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Original Article

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Continuous Erythropoietin Receptor Activator Ameliorates Diabetic Kidney Disease in Rats

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Abstract

Objective: Diabetic kidney disease (DKD) is still a significant cause of morbidity in patients with diabetes. In this study, we examined the potential effects of continuous erythropoietin receptor activator (CERA) on oxidative stress, inflammation, and the renin-angiotensin system in a rat model of DKD induced by streptozocin.

Materials and Methods: A single intraperitoneal injection of streptozocin (65 mg/kg) was used in male Sprague Dawley rats to induce diabetes. The rats were randomly divided into 4 groups (n=8/group): diabetic (group D), CERA (group CR), CERA-treated diabetic group (group D+CR), and the control group (group C). The oxidative stress biomarkers, renal function parameters, messenger-ribonucleic acid expression of renin-angiotensin system parameters, and kidney histology were investigated.

Results: Creatinine clearance was found to be increased and the urinary albumin-to-creatinine ratio decreased in group D+CR compared with that of group D. Serum malondialdehyde levels were significantly lower, and glutathione and glutathione peroxidase levels were significantly higher in group D+CR than those of group D. Serum interleukin-1β, tumor necrosis factor-α, and interferon-ɣ levels were significantly lower; and monocyte chemoaatractant protein 1 levels were significantly higher in group D+CR than those in group D.

Conclusion: CERA can protect against DKD, in part, and is related to the suppression of the renal oxidative and inflammatory response.

Keywords: Continuous erythropoietin receptor activator, diabetic kidney disease, inflammation, oxidative stress, experimental model, renin-angiotensin system

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INTRODUCTION

Diabetic kidney disease (DKD) is still a significant cause of morbidity in patients with diabetes, ultimately leading to end-stage renal disease (1). The increasing number of patients affected by DKD parallels the dramatic rise in the prevalence of diabetes, and DKD is considered to be a medical disaster worldwide (2). Development of DKD is associated with metabolic and hemodynamic changes that lead to glomerular hypertrophy, glomerulosclerosis, tubulointerstitial inflammation, and fibrosis. Activation of the renin-angiotensin system (RAS), oxidative stress, and proinflammatory cytokines increase in the course of diabetes and contribute to the appearance and improvement of DKD (3). The RAS plays a critical role in the evolution of DKD; therefore, the pharmacological blockade of the RAS has been recently used for the preservation of renal function in patients with DKD (4, 5). Oxidative stress and inflammation play a significant role in the pathogenesis of diabetes-induced renal injury. The mechanisms, such as dysfunction of the antioxidant defense system and an increase in reactive oxygen species (ROS) formation, are responsible for the increase in oxidative stress in these patients (6). The link between inflammation and the improvement of

diabetic nephropathy includes complicated molecular systems and processes (7).

The current therapeutic approach, unfortunately, only slows the progression of the disease but is not able to prevent diabetic renal insufficiency in clinical settings. Therefore, new therapeutic approaches are needed to prevent the progressive loss of renal function in patients with diabetes. Experimental data have pointed to oxidative stress, inflammation, growth factors, and cytokines as the targets of potential therapeutic interventions (8, 9).

Recent studies have shown that erythropoiesis-stimulating agents (ESAs) have a protective effect on many nonerythroid cells apart from the effects on erythroid progenitor cells. Recombinant human erythropoietin (epoetin) is one of the first molecules used to stimulate erythropoiesis. More recently, continuous erythropoietin receptor activator (CERA) has been developed. CERA has a considerably longer half-life, different receptor-linking features and pharmacology from other erythropoietic agents, and also slows clearance rate (10). Recent experimental data demonstrated that CERA has healing effects on diabetic renal injury (9, 11).

In this study, we aimed to explore the potential effects of CERA on inflammation, oxidative stress, and the RAS in a rat DKD model induced by streptozocin.

MATERIALS AND METHODS

Ethics

The Animal Ethics Review Committee of Yeditepe University School of Medicine approved this study (Approval Date: July 04, 2020; Approval Number: 2015-87), and the experiments were executed in compliance with the international guidelines on the ethical use of animals.

Animals and Experimental Design

In the study, we used male Sprague Dawley rats (n=32), weighing ~240-260 g (10-weeks-old), fed ad libitum, and housed in controlled conditions at a temperature of 22°C±1°C with a 12-hour light/dark cycle. We utilized separate metabolic cages for collection of 24-hour urine.

After an overnight fast, we gave the rats a single-dose streptozocin (Sigma Aldrich Co.; St Louis, MO, USA) intraperitoneally. We measured blood glucose levels after 48 hours using the Optimum Exceed test strip glucometer (Abbott Laboratories; Abbott Park, Illinois, USA) and considered glucose levels above 300 mg/dL as diabetes.

We administered 1-8 U insulin (Levemir; Novo Nordisk, Bagsvaerd, Denmark) to the diabetic rats for prevention of weight loss and ketotic status every day. At the end of the 4th week after the formation of diabetes, we divided the rats into 4 groups randomly (n=8/group).

1. The control group (group C): nondiabetic rats without streptozocin or any drug treatment
2. Diabetic group (group D): diabetic rats
3. CERA group (group CR): nondiabetic rats administered 50 µg/kg/biweekly CERA
4. CERA-treated diabetic group (Group D+CR): diabetic rats administered 50 µg/kg/biweekly CERA.

At the end of 12 weeks after randomization, all the rats were sacrificed by decapitation under anesthesia.

