PATHOPHYSIOLOGICAL EVALUATION OF THE PROTECTIVE ROLE OF ALPHA LIPOIC ACID ON DIABETES MELLITUS NEPHROPATHY IN MALE RABBITS

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ABSTRACT

In order to estimate the ameliorating effects of alpha lipoic acid (ALA) on the nephropathy of diabetes mellitus (DM) associated with oxidative stress, this study performed in 18 adults male rabbits were divided into 3 equal groups, the group-I was regard as control while group-II Induced DM only by intravenously single dose (150 mg/kg b.w.) of alloxan-monohydrate; group-III was induced DM by intravenously single dose of (150mg/kg b.w.) of alloxan-monohydrate and then treated by (10mg/kg b.w.) daily of ALA intraperitoneally for 4 weeks. The biochemical results showed significant ($P \leq 0.05$) decreased of the serum values of GSH, CAT and SOD as well, significantly ($P \leq 0.05$) increased of MDA, peroxynitrate, creatinine and BUN of group-II when compared to control; also the result of group-III showed non-significant ($P > 0.05$) differences of GSH, SOD, MDA, creatinine, BUN and peroxynitrate when compared to control. The histopathological and histochemical results of kidney of group-II showed moderate thickness of basement membrane of glomeruli with compressed capillaries as well infiltration of glyco-proteinaceous materials in glomeruli and around renal tubules with some vacuolation of these tubules and mesangial cells; while the results of group-III showed normal architectures with very mild degree of vacuolation of few renal tubules in addition to disappearance of glyco- proteinaceous materials in this group; in conclusion, ALA had a dual protective effects on nephropathy by scavenging the oxidative stress free radicals and enhanced insulin metabolism.

INTRODUCTION

Diabetes mellitus (DM) has metabolic disorders that characterized increase in blood glucose level by alterations in the carbohydrates, lipids and proteins metabolisms leading to cardiovascular, nephropathy and neuropathic complications, it remains the most public health problems worldwide (1).

Among all human diabetics' complications, about an 85% of all diabetics developed retinopathies, 25–50% that developed nephropathies and 60–70% had neuropathies (2).

Experimental models of diabetes induction with alloxan ($C_4H_2N_2O_4.H_2O$ 10%) has been mostly used, administration of alloxan to different animal models caused necrosis to pancreatic islets, therefore several features of common to these observations in human diabetes (3).

Alpha lipoic acid (ALA) also termed thioctic acid, which firstly isolated from bovine livers in 1950, ALA contains two thiol groups that regards as a part of a redox pairs, that being the oxidized partners of the reducing forms of dihydrolipoic acid (DHLA) (4). The ALA is a natural occurring compounds that was known as 1,2-dithiolane-3-pentanoicacid or thioctic acid. It synthesized by enzymatic way in plants and animals mitochondria from octanoic acid and cysteine as a sulfur sources (5). ALA can elicits its antioxidant actions against oxidative stresses in the cytosol and in the plasma membranes (aqueous plus lipid media of the cell), also in serum and lipoproteins (aqueous plus lipid media of blood) in contrast to vitamin C (which is hydrophilic) and vitamin E (which is hydrophobic) (6).

ALA appeared in engaging the insulin signaling pathways by increasing glucose uptakes into muscles and fat cells, as well several clinical studies points to a beneficial effects of ALA on entire body glucose metabolisms in patients of type 2 DM (7). Oxidative stress proposed to be early events in the pathophysiology of DM and may influenced on onset and progression of late complications, therefore, this study aimed to estimated the protective effects of ALA in nephropathy as a complication to DM.

MATERIALS AND METHODS

Eighteen adult healthy male rabbits of local bread were used in this study, weighing about (1400 ± 50 grams). The animals were acclimatized under the standard conditions of temperature (25±2^{o}C) and humidity (45±5%). The rabbits were kept under observation for a week in the animals house of college of veterinary medicine at university of Basrah before beginning of experiment.

DM induced by using a single intravenous injection dose of 10% alloxan-monohydrate (150mg/kg, b.w.) manufactured by (Sigma-Aldrich/France). Blood glucose concentrations when exceed to 300 mg/dl for 3 days after treatment were considered as DM and used in experiments, moreover, in order to reduce death because of hypoglycemic shock, alloxan-treated groups administered 5% of glucose instead of water for 24 hours after DM induction (8).

The animals were divided into equal three groups in which each group composed of 6 male rabbits and treated for 4 weeks as the following:

1. Group I: served as negative control group (untreated group).
2. Group II: Induced DM only by single dose of (150 mg / kg b.w.) of 10% alloxan-monohydrate.
3. Group III: Induced DM by single dose of (150mg / kg bw.) of 10% alloxan-monohydrate and then treated with (10mg / kg bw) daily of ALA intraperitoneally for 4 weeks.

The ALA (10mg / kg bw.) manufactured by (Vita cost / USA), the solution was suspended in 1ml of sterile saline, until completely dissolved, then carefully applied intraperitoneally daily for 4 weeks.

In the end of experiment, blood sample was collected from animals via cardiac puncture using disposable syringe (5ml). The serum was prepared by centrifugation of blood at 3000 rpm for 10 minutes and frozen at (-20C$^{\circ}$) until it used to determination the oxidants and antioxidant levels between groups as the following:

The determination of serum glutathione concentration (GSH) by method of (9).

