison, WI, USA), reverse transcribed into cDNA, and amplified by PCR using RT-PCR kit (Stratagene, NJ, USA). The oligonucleotide sequences of forward and reverse primers are as follows:

TGF-β1 (Forward primer: **5'-TCACTTGTTTTGGTGGATGC-3'**; Reverse primer: **5'-TTCTGTCTCTCAAGTCCCCC-3'**)

TNF-α (Forward primer: **5'-GGCAGGTCTACTTTGGAGTCATTGC-3'**; Reverse primer: **5'-ACATTCGGGGATCCAGTGAGCTCCG-3'**)

β-actin (Forward primer: **5'-ACTGCCGCATCCTCTTCCTC-3'**; Reverse primer: **5'-ACTCCTGCTTGCTGATCCACAT-3'**)

The semi-quantitative determination of PCR products was performed using the gel documentation system (BioDO, Analyser) supplied by Biometra. According to the following amplification procedure, relative expression of each studied gene (R) was calculated according to the formula:

R= Densitometrical Units of each studied gene/ Densitometrical Units of β-actin.

III. RESULTS

A. Metabolic Parameters

Diabetic control group (DC) demonstrated significant increase in serum glucose, fructosamine, creatinine, urea and CRP in comparison to normal group (NC). Treatment with curcumenoids for 6 weeks induced a significant decrease of these markers as compared to the DC group ($P<0.05$) Table 1.

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TABLE 1 COMPARISON OF RESULTS FOR ALLOXAN-DIABETIC RATS VS. NORMAL RATS AND SUMMARY EFFECT OF CURCUMENOIDS TREATMENT IN DIABETIC RATS

Parameters	NC	DC	Curc.
Serum glucose (mg/dl)	94 (11)	480 (13)*	136 (2)#
Fructosamine (mmol/l)	48 (7)	252 (10) *	94 (4) #
Creatinine (mg/dl)	0.23 (0.04)	2.5 (0.30) *	0.37 (0.03)#
Urea (mg/dl)	29 (3)	80 (8) *	43 (1) #
CRP (ng/ml)	3.4 (0.2)	12.1 (0.6) *	4.0 (0.1) #
MDA (nmol/g)	9 (3)	43 (3) *	16 (3) #
Microalbumin (μg/ml)	2.7 (0.4)	18.0 (1.3) *	5.0 (0.1) #
Total protein (mg/dl)	40 (9)	288 (12) *	71 (2)#
TNF-α/β-actin (units[1])	0.2 (0.1)	1.2 (0.3) *	0.6 (0.1) #
TGF-β-1/ β-actin (units[1])	0.3 (0.1)	1.8 (0.4) *	0.7 (0.2)#
CD68 activity (units[2])	3.1 (0.1)	5.3 (1.1) *	3.3 (0.1) #
Actin activity (units[2])	2 (0.3)	6.5 (0.9) *	2.5 (0.1) #
Bowman's space (μm x104)	5.2 (0.9)	1.7 (0.3) *	4.3 (0.2) #

NC = normal rat group, DC =alloxan diabetic rat group, Curc. = curcumenoids treated diabetic rat group, n=8 in each case. 1Gene expression in densitometric units (R), 2Relative intensity of activity x107. Significant differences are shown: *p<0.05 vs. NC group, #p<0.05 vs DC group.

B. Kidney MDA, Urinary Microalbumin and Total Protein Contents in Urine

Urinary microalbumin (Fig. 1a) and glucose (Fig. 1b) showed progressive increase over 2 days to 6 weeks following alloxan administration.

Fig. 1

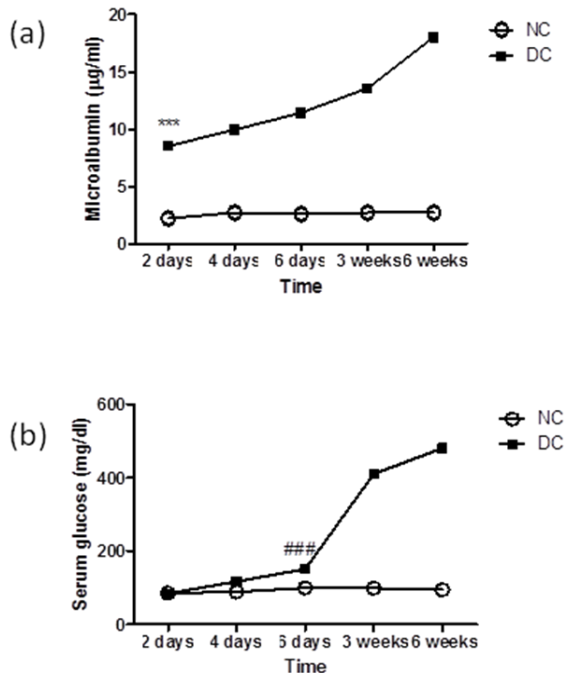

Fig. 1 Onset of urinary microalbumin (a) and its subsequent increase following alloxan administration compared with serum glucose (b) and its subsequent increase. ***significantly different (p<05, n=8) vs. NC after 2 days and ###significantly different (p<05, n=8) vs. NC after 6 days.

