Histopathological Study of Liraglutide on Renal Deterioration Progression Induced by Doxorubicin

Hayder Hussein Luaibi*, Mustafa Ghazi Alabbassi, Israa Burhan Raoof***
Department of Clinical Laboratory Science, College of Pharmacy, Mustansiriyah University / Iraq
****Department of Pharmacology and Toxicology***

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Corresponding Author email:
pharm.mustafa-alabbassi@uomustansiriyah.edu.iq
orcid: https://orcid.org/0000-0001-8923-0264

Abstract:

Podocyte injury is a major factor in many renal diseases leading to proteinuria that causes the risk of developing kidney deterioration. Glomerulosclerosis and tubulointerstitial fibrosis are the main histopathological feature as consequence of inflammation and apoptosis a result of long period of tubular protein load. Liraglutide an incretin hormone (GLP-1) analogue has effective as glycemic control in patient with type 2 diabetes. In recent years, liraglutide appear protected mechanism against inflammation and apoptosis for many tissues through GLP-1 receptor (GLP-1R) activation unrelated with glycemic control. 36 animal Wister rats used in this experiment, first group include 12 rats set as control group received just the normal saline, while second group include 24 rats induced podocyte injury by doxorubicin single dose and third group treated either normal saline or liraglutide (200 µg /kg/day I.P) for 28 days. Histopathological study is used to assess the protected effect of liraglutide on podocyte injury induced in male rats through three main histopathological changes (glomerulosclerosis, tubular damage, inflammatory infiltration) by Hematoxylin and eosin staining. In this study treatment group(C) with liraglutide appeared significant decreased ($P< 0.05$) in glomerulosclerosis, tubulointerstitial, and inflammatory infiltration after 28 day of treatment. In conclusion, liraglutide is effective in reducing inflammation and apoptosis which associate with chronic renal development as well as renoprotection by glycemic control which is indirectly effect.

Key words: Glomerulosclerosis, liraglutide, podocyte injury, tubulointerstitial inflammation.

دراسة التشريح المرضي لدواء الليراغلوتايد على تطور التدهور الكلوي المستحث بالدوكسوروبيسين

حيدر حسين لعيبي* .مصطفى غازي العباسي .أسراء برهان رؤوف***
**** فرع العلوم المختبرية السريرية/كلية الصيدلة -الجامعة المستنصرية***
******فرع الادوية والسموم/كلية الصيدلة -الجامعة المستنصرية***

الخلاصة:

تعد أصابة الخلية podocyte injury عاملا رئيسيا لكثير من أمراض الكلى التي تؤدي الى ظهور بروتين البول وتشكل خطرا كبيرا في تدهور الكلى. يعتبر التصلب الكبيبي الكلوي والتليف الانبوبي السمة المرضية الرئيسة لهذه الإصابة نتيجة الالتهاب وموت الخلايا المبرمج الذي سببه بقاء البروتينات لفترات طويلة داخل الانبوب الكلوي .دواء الليراغلوتايد مشابه لهرمون (GLP-1) له تأثير فعال في السيطرة على نسبة السكر في الدم لدى المرضى الذين يعانون من مرض السكري من النوع الثاني . في السنوات الأخيرة ، أظهر الليراغلوتايد آلية حماية ضد الالتهاب وموت الخلايا المبرمج لكثير من الأنسجة من خلال تنشيط مستقبلات (GLP-1R) بألية ليست لها علاقة بالتحكم في نسبة السكر في الدم. ٣٦ جرذ من نوع

ويستر تم استخدامها في هذه التجربة ،المجموعة الاولى شملت ١٢ جرذ كمجموعة سيطرة وتتلقى فقط المحلول الملحي ، في حين المجموعة الثانية شملت ٢٤ جرذ باصابة (podocyte) بجرعة واحدة من دوكسوروبيسين والمجموعة الثالثة تعطى إما محلول الملحي أو ليراغلوتيد (٢٠٠ ميكروغرام / كغ / يوم) داخل الصفاق لمدة ٢٨ يومًا . تُستخدم دراسة التشريح المرضي لتقييم تأثير الليراغلوتايد في الحماية من الأثار المترتبة بعد اصابة(podocyte injury) في ذكور الجرذان من خلال تقييم ثلاثة متغيرات رئيسية في التشريح المرضي (تصلب الكبيبات والتلف الأنبوبي والتسلل الالتهابي) بواسطة صبغة (H&E). في هذه الدراسة مجموعة المعالجة بالليراغلوتيد تظهر انخفاض واضح في تصلب الكبيبات ، تسلل الأنبوب الخلالي ، والالتهابات بعد ٢٨ يومًا من العلاج. الاستنتاجات ،الليراغلوتايد فعال في الحد من الالتهاب وموت الخلايا المبرمج مع تطور مرض الكلى المزمن بالاضافة الى حمايته للكلى بشكل غير مباشر عن طريق التحكم في نسبة السكر في الدم.

