

Differential Effects of Low-Dose Erythropoietin in Rat Model of Diabetic Nephropathy

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ABSTRACT

Background. Previous reports on the renoprotective effect of erythropoietin (EPO) in the setting of chronic kidney disease (CKD) have yielded conflicting results. The aim of this study is to clarify the effect of low, non-hematopoietic dose of EPO on the evolution of diabetic nephropathy (DN) in rat model. **Methods.** Low dose of recombinant human EPO (150 U/kg, s.c. three times/week) was given to streptozotocin (STZ)-induced diabetic rats in two schedules; in the first one, EPO was given from day 2 after STZ injection till the end of the study (28 weeks) as prophylactic treatment; and in the other schedule EPO was given after development of DN (last 8 weeks) as therapeutic treatment. Albuminuria, blood pressure, creatinine clearance, renal venous oxygen tension (vPO₂), plasma EPO, hematocrit and renal histopathology were assessed. **Results.** Unexpectedly, 28 weeks administration of EPO to diabetic rats led to aggravation of albuminuria and worsening of histopathological damage in spite of partial correction of renal hypoxia. Contrary to this, terminal 8 weeks EPO therapy of DN reduced albuminuria and demonstrated some favorable effects on biochemical changes and histologic picture. **Conclusion.** Low dose EPO exerted differential effects in rat model of DN according to treatment duration. In addition, findings of the present study warrants further investigations of the exact renoprotective role of EPO in diabetic patients with CKD who receive EPO therapy for long periods.

Key words: Erythropoietin; Diabetic nephropathy; Renal hypoxia; Albuminuria.

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INTRODUCTION

Diabetic nephropathy (DN) is a serious complication of diabetes mellitus (DM) and a leading cause of end-stage renal disease (ESRD) worldwide [1]. The pathogenesis of DN is complex and implies interactions between different metabolic and hemodynamic factors that concomitantly activate several growth factors such as connective tissue growth factor and profibrotic cytokines [2]. In addition, the hypoxic hypothesis of chronic kidney disease (CKD) had an evident role in the progression of renal injury. Renal hypoxia leads both to desired and undesired effects that in most cases maintain renal disease advancement through an ongoing vi-

cious circle [3]. Despite the beneficial effects of the current strategies for the treatment of DN, based mainly on adequate glycemic control, albuminuria and evolution of renal disease are not completely halted. Therefore, it is necessary to explore potential new renoprotective therapies.

Erythropoietin (EPO) is an acidic glycoprotein hormone that controls erythropoiesis and primarily, but not entirely, produced by renal interstitial fibroblasts [4]. Recombinant human EPO has been used clinically to accelerate erythropoiesis in anemia of CKD and to protect tissues in certain clinical settings [5]. Recently, much attention has been directed toward tissue protective properties of EPO beyond

stimulating erythropoiesis [6]. Although the possible renoprotective effect of EPO in the setting of CKD is of major clinical importance, previous reports have yielded conflicting results; yet, while few reports have revealed its protective effects on chronic renal injury models [7], at least 13 others have failed to establish any effect [8].

Moreover, the tissue-protective effect of EPO was linked to the use of low-dose than that used for anemia correction. In an animal model of type 2 diabetes, chronic administration of long-acting erythropoiesis-stimulating agent had remarkable dose-dependent effects on cellular signaling of diabetic kidney injury; with low-dosage therapy totally displayed the tissue-protective effect [9]. Additionally, one-month treatment with the low dose recombinant EPO has attenuated renal injury beyond its hematopoietic effect in streptozotocin (STZ)-induced diabetic rats [10]. Whether this tissue-protective effect of low-dose EPO could be maintained for longer periods without adverse effects is not known. So, the idea behind this study is to assess the effects of low-dose EPO on the evolution of DN in rat model. We used EPO in two schedules, in the first one we used EPO from the beginning of diabetes mellitus till the end of the study (28 weeks) as a prophylactic 'preventive' treatment; and in the other schedule we gave EPO after development of DN (the last 8 weeks only) as therapeutic treatment.