Urinary microalbumin and total protein content showed higher levels as compared to the NC group additionally, MDA content of diabetic kidney tissues. Treatment with curcumenoids for 6 weeks induced a significant decrease of these parameters as compared to DC group (P<0.05) Table 1.

C. Kidney TNF-α and TGF-β1 Expression

Diabetic group demonstrated a significant increase in gene expression of TNF-α and TGF-β1 after 1, 2 and 6 weeks of alloxan administration (Figs. 2a and 2b) in comparison to the NC group. Treatment with curcumenoids for 6 weeks induced a significant decrease in TNF-α and TGF-β1 gene expression in comparison to DC group (Table 1).

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Fig. 2

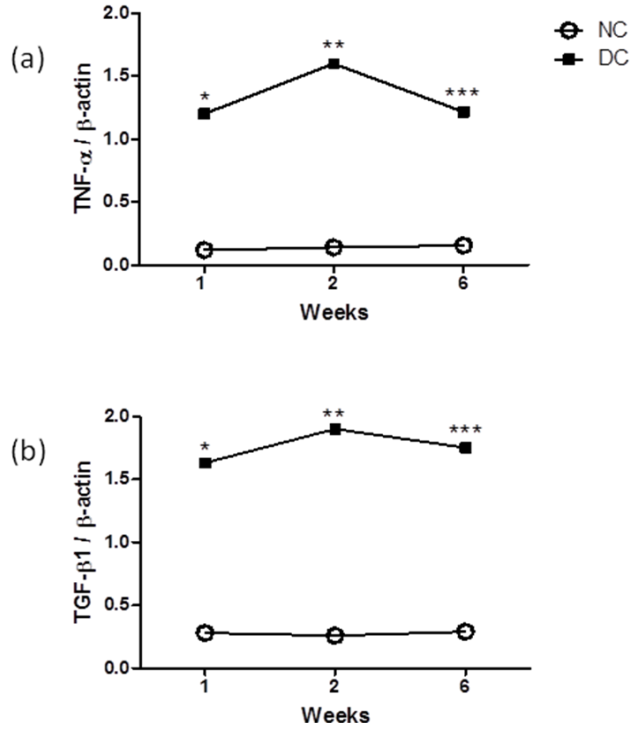

Fig. 2 Onset of TNF-α (a) and TGF-β1 (b) gene expression following alloxan administration. Significant differences are shown: * P<0.05 vs. NC at 1 week, **p<0.05 vs. NC at 2 weeks and ***p<0.05 vs. NC at 6 weeks. NC and DC rats, n=8 in each case.

In the diabetic group (DC) image analysis for CD68 activity (Fig. 3b) and actin staining (Fig. 4b) showed darker color density than non-diabetic rats (Figs. 3a and 4a) and Bowman's space (Fig. 5) was decreased in the diabetic group (Fig. 5b) as compared to non-diabetics (Fig. 5a). Curcumenoids group on the other hand, showed marked changes as compared to the DC control, where decreased color density of CD68 (Fig. 3d) and actin (Fig. 4d) additionally, increased Bowman's space was observed (Fig. 5d). Summary of results are given in Table 1.

Fig. 3

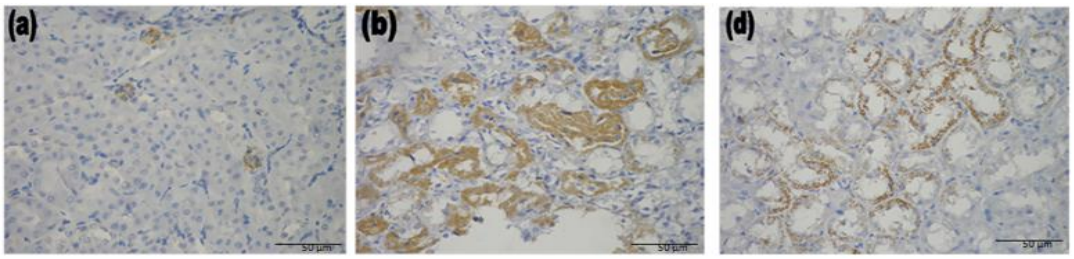

Fig. 3 Microphotograph of normal rat kidney (a) for CD 68 activity, diabetic rat kidney (b) showing intense CD 68 reaction in association with tubular injury and Curcumenoids-treated rat kidney (d) showing strong CD68 reaction in the injury tubular epithelial cytoplasm cells and absent in most regenerative one. Paraffin sections were used.

