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ORIGINAL ARTICLE

Possible Synergistic Effect of Curcumin on Captopril /Losartan Combined Therapy in Diabetic Nephropathy

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ABSTRACT

Background: Diabetic nephropathy (DN) is one of the most serious microvascular consequences of diabetes, and it is the leading cause of end-stage renal disease. Angiotensin converting enzyme inhibitor (ACEI) / angiotensin receptor blocker (ARBs) combined therapy is more effective than monotherapy in treating DN. Curcumin acts as an anti-inflammatory agent and also affects apoptotic and fibrotic pathways. We hypothesized that adding curcumin to the standard combined ACEI/ARBs treatment of DN could have a more beneficial effect on renal functions.

Methods: Type 1 diabetes was prompted by intraperitoneal injection of streptozotocin (STZ) in thirty adult male Albino rats. They were divided into five groups; control group, diabetic, curcumin treated, captopril/losartan treated and triple therapy treated group. Then, biochemical and histological investigations were conducted.

Results: Captopril/Losartan treated group and curcumin treated group showed partial restoration of shape of some tubules, glomeruli and decrease in apoptosis, congestion of blood vessels, fibrosis and inflammation induced by diabetes. And also, it showed partial improvement in renal function. The triple therapy group showed better restoration of renal architecture and more improvement in renal function.

Conclusions: Interestingly, triple therapy group showed better reno-protective effect and improved both urinary and serum biochemical profiles more than curcumin or Captopril/Losartan treated groups alone.

Keywords: Diabetic nephropathy; Curcumin; ACEI; ARB.

INTRODUTION

Diabetes mellitus type 1 is a metabolic disease characterized by hyperglycemia caused by insulin insufficiency. Diabetic nephropathy is the most prevalent cause of end-stage kidney damage and is a serious microvascular complication of diabetes. Diabetes impacts the kidney in the form of increased apoptotic cells, blood vessel congestion, congested glomeruli, vacuolated cell lining of tubules, and induction of cell fibrosis and inflammation. Diabetes also caused a decline in renal function [1].

In the therapy of DN, ACEI and ARB are extensively utilized. The numerous mechanisms that inhibit angiotensin II and aldosterone are at the core of the pathophysiology for dual Renin-angiotensin-aldosterone system (RAAS) inhibition. Blocking the RAAS reduces renal perfusion and slows mesangial cell proliferation, as well as increasing extracellular matrix synthesis and glomerulosclerosis. Because other enzymes, such as chymase, can generate angiotensin II in the absence of ACE, monotherapy with ACEI or ARB provides a partial blockage of the RAAS [2].

The combination of ACEIs and ARBs is likely to be more effective than monotherapy in combating the RAAS. However, it is important to note that ACEI/ARBs combined therapy causes hyperkalemia, hypotension, reduced renal function, and in some cases is associated with acute kidney injury (AKI) [2].

Curcuma longa (turmeric or curcuma) is an herbaceous plant member of the ginger family [3]. Curcumin acts as an anti-inflammatory agent [4].

And also affect apoptotic and fibrotic pathways and thus prevent diabetes- induced tissue injury [5].

Therefore, the present work was designed to study the pathological effects of type 1 diabetes on the kidney of albino rats and the possible synergistic protective effects of triple therapy.

Streptozotocin has been usually used for prompting diabetes mellitus in animals, as it has β-cell cytotoxic effect and causes pancreatic β-cells degeneration and necrosis [6].

Sirius red stain is used to assess fibrous tissue [7].Caspase-3 immunohistochemical stain is used to detect cell apoptosis induced by diabetes [8].

METHODS

Drugs and chemicals:

Streptozotocin (Sigma Aldrich, St. Louis, MO, USA) was newly dissolved in 0.9% ice-cold sterile saline [9].

Captopril (ACEI) (AMRIA Pharm. Ind) was dissolved in 0.9% saline.

Losartan (ARB) was obtained from (ALEXANDRIA Company for pharmaceuticals)

Curcumin was purchased from Acros Organics Product in the United States and suspended in 0.5-riboximethyl cellulose [10]. The curcumin suspension has been newly prepared.

Study design; (figure 1) For two weeks prior to the experiment, thirty adult male Albino rats weighing 150-200 grams were maintained in steel cages with soft wood chips for bedding and fed a commercial basal meal and water ad libitum. All animals were bred in controlled environmental conditions (12 hour light-dark cycle, temperature 24 ° C) and were free to feed standard laboratory food and water. Rats were divided into five groups,

- ***Control group:*** animals received the vehicle daily by oral gavage.
- ***Diabetic group:*** Vehicles were received by gavage and sacrificed 20 weeks after the onset of diabetes.
- ***Curcumin treated group:*** animals were treated 8 weeks after induction of diabetes with curcumin (150 mg/kg/day) by oral gavage.
- ***Captopril/losartan treated group:*** animals were treated 8 weeks after the onset of diabetes with 50 mg/kg/day captopril [11], and losartan (20 mg/kg/day) [12] by oral gavage.
- ***Triple therapy (curcumin, captopril and losartan) treated group:*** animals were treated with the same doses by oral gavage.