The determination of serum catalase (CAT) activity was measured by method of (10), the determination of superoxide dismutase (SOD) activity by method of (11), the assessment malondialdehyde (MDA) concentration by method of (12). The determination of serum peroxynitrate concentration by method of (13). The determination of kidney function test of creatinine and blood urea nitrogen (BUN) by method of (14).

The histopathological samples of kidney were taken immediately, fixed with 10% neutral buffered formalin, dehydrated, embedded in paraffin and sectioned at 4-5µ, then stained with hematoxyline and eosin, and other sections stained by periodic acid schiff (PAS) stain. Finally examined under light microscopy (15).

The statistical analysis of data was performed in the bases of one-way analysis in variance (ANOVA) depending on the experimental design; the significant ($P \leq 0.05$) differences were determined using least significant differences (16).

RESULTS

The biochemical tests of the serum glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) were significantly ($P \leq 0.05$) decreased in group II (DM group) with mean values 3.48 ± 0.06, 1.87 ± 0.03 and 3.39 ± 0.05 respectively when compared to the values in the group I (control group) which were 4.52 ± 0.03, 3.32 ±0.04 and 5.31 ± 0.02 respectively. While the mean values of malondialdehyde (MDA) and peroxynitrite showed significantly ($P \leq 0.05$) increased in the serum of group II (DM) which the values were 6.35 ± 0.07 and 15.1 ± 0.03 respectively when compared to the group I (control group) were 3.62 ± 0.01 and 9.8 ± 0.16 respectively, as in table (1). Whereas, the results of biochemical tests of group III (DM+ALA group) showed there were non-significant ($P > 0.05$) differences in the mean values of GSH, SOD, MDA and peroxynitrate which were 4.4 ± 0.02, 5.23 ± 0.06, 3.49 ± 0.19 and 9.91 ± 0.06 respectively when compared to those values of group I (control group), while the CAT mean value showed significantly ($P \leq 0.05$) increased which was 2.95 ± 0.15 when compared to the value in the group II was 1.87 ± 0.03, as in table (1).

Table (1): Biochemical results of serum antioxidant and oxidant biomarkers.

Parameters / Groups	GSH nmol/g Mean±SE	CAT u/mg Mean±SE	SOD u/mg Mean±SE	MDA nmol/ml Mean±SE	Peroxynitrate m/l Mean±SE
Group I Control	**4.52 ± 0.03 a**	**3.32 ± 0.04 a**	**5.31 ± 0.02 a**	**3.62 ± 0.01 b**	**9.8 ± 0.16 b**
Group II DM only	**3.48 ± 0.06 b**	**1.87 ± 0.03 c**	**3.39 ± 0.05 b**	**6.35 ± 0.07 a**	**15.1 ± 0.03 a**
Group III DM + ALA	**4.4 ± 0.02 a**	**2.95 ± 0.15 b**	**5.23 ± 0.06 a**	**3.49 ± 0.19 b**	**9.91 ± 0.06 b**

*Different letters vertically refers to presence a significant differences between groups.

The results of kidney function tests showed significant ($P \leq 0.05$) increases in the values of serum creatinine and BUN in group II (DM group) with mean values 1.29 ± 0.02 and 21.76 ± 0.28 respectively when compared to the values in the group I (control group) which were 0.86 ± 0.02and 17.61± 0.20 respectively. Whereas, the results of group III (DM+ALA group) showed there were non-significant ($P>0.05$) differences in the mean values of serum creatinine and BUN which were 1.0 ± 0.02 and 17.75 ± 0.07 respectively when compared to those values of group I (control group) as in table (2).

Table (2): Biochemical results of kidney function test.

Parameters / Groups	Creatinine mg/dl Mean±SE	BUN mg/dl Mean±SE
Group I (Control)	**0.86 ± 0.02 b**	**17.61 ± 0.20 b**
Group II (DM only)	**1.29 ± 0.02 a**	**21.76 ± 0.28 a**
Group III DM + ALA	**1.0 ± 0.02 b**	**17.75 ± 0.07 b**

*Different letters vertically refers to presence significant differences between groups.

The histopathological and histochemical results of group I (control group) showed normal architectures of the kidney as in (figures.1 and 2) as well not presence of any deposition of abnormal glyco-proteinaceous materials (figures 7 and 8). Whereas, the histopathological results of group II (DM group) showed moderate thickness in the basement membrane of the Bowman capsules with compressed of capillaries were observed because of severe infiltration of proteinaceous materials in the glomeruli and in or around renal tubules as in (figure.3), in addition present a moderate vacuolation in the mesangial cells of glomeruli with present of some vacuolated proximal and distal convoluted tubules as in (figure.4), also the histochemical results showed moderate deposition of glyco-proteinaceous materials in glomerulus and around renal tubules (figures 9 and 10) .

The histopathological results of group III (DM+ALA group) showed very mild degree of vacuolation of some renal tubules, also there is normal appearance of glomeruli and other renal tubules as in (figures. 5 and 6), in addition, there weren't present of the protein like materials in the entire renal architectures as that shown in group II. Also the histochemical results showed clearance of renal parenchyma from any deposition of abnormal glyco-proteinaceous materials (figures 11 and 12).