Renal Function Parameters

We used the immunoperoxidase assay to measure the urinary albumin levels (GenWay Biotech; San Diego, CA, ABD). We measured the serum and urine creatinine levels according to the manufacturer's instructions using clinical assay kits (COBAS Integra 400 Plus; Roche Diagnostic, Rotkreuz, Switzerland) and calculated creatinine clearance (Ccr) applying the following formula (12):

$$Ccr = \left[\frac{urinary\ Cr \times urinary\ volume}{serum\ Cr}\right] \times \left[\frac{1000}{body\ weight \times 1440}\right]$$

Oxidative Stress Parameters in Kidney

We homogenized the kidney samples in ice-cold 0.15 M KCl (10% w/vol) before all analyses. We determined malondialdehyde (MDA) levels according to the methods of Ohkawa et al. (13), and we used MDA to evaluate the degree of lipid peroxidation. We measured the kidney glutathione (GSH) levels (14), kidney superoxide dismutase (SOD) activities (15), and glutathione peroxidase (GSH-Px) activities (16) according to the recent studies using related procedures. We used bicinchoninic acid to determine the protein levels (17).

Serum Cytokines

We measured interleukin (IL)-1β, IL-4, interferon (IFN)-ɣ, monocyte chemoattractant protein (MCP)-1, and tumor necrosis fac-

Main Points

- Diabetic kidney disease is still a significant cause of morbidity in patients with diabetes, ultimately leading to end-stage renal disease.
- Activation of the renin-angiotensin system, oxidative stress, and proinflammatory cytokines increase in the course of diabetes and contribute to the appearance and improvement of diabetic kidney disease.
- Studies have shown that erythropoiesis-stimulating agents (ESAs) have a protective effect on many nonerythroid cells apart from the effects on erythroid progenitor cells.
- CERA has a renoprotective effect on DKD, demonstrated by the improvement in Ccr, reduced proteinuria, intrarenal oxidative stress, inflammation, and interstitial fibrosis.

tor (TNF)-α levels in serum using the rat cytokine kit (BMS826FF, E-Bioscience, Belgium).

Real-Time Polymerase Chain Reaction for Renin Receptor, Renin, Angiotensin Type 1 Receptor, and Angiotensin

We applied the method described previously for real-time polymerase chain reaction (RT-PCR) evaluations (18). We put 25 mg of the kidney tissue containing both cortex and medulla sections into ribonucleic acid (RNA) later (QIAGEN, Germany). Total RNA was isolated using the Roche High Pure RNA Isolation kit (Roche Diagnostics; Switzerland). Isolations were performed according to the manufacturer's instructions. The tissues were homogenized using magnetic pellet motor pestle (Sigma; USA). Supernatants containing nucleic acids were taken into 1.5 mL microcentrifuge tubes containing 200 µL of 100% ethanol. Samples were then moved into the spin columns and centrifuged at 13,000× g for 2 minutes. After washing, they were quantified in the NanoDrop spectrophotometer (Thermo Scientific; USA) before complementary deoxyribonucleic acid (cDNA) synthesis.

For single-strand cDNA synthesis, Transcriptor High Fidelity cDNA Synthesis Kit (Roche; Switzerland) was used according to the manufacturer's instructions. The quality of samples was evaluated using NanoDrop Spectrophotometer (Thermo Scientific; USA).

RT-PCR was performed using TaqMan Master Kit (Roche; Switzerland). Primers used in RT-PCR were angiotensin, angiotensin receptor 1a, renin, and renin receptor, and β-actin as internal control and were acquired from Roche as Realtime Ready Primers with an optimized annealing temperature of 60°C. Roche Light Cycler 480 was used to acquire the results with following PCR conditions: 10 minutes at 95°C, 95°C for 10 s, 60°C for 30 s, 72°C for 1 s for 45 cycles, and 40°C for 30 s. All samples were evaluated in triplets, and the results were analyzed on the Light Cycler 480 Analysis Program v5.0 by 2-ddCP relative quantification.

Kidney Histology and Morphometry

The dissected kidneys were fixed overnight with 4% formaldehyde in phosphate buffered saline (pH 7.2), processed, embedded in paraffin according to standard protocols, and sectioned at 4 µm. The slides were stained with hematoxylin and eosin and Massone Trichrome using standard histologic procedures. Glomerular sclerosis, interstitial fibrosis, and tubulointerstitial damage were evaluated in a blinded fashion by an experienced pathologist. Glomerular sclerosis was graded semiquantitatively in 4 grades: 0=normal glomerulus, 1±sclerosis involving less than 25% of the glomerular surface area, 2±sclerosis involving 25% to 50% of the glomerular surface area, and 3±sclerosis involving more than 50% of the glomerular surface area. Tubulointerstitial damage (tubulointerstitial inflammation, tubular injury, and interstitial fibrosis) was also scored semiquantitatively as described previously: 0=normal interstitium and tubules; 1±minimal injury (<25% of tissue section affected); 2±mild injury (25%–<50% of tissue section affected); 3±moderate injury (50% to 75% of tissue section affected); and 4±severe injury (>75% of tissue section involved). Histomorphometric analysis was performed on a Masson's trichrome stained slide at 100× magnification. For each case, 10 microscopic fields were randomly selected. Interstitial fibrosis volume was quantified using an Olympus BX 51 with a DP2-BSW Soft Imaging System (Olympus Co.; Hamburg, Germany) and expressed as μm^2 (18).

Statistical Analysis

For statistical data comparisons, IBM Statistical Package for Social Sciences version 22 for Windows (IBM SPSS Corp.; Armonk, NY, USA) was used. Analysis of variance and Bonferroni's tests were used to compare the means of continuous variables that were normally distributed and had equal variances. The Welch and Games-Howel tests were used to compare the means of continuous variables that were normally distributed and did not have equal variances. The Kruskal-Wallis and Mann-Whitney U tests were used to compare the medians of non-normally distributed continuous variables. All the values are given as mean±standard error of mean. Values of $p<0.05$ (two-tailed) were considered significant.

RESULTS

Assessment of Renal Functions and Hematological Parameters

The urinary albumin-to-creatinine ratio was significantly lower ($p=0.04$) and Ccr was significantly higher ($p=0.03$) in group D+CR than in group D. Furthermore, CERA had no significant effect on platelet count, hemoglobin levels, and the kidney-to-body weight ratio (Figure 1).