Induction of diabetes: The rats were starved for 12 hours prior to receiving STZ. A single intraperitoneal (IP) injection of STZ at a dose of 55 mg/kg body weight freshly dissolved in ice-cold sterile saline 0.9 % was used to establish type 1 diabetes. After injection, rats were fed with an oral 10% glucose solution next to a normal diet for the next 2 days to prevent hypoglycemia [13].

Rats were confirmed diabetic when fasting blood glucose (FBG>250mg/dl) for 2 consecutive days [14]. Animals were followed up and development of nephropathy was confirmed by detecting the degree of proteinuria. Dosage and duration were chosen based on previous research that showed therapeutic efficacy without harm. [11, 15] All groups were treated 8 weeks after induction of diabetes. The treatment with either curcumin, Captopril/Losartan combined therapy or triple therapy lasted for 12 weeks [16]. So, the study lasted for 20 weeks

The ethical committee at Mansoura Experimental Research Center accepted the research protocol based on the “Guidelines of Experiments on Animals” (MERC).

All animal experiments comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals.

Samples' collection

The rats were weighted every two weeks till the end of the experiment which lasted for 20 weeks. At the assigned times, the rats were anaesthetized with diethyl ether; samples of blood were drawn from the eye sockets of all rats and collected in polyethylene tubes and centrifuged for separation of serums. Serums were used for estimation of glucose, blood urea and creatinine. Reduced glutathione (GSH) and Malondialdehyde (MDA) lipid peroxidation were measured. Twenty-four hour urine samples were collected via metabolic cages every 2 weeks to study changes in the excretion of proteinuria.

The rats were then sacrificed by cervical dislocation and their abdomens were opened and the kidneys were carefully dissected out and were preserved in 10% buffered formalin and processed for paraffin sections

Histopathological Examination

Preparation of samples for light microscopy: Kidney specimens were processed for paraffin sections by gradual dehydration with increased alcohol concentration, clarified with xylene, and embedded in soft and hard paraffin wax. [17]. Five µm thickness longitudinal sections were cut using the microtome and stained with hematoxylin and eosin stain (H&E) for routine histopathological examination, sirius red stain to

assess fibrous tissue and Caspase- 3 for detection of apoptosis, as indicated by positively reactive cells.

Morphometric study

In Heamatoxylin and eosin stained sections: Measurement of the glomerular area/renal corpuscular area ratio: (Figure 2)

Digital images were taken under objective lens magnification 400x by using Olympus CX41 light microscope and photographed with Olympus SC100 digital camera**.** Morphometric analysis was done using computerized image analysis system image J. At least 30 glomeruli were randomly chosen for each animal. The glomerular area was measured and divided by the renal corpuscular area.

In Sirius red stained sections: Image analysis of the area occupied by collagen fibers [18].

The Sirius red stained sections of all groups were subjected to image analysis. Quantitative assessment of kidney fibrosis was performed with morphometry on sections processed with 0.1% Picrosirius red, which specifically stains collagen.

These measurements were made using an objective lens with a total magnification of 200. The Image Analyzer was initially automatically tuned to convert the measurement units (pixels) generated by the Image Analyzer program to the actual micrometer units. The patch menu was used to measure the area percentage of the red picrosirius region.

Detection of positive immune reaction in Immunohistochemical staining:

The area percentage of immunoreactive cells stained with caspase3 was used as a criterion of cellular activity after subtracting backgroud noise. The color brown of diaminobenzidine was highlighted using the command image/adjustment/color threshold and then quantified the percentage of area using the analyze/analyze particles command. From each slide of all experimental groups, fields were randomly selected [19].

STATISTICAL ANALYSIS

the computer application SPSS (Statistical package for social science) version 17.0 was used to tabulate, code, and analyze the data. Descriptive statistics were calculated in the form of mean ± Standard deviation. To compare more than two sets of parametric data, we used analysis of variance (ANOVA), followed by post-hoc tukey /LSD for multiple comparisons. The Kruskal-Wallis test was used for comparisons between three or more groups of nonparametric data, followed by the Mann-Whitney test for multiple comparisons.

RESULTS

Diabetic animals received the triple therapy showed faster and better weight gain:

At the end of the 8th week, all groups showed highly significant decrease in the body weight compared with control (p<.000), however, there was no significant difference between diabetic, curcumin, captopril/losartan and triple therapy groups. At the 12th week, triple therapy group had a significant increase in the body weight when compared with diabetic group (p<.01), curcumin group had also significant increase in the body weight compared with diabetic group (p<.05), while captopril /losartan group showed no significant difference in the body weight compared with diabetic group.