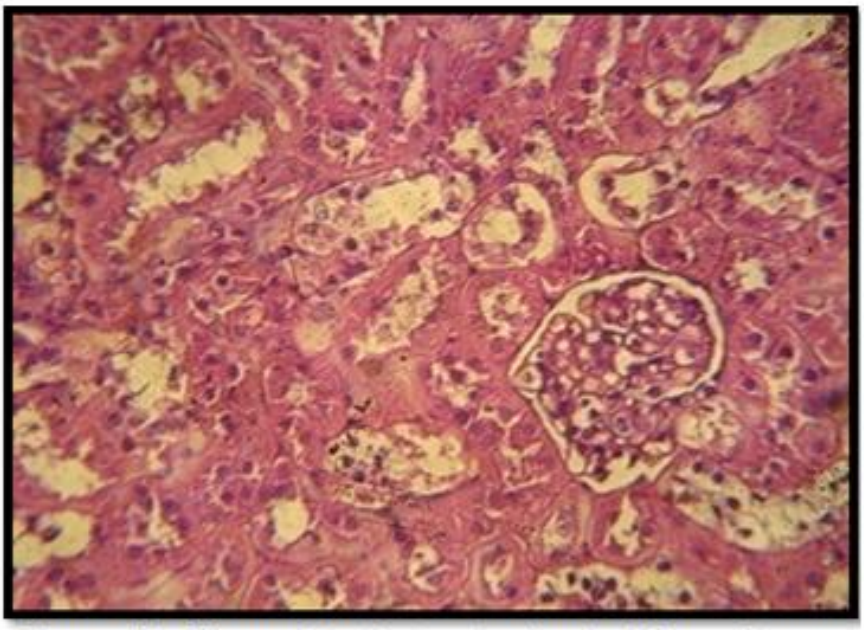

Figure (1): Histopathological section in the kidney of group I (control) showed normal architecture of renal parenchyma. H&E stain. 40X.

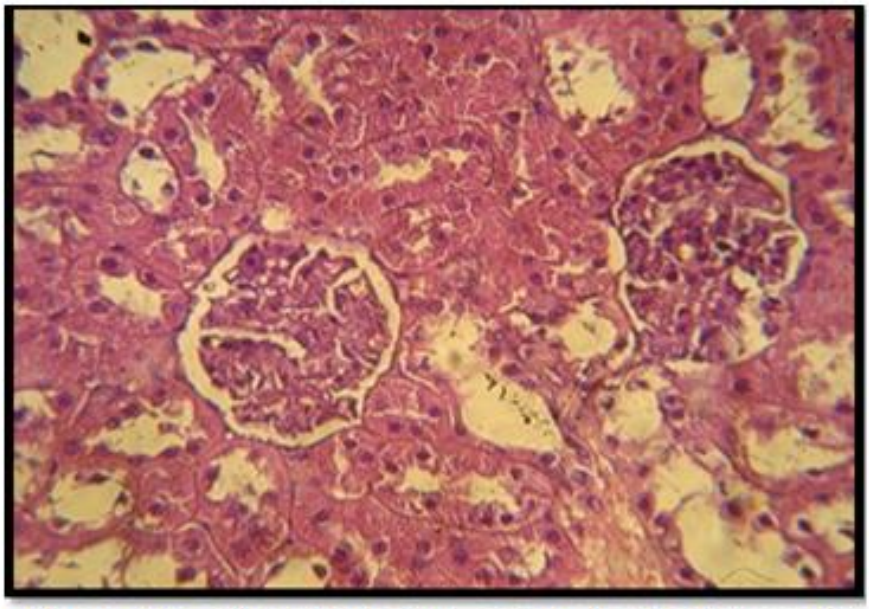

Figure (2): Histopathological section in the kidney of group I (control) showed normal glomeruli and renal tubules. H&E stain. 40X.

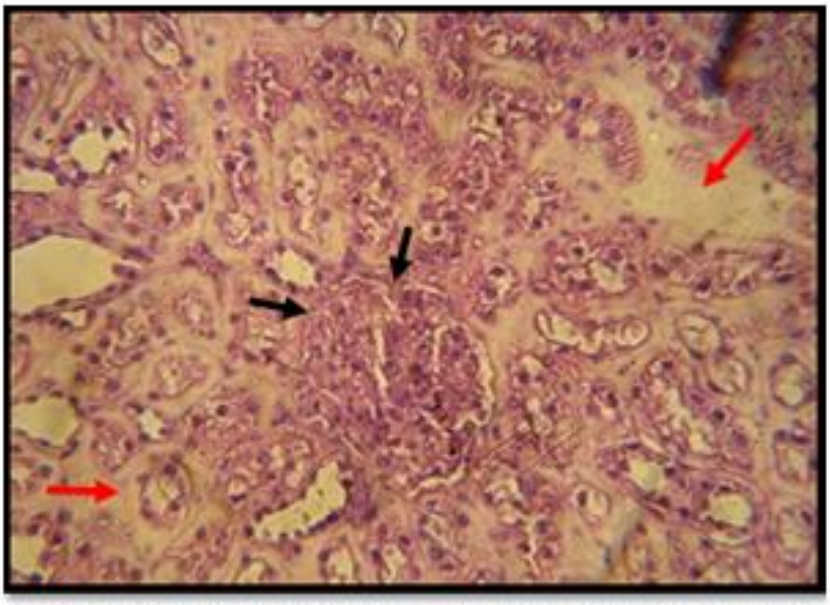

Figure (3): Histopathological section in the kidney of group II (DM) showed thickened basement membrane of Bowman's capsule and compressed capillaries of glomeruli (black arrows), also infiltration of proteinaceous materials in the interstitium of renal tubules (red arrows). H&E stain. 40X.