Assessment of Oxidative Stress Parameters in Kidney

There was a significant increase in the renal MDA levels in group D compared with group C ($p=0.00$), which indicates an excess lipid peroxidation in DKD. CERA protected the kidney from lipid peroxidation ($p=0.00$ for group D+CR vs. group D). Figure 2 depicts significantly decreased levels of kidney MDA and increased levels of kidney GSH and kidney GSH-Px activity in the treatment group ($p=0.00$, $p=0.02$, $p=0.00$, respectively, for D+CR group vs. group D). A decrease was observed in the SOD activities of the kidney tissue in the diabetic group, whereas it increased in the CERA-treated diabetic rats. However, these changes are not statistically significant.

Assessment of Inflammatory Cytokines in Serum

Serum IL-1β, IFN-ɣ, TNF-α, and IL-4 were significantly increased, and MCP1 levels were reduced in diabetic rats compared with those in control rats. CERA treatment decreased IL-1β, IFN-ɣ, TNF-α, and IL-4 and increased MCP1 levels. These changes were significant for MCP1 ($p=0.04$), IL-1β ($p=0.00$), TNF-α ($p=0.01$),

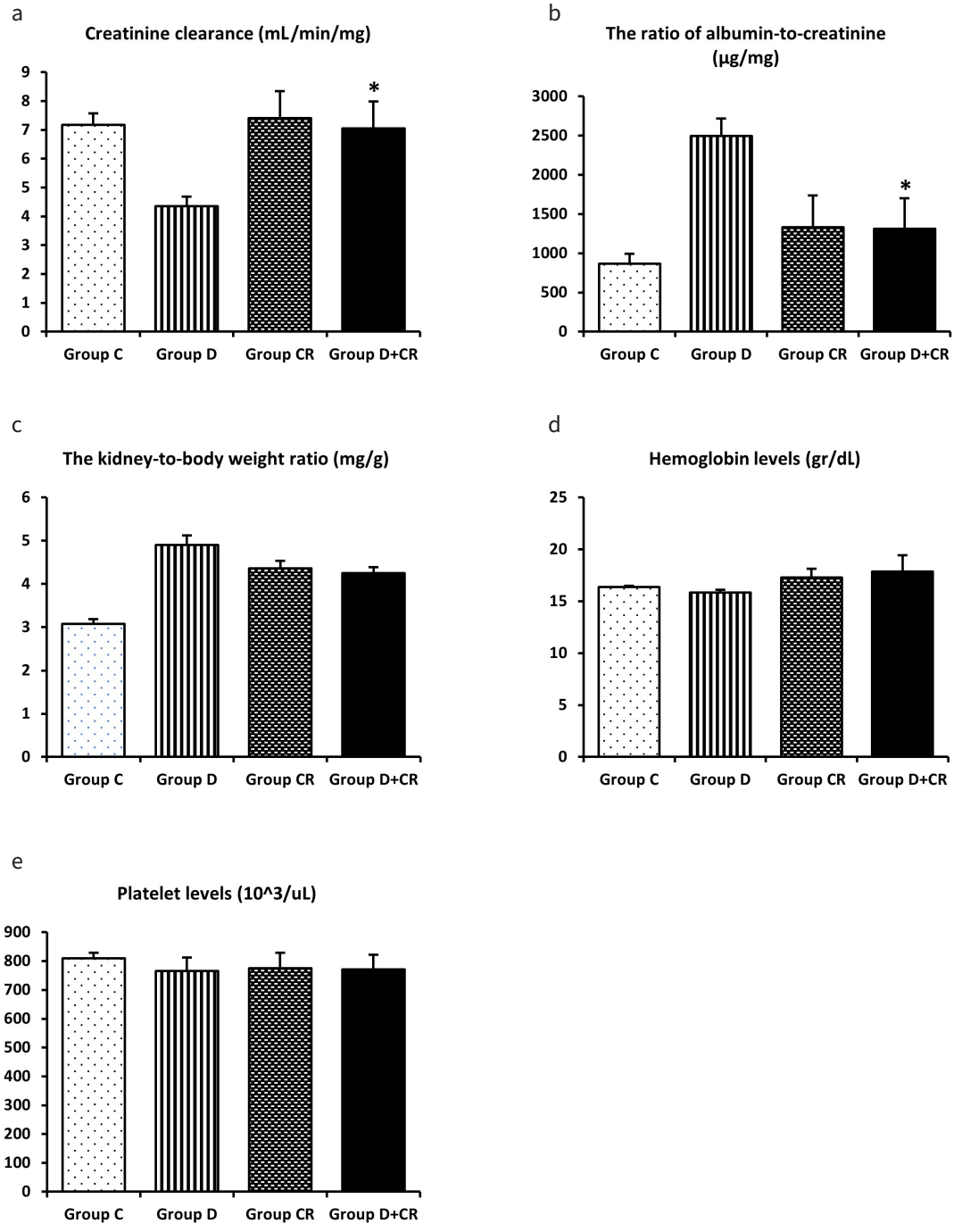

Figure 1. a-e. Assessment of renal functions and hematological parameters. a) Creatinine clearance, b) albumin-to-creatinine ratio, c) kidney-to-body weight ratio, d) hemoglobin levels, and e) platelet levels. Group C: control group; group D: diabetic group; group CR: continuous erythropoietin receptor activator (CERA)-treated nondiabetic group; group D+CR: CERA-treated diabetic group. Data are mean±standard error of mean. *p<0.05 compared with group D

and INF-ɣ (p=0.04) (Figure 3). The change in IL-4 levels had no statistical significance.

Assessment of Renal Messenger-Ribonucleic Acid Expression Levels

CERA decreased the messenger-ribonucleic acid (mRNA) levels of renin and renin receptor, but this decrease did not reach the level of significance (Figure 4).

Immunofluorescence for Renin Receptor, Renin, Angiotensin Type 1 Receptor, and Angiotensin

CERA did not cause statistically significant changes in the semi-quantitative scoring (Figure 5).