MATERIALS AND METHODS

Animals and experimental design

All experiments were conducted in accordance with Mansoura University Institutional Research Board and the Guide to the Care and Use of Laboratory Animals (8th edition, 2011, The National Academic Press, Washington, DC, USA). Fifty-seven male Sprague-Dawley rats (8 weeks old, 170–190 g body wt) were acclimatized upon arrival for 5 days in a naturally controlled lab with 12h dark/light cycle and had free access to standard food and water. Rats were randomized into five groups (Fig 1) as follows – (1) Naïve control group (n=10): healthy rats injected only with citrate buffer; (2) EPO control group (n=10): healthy rats received EPO for 28 weeks; (3) Untreated diabetic group (n=17; D-UT-28W): diabetic rats without treatment for 28 weeks after diabetes induction; (4) EPO prophylactically-treated diabetic group (n=10; D-EPO-28W): diabetic rats received EPO starting on day 2 after STZ injection and continued for 28 weeks (pro-

phylactic schedule); (5) EPO therapeutically-treated diabetic group (n=10; D-EPO-8W): diabetic rats received EPO starting from the end of week 20 till the end of week 28 (therapeutic schedule). After 20 weeks, 7 rats from the untreated diabetic group were sacrificed and subjected for biochemical and histopathological examination to confirm development of DN while the residual 10 rats were allowed to continue to the end of the study. The type of EPO used is of recombinant human type and the dose in all schedules is 150 U/kg s.c. three times/week (epoetin alfa [Eprex®], Janssen-Cilag Co., NJ, USA).

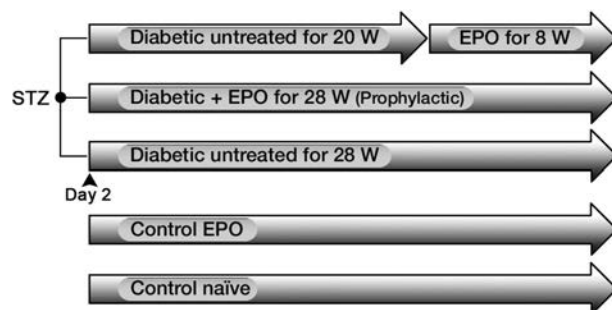


Fig 1. Schematic representation of the experimental design.

Every 4 weeks, plasma glucose, creatinine (Cr) and insulin were measured from tail vein samples then rats were individually housed in metabolic cages to allow for 24 h urine collection and estimation of creatinine clearance (CrCl) and albuminuria. The mean blood pressure (MBP) was measured by indirect tail-cuff plethysmography (LE5001 pressure meter, PanLab, Harvard Apparatus, Spain). At the end of the study, laparotomy was done under thiopental anesthesia. Renal venous oxygen tension (vPO₂) was analyzed from left renal vein blood samples (Rapid Point 400, Bayer). The kidneys were harvested to assess kidney/body wt (K/B) ratio and histopathological examination. Blood samples were collected from the heart for estimation of hematocrit (Hct) value while the other portions of blood were centrifuged with EDTA for 10 minutes to separate plasma for further assays.

Induction of type 1 diabetes mellitus (T1DM)

Type 1 DM was induced in rats by single intraperitoneal injection of 60 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA), dissolved in citrate buffer (0.05 mol/l, pH 4.5). Hyperglycemia was confirmed in all rats after 48 h by measuring blood glucose level from a tail vein sample after 6 h fasting

using a One Touch Basic blood glucose monitoring system. Animals with blood sugar of more than 250 mg/dl were considered diabetic and used for the study [11]. To avoid animal loss, severely diabetic rats with blood glucose level ≥ 500 mg/dl were treated daily with low-dose NPH insulin at 4.1 ± 1.4 IU/kg s.c. to maintain body wt and prevent ketosis without normalizing hyperglycemia [12].

Blood biochemistry

Plasma glucose (BioMed Glucose L.S, Hanover, Germany), plasma insulin (DRG Diagnostics, Marburg, Germany), plasma creatinine (Cr; Diamond Diagnostics, Hanover, Germany), and plasma EPO levels (Lifespan Bioscience Inc., WA, USA) were determined by using the appropriate assay kits according to the manufacturers' protocols.