الكلمات المفتاحية: تصلب كبيبات الكلى , الليراغلوتايد ,أصابة خلية الخلاء ,التليف الانبوبي.

Introduction

Podocyte injury is a major factor in many renal diseases leading to proteinuria that causes the risk of developing kidney deterioration [1]. The glomerular filtering barrier (GFB) is composed of three crucial parts endothelium, glomerular basement membrane (GBM) and podocytes. Renal tubular absorption, secretion and final composition of urine are mainly depending on the stability of GFB [2]. Podocytes are terminally separated natural cells that adhere strongly to GBM and supply epithelial insurance coverage to the surface of glomerular capillaries[3].it is composed from system of connected fiber protein and attached each other by slit diaphragms that is consider avital part for filtration selectivity in glomerular filtration barrier (GFB) system [4] .In addition, podocyte have a role in maintenance of GBM integrity by regulating turnover of extracellular matrix, intracapillary pressure, endothelial modulation and mesangial cell by vascular endothelial growth factors (VEGF)[5]. Podocyte injury is the major contributor of proteinuria in diabetic nephropathy pathogenesis which mostly created by excessive formation of reactive oxygen species [6,7]. Several Study showed the inverse correlation between Podocyte number and proteinuria in patient with early diabetic type 1&2[8,9]. Long period of protein load in the tubule especially albumin is the major factor that responsible of renal deterioration [10]. One of the suggested mechanisms for the harmful effects of proteinuria is the high albumin loaded in the renal tubule stimulating epithelial cells to generate fibrogenic and pro-inflammatory molecules [11]. Furthermore, albumin toxicities which induce to conjugate fatty acid with abumin in the lumen viewd high levels of fatty acids an essential factor of tubulointerstitial injury and albumin overload [12]. Glomerulosclerosis and tubulointerstitial fibrosis are the main histopathological feature in progression of chronic kidney disease characteried by exceessive deposition of protein in the extracellular matrix (ECM) of interstitial tubule and glomeruli as consequence of inflammation and apoptosis as result of long period of tubular protein load which ended mainly with renal failure [13,14]. Myofibroblast is the main sources of ECM in the tissue scar which is express α-smooth muscular tissue actin (α-SMA) on surface[15] . It was found mainly in the interstitium come from fibroblasts and pericytes in the interstitium[16]. Significant cellular changes in tubulointerstitial fibrosis includes:peritubular infiltration of inflammatory cell ,myofibroblast activation and ECM accumulation and finally tubular atrophy [17]. In vitro model, incubated proximal tubule cells with high concentration of albumin can stimulate profibrotic proinflammatory molecules TNF-alpha,TGF-β, MCP-1 and interleukin-8 (IL-8)[18]. These pro-inflammatory cell recruitment the signal of inflammation by chemtotactic inflam-matory molecule from vasular such as lymphocytes, monocytes/macrophages, dendritic cells (DCs) as well as mast cells

caused increased oxidative stress and fibrogenic cytokines with growth factors [18] Complying with profibrotic cytokine and mechanical tension, the interstitial fibroblasts undergo phenotypic change in to myofibroblasts by expressing αSMA, as well as they begin to create a huge amount of ECM components, when stimulated with cytokines, both myofibroblasts and fibroblasts have the capability to proliferate [19]. The compostion of extracelluar matrix protein are Fibronectin and type1 and 3 of collagen[20].doxorubicin as toxic agent used for induction of podocyte injury by direct effect on podocyte with proteinurea after 48 hour and peak after 14 day [21]. Liraglutide as GLP-1 analogue used a glycemic control by insulin secretion in response of GLP-1R activation [22]. Regarding of hypoglycemic role, recent studies explored that GLP-1 is anti-inflammatory, antioxidative, neurogenerative and vascular protective in the kidney, it is influenced on the different cellular pathway with protect against apoptosis sequence [23-25].