Histological and Morphometric Evaluation

Glomerular sclerosis: Assessed as “0” for all rats. Therefore, no comparison was carried out between the groups.

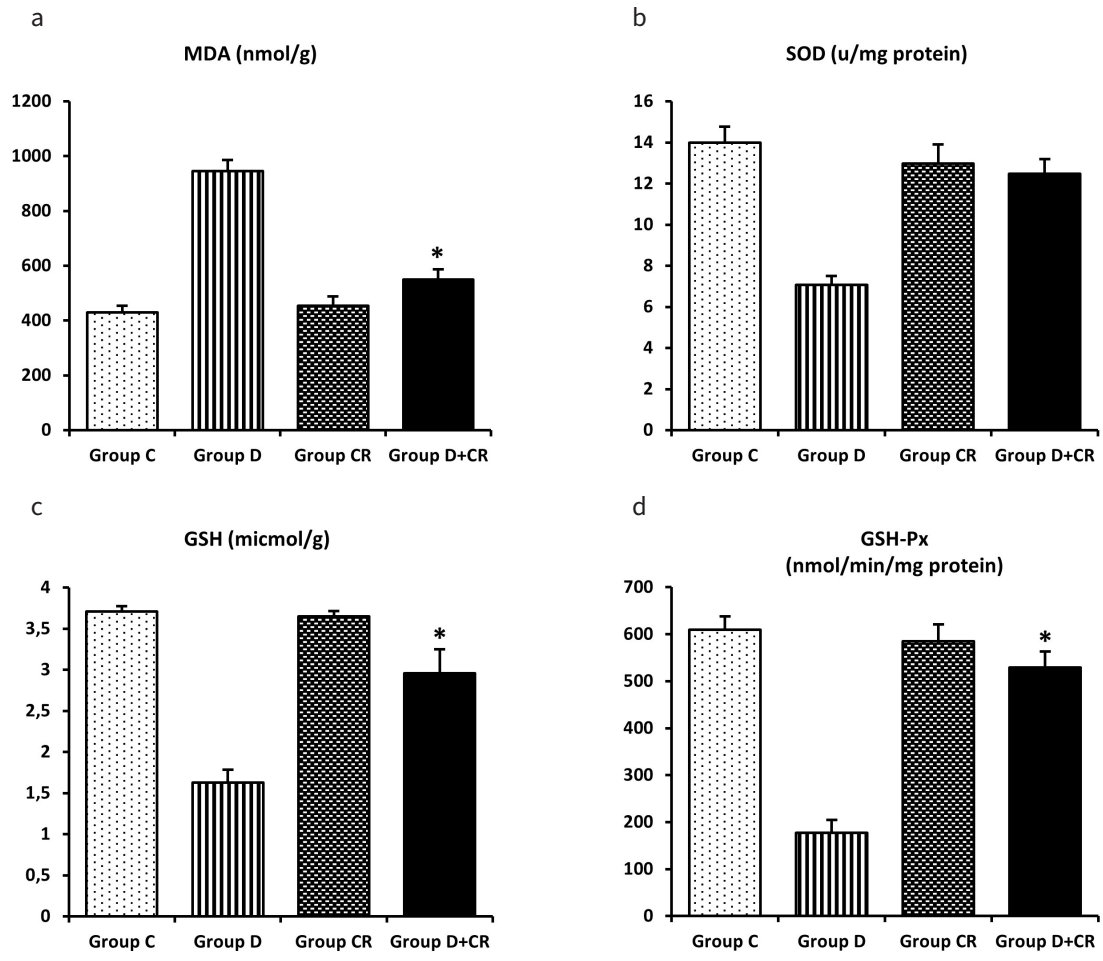

Figure 2. a-d. Oxidative stress parameters in the kidney tissue. a) malondialdehyde levels, b) superoxide dismutase levels, c) glutathione levels, d) glutathione peroxidase levels. Group C: control group; group D: diabetic group; group CR: continuous erythropoietin receptor activator (CERA)-treated nondiabetic group; group D+CR: CERA-treated diabetic group. Data are mean±standard error of mean. *p<0.05 compared with group D

Tubulointerstitial damage: When the groups C and D were evaluated, there were statistically significant differences (p=0.00). CERA (p=0.04) had ameliorating effects on tubulointerstitial damage (Figure 6a).

Interstitial fibrosis volume: A significant difference was observed between the control and diabetic groups (p=0.00), which indicates that CERA (p=0.03) has a reducing effect on interstitial fibrosis (Figure 6b).

Quantitative and qualitative measurements of fibrosis volume show the preventive effect of CERA on kidney fibrosis in diabetic rats.

DISCUSSION

The application dose of CERA varies in the literature. Generally, once a week drug administration is preferred; however, there are studies in which different application ranges are preferred. Recently, studies on CERA have shown that a monthly thesis dose administration (75-100 µg/kg) is effective and safe (19, 20). From this point of view, in our research, we preferred to use a dose of 50 µg/kg every 2 weeks, totaling 100 µg/kg.

For the first time, this study demonstrates the protective effect of CERA against DKD development in an experimental rat model. Treatment with CERA significantly reduced albumin-to-creatinine ration, and increased Ccr in diabetic rats. Furthermore, this treatment improved 2 crucial pathways in the development of DKD, oxidative stress, and inflammation. However, we found that CERA had no significant effect on components of the RAS, a major pathogenetic factor in the course of DKD.