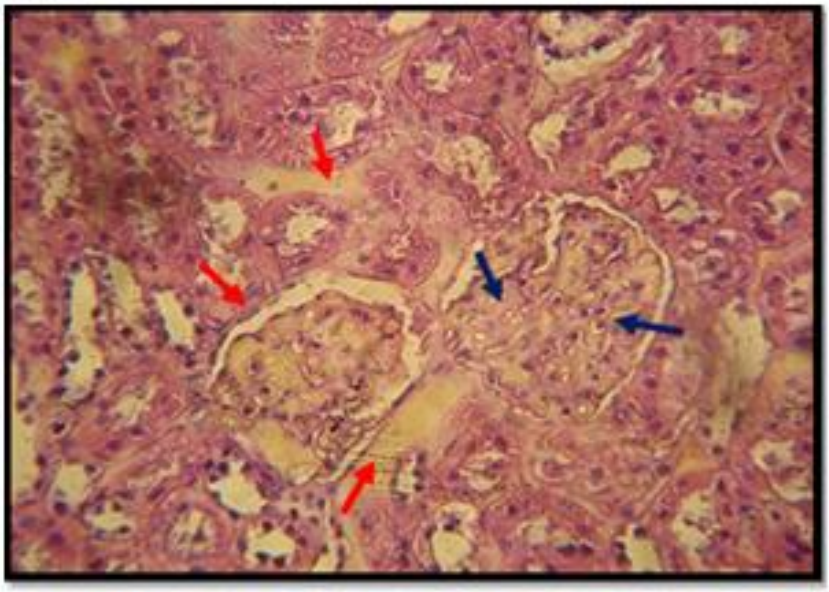

Figure (4): Histopathological section in the kidney of group II (DM) showed vacuolation of mesangial cells and some renal tubules (black arrows), also infiltration of proteinaceous materials inside glomerular tufts and in the interstitium of renal tubules (red arrows) as well increased thickness of

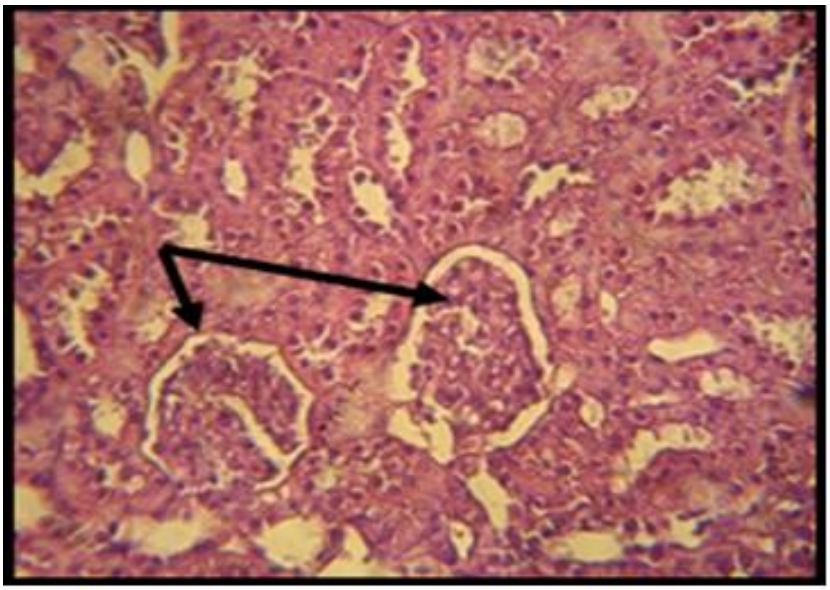

Figure (5): Histopathological section in the kidney of group III (DM+ALA) showed normal architecture of Bowman's capsule and glomerular tufts (black arrows), also no present of any infiltration of proteinaceous materials in the renal parenchyma. H&E stain. 40X.

