Alexandria Journal of Medicine

ISSN: 2090-5068 (Print) 2090-5076 (Online) Journal homepage: https://www.tandfonline.com/loi/tajm20

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To cite this article: Alaa El Din Hassan, Eman A. Shaat, Maha M. Deif, Nesrine M. El Azhary & Eman M. Omar (2014) Effect of erythropoietin hormone supplementation on renal functions and the level of hypoxia-inducible factor-1α in rat kidneys with experimentally induced diabetic nephropathy, Alexandria Journal of Medicine, 50:1, 69-75, DOI: 10.1016/j.ajme.2013.06.003

To link to this article: https://doi.org/10.1016/j.ajme.2013.06.003

Alexandria Journal of Medicine (2014) **50**, 69–75

Alexandria University Faculty of Medicine

Alexandria Journal of Medicine

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Effect of erythropoietin hormone supplementation on renal functions and the level of hypoxia-inducible factor-1α in rat kidneys with experimentally induced diabetic nephropathy

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Received 25 February 2013; accepted 27 June 2013
Available online 31 July 2013

KEYWORDS

Erythropoietin;
Diabetic nephropathy;
Hypoxia-inducible factor-1α

Abstract Erythropoietin (EPO) is a hematopoietic factor with multiple protective effects. The aim of the present study was to investigate the potential effect of EPO administration on renal functions and hypoxia inducible factor 1-alpha (HIF-1α) in diabetic rat kidneys. The current study was carried out on 40 male albino rats divided into four groups ($n = 10$ in each). Group I served as normal control, group II was the diabetic control, group III rats received EPO on the same day of diagnosis of diabetes mellitus (DM), while group IV received the first dose of EPO 2 weeks after the diagnosis of DM. The results showed that EPO supplementation leads to a significant decrease in serum urea, urinary protein and creatinine clearance as well as a significant increase in renal HIF-1α in group III and IV rats compared to the diabetic control group (group II). However, fasting blood glucose was significantly decreased in group III as compared to the diabetic control group in the third week, but no significant difference was reported in the fourth week among groups II, III and IV.
Conclusion: EPO administration leads to the improvement of renal functions and increased levels of HIF-1α in diabetic rats.

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Peer review under responsibility of Alexandria University Faculty of Medicine.

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1. Introduction

Diabetic nephropathy (DN) is the most common cause of end stage renal disease in industrial countries. Proposed mechanisms of DN include hyperglycemia, advanced glycation end-products (AGE), which increased the permeability of the basement membrane and reduced the expression of nephrin.[1] Hyperglycemia was also found to increase the expression of transforming growth factor beta (TGF-ß) in the glomeruli

2090-5068
http://dx.doi.org/10.1016/j.ajme.2013.06.003

which may contribute to both the cellular hypertrophy and enhanced collagen synthesis observed in DN.[2]

Messenger RNA encoding the erythropoietin (EPO) receptor has been detected in several tissues including the kidney, suggesting paracrine extraerythropoietic actions of EPO.[3]

To protect tissues suffering from hypoxia, cells suppress protein synthesis and activate other pathways essential to survival, which results in the expression of genes involved in adaptation to hypoxia. Hypoxia inducible factor alpha (HIF-1α) is an essential corner stone in this adaptive process.[4]

2. Materials and methods

After approval of the Ethics Committee of the Faculty of Medicine, Alexandria University, this study was carried out on 40 male albino rats weighing 200–250 g. Rats were maintained under standard conditions with free access to food and water. Diabetes mellitus was induced in thirty rats by a single intraperitoneal injection of Streptozotocin (STZ) (Sigma, Chemicals, St. Louis, MO) in a dose of 55 mg/kg,[5] dissolved in sodium citrate buffer.[6] Only rats showing glucosuria $\geqslant$50 mg/dl by strip (Medi-Test Combi 3A Strips; Germany) three days after injection of STZ were considered diabetic and were included in the study.[7] No hypoglycemic drugs were given to the rats during the study period. All animals were treated in accordance with the Declaration of Helsinki for treating laboratory animals.