ESAs are recombinant human erythropoietin and related proteins, such as darbepoetin-α, epoetin-α, epoetin-β, epoetin-δ, and methoxy-polyethylene glycol-epoetin-β. They are essential drugs for the cure of anemia in patients with chronic kidney disease (21). Besides their stimulating effect on the erythroid precursor cells, they can stimulate different signaling pathways, such as mitogen activated protein kinase, phosphoinositide 3-kinase, signal transducers and activators of transcription, and RAS, to enhance cel-

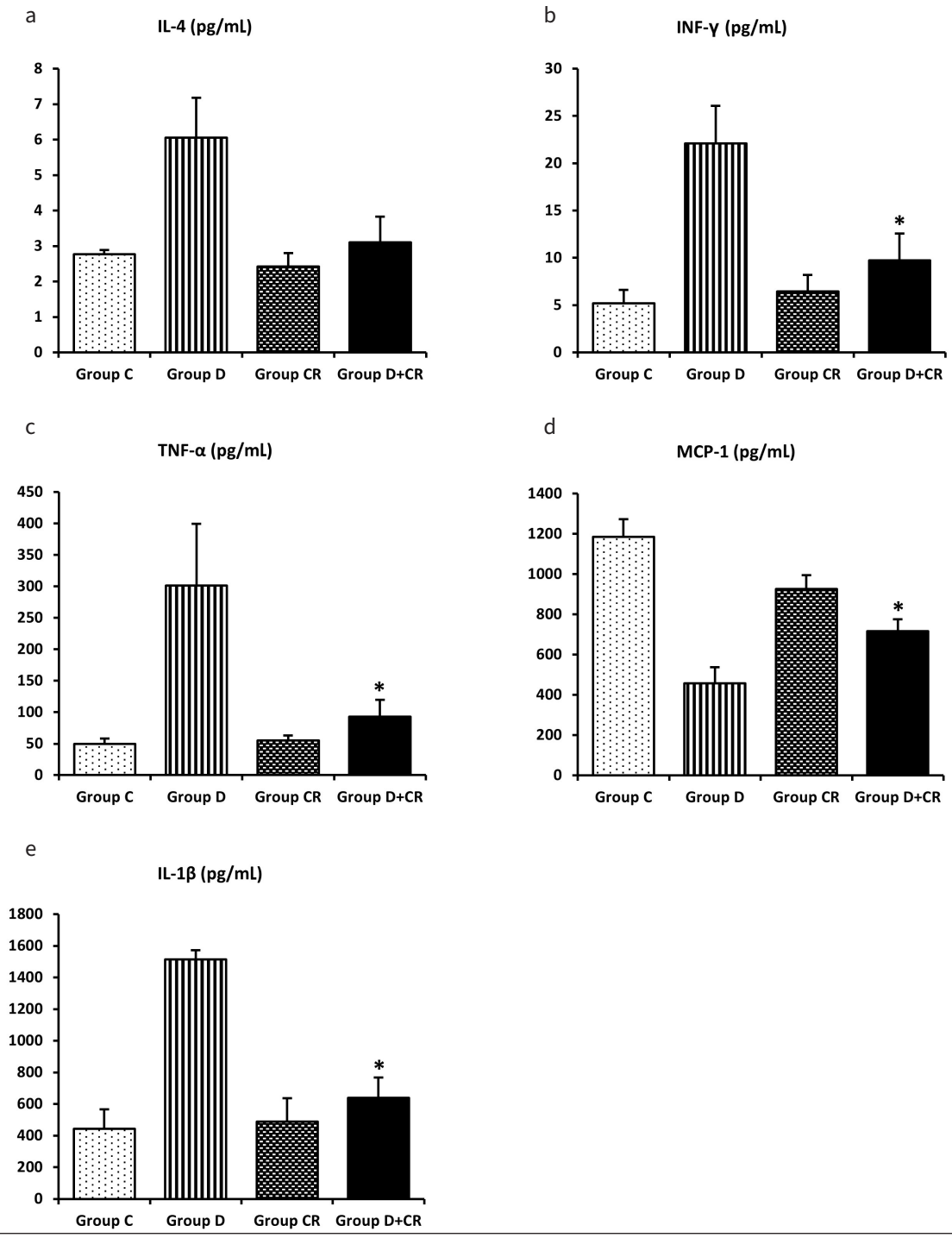

Figure 3. a-e. Serum cytokines levels. a) Interleukin (IL)-4, b) interferon-γ, c) tumor necrosis factor-α, d) monocyte chemoattractant protein 1, e) IL-1β. Group C: control group; group D: diabetic group; group CR: continuous erythropoietin receptor activator (CERA)-treated nondiabetic group; group D+CR: CERA-treated diabetic group. Data are mean±standard error of mean. *p<0.05 compared with group D

lular differentiation or provide antiapoptotic and cytoprotective properties on many nonerythroid cells by attaching to its receptor (22, 23). Emerging evidence from experimental and clinical studies suggests that ESAs have a protective role in the progression of DKD (9, 24). Loeffler et al. (25) showed that erythropoietin could ameliorate podocyte damage and albuminuria in advanced diabetic nephropathy in mice. Numerous similar experimental and clinical studies have shown that ESAs play a protective role in the progression of DKD, but there are studies that argue otherwise. According to the results of a meta-analysis conducted by Covic et al. (26), there is no evidence that ESA treatment affects the renal function in patients with chronic kidney disease. According to another meta-analysis by Elliott et al. (27), the use of ESA did not show significant clinical renoprotective efficacy.