Morphologic analysis of renal tissue

For all groups, the kidneys were perfused in a retrograde manner through the abdominal aorta using 0.9% saline then 10% neutral buffered formalin for in situ fixation. Paraffin sections of 5- μ m thickness were evaluated using hematoxylin and eosin, periodic acid Schiff (PAS), and Masson trichrome stains and examined by light microscopy by two independent pathologists who are blinded to the specimens examined. Glomerular changes developed in untreated diabetic rats at the 20th week after diabetes induction were in the form of mesangial matrix expansion, mesangial hypercellularity, and segmental thickening of the glomerular basement membrane (GBM). These lesions were mild (affecting <25% of glomerulus) and focal (affecting <50% of all glomeruli per kidney). So, these glomerular changes were scored according to their absence or presence as follows – normal (absence of lesions = score 0); presence of any one of the above-mentioned glomerular lesions (score 1); presence of two glomerular lesions (score 2); presence of the three glomerular lesions (score 3). Also, untreated diabetic rats after 20 weeks developed mild tubulointerstitial and vascular lesions in the form of mild interstitial fibrosis, tubular atrophy, interstitial inflammation, and arteriolar hyalinosis. Because these lesions were mild, they were classified and scored according to their absence (score 0) or presence (score 1).

Statistical analysis

Data were represented as mean \pm SD for parametric data or

frequency (number-percent) for histopathological scores. Parametric data were analyzed as appropriate by using the Student t-test or the one-way analysis of variance (ANOVA) followed by Tukey *posthoc* test. Qualitative data were analyzed as appropriate by using the Kruskal-Wallis H test, chi square " χ^2 " or Fischer's exact tests. A P-value < 0.05 was considered significant.

RESULTS

Diabetic model after 20 weeks

The success rate of inducing diabetes (defined as fasting blood glucose of more than 250 mg/dl 48 h after STZ injection) was 94% and the early mortality (within the 1st week after STZ injection) was 7.6% (data not shown). The survived rats were classified and recruited in the study. As compared to naïve rate, non-treated diabetic rats 20 weeks after diabetes induction had significant rise of blood glucose ($P < 0.001$), plasma Cr ($P < 0.001$), MBP ($P < 0.01$), and decreased both CrCl ($P < 0.01$) and plasma insulin ($P < 0.001$), together with heavy albuminuria ($P < 0.001$) and increase K/B ratio ($P < 0.001$). Blood samples from the left renal vein had decreased vPO₂ ($P < 0.001$). Histopathological examination showed mild glomerular changes (score 1) in the form of focal mesangial matrix expansion, mesangial hypercellularity, and patchy thickening of the GMB. Interstitial fibrosis, inflammation, and arteriolar hyalinosis were minimal or non-significant (Fisher's exact test; Table 1 and Fig 3).

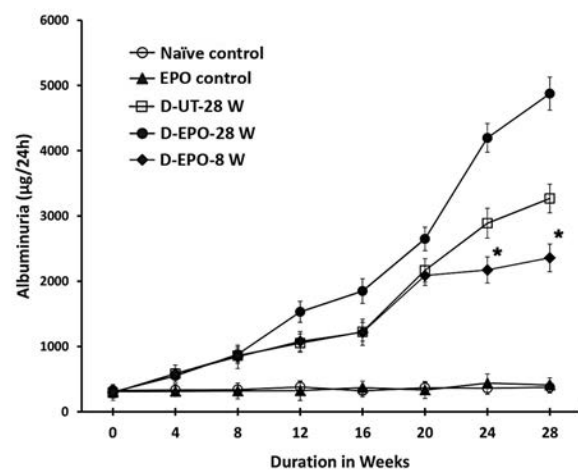


Fig 2. Effect of Erythropoietin (150 U/kg s.c. three times/week) on albuminuria of control and diabetic rats. Significance difference * $P < 0.001$ vs. EPO prophylactically-treated diabetic rats (mean \pm SD, one way ANOVA).