Rats were divided into four groups (10 rats each). Group I (Normal Control): Non-diabetic rats that received a single intraperitoneal dose of solvent (Sodium Citrate, pH = 4.5). Group II (Untreated diabetic): Diabetic rats were given subcutaneous injections of saline every 2 days for 2 weeks (a total of 6 injections) starting the first dose on the same day of diagnosis of DM. Group III (Preventive Diabetic Group): Diabetic rats that received subcutaneous injections of 100 IU/kg recombinant human EPO every 2 days for 2 weeks (a total of 6 injections) starting the first dose on the same day of diagnosis of DM. Group IV (Treated Diabetic Group): Diabetic rats showing proteinuria, were given subcutaneous injections of 100 IU/kg recombinant human EPO every 2 days for 2 weeks (a total of 6 injections) starting the first dose 2 weeks after diagnosis of DM. The study period lasted for 4 weeks. The following parameters were measured weekly:

- Blood glucose level (mg/dl)[8] from fresh samples by glucose oxidase (GOD)-peroxidase (POD) method. (Diamond Diagnostic; Germany).
- Blood urea level (mg/dl)[9] by enzymatic method (modified Berthelot reaction) (dp international; Tuscaloosa: USA).
- Creatinine level in blood and urine (mg/dl)[10] by a colorimetric kinetic method (Bio-diagnostic; Paterson, New Jersey, United States).
- Protein in urine (mg/24 h)[11,12] by Folin–Lowry Colorimetric Method (Bio-diagnostic; Paterson, New Jersey, United States).

In addition, at the end of study period, rats were sacrificed and total Hypoxia Inducible Factor-1 alpha (HIF-1α) was estimated in homogenized renal tissue (pg HIF-1α/mg protein)[13] by immunoassay (R&D systems; USA).

3. Statistical analysis

Data were collected and analyzed using SPSS software package version 17. Quantitative data were analyzed using *F*-test (ANOVA) to compare between the different groups. Pearson coefficient was used to estimate the correlation between different parameters. *P* value was assumed to be significant $\leqslant$0.05.

4. Results

The fasting blood glucose level in groups II, III and IV diabetic rats was significantly higher than in group I control rats in the first week (340 ± 7.04, 310.5 ± 7.12, 310.5 ± 5.56 and 80.4 ± 5.32, respectively) ($P = 0.001$). However, in the third week EPO supplementation in group III significantly lowered the fasting blood glucose as compared with group II (350.00 ± 10.01 and 398.5 ± 17.88 mg/dl) ($P = 0.001$). In the fourth week, no significance was reported between groups II, III and IV (Fig. 1).

The serum urea levels in the diabetic control group (group II) were significantly higher than those of group I (normal control) during the whole study period, while in group III, serum urea levels were significantly higher than in group I in the 2nd, 3rd and 4th weeks (22.12 ± 2.95, 25.25 ± 4.76 and 26.33 ± 4.52 mg/dl in group III versus 19.31 ± 1.12, 20.49 ± 1.85 and 19.60 ± 2.27 mg/dl in group I) with high significance detected on the fourth week ($P = 0.006$, $P = 0.004$, $P = 0.001$, respectively). However, serum urea levels in group III (20.70 ± 1.19, 22.12 ± 2.95, 25.25 ± 4.76 and 26.33 ± 4.52 mg/dl during the four studied weeks, respectively) were significantly lower than in group II (27.90 ± 2.92, 33.40 ± 3.10, 40.10 ± 5.70 and 73.07 ± 10.15 mg/dl, $P = 0.001$). In treated diabetic rats (group IV), serum urea levels were significantly higher than in group I during the four studied weeks (28.36 ± 5.19, 30.28 ± 11.02, 31.59 ± 12.26 and 32.80 ± 9.68 mg/dl in group IV versus 20.70 ± 1.19, 19.31 ± 1.12, 20.49 ± 1.85 and 19.60 ± 2.27 mg/dl in group I, $P = 0.001$, 0.003, 0.006 and 0.001, respectively) and significantly lower than group II in the 3rd and 4th weeks with high significance in the fourth week ($P = 0.031$ and 0.001, respectively) (Fig. 2).