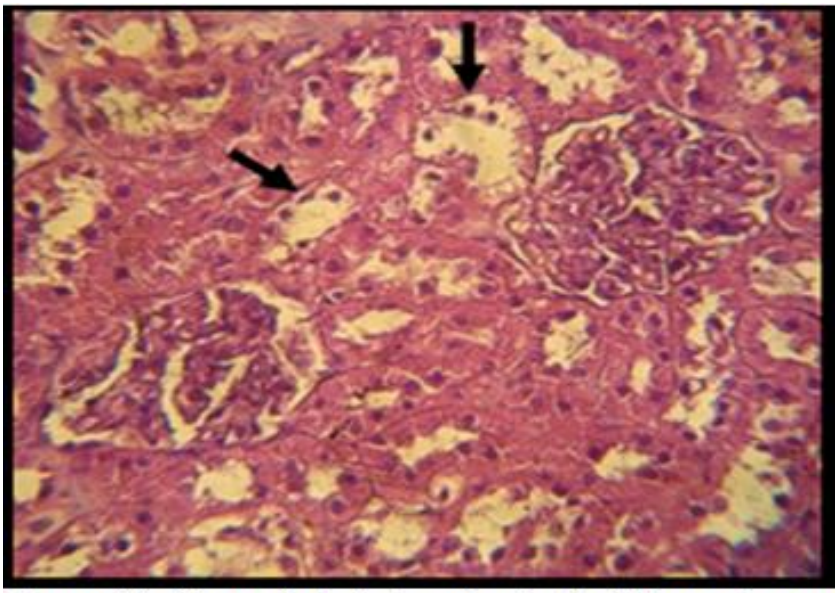

Figure (6): Histopathological section in the kidney of group III (DM+ALA) showed normal architecture of Bowman's capsule and glomerular tufts, with noticed mild degree of vacuolation in some renal tubules (black arrows). H&E stain. 40X.

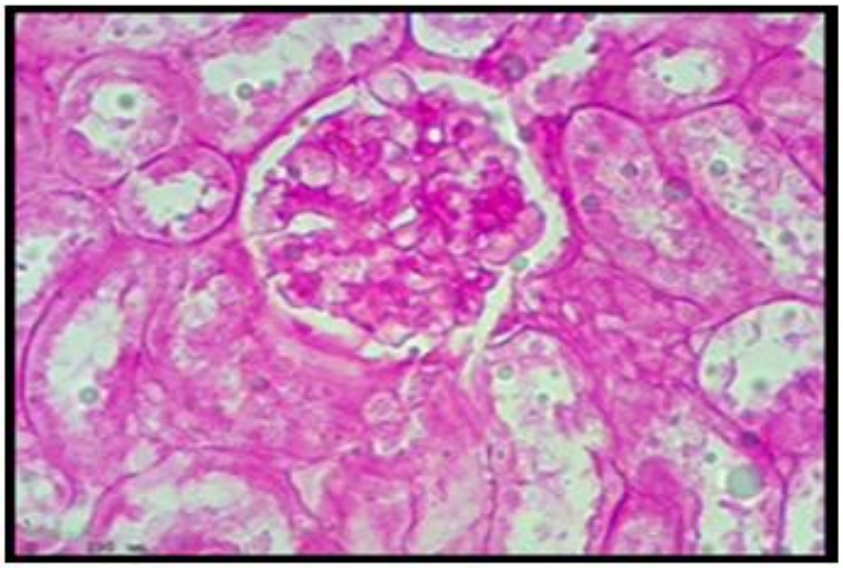

Figure (7): Histochemical section in the kidney of group I (control) showed normal architecture of renal parenchyma without presence of any deposition of abnormal glyco-proteinaceous materials. PAS stain. 40X.

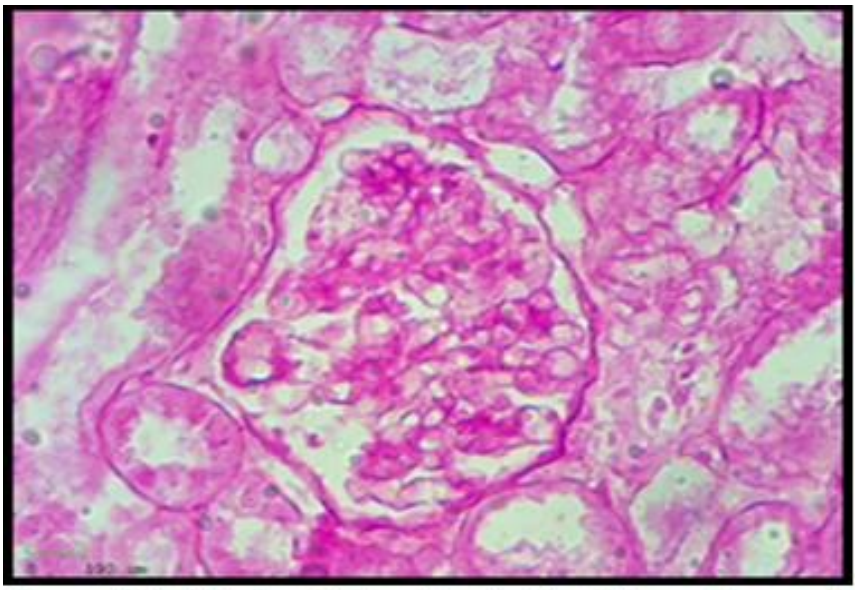

Figure (8): Histochemical section in the kidney of group I (control) showed normal architecture of renal parenchyma not obvious a deposition of abnormal glyco - proteinaceous materials. PAS stain. 40X.