Triple therapy group and curcumin group showed very high significant increase in the body weight compared with diabetic group throughout 16th,18^{th,} and 20th weeks. Captopril/losartan group showed significant (p<.05), high significant (p<.01) and a very high significant (p<.000) increase in the body weight compared with diabetic group throughout 16th, 18th and20th weeks, respectively. At the same time, captopril/losartan group showed a high significant decrease in the body weight compared with triple therapy group at 20th week (p<.01). (Table1)

Triple therapy improved both urinary and serum biochemical profiles in diabetic nephropathy rat model:

By the end of the study both captopril/losartan group and triple therapy group showed no significant difference in the level of proteinuria compared to control group (p> .05), while there was a high significant decrease in the level of proteinuria compared to diabetic group. As regard triple therapy group showed a very high significant decrease in the level of proteinuria compared to curcumin group (Table 2). Triple therapy group showed high significant decrease in blood glucose level compared with diabetic group (P<.001) and show no significant difference compared with the control group and significant decrease compared with curcumin group and captopril/losartan group (P<.05). There was a significant increase in serum creatinine level in diabetic and captopril/losartan groups in comparison with control group (p<.05), while curcumin group and triple therapy group showed significant decrease in serum creatinine level compared with diabetic group (p<.05). Diabetic group showed significant increase in serum urea level compared with other groups (p<.05).

While all treated groups showed no significant difference compared with control group (p>.05). Captopril/losartan, diabetic and curcumin groups showed significant increase in serum potassium level compared with the control group (p<.05). Triple therapy group showed significant decrease compared with captopril/losartan, diabetic and curcumin groups (p<.05). Level of MDA showed high significant increase in diabetic and captopril/losartan groups compared with control group (p<.01). While had significant decrease in curcumin and triple therapy groups compared with diabetic group (p<.05). There was high significant decrease in the level of GSH in diabetic group and captopril/losartan group compared with control group (p<.01), while there was no significant difference between curcumin group and triple therapy when compared with control group (p>.05) (Table 3).

Histopathological and Morphometric Results:

Hematoxylin and eosin-stained kidney sections of control group showed normal architecture. The renal cortex showed proximal convoluted tubules which appeared deeply stained with narrow lumen, cuboidal cell lining and acidophilic granular cytoplasm. Distal convoluted tubules appeared with thin wall, wide lumen and more lightly stained than the proximal tubules. The glomeruli were surrounded by Bowman's capsule formed of two layers separated by Bowman's space. The parietal layer was lined with simple squamous epithelium and visceral layer was lined by podocyte (figure 3a). Distorted kidney architecture appeared in diabetic group. The glomeruli were distorted and congested with mesangial expansion. There was inflammatory cell infiltrate in the interstitium. Proximal and distal convoluted tubules were degenerated with loss of their lining epithelium**.** Most tubules showed hypertrophied and vacuolated lining cells (figure 3b). Curcumin treated group& Captopril/losartan treated group showed partial restoration of the normal architecture of PCT. Other tubules appeared with vacuolated cytoplasm. Glomerulus with irregular capsular space and mild inflammatory cells in the interstitium were observed and few congested blood vessels could be seen (figure 3c&3d). The histological structure of the kidney of triple therapy group appeared similar to the control group to some extent. The shape of most PCT and DCT appeared nearly normal. However, some tubules with vacuolated cell lining were shown. Glomerulus with wide capsular space was seen. Dilated blood vessels could be seen (figure 3e). The glomerular area/renal corpuscular area ratio in all groups showed very high significant decrease when compared with the control group (P<.000). Compared with diabetic group; captopril/losartan group showed a high significant increase (p<.001). Both curcumin and triple therapy groups showed significant increase (P<0.05) when compared with the diabetic group as well. Interestingly, there is insignificant difference between the three treated groups (figure3f).

Triple therapy reduced fibrosis in diabetic nephropathy rat model:

Sirius red stained sections of Control group showed thin collagen fibers around glomeruli, renal tubules and around Blood vessels (figure 4a). The amount of the collagen fibers around glomeruli, renal tubules and around blood vessels appeared markedly increased in diabetic group (figure 4b). All the treated groups showed thin collagen fibers around glomeruli, renal tubules and around blood vessels (figure 4c, d, e). By quantification of Sirius red stained area percentage in the cortex and medulla, diabetic group showed highly significant increase as compared with control group (P<.001). Curcumin treated group showed significant decrease as compared with diabetic group (P<.05). Both of captopril/losartan treated group and triple therapy treated group showed high significant decrease as compared with diabetic group (P<.001) (figure 4f).

Caspase-3- stained sections

Caspase-3- stained sections of control group Showed very scanty cytoplasmic immune reactivity for caspase-3 in glomerular cells and cells of renal tubules (figure 5a). There was highly strong brown positive cytoplasmic immune reaction for caspase-3 in diabetic group (figure 5b).There were a few positive immunoreactions for caspase-3 especially in cells of renal tubules in all treated groups(figure 5c, d, e). By quantification of caspase-3-stained area percentage in the cortex and medulla, diabetic, captopril/losartan and triple therapy groups showed highly significant increase as compared with the control group (P<.001). Curcumin group showed significant increase as compared with the control group. At the same time all the three treated groups showed highly significant decrease as compared with diabetic group (figure 5f).