The urinary protein levels of groups II and IV were significantly higher than group I during the whole study period. In group III rats that received EPO for 2 weeks since the diagnosis of DM, urinary proteins were significantly lower than group II untreated diabetic rats in the third and fourth weeks (10.25 ± 5.12 and 12.86 ± 3.46 mg/24 h in group III versus 28.32 ± 5.67 and 40.06 ± 8.86 mg/dl in group II, $P = 0.001$). However, in group IV rats that received EPO two weeks after the diagnosis of DM, urinary protein levels were significantly lower than group II (untreated diabetic) in the 3rd and 4th weeks only (17.10 ± 0.88 and 20.43 ± 3.48 mg/24 h in group IV versus 28.32 ± 5.67 and 40.06 ± 8.86 mg/24 h in group II, $P = 0.001$) (Fig. 3).

Creatinine clearance in group II diabetic rats was significantly higher than group I rats in the 2nd, 3rd and 4th weeks (1.41 ± 0.06, 2.09 ± 0.06 and 2.17 ± 0.24 ml/min in group II versus 1.13 ± 0.04, 1.14 ± 0.19 and 1.04 ± 0.15 ml/min in group I, $P = 0.001$). In the preventive diabetic group (group III), creatinine clearance was significantly higher than in group I mainly in the 3rd and 4th weeks (1.32 ± 0.15 and 1.44 ± 0.07 ml/min in group III versus 1.14 ± 0.19 and

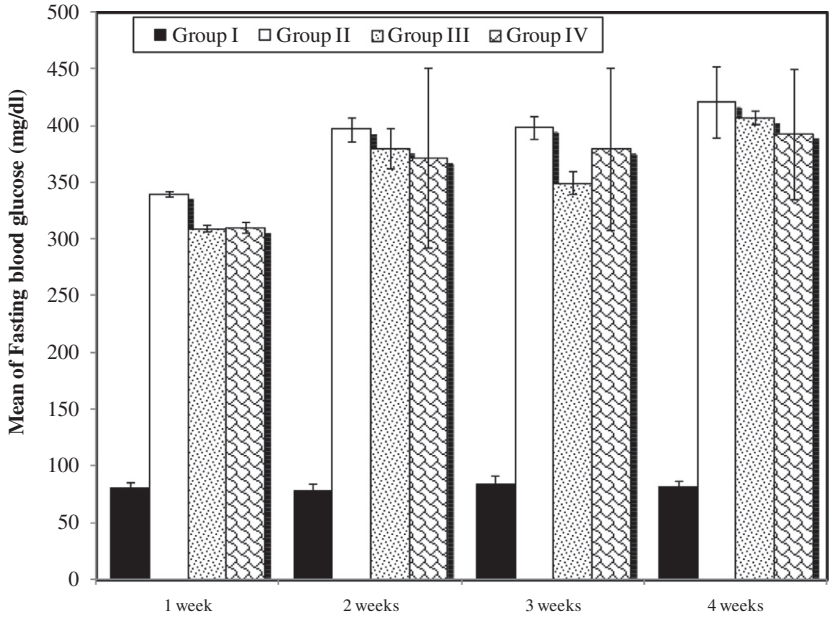

Figure 1 Fasting blood glucose level (mg/dl) in the different studied groups.