Material and Methods:

Thirty-six of wister rats with age over eight weeks were used and placed in the animal house of pharmacy college in Al - Mustansiriyah university after applied ethical committee. Each four animal was kept in the plastic cage with dimensions (20x25x35cm) and colored for distinguished. Acclimatization of animals at room temperature 20-22 ^{0}C and normal dark / light for 1 week in addition to animal were checked by general examination before beginning. Rats were divided into three groups (12 rats / group), see table (1) for protocol of research design

Table (1): The protocol of research design

Group	No.	Treatment	Duration
A (control)	12	Single dose 0.5ml/kg normal saline intraperitoneal wait for 14 days then for four weeks.	Six weeks
B (Induction)	12	Single dose 7.5mg/kg of doxorubicin administered intraperitoneal wait for 14 days then administered 0.5ml/kg normal saline intraperitoneal for four weeks	Six weeks
C (treatment)	12	Single dose 7.5mg/kg of doxorubicin administered intraperitoneal wait for 14 days then received 200µg/kg/day intraperitoneal liraglutide in 0.5ml/kg normal saline four weeks.	Six weeks

At the end , animal aneasthesized with ketamine and xylazine as coctail in dose (100 mg/kg ketamine plus 10 mg/kg xylazine) injected intramuscular and kidneys removed and euthanized by decapitation and placed tissue in formalin10% as neutral buffer .finally, pass tissue in process and paraffin ended with tissue block then section with ((4um) thickness and staining with haematoxyllin & eosin . Renal histology is assessed according to the modified scoring system [26] at x40 magnification using an Olympus BX-41 microscope (Olympus optical

co.Ltd.,japan) for three models (glomerulosclerosis , tubulointerstitial damage and inflammatory infiltration), illustrated in the Table (2) :

Table (2) [26]: scoring of renal morphology.

Scoring	Glomerulosclerosis	Tubular damage	Inflammatory infiltration
0	Normal glomerulus	Normal tubules and interstitium	Normal glomeruli, tubule and interstitium
1	Thickening of the basement membrane	Brush boarder loss or tubular dilation in < 25% of the field view (fv)	Inflammatory cell < 25% fv
2	mild<25%	Tubular dilation, atrophy and cast in < 50% fv	Inflammation < 50% fv
3	Severe segmental >50%	Tubular and interstitial damage < 75% fv	Inflammation < 75%
4	Diffuse hyalinosis and collapse	Tubular atrophy, dilation, cast and fibrosis in >75% fv	Inflammation > 75%

- Glomerular score for each animal as the mean of 100 glomeruli.
- Tubular was assessment as score of mean for 10 field view (fv) in semi quantitative way.
- Inflammatory score as mean of 10 field view (fv)

Statistical analysis

Data was analyzed by multiple software program like medical, microsoft excel 2010 and statistical packages for social sciences (Spss-23). It was including Mean± Standard error, mean value compared between groups by ANOVA followed by Tukey test [27]. P-value expressed as significant when it was equal to or less than 0.05 and highly significant when it was equal to or less than 0.01 [28].

Results :

Histopathological study is used to assess the effect of liraglutide on podocyte injury induced in male Rats through three main features (glomerulosclerosis, tubular damage, inflammatory infiltration). Hematoxylin and eosin were used for staining, all data of scoring was expressed as M±SEM. In glomerulosclerosis score, induction group(B) appeared significant increased (P< 0.05) in thickness of basement membrane of glomerulus as compared with control(A) and treatment(C) groups while after 28 days of liraglutide treatment group(C) was showed significant decreased (P< 0.05)) in the thickness of basement membrane of some animal group when compared with induction group(B) as positive control however mean value of treatment group still significantly higher (P< 0.05) as comparison with control group (A), Table (3) figure (1,4,5,6). In the other hand, tubular damage score in the group B showed significant increased (P< 0.05) deterioration in the tubule as cast ,tubular atrophy and tubular damage when compared with other groups while group C appeared significant improvement (P< 0.05) than group B after 4 week of I.P liraglutide treatment however mean value of group C still significantly higher(P< 0.05)as comparison with group A, Table (3) and Figure (2,4,5,6).

Inflammatory infiltration concen-tration in the group B was noticed significant increased (P< 0.05) as compared with other groups , while treatment with I.P liraglutide for 28 days was appeared significant decreased (P< 0.05) in the inflammatory cell than group B but group C still significant higher (P< 0.05) as

comparison with group A, Table (3) , Figure (3,4,5,6).

Table (3): Histopathological scoring of glomerulosclerosis, tubular damage, and inflammatory infiltration.