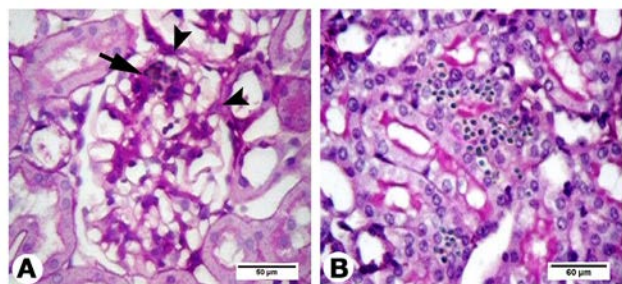


Fig 3. H&E staining of glomerular and tubular changes developed in untreated diabetic rats at the 20th week after diabetes induction. (A) Glomerular changes in the form of mesangial matrix expansion and hypercellularity (arrow), and segmental thickening of the glomerular basement membrane (arrow heads). These lesions were mild (affecting <25% of glomerulus) and focal (affecting <50% of all glomeruli per kidney). (B) Mild tubulointerstitial lesions in the form of mild interstitial fibrosis, tubular atrophy, interstitial inflammation, and hyalinosis.

General and biochemical characteristics after 28 weeks

The number of animals died before completion of the 28 weeks were as follows: 2 rats were lost from the untreated diabetic group (20% mortality); 3 rats were lost from the diabetic EPO prophylactically-treated group (30% mortality), and one rat was died in the diabetic EPO therapeutically-treated group at the 20th week before initiation of EPO administration (10% mortality). No rats were lost from either the control naïve or control EPO groups. The number of rats survived the 28 weeks duration of the study is mentioned in Tables 2 and 3.

All survived diabetic rats remained hyperglycemic throughout the study, and EPO administration to the healthy control rats did not significantly affect blood glucose levels, kidney wt, plasma insulin or CrCl but caused significant increase of both Hct % and the final MBP ($P<0.001$ and $P<0.05$ respectively (Table 2). Untreated diabetic and 28 weeks EPO prophylactically-treated diabetic groups had significant hyperglycemia, insulinopenia, high plasma Cr, decreased CrCl, and increased K/B ratio as compared to naïve group ($P<0.001$ for all). Diabetic rats received EPO only for the last 8 weeks (therapeutic schedule) had significant decrease of all these biomarkers as compared to both untreated diabetic and EPO prophylactically-treated groups. Likewise, untreated diabetic rats had significant decrease of renal vPO2 compared to naïve rats ($P<0.001$). EPO administration to diabetic rats (in both prophylactic and therapeutic schedules) partially restored renal vPO2 ($P<0.001$ and $P<0.01$ respectively). The restoration of renal vPO2 was significantly greater in therapeutically-treated rats than prophylactically treated ones ($P<0.01$; Table 2).

Although diabetic rats that were prophylactically-treated with EPO for 28 weeks had the highest final plasma EPO of all groups ($P<0.001$ for all), their Hct value remain comparable to naïve and non-treated diabetic rats. Contrary to this, EPO therapeutically-treated rats showed significantly lower final plasma EPO with high Hct value as compared to prophylactically-treated ones ($P<0.001$ for both). The final

	Naïve (n=10)	After 20 weeks (n=7)
Plasma glucose (mg/dl)	148.8 ± 17.3	560.8 ± 71.1‡
Plasma insulin (µg/l)	2.63 ± 0.23	0.22 ± 0.03‡
Plasma Cr (mg/dl)	0.43 ± 0.03	0.74 ± 0.03‡
CrCl (ml/min)	1.65 ± 0.17	1.37 ± 0.21†
Albuminuria (µg/24h)	338 ± 70	2126 ± 155‡
Renal vPO2 (mmHg)	38.5 ± 2.7	25.2 ± 2.04‡
MBP (mmHg)	104.2 ± 4.58	112.3 ± 4.82†
Glomerular score (median: Min–Max)	0.00 (0–0)	1.00 (1–2)*
Interstitial fibrosis–tubular atrophy score (%)	0.0 (n=0/10)	42.8% (n=3/7)*
Interstitial inflammation (%)	0.0 (n=0/10)	42.8% (n=3/7)*
Arteriolar hyalinosis (%)	0.0 (n=0/10)	0.0 (n=0/10)

Table 1. Laboratory and histopathological characteristics of diabetic rat model after 20 weeks of diabetes induction.

Data are presented as mean ± SD for biochemical parameters and as median (Min–Max) for glomerular score or percentage of lesion for interstitial fibrosis–tubular atrophy, interstitial inflammation, and arteriolar hyalinosis.