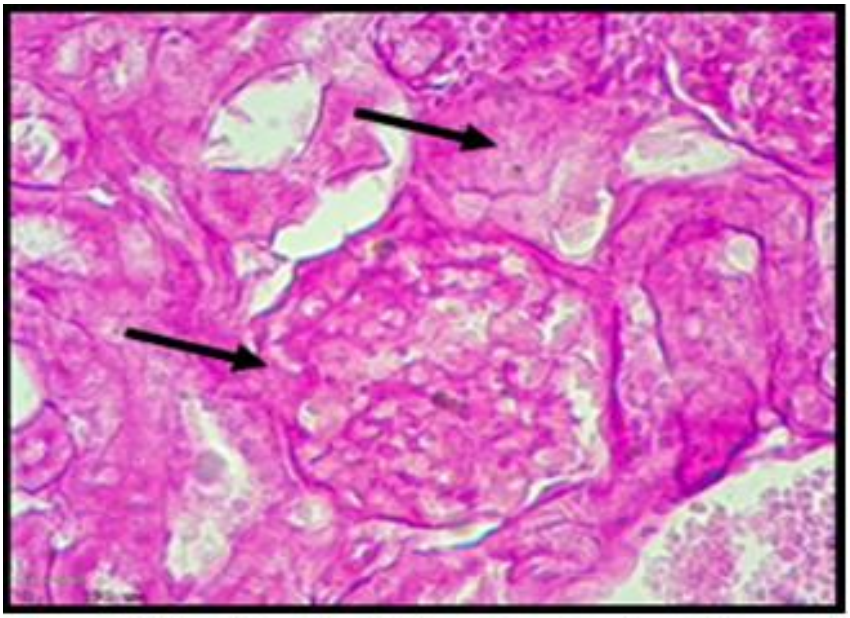

Figure (9): Histochemical section in the kidney of group II (DM) showed moderate deposition of glyco-proteinaceous materials in glomerulus and around renal tubules (black arrow). PAS stain. 40X.

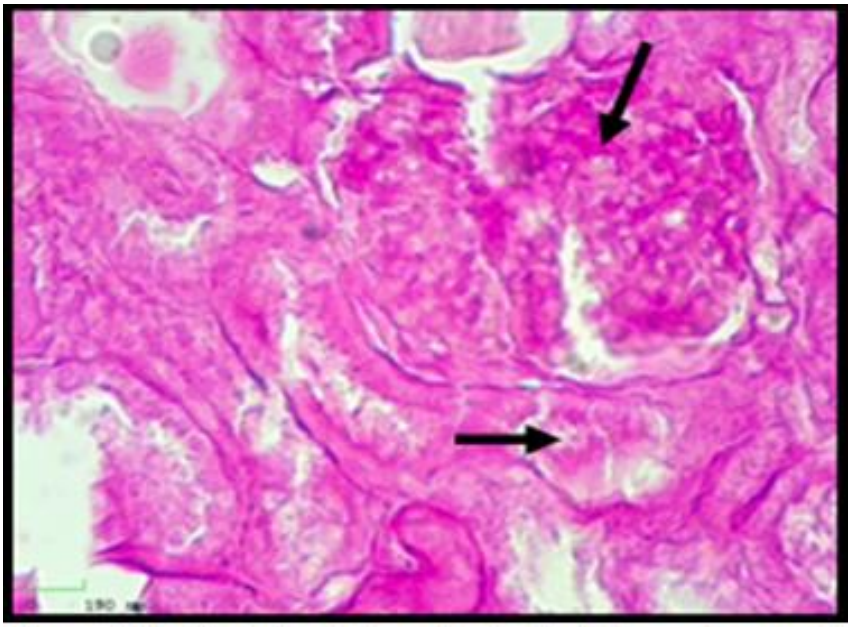

Figure (10): Histochemical section in the kidney of group II (DM) showed obvious deposition of glyco-proteinaceous materials in glomerulus and around renal tubules (black arrow). PAS stain. 40X.

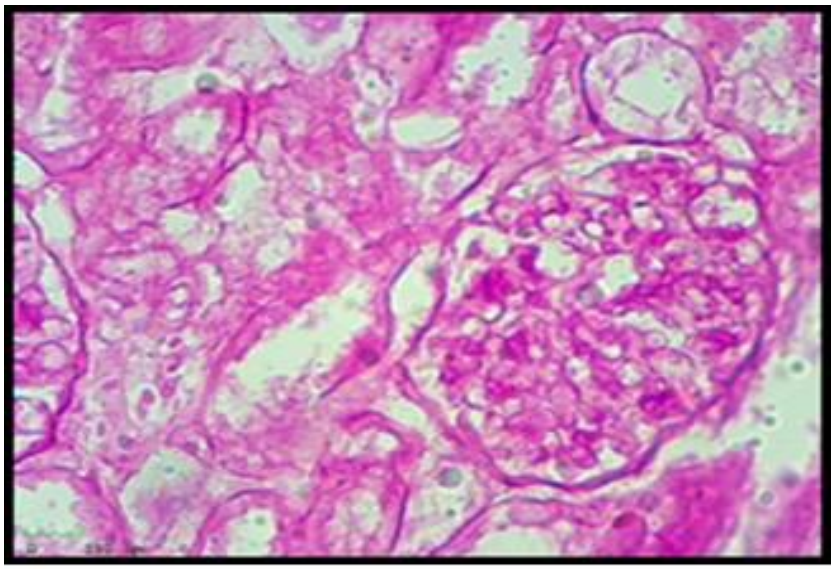

Figure (11): Histochemical section in the kidney of group III (DM+ALA) showed clearance of renal parenchyma from any deposition of abnormal glyco-proteinaceous materials. PAS stain. 40X.