Table (1): The Mean body weight in experimental groups throughout 20 weeks.

Body weight (gm)						
	Control	**Diabetic**	**Curcumin**	**Captopril/Losartan**	**Triple therapy**	**ANOVA**
2nd week	**225.5±10.23**	**216±10.54**	**221±12.17**	**215±7.6**	**222±7.26**	**.371**
4th week	**233.17±10.26**	**211±7.5**	**215.17±10.8**	**207.75±10.3**	**214.4±8.14**	**.002**
P1		.02	.061	.011	.063	
P2			.970	.992	.998	
P3			-	.833	1	
P4			1	.894		
6th week	**241±9.98**	**204±7.3**	**205.5±18.6**	**202.25±9.9**	**207.2±10.98**	**.000**
P1		.002	.002	.002	.005	
P2		-	1	.999	.998	
P3		-	-	.997	1	
P4		-	-	.985	-	
8th week	**248.83±10.12**	**200.25±6.9**	**210±6.04**	**203.5±11.2**	**211.8±9.68**	**.000**
P1		.000	.000	.000	.000	
P2		-	.637	.992	.481	
P3		-	-	.883	.999	
P4		-	-	.759	-	
10th week	**257±12.06**	**193±6.78**	**210±5.09**	**184±44.85**	**217±9.6**	**.001**
P1		.006	.054	.002	.095	
P2			.847	.983	.568	
P3				.543	.992	
P4				.268		
12th week	**266±11.66**	**185.75±5.7**	**212.5±5.2**	**204±11.6**	**220.5±11.8**	**.000**
P1		.000	.000	.000	.000	
P2			.026	.196	.003	
P3				.825	.854	
P4				.278		
14th week	**274.20±13.68**	**177.5±6.13**	**215.25±4.64**	**203.75±9.9**	**225.5±13.1**	**.000**
P1		.000	.000	.000	.000	
P2			.003	.043	.000	
P3				.662	.748	
P4				.118		
16th week	**284±15.9**	**171.67±4.9**	**217.75±5.56**	**204.33±6.02**	**225.5±7.14**	**.000**
P1		.000	.000	.000	.000	
P2		-	.001	.022	.000	
P3		-	-	.555	.870	
P4		-	-	.158	-	
18th week	**294.8±15.8**	**160.3±1.5**	**221.3±3.2**	**202.67±8.02**	**231.5±7.4**	**.000**

Body weight (gm)						
	Control	**Diabetic**	**Curcumin**	**Captopril/Losartan**	**Triple therapy**	**ANOVA**
P1		.000	.000	.000	.000	
P2			.000	.004	.000	
P3				.326	.781	
P4				.038		
20th week	**311.4±15.07**	**150.67±2.08**	**220±3**	**199±1**	**235.5±6.85**	**.000**
P1		.000	.000	.000	.000	
P2			.000	.000	.000	
P3				.155	.342	
P4				.003		

P: Probability
P1: significance relative to control group
P2: significance relative to diabetic group
P3: significance relative to curcumin group
P4: significant relative to triple therapy group

Table (2): The mean protein level ±SD in the urine of experimental groups after 6 weeks of receiving drugs.

	Protein in urine (g/dl)					
	Control	**Diabetic**	**Curcumin**	**Captopril/Losartan**	**Triple therapy**	**ANOVA**
14th week	**3.77±0.13**	**6.73±0.34**	**4.97±0. 15**	**4.71±0.48**	**4.09±0.13**	**.000**
P1	-	.000	.003	.017	.654	
P2	-	-	.000	.000	.000	
P3	-	-	-	.785	.025	
P4	--	-	-	.141	-	
16th week	3.79±0.03	6.81±0.23	4.76±**0.**18	4.59±0.39	3.97±0.13	.000
P1	-	.000	.003	.01	.854	
P2	-	-	.000	.000	.000	
P3	-	-	-	.884	.011	
P4	-	-	-	.046	-	
18th week	3.88±.015	6.87±0.14	4.73±0.10	4.96±1.03	4±0.22	.000
P1	-	.000	.26	.112	.998	
P2	-	-	.002	.004	.000	
P3	-	.-	-	.975	.387	
P4	-	-	-	.177	-	
20th week	3.91±0.10	7.07±0.14	4.77±0.40	4.81±1.04	3.98±0.17	.000
P1	-	.000	.306	.270	1.00	
P2	-	-	.002	.002	.000	
P3	-	-	-	1	.375	
P4	-	-	-	.333	-	

P: Probability

P1: significance relative to control group
P2: significance relative to diabetic group
P3: significance relative to curcumin group
P4: significant relative to triple therapy group

Table (3): the mean biochemical results ± SD of the experimental groups sacrificed at 20th week:

	Control	Diabetic	Curcumin	Captopril/Losartan	Triple therapy	ANOVA
Blood glucose (mg/dl)	**88.5 ±2.5**	**392±36**	**169±33.5**	**217±16.8**	**105±9.07**	**.000**
P1	-	.000	.002	.000	.901	
P2	-	-	.000	.000	.000	
P3	-	-	-	.809	.006	
P4	-	-	-	.001	-	
Serum Urea (mg/dl)	**22.8±3.6**	**128±40.6**	**32.1±13.3**	**55.26±16.3**	**29.8±6.1**	**.001**
P1	-	.001	.979	.368	.993	
P2	-	-	.001	.011	.001	
P3	-	-	-	.661	1.0	
P4	-	-	-	.583	-	
Serum creatinine (mg/dl)	**.61±.02**	**1.17±.43**	**.72±.18**	**1.06±.15**	**.68±.03**	**.040**
P1	-	.011	.534	.032	.694	
P2	-	-	.033	.546	.022	
P3	.-	-	-	.096	.816	
P4	-	-	-	.064	-	
Serum potassium level (mmol/L)	**5.03±.64**	**7.14±.40**	**6.4±.44**	**6.96±.12**	**5.03±.48**	**.000**
P1	-	.001	.026	.003	1.00	
P2	-	-	.330	.986	.001	
P3	-	-	-	.581	.026	
P4	-	-	-	.003	-	
Serum MDA (nmol/mg protein)	**2.35±.15**	**3.91±.80**	**2.99±.55**	**3.98±.20**	**2.87±.42**	**.009**
P1	-	.003	.137	.002	.220	
P2	-	-	.044	.871	.026	
P3	-	-	-	.033	.764	
P4	-	-	-	.020	-	
Serum GSH (nmol/mg protein)	**.15±.04**	**.05±.02**	**.09±.04**	**.04±.006**	**.09±.02**	**.019**
P1	-	.005	.062	.003	.070	
P2	-	-	.158	.768	.142	
P3	-	-	-	.097	.946	
P4	-	-	-	.087	-	

P: Probability
P1: significance relative to control group
P2: significance relative to diabetic group
P3: significance relative to curcumin group
P4: significant relative to triple therapy group

figure1

Figure (1): Diagram for study design.

figure2

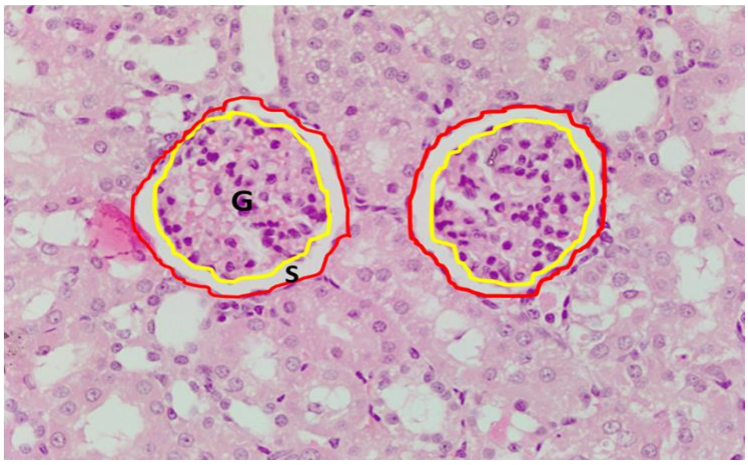

Figure (2): show measurement of the glomerular area/renal corpuscular area ratio.

Figure 3

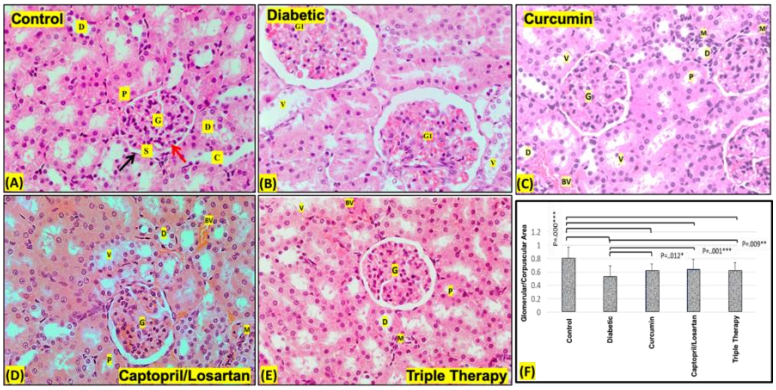

Figure (3): (a) show a photomicrograph of H&E stained kidney section of control group showing renal glomerulus (G) surrounded by Bowman's capsule having two layers, parietal layer (black arrow) and visceral layer (red arrow), separated by Bowman's space (S),the PCT(P), DCT (D).and CT(C).**(b)** Diabetic group showing distorted glomeruli with mesangial expansion and marked congested glomeruli (G1) and many tubules show hypertrophic cell lining with vacuolated cytoplasm (v).**(c, d, e)** The kidney sections of curcumin treated group, captop ril/losartan treated group and triple therapy treated group showing restoration of the shape of some of PCT (P), DCT (D). Other tubules show vacuolated and degenerated cell lining (V). Slightly congested glomerulus (G), Minimal mononuclear cell infiltration (M) and congested blood vessel (BV) could be seen. **(f)** Quantification of glomerular area/ corpuscular area ratio of all groups