Fig. 4

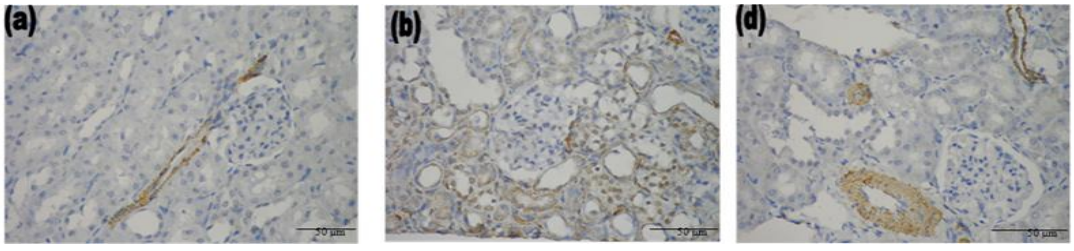

Fig. 4 Microphotograph of normal rat kidney (a) showing weak immunostaining of actin, diabetic rat kidney (b) showing actin immunostaining concentrated in the basement membrane of injured tubules and weak content in a few mesengeal cells of glomeruli and Curcumenoids-treated rat kidney (d) showing actin immunostaining concentrated in the proliferating endothelial cell of an artery and negative staining in Bowman's capsule and weak stain in regenerative tubules. Paraffin sections were used.

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Fig. 5

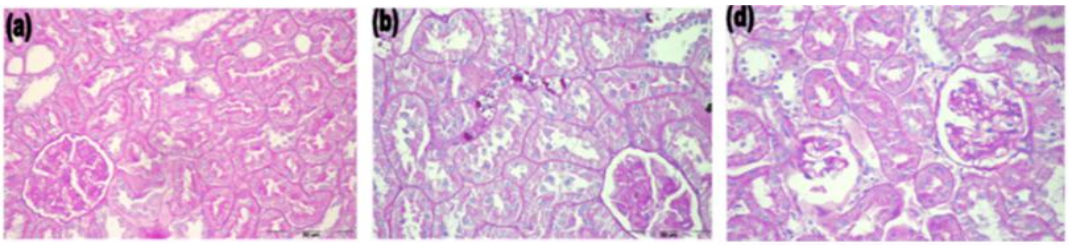

Fig. 5 Normal Bowman's space from normal kidney rats (a), Bowman's space from kidney of diabetic control rats (b) and Bowman's space from kidney of Curcumenoids-treated rats (d)

IV. DISCUSSION

As reported before hyperglycemia can activate polyol pathway, NADPH oxidase leading to increased oxidative stress, endothelial dysfunction, proliferation of vascular smooth muscle cells and induction of cellular damage [19-21].

Alloxan administration induced significant increase in serum glucose, fructosamine, CRP, urinary contents of microalbumin, total protein, gene expression of TNF-α and TGF-β1. This is mostly attributed to an enhancement of oxidative stress additionally activation of nuclear factor-κB (NF-κB). The latter can modulate gene transcription of proinflammatory cytokines such as interleukine-1α (IL-1α), IL-6 and TNF-α [22, 23]. These inflammatory cytokines are mostly responsible for initiation of renal injury development [24-26]. The observed increase in TGF-β1 gene expression is mostly attributed to hyperglycemia [27] which can induce PKC activation leading to expression of TGF-β1 [10, 11, 28, 29].This may lead to basement membrane thickening and increased vascular permeability [30] which make it a powerful stimulator for the synthesis and deposition of collagen and other ECM proteins [22].

A few years ago researches have shown that TGF-β1 enhanced the acetylation of p65 subunit of NF-κB by p300 in Hela cells [31, 32]. Taken all together, these findings suggest that p300 and NF-κB may be important in mediating or development and progression of diabetic induced renal injury, possibly through upregulating of vasoactive factors and extracellular matrix (ECM) proteins [32].

Therefore, increased expression of TGF-β1 in diabetes may further exacerbate this pathologic process through p300 and NF-κB [32].

Curcumenoids treatment of diabetic rats resulted in significant decrease in serum glucose, fructosamine, urea, CRP, urinary microalbumin and the expression of TNF-α and TGF-β1. These effects are mostly attributed to an inhibition of inducible nitric oxide synthase (iNOS) [14], NF-κB, activated protein-1 (AP-1) [15] and down-regulation of the production of pro-inflammatory cytokines [16].

Here we can also add that curcumenoids may act on gene expression through its interaction with p300 and NF-κB to attenuate or upregulate ECM proteins [32].

Image analysis for CD68, actin staining and estimated Bowman's space showed marked and significant improvement in Curcumenoids-treated groups as compared to diabetic controls. This may reflect the potential of curcumenoids to improve kidney tissues at cellular level and may add great support to the observed biochemical results. Present findings may exemplify the beneficial effects of curcumenoids on diabetes induced renal injury through inhibition of TNF-α as a novel therapeutic target regarding treatment of renal diabetic complications.

V. CONCLUSION

Curcumenoids is a natural extract from Curcuma Longa family Zingebracae which acts as antioxidant anti-inflammatory agent, so the administration of curcumenoids individually to alloxan-induced diabetic rats induced significant decrease of serum glucose, fructosamine, release of pro-inflammatory cytokines (TNF-α and TGF-β1), CRP and oxidative stress. Modulation of serum urea, creatinine, urinary microalbumin and total protein content in addition to an improvement in histopathological patterns of kidney tissues were observed. This may refer to its therapeutic value in modulation of diabetic renal injury induced in experimental rats.

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