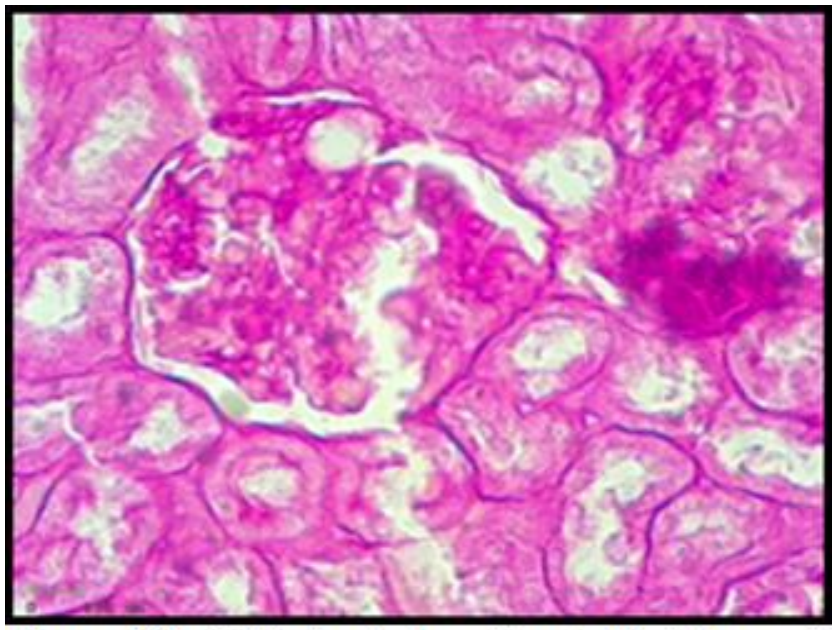

Figure (12): Histochemical section in the kidney of group III (DM+ALA) showed clearance of renal parenchyma from the deposition of abnormal glyco-proteinaceous materials. PAS stain. 40X.

DISCUSSION

It was obvious that hyperglycemia leading to oxidative stresses, which consider as main causations of DM complications, in addition these oxidative stresses, play crucial roles in developments of DM complications like nephropathies, that resulting from augmenting the reactive oxygen species generation via NADPH oxidase (17). These ideas was agreed with the present investigations that showed a significant ($P \leq 0.05$) decreasing in antioxidant values like GSH that may as a result of diabetes enhanced severe lipid peroxidation by generation of free radicals, where excessive lipid peroxidation caused an increased in GSH consumption that leaded to significant ($P \leq 0.05$) decreased of GSH in group II ; also when diabetes increased the lipid peroxidation that leaded

to decreased the activities of other antioxidants enzymes like CAT and SOD in fact that these enzymes responsible to work together which CAT hydrolyzed H_2O_2 that formed by superoxide anion disputation via activity of SOD, theses investigation may agreed with the results that mentioned by (18) who reported that a significant increased in hepatic lipid peroxidation resulted in a significant decreases in hepatic antioxidants including SOD and CAT activities due to alloxan induce diabetic animals.

These diabetic group (group II) also showed significant ($P \leq 0.05$) increased of value of MDA and peroxynitrate that may due to excess formation of free radicals as reactive oxygen species ROS and reactive nitrogen species RNS caused damages to the cellular membranes of vital

organs like liver resulted in peroxidation of bi-layers lipid membranes then caused cellular dysfunction especially when the MDA regarded as a main end-product of free radicals action in fatty acids membranes, these investigation may agreed with the idea that mentioned by (18) who documented that the free radicals can form peroxynitrite which is a potent oxidant caused cellular damages, in addition, MDA is the major end-product of free radical reaction on membrane fatty acids that resulting in structural alterations of membranes, loss of essential fatty acid which able to form peroxide products including MDA (20).

In other hand, the group III (DM+ALA) showed non-significant ($P>0.05$) differences of antioxidant enzymes like GSH and SOD as well oxidant parameters as MDA and peroxynitrate, these may due to the ALA increased the scavenging of free radicals that peroxide the cellular components in association with diabetes in this group, which can augment the values of GSH and SOD as well CAT that appeared superior to those values in diabetic group, this study suggest that the ALA had a synergic effect with self antioxidant enzymes to reduce free radicals formation by increased it scavenging from the body, these results agreed with the reported results of (21) they mentioned that ALA had a direct action against free radical and regarded as a scavenger, also ALA induced decreasing in lipids peroxidation of livers, pancreases, kidneys, brains and blood vessels of DM animals as well in blood of patients of DM neuropathies therefore, ALA appeared in prevents DM evoked by disturbance in antioxidant enzymes homeostasis in blood, liver and

kidney, then spontaneously decreased the MDA and peroxynitrate formation.

The results of kidney function tests showed significantly ($P \leq 0.05$) increases in the values of creatinine and BUN of the group II (DM group) that may due to the oxidative stress radicals could caused a pathological disorders to the nephrons which it un able to filtrate the protein end products properly then that causing these elevation in the renal biomarkers, these investigation may agreed with (22) that mentioned it has been shown that high blood concentrations of BUN and creatinine in rabbits are usually associated with renal disease, also in clinical diabetic nephropathies, tests for creatinine and BUN are routines to confirm the kidney's involvement and to assess the associated pathology. Also the results showed non-significant ($P > 0.05$) differences in the values of creatinine and BUN between control and the group III (DM+ALA) that may the alpha lipoic acid act as a scavenger to free radicals then caused marked improvement in the nephrons function especially when compared these idea with the current results of alpha lipoic acid as antioxidant for ROS and RNS in this study, moreover, these may agreed with the results of (23) when they mentioned that antioxidants protect cell and tissue injury against ROS and free radicals damaging effect, therefore, alpha lipoic acid can prevent the damage of renal brush borders in proximal tubules.