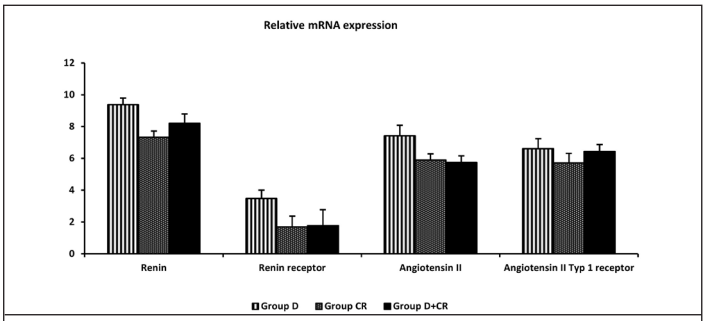

Figure 4. Effects of continuous erythropoietin receptor activator (CERA) on renal messenger-ribonucleic acid expression. Group D: diabetic group; group CR: CERA-treated nondiabetic group; group D+CR: CERA-treated diabetic group. Data are mean±standard error of mean

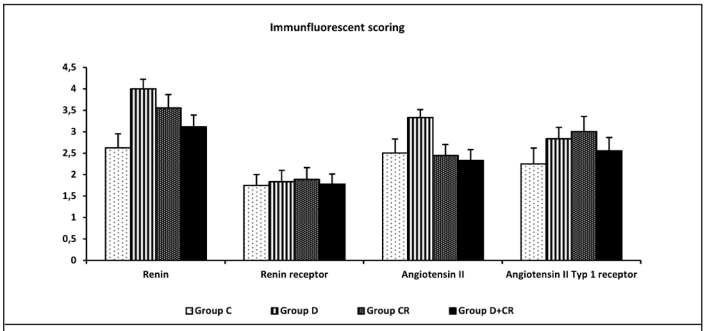

Figure 5. Semiquantitative immunfluorescence scores. Group C: control group; group D: diabetic group; group CR: continuous erythropoietin receptor activator (CERA)-treated nondiabetic group; group D+CR: CERA-treated diabetic group. Data are mean±standard error of mean

In this study, we found that CERA significantly attenuated renal dysfunction and decreased albumin-to-creatinine ratio in streptozocin-induced DKD. There was no statistically significant difference in the platelet count or hemoglobin levels among the study groups, leading us to suggest that the observed effect was most likely because of the nonerythropoietic effect of CERA. We interpreted that this discrepancy with the expected hematopoietic effects may be related to the dose of CERA that we used in the study.

Oxidative stress plays an important role in the increased risk of DKD. The mechanisms responsible for enhanced oxidative stress in patients with DKD include increased creation of ROS and dysfunction of the antioxidant defense system (6, 28). In our study, increased concentration of the main lipid peroxidation product, malondialdehyde, in the kidney indicates an overproduction of ROS in diabetic rats. However, the dysfunction of the antioxidant defense system decreases the activity of SOD, GSH-Px, and GSH. Conversely, after CERA treatment, the activity of GSH-Px and the GSH levels demonstrated a serious enhancement accompanied by an apparent decrease in the MDA level. Our outcomes are similar to those of Bartnicki et al. (24), who showed that CERA increases the systemic oxidative stress in patients with chronic kidney disease predialysis.

Another study showed that epoetin beta averts the generation of ROS and also prevents high-glucose-induced renal cell apoptosis in the diabetic kidney (29). A study of Serizawa et al.

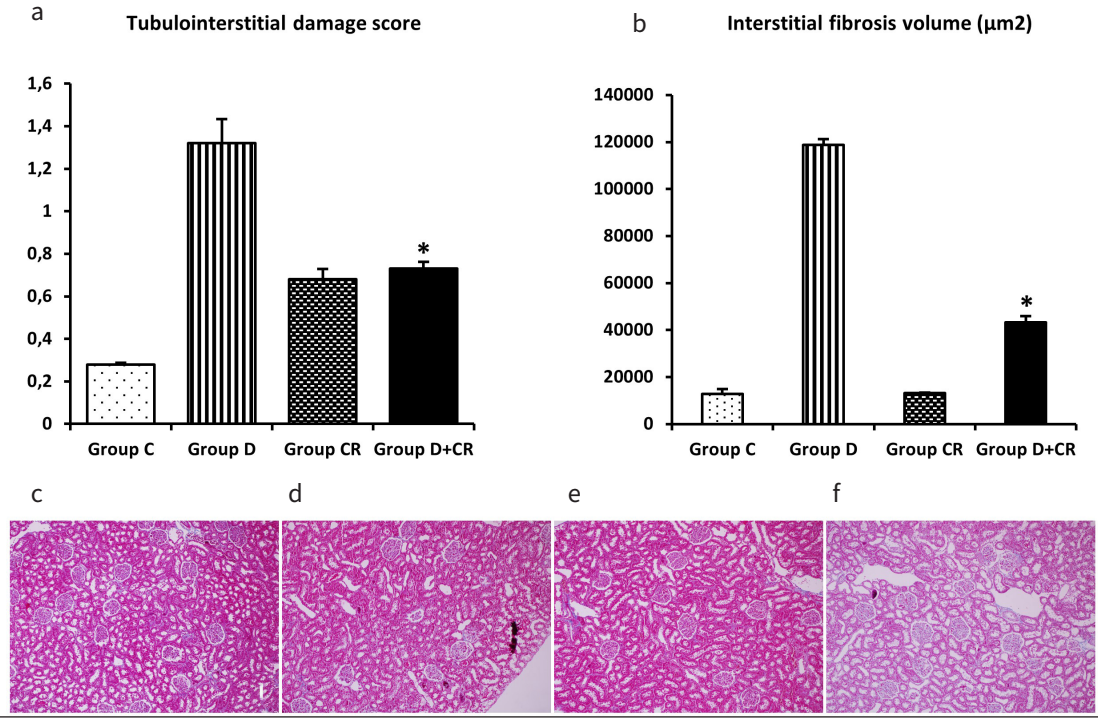

Figure 6. a-f. Histological and morphometric evaluation of the kidney tissue. a) Tubulointerstitial damage score, b) interstitial fibrosis volume. Histopathological examinations of renal tissue sections (magnification 100×); c) for Group C; d) for group D; e) for group CR; f) for group D+CR. Data are mean±standard error of mean. *p<0.05 compared with group D

(30) also provides the evidence that CERA prevented endothelial dysfunction in chronic kidney disease model rats, possibly through reduction of local oxidative stress and enhancement of endothelial nitric oxide synthase phosphorylation in the arteries. These results suggested that CERA treatment had antioxidant activity and could improve the antioxidative defense system of DKD.