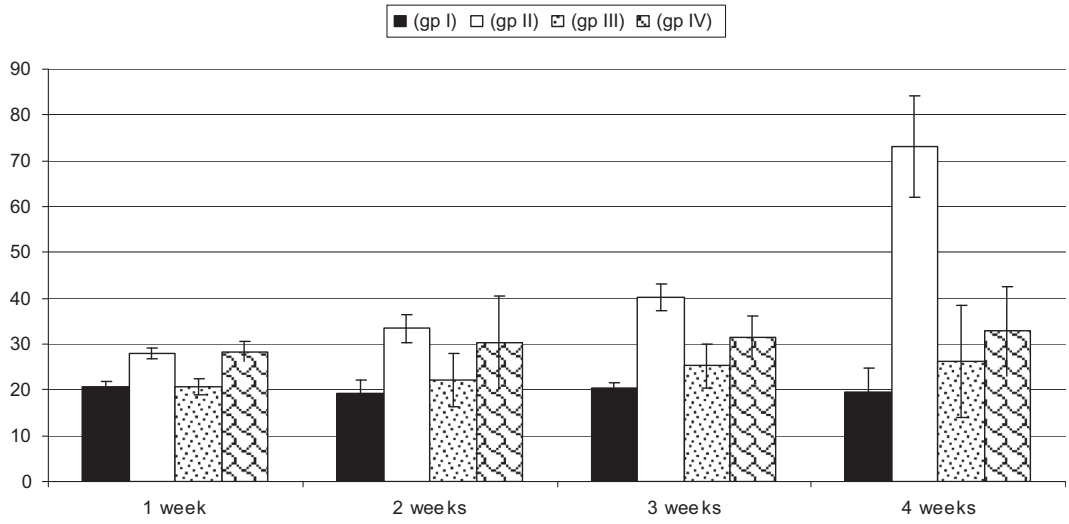

Figure 2 Serum urea levels in (mg/dl) in the different studied groups.

1.04 ± 0.15 ml/min in group I, $P = 0.014$) with high significance detected in the fourth week ($P = 0.001$). However, in the 2nd, 3rd and 4th weeks, the creatinine clearance level was significantly lower in group III (1.12 ± 0.05, 1.32 ± 0.15 and 1.44 ± 0.07 ml/min) than in group II (1.41 ± 0.06, 2.09 ± 0.06 and 2.17 ± 0.24 ml/min) and group IV rats (1.41 ± 0.21, 1.63 ± 0.22 and 1.63 ± 0.13 ml/min ($P = 0.001$). In treated diabetic group (group IV), creatinine clearance level was significantly lower than the diabetic control group in the 3rd and 4th weeks ($P = 0.001$) (Fig. 4).

HIF-1α in renal tissue showed a highly significant increase 4 weeks after the induction of diabetes in groups II, III and IV (114.04 ± 31.58, 547.86 ± 218.23 and 380.30 ± 101.27 pg/mg protein, respectively) when compared to the normal control group I (26.38 ± 8.78 pg/mg protein) ($P = 0.001$). However, erythropoietin-supplied groups (preventive group III and treated diabetic group IV) showed significantly higher levels than the diabetic control group ($P = 0.001$). Group III rats reported a significantly higher level of HIF-1α than group IV ($p = 0.02$) (Fig. 5, Table 1). See (Fig. 6, Table 2).

5. Discussion

Diabetes mellitus (DM) is a chronic disease resulting in multiple complications, including nephropathy.[14] Numerous studies reported the association of DM with several changes in the structure and function of the kidney.[15] Strict control of blood glucose and blood pressure levels sometimes fail to delay the development of diabetic nephropathy (DN), and an effective therapy is not yet available.[16]