The histopathological results of group II (DM group) showed a present of nephropathy associated with infiltration of glyco-proteinaceous materials in the parenchyma of kidney, these may due to the diabetes impaired the function of capillaries (glomeruli) to filtrate the blood

components by reduced glomerular filtration rate (GFR) especially when the diabetes causing endothelial dysfunction by slowing and disrupt the blood flow as a result of increased thickness of basement membranes of Bowman capsule causing disturbances in GFR and then disturbed re-absorption of renal tubules that leaded to accumulation of these glyco-proteinaceous materials in the renal parenchyma as a sequels to diabetes in addition showed degrees of vacuolation in glomeruli and tubules, these idea may agreed with (24) who mentioned that the endothelial function often impaired in diabetic patients, who at high risk for vascular disease, therefore, the severity of glomerular damage is proportional to GFR value and blood glucose regulation as well the main pathological changes in DM nephropathies includes thickening of basement membranes of glomeruli, mesangial vacuolation, diffuse glomerular sclerosis and hyalinosis of renal blood vessels, then developed to appearance of micro-albuminuria.

The histopathological results of kidney of group III (DM+ALA) showed present of normal appearance of glomeruli and other renal tubules as well there weren't present of abnormal proteinaceous materials, these may due to the dual actions of ALA in scavenging of free radicals and to improved the pathway of insulin metabolism and decreased it peripheral resistance then it reduced the micro-complications on capillaries, renal GFR and tubules of diabetic animals, these results may agreed with the results reported by (25) who mentioned that ALA seem to be promised in term of DM therapy, which reported that ALA doesn't showed effective as antioxidant only but also it seem excellent regulator agent of glucose metabolism and prevent insulin resistance in DM type-2

and lead to decrease risk of micro-albuminuria, that appeared to be most interests DM therapy. Moreover, the recently reports indicated that ALA had

direct binding sides at insulin receptor tyrosine-kinase domain, therefore, ALA can applicant it in diabetes therapy (26).

In conclusion, the ALA has a dual actions to prevent the nephropathy complication by scavenger of oxidative stress initiated via free radicals and decreasing the diabetes complications risk by enhances insulin metabolism as well decrease it peripheral resistance.

التقييم المرضي الوظيفي للدور الوقائي لحامض اللايبويك ألفا على الاعتلال الكلوي السكري في ذكور الأرانب

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الخلاصة

لغرض تقييم التأثير الوقائي لحامض اللايبويك ألفا على الاعتلال الكلوي بفعل الجهد التأكسدي المصاحب لداء السكري، أجريت هذه الدراسة على 18 أرنب ذكر بالغ حيث قسمت إلى 3 مجاميع متساوية، المجموعة الأولى اعتبرت مجموعه سيطرة؛ المجموعة الثانية تم استحداث داء السكري فيها باستخدام 150 ملغم/كغم وزن الجسم من ماده الالوكسان؛ المجموعة الثالثة تم استحداث داء السكري فيها باستخدام 150 ملغم/كغم وزن الجسم من ماده الالوكسان وعولجت ب 10 مغلم/كغم وزن الجسم من حامض اللايبويك ألفا حقنا بالخلب ولمده 4 أسابيع.

أظهرت النتائج الكيموحيويه وجود انخفاض معنوي في مستويات أنزيمات GSH, CAT, SOD بالاضافه إلى وجود ارتفاع معنوي في MDA و Peroxynitrate و الكرياتينين و BUN في المجموعة الثانية (مجموعه السكري) عند مقارنتها مع مجموعه السيطرة. بينما أظهرت نتائج المجموعة الثالثة (مجموعه السكري مع حامض اللايبويك ألفا) إلى عدم وجود أي فروقات معنوية في مستويات GSH , SOD, MDA و Peroxynitrate و الكرياتينين و BUN عند مقارنتها مع مجموعه السيطرة.

أشارت النتائج النسجية المرضية النسجيه الكيميائيه للكلية في المجموعة الثانية إلى وجود تثخن في الغشاء القاعدي للكبيبات مع انضغاط ألاوعيه الدموية بالاضافه إلى ارتشاح مواد شبيه بالبروتينيه السكريه في الكبيبات

وداخل وحول النبيبات الكلوية مع تفجي متوسط الشدة في الخلايا الميزنكيه والنبيبات الكلوية؛ بينما أشارت النتائج

في كليه المجموعة الثالثة إلى تركيب طبيعي للكلية ومحتوياتها مع وجود حالات خفيفة جدا من التفجي لبعض النبيبات الكلوية بينما لم توجد ارتشاحات للمواد البروتينيه السكريه في هذه المجموعة. نستنتج من خلال هذه الدراسة إن لحامض اللايبويك ألفا دورا مزدوجا لحماية الكلية من خلال أزاله التأثير التأكسدي للجذور الحرة و تحسين أيض هرمون الأنسولين وبذلك له القابلية على تقليل الاعتلال الكلوي.

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