Significant difference: * $P<0.05$, † $P<0.01$, ‡ $P<0.001$ (Student's t-test for parametric data and Kruskal–Wallis H test, chi-square or Fisher's exact tests for qualitative data).

Abbreviations: Cr = creatinine; CrCl = creatinine clearance; MBP = mean blood pressure; vPO2= venous oxygen tension.

Table 2. Final laboratory characteristics of experimental groups after 28 weeks.

	Naïve (n=10)	EPO control (n=10)	D-UT-28W (n=8)	D-EPO-28W (n=7)	D-EPO-8W (n=9)
K/B ratio (mg/g)	5.48 ± 0.25	5.47 ± 0.2	10.28 ± 1.17 ‡ab	11.03 ± 1.07 ‡ab	8.04± 0.63 ‡abcd
Plasma glucose (mg/dl)	148.8 ± 17.3	145.5 ± 23.7	556.9 ± 86.7 ‡ab	542.5±105.4 ‡ab	523.6±75.2 ‡ab
Plasma insulin (µg/l)	2.63 ± 0.23	2.45 ± 0.2	0.18 ± 0.10 ‡ab	0.16 ± 0.12 ‡ab	0.17 ±0.08 ‡ab
Plasma Cr (mg/dl)	0.43 ± 0.03	0.39 ± 0.03	1.04 ± 0.07 *ab	1.05 ±0.07 *ab	0.70 ±0.02 *abcd
CrCl (ml/min)	1.65 ± 0.17	1.43 ± 0.14	1.1 ± 0.10 ‡ab	1.21 ± 0.13 ‡a,*b	1.35±0.12 ‡a, †c
Albuminuria (µg/24h)	377 ± 90	410 ± 112	3286 ± 220 ‡ab	4875 ± 252 ‡ab, *c	2359 ± 214 ‡ab, *cd
Renal vPO2 (mmHg)	38.5 ± 2.7	42.6 ± 4.57	14.9 ± 2.62 ‡ab	22.2 ± 2.77 ‡ac, †b	28.4±2.85 ‡abc, †d
Plasma EPO (pg/ml)	39.7 ± 4.81	47.1 ± 5.85	43.5 ± 6.56	80.0 ± 12.4 ‡abc	46.5±4.88 ‡d
Hct (%)	45.2 ± 2.73	58.5 ± 3.69 ‡a	41.7 ± 2.88 ‡b	43.4 ± 3.11 ‡b	55.8±3.41 ‡abcd
MBP (mmHg)	104.2 ± 4.58	118 ± 5.89 *a	97.5 ± 6.21	115.6 ± 5.26 *a, ‡c	107.5±4.86 †bc, *d

a= significance difference vs. naïve control; b = significance difference vs. EPO control group; c = significance difference vs. untreated diabetic (D-UT-28W) group; d = significance difference vs. EPO prophylactically-treated (D-EPO-28W) group.

Significance levels: *P<0.05; †P<0.01; ‡P<0.001 (Mean ± SD, one way ANOVA and Tukey posthoc test).

Abbreviations: D = diabetic; EPO = Erythropoietin; UT = untreated; K/B = kidney/Body wt; Cr = creatinine; CrCl = creatinine clearance; vPO2 = venous oxygen tension; Hct= hematocrit; MBP = mean blood pressure.

Table 3. Final histopathological scores of experimental groups after 28 weeks.

	Naïve (n=10)	EPO control (n=10)	D-UT-28W (n=8)	D-EPO-28W (n=7)	D-EPO-8W (n=9)
Glomerular score	5.48 ± 0.25	5.47 ± 0.2	10.28 ± 1.17 ‡ab	11.03 ± 1.07 ‡ab	8.04± 0.63 ‡abcd
Median (Min-Max)	0.00 (0 –0)	0.00 (0 –1)	1.00 (1–2) *a	2.00 (1–2) †a	0.50 (0 –1) *bc
Interstitial fibrosis - tubular atrophy score (%)	0.0 (n=0/10)	0.0 (n=0/10)	37.5 (n=3/8)	71.4 (n=5/7) *a	0.0 (n=0/9) *c
Interstitial inflammation (%)	0.0 (n=0/10)	0.0 (n=0/10)	37.5 (n=3/8)	100.0 (n=7/7) †a, *b	44.4 (n=4/9) *c
Arteriolar hyalinosis (%)	0.0 (n=0/10)	0.0 (n=0/10)	25.0 (n=2/8)	0.0 (n=0/7)	33.3 (n=3/9)

Data are expressed as median (Min–Max) for glomerular score and percentage of lesion for interstitial fibrosis-tubular atrophy, interstitial inflammation, and arteriolar hyalinosis.

a = significance difference vs. EPO control group; b = significance difference vs. untreated diabetic (D-UT-28W) group; c = significance difference vs. EPO prophylactically-treated (D-EPO-28W) group.