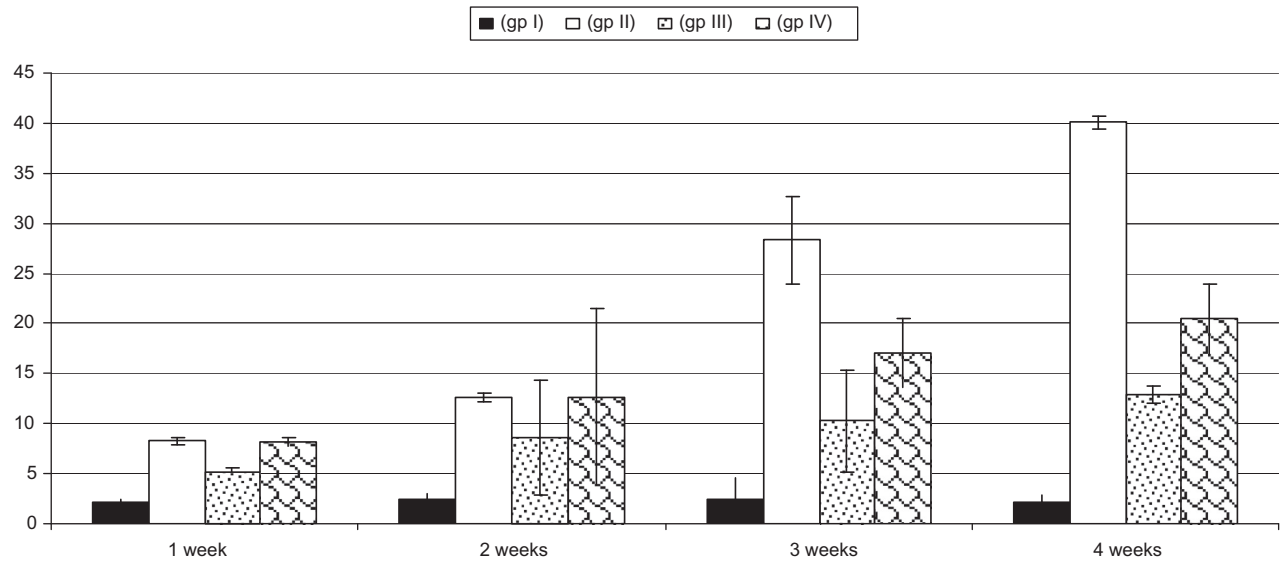

Figure 3 Protein in urine (mg/24 h) in the different studied groups.

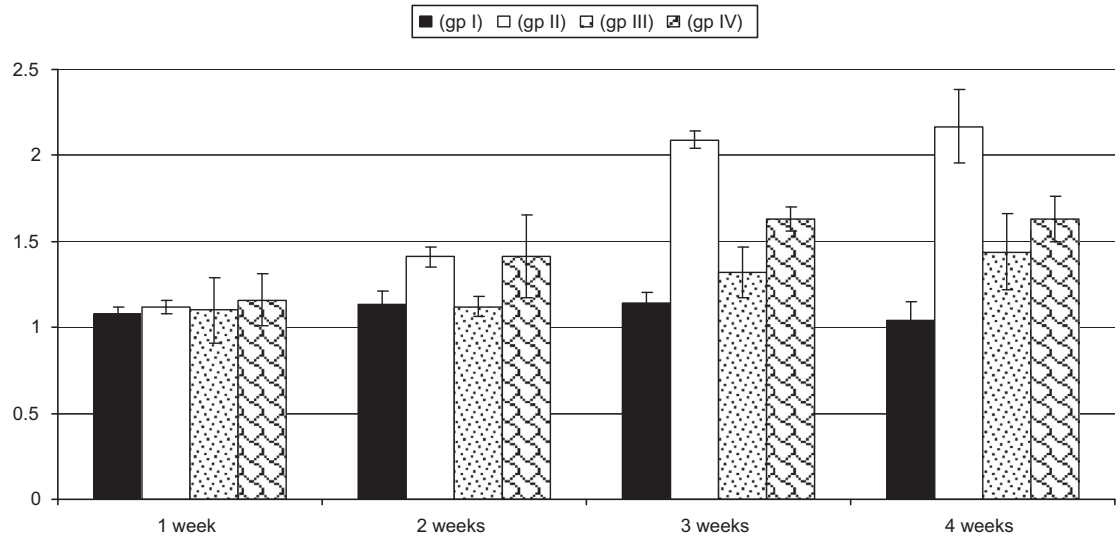

Figure 4 Creatinine clearance (ml/min) in the different studied groups.