A. Glomerulosclerosis						
Score of histopathology		0	1	2	3	4
Group A	N=10	10	0	0	0	0
	M±SEM	0 [b,c]				
Group B	N=10	0	9	1	0	0
	M±SEM	1.10± 0.1 [a,c]				
Group C	N=10	3	7	0	0	0
	M±SEM	0.7±0.15 [a,b]				
B. Tubular damage						
Score of histopathology		0	1	2	3	4
Group A	N=10	10	0	0	0	0
	M±SEM	0 [b,c]				
Group B	N=10	0	0	1	6	3
	M±SEM	3.20± 0.20 [a,c]				
Group C	N=10	0	4	6	0	0
	M±SEM	1.6± 0.16 [a,b]				
C. Inflammatory infiltration						
Score of histopathology		0	1	2	3	4
Group A	N=10	10	0	0	0	0
	M±SEM	0 [b,c]				
Group B	N=10	0	0	4	5	1
	M±SEM	2.40± 0.22 [a,c]				
Group C	N=10	0	5	5	0	0
	M±SEM	1.50± 0.16 [a,b]				

Data expressed as Mean± Standard error of mean (SEM), Group A = control, Group B = induction, Group C = treatment. Number of animals for each group = 10. [a] Significantly different when compared with group A at P < 0.05.[b] Significantly different when compared with group B at P < 0.05.[c] Significantly different when compared with group C at P < 0.05.

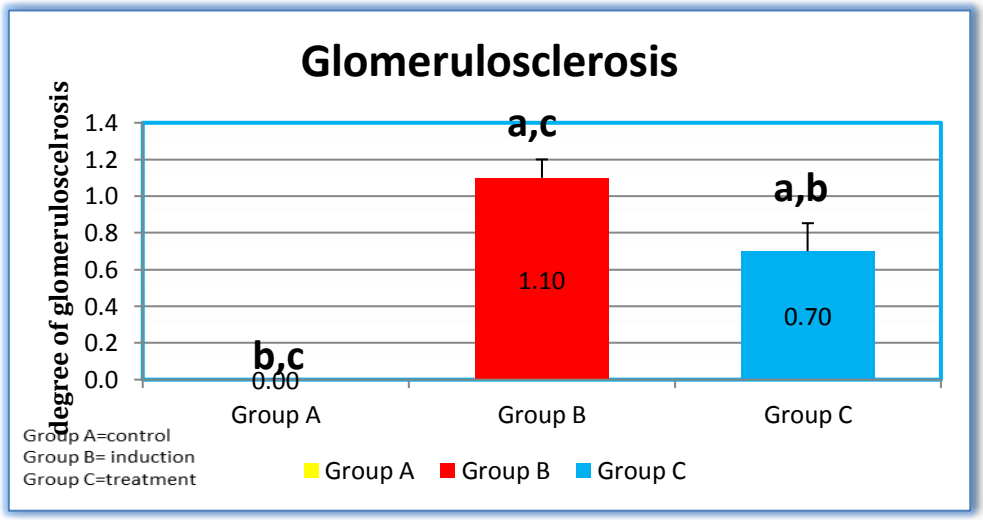

Figure (1) : Bar chart display the differences in the mean of glomerulosclerosis score for all studied groups, data expressed as mean ±SEM. a Significantly different when compared with group A at P < 0.05,b Significantly different when compared with group B at P < 0.05, c Significantly different when compared with group C at P < 0.05.

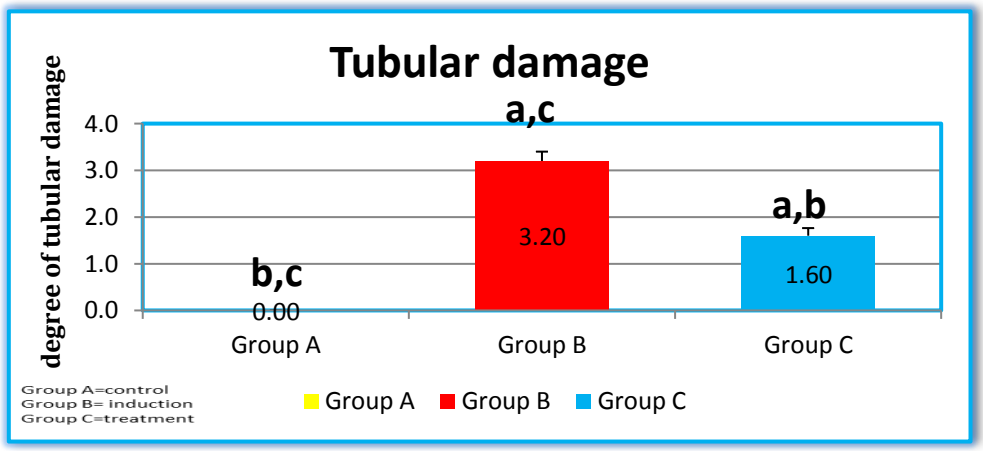

Figure (2): Bar chart display the differences in the mean of Tubular damage score for all studied groups, data expressed as mean ±SEM. [a] Significantly different when compared with group A at P < 0.05,[b] Significantly different when compared with group B at P < 0.05, [c] Significantly different when compared with group C at P < 0.05.