Significance levels: *P<0.05; †P<0.01 (Kruskal-Wallis H test for glomerular score; Fisher's exact tests for interstitial fibrosis-tubular atrophy, interstitial inflammation, and arteriolar hyalinosis scores).

MBP of EPO therapeutically-treated rats was significantly lower than that of the EPO prophylactically-treated and EPO control (P<0.05 and P<0.01 respectively); however both had higher MBP than untreated diabetic group (P<0.001 and P<0.01 respectively; Table 2).

Albuminuria

Compared to both naïve and EPO control groups, all diabetic groups (treated and untreated) showed significant escalation of albuminuria starting from the 4th week and thereafter (Fig 2; significance marks vs. controls not shown). However, diabetic rats received EPO for 28 weeks (prophylactic sched-

ule) had significantly higher albuminuria starting from the 12th week till the end of the study in comparison with other groups. Diabetic rats received EPO for the last 8 weeks (therapeutic schedule) had significantly less albuminuria at the 24th and 28th weeks as compared to both untreated and EPO prophylactically-treated diabetic groups (P<0.001 for both, Fig 2).

Renal histopathology

As shown in Fig 4, administration of EPO for 28 weeks did not significantly induce histopathological abnormalities in control rats. Untreated diabetic rats showed significant in-

crease of glomerular changes score ($P < 0.05$, K-W) with mild interstitial fibrosis, inflammation, and arteriolar hyalinosis as compared to naïve and EPO control groups (Table 3 and Fig 4C, Fisher's exact test). EPO prophylactically-treated diabetic rats had significant increase in scores of glomerular changes ($P < 0.01$), interstitial fibrosis–tubular atrophy ($P < 0.05$), and interstitial inflammation ($P < 0.01$) as compared to EPO control groups. In addition, EPO prophylactically-treated diabetic group showed significant picture of acute tubular injury and degeneration that was not included in DN pathological scoring system (Fig 4D). Contrary to this, EPO therapeutically-treated rats showed significant decrease in glomerular changes ($P < 0.05$, K-W), interstitial fibrosis, and inflammation as compared to both untreated diabetic and EPO prophylactically-treated diabetic rats ($P < 0.05$, chi square test; Fig 4E).

DISCUSSION

Recombinant human EPO has long been used for correction of anemia due to CKD since it was first synthesized in 1987 [13]. In this study we aimed at studying the possible role of recombinant EPO on the progression of DN in rat model since the available data on this topic are not consistent and leave many open questions [8,14]. We used EPO in low-dose to dissociate its tissue protective effect from its hematopoietic one. In the first schedule we gave EPO to diabetic rats from the beginning of hyperglycemia till the end of the study (28 weeks) as a possible preventive treatment of DN; and in the second one, we employed EPO only for the last 8 weeks (after development of DN) as a possible therapeutic treatment. Unexpectedly, prophylactic administration of low-dose EPO to diabetic rats all over 28 weeks led to marked progression of albuminuria which is commonly considered a good predictor for progressive nephropathy [15]. This finding was also associated with elevation of the final MBP and aggravation of renal histological damage. Contrary to this, administration of EPO to diabetic rats in the last 8 weeks after the development of DN led to some favorable effects in the form of decreased albuminuria, lowering of final MBP and improvement of the renal histological deterioration. In addition, the EPO prophylactically-treated diabetic group had the highest mortality proportion among all experimental groups where 3 out of 10 rats died before the end of the study while only one rat was died in the therapeutically-treated group during