In the present study, the fasting blood glucose level was significantly higher in groups II, III and IV than in group I rats throughout the study period. This is explained by the cytotoxic effect of streptozotocin on beta cells of the islets of Langerhans, liberation of free radicals and toxic amount of nitric oxide causing necrosis of these cells.[17] Moreover, in the third week of the study, a significant decrease was noticed in the preventive group (III) as compared with the diabetic control group (II), however no significant change was reported in the fourth week between groups III and IV. This hypoglycemic effect in group III in the third week may be explained by the increase in the erythrocyte counts and their consequent uptake of glucose previously reported by Montel-Hagen et al.[18] Another work that supports the previous result reported an increase in the circulation volume of total red blood cells after twelve days of recombinant erythropoietin injections into nephrectomized rats.[19] By the fourth week, no significant differences were reported among groups II, III and IV. The possible reason for this was that the beta cells have already been destroyed by streptozotocin and the possible improvement of the function of the remaining beta cells was not sufficient to return the blood glucose level to the normoglycemic state in spite of the antioxidant effect of erythropoietin.[20] Similar results were reported by other researchers who found that after 5, 8, or 11 weeks, all diabetic rats remained hyperglycemic, and EPO treatment did not significantly affect blood glucose levels.[21] Katz et al.[22] reported that after one week of treatment, all EPO-treated mice had lower blood glucose levels, and these levels were further reduced during the second week of treatment, which may be explained by the reduction of insulin resistance; as EPO increased sensitivity to insulin and also the beta cells were not yet totally destroyed.[22]

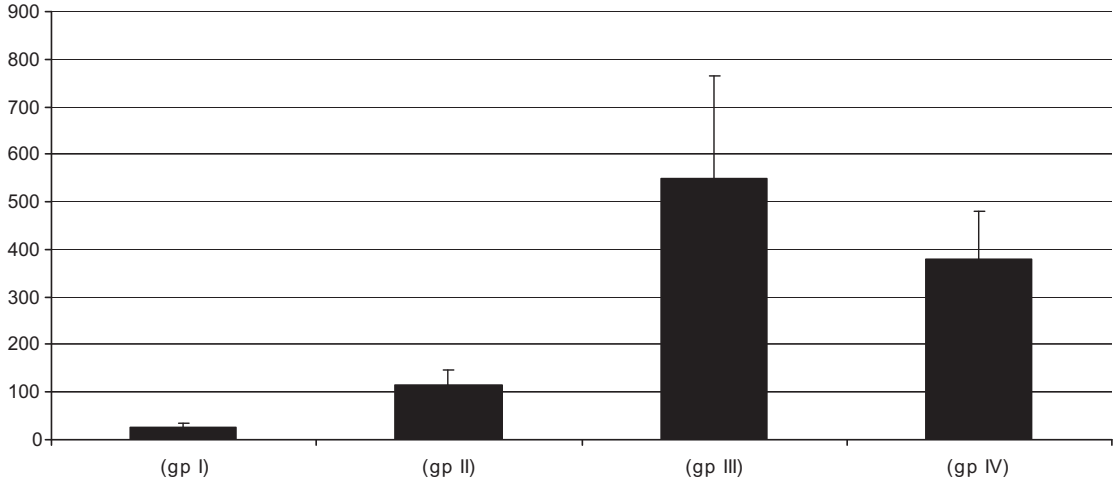

Figure 5 Hypoxia inducible factor 1-alpha (HIF-1α) levels (pg/mg protein) in the different studied groups.

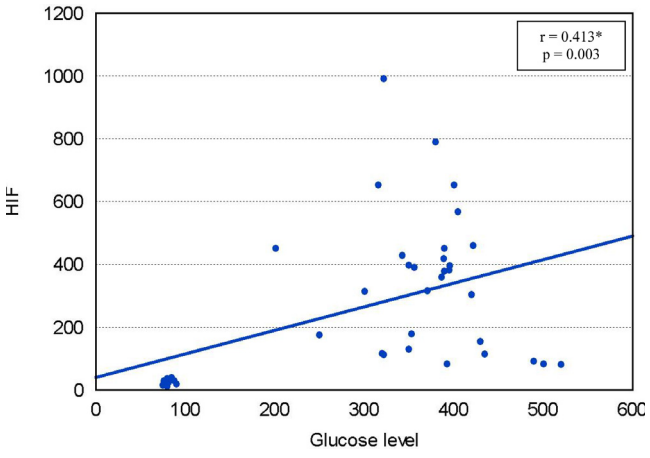

Figure 6 Correlation between HIF-1α and glucose in all groups.