Recent studies have reported that CERA has potent anti-inflammatory effects (31). Recently, Rodrigues et al. (31) noted that CERA lowered the plasma levels of IL-1β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ in the cecal ligation and puncture model of sepsis-induced acute kidney injury (AKI). Moreover, they demonstrated that pretreatment with CERA preserved Ccr and tubular function as well as the expression of Aquaporin2 and Na-K-Cl cotransporter2. Furthermore, CERA sustained plasma lactate at normal levels in addition to conserving the plasma levels of transaminases and lactate dehydrogenase. They concluded that CERA protects against sepsis-induced AKI, and this protective effect is, in part, attributable to the suppression of the inflammatory response.

Furthermore, in the model of acute cyclosporine A-mediated renal injury, low-dose CERA treatment was linked with anti-inflammatory effects and protection of pancreatic islet cell viability (32). We demonstrated that CERA treatment decreases the serum levels of inflammatory cytokines and increases the serum levels of MCP-1 on the experimental DKD model. Our results also showed that the inflammatory response that emerged from hyperglycemia in the diabetic kidney might be markedly suppressed by CERA treatment.

On the basis of the understanding of the crucial role of the RAS in pathogenesis of DKD (3, 4), in this study, we aimed to investigate the components of RAS in detail. We evaluated the mRNA expression of angiotensin, angiotensin type 1 receptor, renin, and renin receptor in the kidney. Moreover, we performed immunostaining of the kidney sections with antirenin, antirenin receptor, antiangiotensin, and antiangiotensin type 1 receptor antibodies to demonstrate the immunofluorescence score of RAS components. Using both the methods, we could not show the effect of CERA on angiotensin, angiotensin type 1 receptor, renin, and renin receptor. We could not interpret whether this observation was an effect of all ESAs or specific to CERA. The literature review showed no study that provided information, even negative, on the effect of ESAs on the RAS system. Further studies are needed to understand the interaction between ESAs and the RAS system.

By histomorphometric analysis, we illustrated that CERA improves renal interstitial fibrosis. Recently, Lu et al. (33) reported that epoetin beta inhibits cardiac fibrosis on an experimental diabetes model. Fischer et al. (34) reported the effects of CERA on tubulointerstitial fibrosis as well as on the generation of the matrix-producing myofibroblasts. They found that the mechanisms in which CERA acts as an antifibrotic agent/drug seem to be multifaceted: first, CERA inhibits the generation of matrix-producing myofibroblasts, and second, CERA increases the ability for tissue repair. Finally, they concluded that the finding could explain many of these CERA effects and that CERA inhibits the renal expression of the cytokine TGF-β1. Although we showed renal fibrosis attenuation by CERA, the mechanism is unclear. It might be speculated that interstitial fibrotic alterations are, in part, related to the renal oxidative system and inflammation. Further studies are needed to shed more light on this issue.

This study has several limitations. The small sample size and lack of information about molecular mechanisms are an obvious limitation. This is an experimental study of DKD treatment in the rat, and it verifies the concept and could have implications for further research. Despite these limitations, we demonstrated for the first time that administration of CERA may improve the renal function parameters, oxidative stress, and inflammation in streptozocin-induced DKD.

CONCLUSION

In summary, we found that CERA has a renoprotective effect on DKD, demonstrated by the improvement in Ccr, reduced proteinuria, intrarenal oxidative stress, inflammation, and interstitial fibrosis.

Ethics Committee Approval: Ethics committee approval was received for this study from the Animal Ethics Review Committee of Yeditepe University School of Medicine (Approval Date: July 04, 2015; Approval Number: 2015-87).

Informed Consent: Informed consent was not obtained due to the nature of this study

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - Z.E., M.Y.G., E.A.B.; Design - M.Y.G., Z.E.; Supervision - M.Y.G.; Resources - Z.E., M.Y.G., E.A.B.; Materials - M.Y.G.; Data Collection and/or Processing - M.Y.G., Z.E.; Analysis and/or Interpretation - M.Y.G., Z.E., E.A.B.; Literature Search - Z.E., E.A.B.; Writing - Z.E., M.Y.G., E.A.B.; Critical Reviews - Z.E., M.Y.G., E.A.B.

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REFERENCES

1. Mogensen CE. Twelve shifting paradigms in diabetic renal disease and hypertension. Diabetes Res Clin Pract 2008; 82: S2-9. [Crossref]