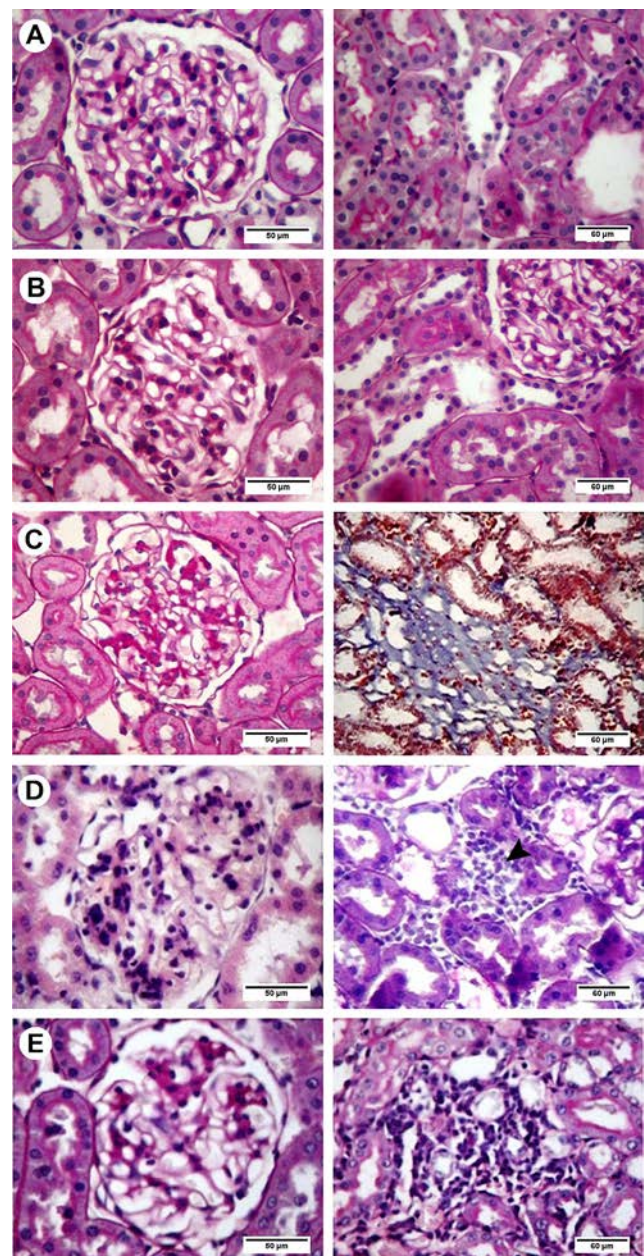


Fig 4. Representative glomerular (left) and tubulointerstitial (right) changes after 28 weeks in 5 groups of rats. (A) Control naïve rat with normal glomerular and tubulointerstitial architecture. (B) Control EPO rats without significant changes than naïve rats. (C) Diabetic untreated rats showing axial mesangial expansion and interstitial fibrosis with tubular atrophy (trichrome stain). (D) Diabetic EPO prophylactically-treated rats (for 28 weeks) showing mesangial hypercellularity and interstitial inflammatory cell infiltrations (arrow head). (E) Diabetic EPO therapeutically-treated rats (for the last 8 weeks) showing mild mesangial expansion and hypercellularity with some focal interstitial inflammation (PAS stain).

the first 20 weeks after diabetes induction, and before initiation of EPO treatment.

The concern that recombinant human EPO might induce progressive deterioration of renal function in patients

with CKD was previously arisen from experimental data in the partially nephrectomized uremic rat model where Gretz and co-workers reported that EPO administration to the 5/6 nephrectomized rats caused progression of the renal deterioration and increased mortality as compared to control rats [16]. However, such deleterious effect has not been reported in clinical setting where EPO is commonly used by patients with CKD for anemia correction [17]. In addition, we reported that the final plasma EPO concentration in the prophylactically-treated diabetic rats was noticeably higher than its level in other groups, such finding that could be attributed to decline of the urinary excretion of EPO as a part of the general picture of deteriorated nephropathy. Supporting evidence of this finding also exists to denote that prolonged EPO administration resulted in an inhibition of the urinary excretion of endogenous EPO [18].