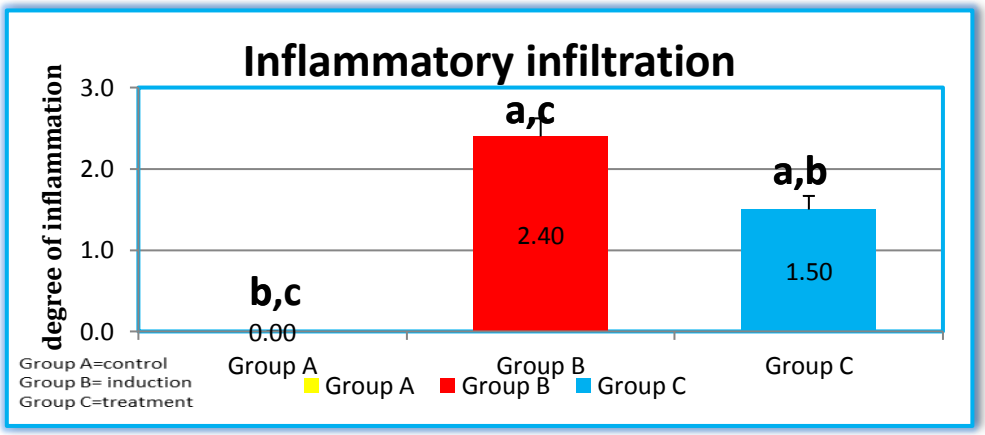

Figure (3): Bar chart display the differences in the mean of inflammatory infiltration score for all studied groups, data expressed as mean ±SEM. [a] Significantly different when compared with group A at P < 0.05,[b] Significantly different when compared with group B at P < 0.05, [c] Significantly different when compared with group C at P < 0.05.