2. Alicic RZ, Rooney MT, Tuttle KR. Diabetic kidney disease challenges, progress, and possibilities. Clin J Am Soc Nephro 2017; 12: 2032-45. [Crossref]
3. Reidy K, Kang HM, Hostetter T, Susztak K. Molecular mechanisms of diabetic kidney disease. J Clin Invest 2014; 124: 2333-40. [Crossref]
4. Balakumar P, Bishnoi HK, Mahadevan N. Telmisartan in the management of diabetic nephropathy: A contemporary view. Curr Diabetes Rev 2012; 8: 183-90. [Crossref]
5. Burnier M, Zanchi A. Blockade of the renin-angiotensin-aldosterone system: A key therapeutic strategy to reduce renal and cardiovascular events in patients with diabetes. J Hypertens 2006; 24: 11-25. [Crossref]
6. Ishimoto Y, Tanaka T, Yoshida Y, Inagi R. Physiological and pathophysiological role of reactive oxygen species and reactive nitrogen species in the kidney. Clin Exp Pharmacol P 2018; 45: 1097-105. [Crossref]
7. Navarro-Gonzalez JF, Mora-Fernandez C, de Fuentes MM, Garcia-Perez J. Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. Nat Rev Nephrol 2011; 7: 327-40. [Crossref]
8. Toba H, Nakashima K, Oshima Y, Kojima Y, Tojo C, Nakano A, et al. Erythropoietin prevents vascular inflammation and oxidative stress in subtotal nephrectomized rat aorta beyond haematopoiesis. Clin Exp Pharmacol P 2010; 37: 1139-46. [Crossref]
9. Schiffer M, Park JK, Tossidou I, Bartels J, Shushakova N, Menne J, et al. Erythropoietin prevents diabetes-induced podocyte damage. Kidney Blood Press R 2008; 31: 411-5. [Crossref]
10. Topf JM. CERA: Third-generation erythropoiesis-stimulating agent. Expert Opin Pharmaco 2008; 9: 839-49. [Crossref]
11. Menne J, Park JK, Shushakova N, Mengel M, Meier M, Fliser D. The continuous erythropoietin receptor activator affects different pathways of diabetic renal injury. J Am Soc Nephrol 2007; 18: 2046-53. [Crossref]
12. Kitada M, Takeda A, Nagai T, Ito H, Kanasaki K, Koya D. Dietary restriction ameliorates diabetic nephropathy through anti-inflammatory effects and regulation of the autophagy via restoration of sirt1 in diabetic wistar fatty (fa/fa) rats: A model of type 2 diabetes. Exp Diabetes Res 2011; 2011: 908185. [Crossref]
13. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-8. [Crossref]
14. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963; 61: 882-8.
15. Mylroie AA, Collins H, Umbles C, Kyle J. Erythrocyte superoxide-dismutase activity and other parameters of copper status in rats ingesting lead acetate. Toxicol Appl Pharm 1986; 82: 512-20. [Crossref]
16. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun 1976; 71: 952-8. [Crossref]
17. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 1985; 150: 76-85. [Crossref]
18. Eren Z, Gunal MY, Bakir EA, Coban J, Caglayan B, Ekimci N, et al. Effects of paricalcitol and aliskiren combination therapy on experimental diabetic nephropathy model in rats. Kidney Blood Press R 2014; 39: 581-90. [Crossref]
19. Vega A, Abad S, Verdalles U, Aragoncillo I, Velazquez K, Quiroga B, et al. Dose equivalence between continuous erythropoietin receptor activator (CERA), darbepoetin and epoetin in patients with advanced chronic kidney disease. Hippokratia 2014; 18: 315-8.
20. Duman N, Uyanik A, Unsal A, Sezer S, Camsari T, Cirit M, et al. Once-monthly continuous erythropoietin receptor activator (CERA) for haemoglobin maintenance in haemodialysis patients with chronic renal anaemia. Clin Kidney J 2014; 7: 464-9. [Crossref]
21. Palmer SC, Saglimbene V, Mavridis D, Salanti G, Craig JC, Tonelli M, et al. Erythropoiesis-stimulating agents for anaemia in adults with chronic kidney disease: A network meta-analysis. Cochrane Database Syst Rev 2014; 2014: CD010590. [Crossref]
22. Siren AL, Fratelli M, Brines M, Goemans C, Casagrande S, Lewczuk P, et al. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and metabolic stress. P Natl Acad Sci USA 2001; 98: 4044-9. [Crossref]
23. Junk AK, Mammis A, Savitz SI, Singh M, Roth S, Malhotra S, et al. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. Proc Natl Acad Sci USA 2002; 99: 10659-64. [Crossref]
24. Bartnicki P, Fijalkowski P, Majczyk M, Blaszczyk J, Banach M, Rysz J. Effect of methoxy polyethylene glycol-epoetin beta on oxidative stress in predialysis patients with chronic kidney disease. Med Sci Monitor 2013; 19: 954-9. [Crossref]
25. Loeffler I, Ruster C, Franke S, Liebisch M, Wolf G. Erythropoietin ameliorates podocyte injury in advanced diabetic nephropathy in the db/db mouse. Am J Physiol-Renal 2013; 305: F911-F8. [Crossref]
26. Covic A, Nistor I, Donciu MD, Dumea R, Bolignano D, Goldsmith D. Erythropoiesis-stimulating agents (ESA) for preventing the progression of chronic kidney disease: A meta-analysis of 19 studies. Am J Nephrol 2014; 40: 263-79. [Crossref]
27. Elliott S, Tomita D, Endre Z. Erythropoiesis stimulating agents and reno-protection: A meta-analysis. Bmc Nephrol 2017; 18: 14. [Crossref]
28. Singh DK, Winocour P, Farrington K. Oxidative stress in early diabetic nephropathy: Fueling the fire. Nat Rev Endocrinol 2011; 7: 176-84. [Crossref]
29. Dang J, Jia R, Tu Y, Xiao S, Ding G. Erythropoietin prevents reactive oxygen species generation and renal tubular cell apoptosis at high glucose level. Biomed Pharmacother 2010; 64: 681-5. [Crossref]
30. Serizawa K, Yogo K, Tashiro Y, Aizawa K, Kawasaki R, Hirata M, et al. Epoetin beta pegol prevents endothelial dysfunction as evaluated by flow-mediated dilation in chronic kidney disease rats. Eur J Pharmacol 2015; 767: 10-6. [Crossref]
31. Rodrigues CE, Sanches TR, Volpini RA, Shimizu MHM, Kuriki PS, Camara NOS, et al. Effects of continuous erythropoietin receptor activator in sepsis-induced acute kidney injury and multi-organ dysfunction. Plos One 2012; 7: e29893. [Crossref]
32. Meerwein C, Korom S, Arni S, Inci I, Weder W, Jungraithmayr W. The effect of low-dose continuous erythropoietin receptor activator in an experimental model of acute Cyclosporine A induced renal injury. Eur J Pharmacol 2011; 671: 113-9. [Crossref]
33. Lu J, Yao YY, Dai QM, Ma GS, Zhang SF, Cao L, et al. Erythropoietin attenuates cardiac dysfunction by increasing myocardial angiogenesis and inhibiting interstitial fibrosis in diabetic rats. Cardiovasc Diabetol 2012; 11: 105. [Crossref]
34. Fischer C, Deininger N, Wolf G, Loeffler I. CERA attenuates kidney fibrogenesis in the db/db mouse by influencing the renal myofibroblast generation. J Clin Med 2018; 7: 15. [Crossref]