Figure 4

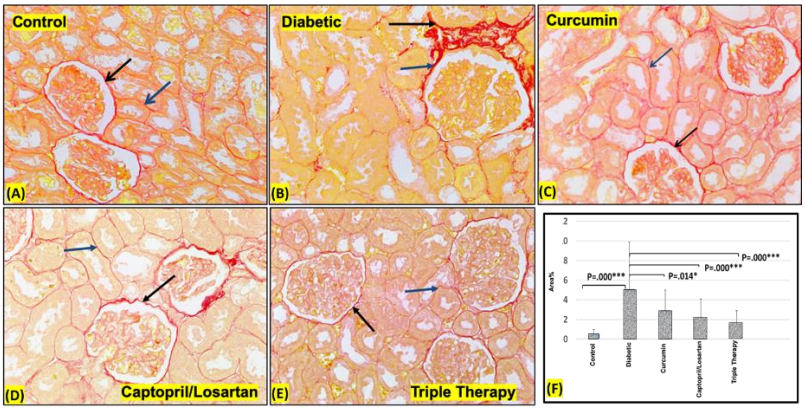

Figure (4): (a) show a photomicrograph of Sirius red stained kidney section of control group showing thin collagen fibers around glomeruli (black arrows), and around renal tubules (bleu arrows), **(b)** Diabetic group showing more thick collagen fibers around glomeruli(bleu arrow) , and around renal tubules(black arrow). **(c, d, e)** The other three treated groups showing thin collagen fibers around glomeruli, and around renal tubules. **(f)** Quantification of Sirius red stained area percentage in all groups.

Figure 5

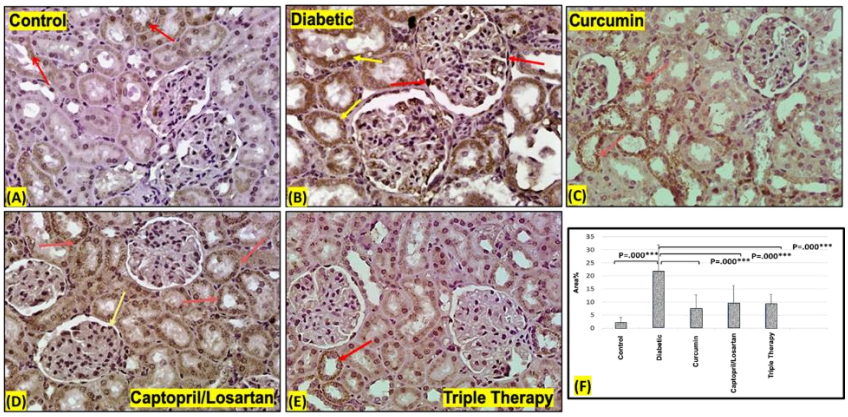

Figure (5): (a) show a photomicrograph of caspase-3 stained kidney section of control group showing very scanty immune reaction for caspase-3 in glomeruli and renal tubule (red arrow),**(b)** Diabetic group showing strong positive brown immunoreaction for caspase-3 in the glomerular podocytes (red arrow) and renal tubular cells (yellow arrow). **(c ,d)**Curcumin treated group and Captopril/Losartan treated group showing brownish cytoplasmic immunoreaction for caspase-3 in many renal tubules (red arrow) and few glomerular cells (yellow arrow), **(e)** triple therapy treated group shows faint immunoreaction for caspase-3 in both glomerular and tubular cells.**(f)** Quantification of caspase-3 stained area percentage in all groups.

DISCUSSION

Diabetic nephropathy is major microvascular complications of diabetes. Oxidative stress, lipid abnormalities, renal hemodynamic alterations, increased non-enzymatic glycosylation of proteins, and activation of the polyol pathway are all factors in the development and outcome of DN [1]**.** In this regard, we used in the current investigation male animals to avoid hormones of the estrous cycle in potential effect on the results [20]

The combination of ACEI/ARBs is predicted to be more effective than monotherapy in preventing the development of diabetic nephropathy, but has serious side effects. Moreover curcumin has renoprotective effect [2]. The current study was of interest in investigating if adding curcumin to the standard combined ACEI/ARBs treatment of DN could have a more beneficial effect on renal functions or not.

Streptozotocin was injected intraperitoneally to generate type 1 diabetes in the current study. Rats were confirmed diabetic when fasting blood glucose (FBG) > (250mg/dl), 72 h after STZ injection [21].