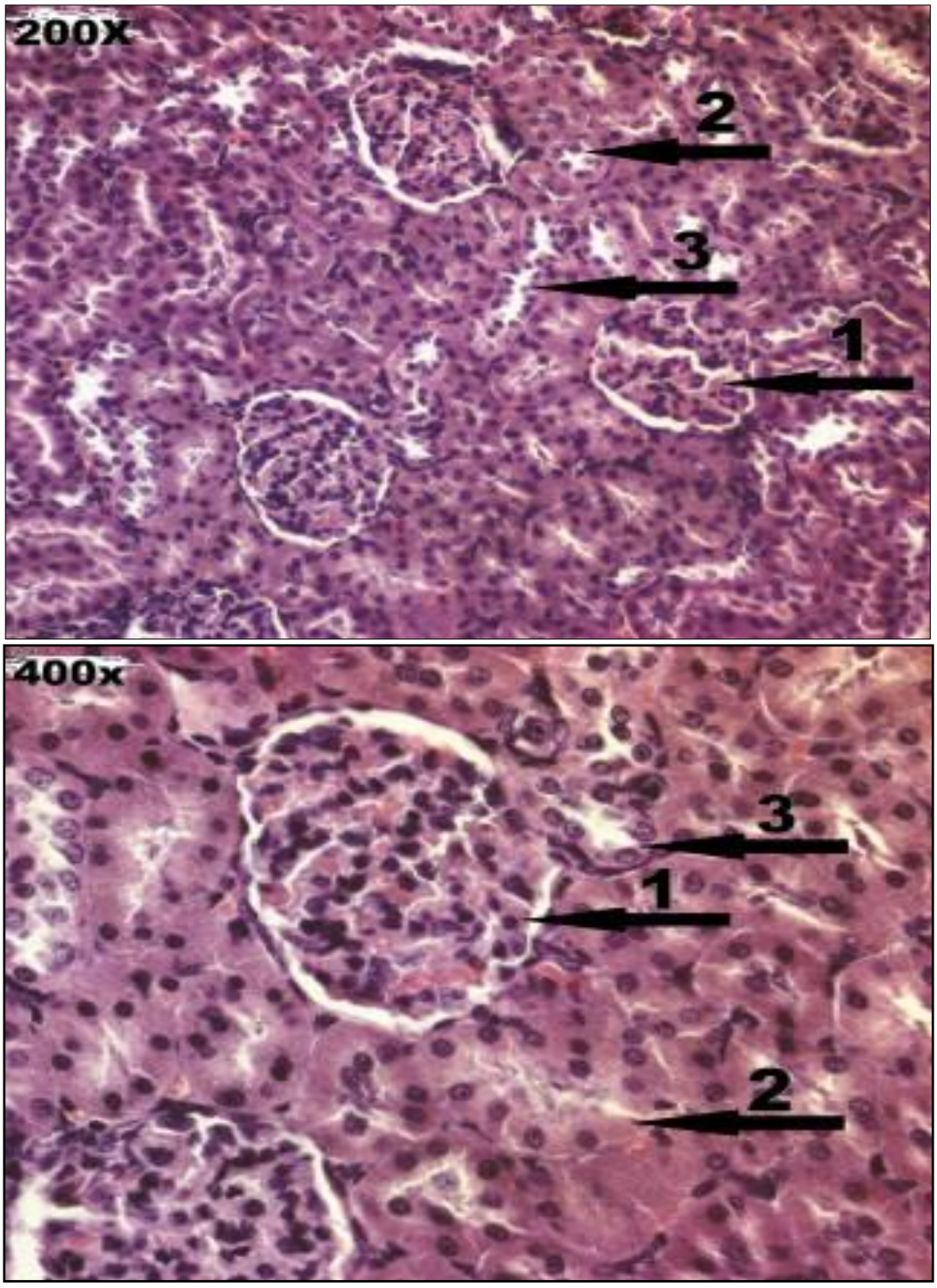

Figure (4): light microscopic section of group A stained by by H &E which apparently normal black arrow (show 1: glomerulous, 2: proximal tubule, 3: distal tubule) at magnification 200X, 400x respectively.