We used EPO in the same dose used by Toba and co-workers [10] who reported that neither blood pressure nor Hct was affected by the administration of EPO in a dose of 150 U/kg three times/week for 4 weeks. However, our results showed that 28 weeks administration of this low dose led to rise of blood pressure and Hct in non-diabetic rats; so, longer treatment duration would indeed allow EPO-side effects to show up and confirms other reports which indicate that hypertension develops in approximately 30% of patients with CKD maintained on EPO therapy [19]. Mechanisms of this hypertension have been proposed to include increased blood viscosity, endothelin release, and inhibition of endothelial nitric oxide production [20-21]. Systemic hypertension could further aggravate glomerular dysfunction through augmentation of renal hypoxia and aggravation of oxidative stress in the kidney [22].

The chronic hypoxia hypothesis proposed that hypoxic milieu precedes and triggers a fibrotic response leading to renal fibrosis and progression of glomerular injury [23-24]. Likewise, it was postulated that hypoxia-induced tubulointerstitial injury may provoke interstitial fibrosis and weakness of peritubular capillaries that would impair the tubular and interstitial oxygen supply [25]. These hazards would definitely constitute a vicious circle that intensifies renal injury. In the present study, we measured renal vPO₂ as an indicator of renal tissue hypoxia and we found that, in diabetic rats, vPO₂ was significantly lower than control rats denoting an increased intrarenal consumption of oxygen probably as a reflection of increased intrarenal oxidative stress [26]. Al-

though the administration of EPO to diabetic rats in both the prophylactic and therapeutic schedules has partially improved renal vPO₂ when compared to non-treated diabetic rats, but prophylactically-treated animals suffered both exaggerated albuminuria and worsened histological picture than non-treated diabetic animals, suggesting that the pathogenesis of DN is a multifactorial process that relies on complex signaling networks rather than hypoxia per se. However, in the therapeutically-treated rats, the improvement of vPO₂ was greater than that found in the prophylactically-treated ones suggesting that administration of EPO at this precise point might disrupt the renal hypoxia-induced endogenous EPO secretion cycle and possibly affect other signaling networks involved in the evolution of DN, consequently attenuated the progression of DN.

We also found that administration of EPO for 28 weeks in control non-diabetic rats produced significant increase of Hct value. This result proves that prolonged administration of such low-dose of EPO is hematologically effective; however, this hematopoietic effect failed to be similarly reproduced in diabetic rats prophylactically-treated with EPO for 28 weeks which showed hematological profile nearly similar to the untreated diabetic group. A possible explanation might be the development of resistance to both exogenously administered and the endogenously secreted EPO within the hyperglycemic environment as a result of chronic low-grade inflammation with its impact on the hematopoietic process [27] and aggravation of oxidative stress [28]. In support of this assumption, a number of preclinical and clinical studies have reported a major role of several inflammatory cytokines, adhesion molecules, and chemokines in the development and progression of DN [29-30]. These pro-inflammatory cytokines downregulate the EPO receptors' expression on erythroid progenitors and interrupt iron recycling by impeding its release from reticuloendothelial cells [31-32]. In addition, the oxidative stress is a critical factor in the etiology of diabetic complications [33] and may participate in EPO resistance by causing lipid peroxidation of red cell membranes [34].

In conclusion, prophylactic low-dose administration of EPO for 28 weeks to diabetic rats led to marked progression of albuminuria and worsening of the renal histological changes in spite of partial improvement of renal hypoxia. This was joined to increased endogenous EPO secretion and rise of final MBP. By contrast, some benefit was obtained

when EPO was only given for the last 8 weeks after development of DN as a therapeutic intervention. It is probable that renal hypoxia occurs at a particular time during the course of diabetic renal disease might be able to have adverse renal outcome, while the administration of EPO at this particular time could disrupt the renal hypoxia-induced endogenous EPO secretion cycle and hence prevents or slows down the evolution of DN. Moreover, chronic hypertension triggered by long-term administration of EPO could further aggravate glomerular dysfunction and intrarenal oxidative stress. However the precise mechanism(s) of this differential effect of EPO in DN should be investigated in further studies in which precise assessment of EPO-induced renal injury on the molecular level may enhance some mechanistic insight. Likewise, whether the hyperglycemic environment interfered or masked a possible renoprotective role of EPO in diabetic rats should be further investigated in clinical studies.

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Conflict of interest

The authors declare that they have no conflict of interest.

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