The pathological changes start after 4 weeks of STZ administration, and diabetic nephropathy develops after 8 weeks of STZ administration [24]. The significant decrease in the body weight and the highly significant elevation of the blood glucose level of untreated diabetic groups were in agreement with *Reza et al* [23]. Diabetes is accompanied with increased glycogenolysis, gluconeogenesis, lipolysis and these biochemical changes lead to loss of tissue protein and muscles wasting that explain the observed decrease in the body weight [24]. The increase in blood glucose level might be due to β- cells necrosis [25].

Interestingly, untreated diabetic groups in the current study showed a highly significant decrease in the GSH level and a highly significant increase in the MDA level. This result was in agreement with Mestry et al [26] and Reza et al [23].The observed decrease in GSH level in untreated diabetic group in the current study could be due to its increased utilization during diabetes induced- oxidative stress to scavenge free radicals [27]. MDA is an aldehyde product of lipid peroxidation. It is indirect evidence of high free radical generation in diabetes and also a biomarker of intensified lipid peroxidation [28].

Untreated diabetic groups showed a highly significant increase in the serum urea and serum creatinine in agreement with Mestry et al [26]. In diabetes, protein glycation can cause muscle atrophy and an increase in the release of purine, which is the main source of uric acid and urea. [29]

In agreement with Abdel-Wahab et al [23] and Zhu et al [30] the untreated diabetic models showed highly significant increase in protein level in urine compared with control group [23,30]. The increase in the urinary protein level might be due to multiple reasons including increased advanced oxidative protein products, reactive oxygen species and increased glomerular membrane permeability [27&29].

In the current study untreated diabetic group revealed high significant increase in serum potassium level this is in agreement with Kim and Deeks [32]. Hyperkalemia in DN may be due to impaired renal function or being an adaptive response reducing the urinary potassium excretion and increasing the colonic potassium secretion [33&34]

Kidney sections obtained from untreated diabetic rats stained with Hematoxylin and Eosin showed distorted and congested glomeruli with loss of sub-capsular space. These sections showed also glomerular atrophy, in addition to evident tubulointerstitial inflammatory changes as vacuolar degeneration of tubular cell lining, damaged brush border, large number of casts in their lumen and blood vessels were congested as well. These results were consistent with findings of Abdel-Wahab et al [23] and Mestry et al [26]. When compared to the control group, the glomerular area/renal corpuscular area ratio decreased significantly. This might be the result of glomerulosclerosis [35].

In the diabetic kidney, hyperfiltration and increased renal glucose absorption can cause AGE buildup, oxidative stress, and inflammatory alterations [36].

In agreement with Terami et al [18] and Jung et al [37] Sirius red stained renal sections of untreated diabetic model showed relatively thick collagen fibers around glomeruli and renal tubules. Sirius red area percentage of the 20 weeks-diabetic animal group showed highly significant increase compared with the control group. The fibrotic cytokine TGF-β1 was shown to be expressed in renal glomeruli, mediating glomerular matrix accumulation in diabetic kidney [38].

In agreement with Sha et al [39] in the present study; caspase-3 stained section of untreated diabetic model showed high significant increase in expression of caspase-3 protein compared with the control group .Oxidative stress and free oxygen radicals develop during nephropathy and trigger apoptosis of tubular epithelial cells and podocytes of the glomeruli [40].

In agreement with Soetikno et al [41] and Huang et al [42] curcumin treated group in the present study showed a significant increase in body weight compared with diabetic group. It also showed significant decrease in blood glucose, proteinuria, serum urea and serum creatinine. Curcumin's hypoglycemic and anti-oxidant qualities might boost insulin secretion and help with weight gain. Curcumin's hypoglycemic, hypolipidemic, and anti-oxidant characteristics, as well as improved renal function, could explain the observed decrease in blood urea and creatinine.

Curcumin's ability to suppress ACE has recently been discovered [5]. It is thought that an increase in ACE1 could worsen diabetic nephropathy; hence curcumin's suppression of ACE1 could help to enhance kidney function [43].

When compared to the control group, the curcumin-treated group showed a substantial rise in blood potassium levels, but also tended to lower the mean level of serum potassium when compared to

the diabetic group. This may be explained by partial reno-protective action of curcumin in the curcumin treated group.

Curcumin treated group showed significant decrease in levels of MDA and tend to increase the mean level of GSH compared with diabetic group and this is in agreement with Cekmen et al [44]. Unfortunately, the increase in GSH level was not statistically significant and this could be attributed to small number of animals in the group or low dose of curcumin.

Interestingly, Triple therapy(curcumin, captopril and losartan treated group showed significant decrease in MDA level when compared with diabetic group and increased the level of GSH to level of control group. This better finding supports our suggestion that combination of curcumin, captopril and losartan has synergistic anti-oxidant effect.

Curcumin's anti-oxidant capabilities are owing to its ability to react directly with reactive oxygen species, causing anti-oxidant proteins to be up-regulated. [45].

In the present study curcumin succeeded to increase the glomerular area/renal corpuscular area ratio in curcumin treated group as compared with untreated diabetic group. This is in line with the findings of Soetikno et al [41] who found that decreased glomerulosclerosis index (GS), tubulointerstitial (IT) fibrosis, and arteriolopathy indicated a reduction in the development of structural damage.