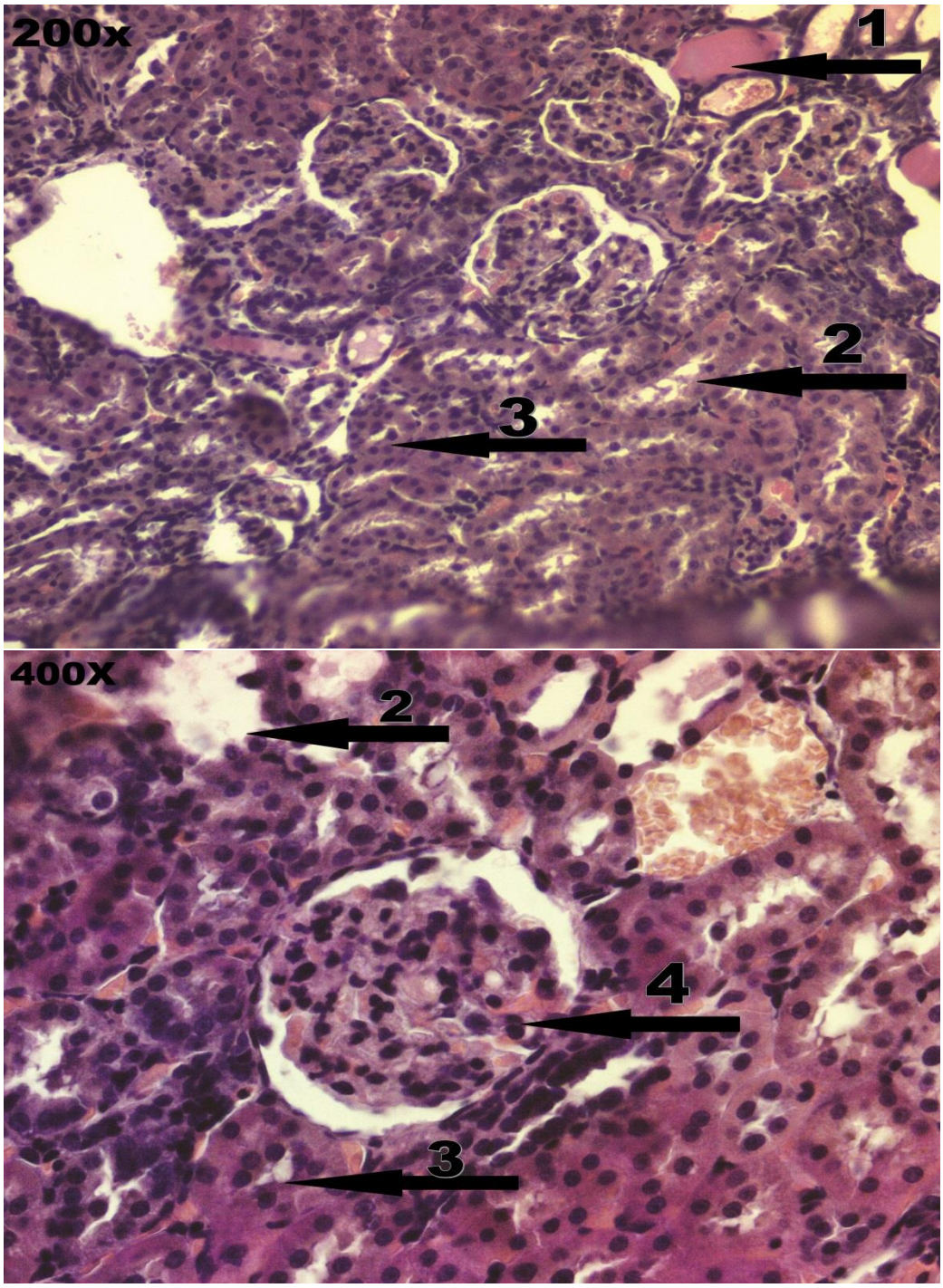

Figure (5) : light microscopic section of group B stained by by H &E which show score 1 as thickening in the basement membrane for glomerulous (arrow 4)and score 3 for tubular damage as cast (arrow 1),tubular dilation (arrow 2)and tubular atrophy (arrow 3) in < 75% of field view at magnification 200X, 400x respectively.