In the present study, group II diabetic rats showed a significantly higher rate of urinary protein excretion one week after the onset of the diabetes when compared with values of normal control rats. Proteinuria progressively increased throughout the whole length of the study period, with maximal level reached at the end of the fourth week. Ronchi et al.[23] reported that diabetic rats showed a significant increase in the excretion of urinary proteins starting as early as 24 h after the induction of DM, which may be related to glomerular dysfunction and damage, while Farvid et al.[24] postulated that proteinuria predicted the onset of overt renal disease in diabetic patients. While, in the third and fourth weeks, groups III and IV showed significantly decreased levels of proteinurea than group II. This is in accordance with the results obtained by Zafirov et al.[25] who explained reduced proteiurea by the reno-protective effect of recombinant human erythropoietin manifested by the stimulation of tubular cell proliferation and regeneration, thus reducing the functional renal failure and renal damage induced by cisplatin.[25]

Moreover, in this study, serum urea level in the preventive diabetic group III rats was significantly lower than the diabetic control group during the four studied weeks. On other hand, in the treated diabetic group IV rats, serum urea level was significantly lower than the diabetic control group in the 3rd and 4th week, with high significance in the fourth week. This denotes improvement of renal function in rats that received EPO. Similar observations were reported by Toba et al.[26] who demonstrated direct reno-protective effects of chronic treatment with low dose of recombinant human EPO (150 IU/kg three

Table 1 Hypoxia inducible factor 1-alpha (HIF-1α) level (pg/mg protein) in the different studied groups.

	Treated diabetic (group IV)	Preventive diabetic (group III)	Diabetic control (group II)	Normal control (group I)
HIF-1α				
Min–Max	179.2–568	303.77–992	81.9–175.1	11.7–40.5
Mean ± SD	380.30 ± 101.27	547.86 ± 218.23	114.04 ± 31.58	26.38 ± 8.78
P1	0.001[b]			
P2	0.001[b]			
P3	0.001[b]			
P4	0.001[b]			
P5	0.001[b]			
P6	0.020[a]			

P1 comparison between normal control group I and diabetic control group II.
[a] Significant at $P \leqslant 0.05$.
[b] Highly significant at $P \leqslant 0.001$.

Table 2 Correlation studies.

HIF	Serum urea	Protein in urine	Fasting glucose	Creatinine clearance
R	−.234	−.040	.413(**)	.033
P	.146	.808	.003	.839

times per week subcutaneously), which included decreased proteinuria and slowing of renal dysfunction and fibrosis in the streptozotocin-induced diabetic rats models.

Several studies reported that preconditioning the kidney with EPO before ischemia reperfusion injury protects the kidney.[27,28] Menne et al.[21] provided evidence that continuous erythropoietin receptor activator (CERA) may exert dose dependent protective effect on diabetic kidneys, decreased albuminuria, reduced expression of TGF-beta, decreased vascular endothelial growth factor, and reduced deposition of collagen IV in the glomeruli and tubulointerstitial area. Overexpression of TGF-B1 is harmful as it stimulates podocyte expression of collagen IV, podocyte detachment and/or apoptosis.[29,30]

Binding of EPO to its receptor triggers the activation of signal transducer and activator of transcription (STAT) 5 proteins, which enters the nucleus, and enhances the transcription of various genes. In fact, EPO may act as a survival factor by inhibiting apoptosis through the anti apoptotic molecule Bcl-x(L), an anti-apoptotic protein of the Bcl-2 family. Yang et al.[27] showed that EPO also induces heat shock protein 70 which possesses antiapoptotic effect by the inhibition of apoptosis protease-activating factor-1 and of apoptosis-inducing factor.