In the present study curcumin lead to highly significant decrease in fibrosis around glomeruli, renal tubules and blood vessels as compared with diabetic group this is in agreement with Zhou et al [46]. Curcumin has been shown to aid in the resolution of renal fibrosis during the priming and activation stages by limiting inflammation, restoring redox balance, suppressing EMT, and removing ECM excess deposition [47].

Curcumin succeeded to highly significant decrease apoptosis in renal tissue. This is in agreement with Sun et al [48] and Hassan et al [49]. Curcumin could prevent HG-induced podocyte death in a dose-dependent manner, in part by restoring both the Bcl-2/Bax balance and the PARP/cleaved PARP balance, as well as reducing caspase-3 activation [48].

Captopril/losartan treatment significantly increased body weight when compared with diabetic group. and also in the present study Captopril/losartan treated group showed high significant decrease in the level of proteinuria and blood glucose level and showed significant decrease in serum urea as compared with untreated diabetic group and this was in agreement with Abd Allah and Gomaa [43].

This could be due to their hypoglycemic and anti-oxidant characteristics, which would boost insulin secretion and help with weight gain [43].

The combined Captopril/ Losartan's lipid-lowering, hypoglycemic and antioxidant actions could be explained by the suppression of ACE, which lowers Ang II levels. Ang II raised blood glucose levels by a variety of processes, including decreased pancreatic islet blood flow, which lowered insulin production by -cells, as well as oxidative stress and inflammation [50].

While there was no statistically significant difference in GSH and MDA level when compared with diabetic group, this could be attributed to small number of animals in the group or low dose of captopril and losartan. In our study, adding of curcumin to captopril/losartan combined therapy in treating rats with DN lead to better anti-oxidant effect. Triple therapy treated group showed statistically significant decrease in MDA level when compared with diabetic group and also increased GSH level to level of control group.

While there was no statistically significant difference in serum creatinine level when compared with diabetic group, but at the same time there was a tendency to decrease in the serum creatinine level mean. This is in agreement with Onozato et al. [51].

Fortunately, in our study triple therapy treated group showed significant decrease in creatinine level when compared with diabetic group. This finding supports our hypothesis that combination of the three drugs adds more curative and protective effects in treatment of DN.

In the present study captopril/losartan treated group significantly increased the serum potassium level as compared with the control group and this was in agreement with Ren et al [2].

Interestingly, in our study adding of curcumin to captopril/losartan combined therapy in treating of rats with DN protected rats from lethal hyperkalemia. Triple therapy treated group showed high significant decrease in serum potassium level when compared with diabetic group and when compared with captopril/losartan treated group also.

Downregulation of some key cytokines, growth factor pathways, and oxidative stress reduction appear to be additional, but important, effects of ACEI treatment [52].

In the present study captopril/losartan combined therapy succeeded to increase the glomerular area/renal corpuscular area ratio in captopril/losartan treated group as compared with untreated diabetic group. And also lead to highly significant decrease fibrosis around glomeruli, renal tubules and blood vessels as compared with diabetic group. This is in agreement with Akbar et al [53].

TGF-ß have been defined as the most potent pro-sclerotic factor that contributes to renal fibrosis [54]. Treatment of the diabetic groups with Captopril significantly decreased kidney tissue miR-192 expression levels. Ebadi et al [55] found a significant positive correlation between miR-192 expression level and the serum TGF-ß concentration in the diabetic group's .Thus, they suggested that therapeutic reinforcement of miR-192 can improve DN and modulate the risk of renal function decline by suppressing fibrogenesis.

In the present study captopril/losartan combined therapy succeeded to highly significant decrease in apoptosis in renal tissue. This is in agreement with Uhal et al [56]who suggested the possibility that captopril and other thiol compounds inhibit lung epithelial cell apoptosis through direct inhibition of caspase-1 and -3 activities and/or other cysteine proteases required for the induction of apoptosis.so captopril may do this effect in the kidney tissue .

In the present study triple therapy group (curcumin, captopril and losartan) showed better reno-protective effect and more improvement in renal function and general condition than curcumin or Captopril/Losartan treated groups alone.

There was better weight gain, more decrease in 24h proteinuria, blood glucose, serum urea, blood creatinine and blood potassium to level of control. Also, there was more decrease in the level of MDA, and more increase in GSH level.

At the same time triple therapy succeeded to increase the glomerular area/renal corpuscular area ratio as compared with diabetic group and succeeded to highly significant decrease apoptosis in renal tissue and highly significant decrease fibrosis around glomeruli, renal tubules and blood vessels as compared with diabetic group to level of control.

CONCLUSIONS

It is concluded that this finding supports our hypothesis that a triple combination of curcumin, captopril, and losartan might be more effective and protective in the treatment of DN, and that adding curcumin to the well-known captopril/losartan combination therapy could protect against hyperkalemia, which can cause acute kidney injury.

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