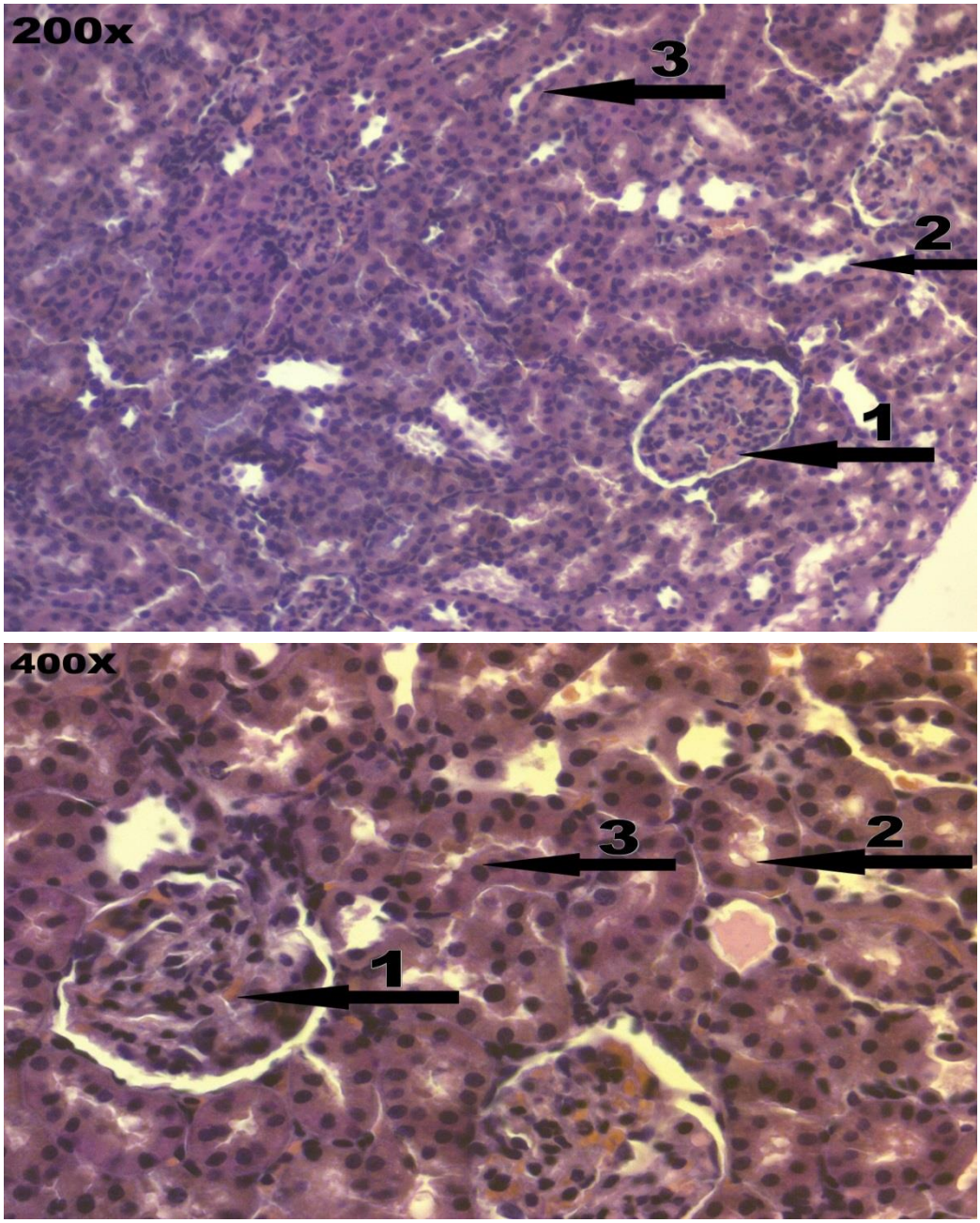

Figure (6): Light microscopic section of group C stained by by H &E which show score 1 as thickening in the basement membrane for glomerulous (arrow 1)and score 2 for tubular damage as some loss brush boarder tubular dilation (arrow 2) in < 50% of field view and some show brush boarder present (arrow 3)at magnification 200X, 400x respectively.

Discussion

Glomerulosclerosis and tubulointerstitial damage are the main feature of CKD progression, different stimulation of these deterioration is reflected by Podocyte injury [29]. histological changes in the glomeruli starting from defect in podocyte number with increase in the thickening of GBM which is the first step for glomerulosclerosis, followed by mesangial expansion and accumulation of ECM ended with haylinosis, these changes can exhibit with many diseases especially in diabetic nephropathy along with several years of disease [30]. The tubulointerstitial playing an essential role in progression

pathway consist of eighty percent of renal [31]. Tubular proteinuria is the one of many stimuli that cause injury for this region as consequence of loss in podocytes [32]. Histological change of (tubular dilation, tubular atrophy and cast, inflammatory infiltration, interstitial damage and fibrosis) were used to determine the tubulointerstitial injury [33]. In this study, Doxorubicin as toxic agent used to simulate these changes as model for CKD in male rats, in histologically, glomerulosclerosis was appeared thickness in the basement membrane and advance stage for tubulointerstitial injury with approximately 70% of field with intense of inflammatory infiltration. The study does not appear complete destruction and even fibrosis because it takes at least 3 months of induction, so the evaluation of treatment depends on the improvement in the parallel induction by doxorubicin model. Administration of liraglutide as treatment for induced glomerulopathy revealed a significantly reduced of glomerulosclerosis and tubular damage score in addition to reduce inflammatory infiltration in renal tissue that agree with Fujita H et al. study(2014) who was found that GLP-1 receptor in the glomeruli has a role in the reduction mesengial expansion and albuminurea when treated with liraglutide as agonist for this receptor [24] and line with Katagiri D *et al.* study (2013) who was reported the effectiveness of GLP-1 in the amelioration of tubular injury induced by cisplatin through upregulation these receptor in proximal tubule [34] and corresponds with Li K.Y *et al.* (2018),who was illustrated the effect of liraglutide in CKD progression through attenuation of renal fibrosis[35].These results compatable with the protective effect of liraglutide on renal tissue in diabetic nephropathy progression [36-38] . however, some clinical cases exposed further study of AKI from treatment with exenatide and liraglutide [39,40]. Glomerulonephritis induced by doxorubicin is characterized by proteinuria, hypoalbuminaemia and dyslipidaemia. Proteinuria is observed after 48 hours of induction and peak is noticed after 14 day with significant increased levels of serum creatinine and blood urea [41]. Doxorubicin is administrated *in vivo* in related to several metabolites and according to generate reactive oxygen specieswhich induced nephrotoxicity by several mechanism such as lipid peroxidation , genomic DNA fragmentation , superoxide dismutase (SOD) and apoptosis [42,43].Also, Doxorubicin playing role as direct toxic effect on the renal which leading to glomerular congestion and acute tubular necrosis may complicated to renal dysfunction[44] . in this study, doxorubicin showed histological changes (tubular dilation, tubular atrophy and cast, inflammatory infiltration, interstitial damage) after 14 day of induction which that agree with previous studies on doxorubicin used as a model for CKD progression.

Conclusion:

Liraglutide showed protective effect on renal tissue deterioration induced by doxorubicin as model of injury by mechanism unrelated with glycemic control such as anti-inflammatory and anti-apoptosis effect.

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