In the current work, creatinine clearance was significantly increased in group II than the control group in the second, third and fourth weeks, this may be due to hyperfiltration which is widely regarded as a contributing factor to the development of microalbuminuria and progressive nephropathy in type 1 diabetes.[31]

Creatinine clearance was significantly lower in diabetic rats that received EPO since the diagnosis of DM (group III) than untreated group II rats. This is also most probably due to the protective antiapoptotic effect of EPO.[27] In group IV, creatinine clearance decreased significantly compared with group II. This may be explained by the hypothesis that EPO was found to decrease the glomerular filtration rate documented in humans.[32]

Hypoxia inducible factor-1α (HIF-1) is a transcriptional factor responsible for cellular adaption to low oxygen tension. It regulates a group of genes involved in angiogenesis, iron metabolism, glucose metabolism as well as cellular proliferation and survival.[33]

In this study, HIF-1α levels showed a significant increase 4 weeks after the induction of diabetes in groups (II, III and IV) when compared to the normal control group (I). However, erythropoietin-supplied groups (preventive group III and treated diabetic group IV) had significantly higher levels than the diabetic control group. This coincides with the findings of other researchers that detected renal HIF- alpha in the glomeruli of rats 30 days after the induction of DM.[34]

In the current study, a significant positive correlation was observed in all the studied groups between blood glucose levels and renal HIF-1α levels. A recent study reported that high glucose exposure induced the production of reactive oxygen species (ROS) and, in parallel, induced glucose 6 phosphatase (G6pc) promoter activity. This, in turn, increased glucose release from hepatocytes. This recent study by Gautier-Stein et al. deciphered a new regulatory mechanism leading to the induction of HIF-1 transcriptional activity which may contribute to the increase of hepatic glucose production during diabetes.[35]

The current data show a delayed onset of nephropathy in diabetic rats which received erythropoietin immediately on the diagnosis of DM (preventive diabetic group). This was manifested by lower urinary protein levels, serum urea and higher creatinine clearance. The results also show the slower progression of nephropathy in diabetic rats that received EPO 2 weeks after the appearance of proteinuria (treated diabetic group). However, urinary protein levels and serum urea levels are much better in the preventive group than the treated group at the end of study. The reno-protective effect of erythropoietin may be due to increased levels of HIF-1α, with its specific target genes that fight oxidative stress, ameliorate blood oxygen, glucose supply and block apoptotic pathways.[33]

Imamura et al.[36] also confirmed the reno-protective effects of EPO treatment in ischemia reperfusion injury (I/R) by the induction of both HIF-1 alpha and vascular endothelial growth factor mRNA under hypoxic conditions, through attenuation of tubular hypoxia. Moreover, erythropoietin could enhance eNOS expression via HIF-2 activation.[37] Recent studies have also revealed that EPO suppresses the up-regulated expression of TGF-β, and may possibly improve the accumulation of extracellular matrix.[38,39]

The protective effects of erythropoietin on tubulointerstitial fibrosis are supported by decreased renal fibrosis in mice by inhibiting TGF-β.[38] Lastly, it has recently been demonstrated that EPO directly ameliorates podocyte injury resulting in the prevention of glomerulosclerosis.[40]

The results of the current study suggest that prophylactic EPO administration in diabetic rats is more beneficial than its administration 2 weeks after the induction of DM. These reno-protective effects of EPO are related to increasing the levels of hypoxia inducible factor-1α. In fact, kidneys of diabetic rats are hypoxic even at an early stage, HIF activation is neither maximal in kidney disease nor in diabetic conditions not receiving EPO.[34]

However, further studies are needed to estimate the proper timing and dosage for EPO administration to maximally stimulate the expression of HIF-1α and delay the occurrence of diabetic nephropathy.

Conflict of interest

None declared.

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