

Anemia and Microalbuminuria in Induced Diabetic Nephropathy in Rats

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Abstract: Animals with diabetic nephropathy may suffer from anemia even before the onset of advanced renal failure and it has been related to erythropoietin deficiency. The present experimental study was carried out on thirty-six Sprague Dawley male Albino rats (Approx 180 g). The rats were randomly divided into 4 groups (n=9) as follows; Group I non-diabetic control animals. Group II diabetic control animals treated with alloxan monohydrate by a single intra-peritoneal injection of 120mg/kg BW. Group III treated with erythropoietin vial in a dose of 300U/kg i.p. once per week. Group IV, rats treated with alloxan then treated with human recombinant erythropoietin (RhEPO) in a dose of 300U/kg i.p. once per week. The experiment lasted for six weeks. Blood samples were collected for studying clinicopathological changes (hematological parameters, blood glucose level, blood urea nitrogen (BUN) and serum creatinine). Urine analysis was assayed in all groups for presence of glucose and protein by urine dipstick, urine protein / creatinine ratio (UP/ C) and microalbuminuria. Results revealed normocytic normochromic anemia in diabetic control group (Group II) which is responds to exogenous EPO administration in the form of recombinant human EPO (Group IV). The observed anemia is due to diabetic nephropathy. UP/C is not a reliable method for detecting albuminuria in the range of 0-300mg/dl. The result suggested that microalbuminuria is a good predictor of proteinuria and is used to predict early renal disease associated with diabetes mellitus.

Key words: Diabetes Mellitus • Erythropoietin • Urine Analysis • Microalbuminuria

INTRODUCTION

Diabetic nephropathy is a worldwide public health concern as it becomes the most common single cause of end-stage renal disease in the U.S.A. and Europe [1]. The quality of life and prognosis of diabetic patients are influenced by nephropathy, which is a severe and chronic disorder. The mortality rate of patients with diabetic nephropathy is 30 times more than diabetic patients without nephropathy [2].

Patients with diabetic nephropathy may also suffer from anemia even before the onset of advanced renal failure and it has been related to erythropoietin deficiency [3]. Furthermore, anemia has been considered a risk factor for progression of renal disease as the low Hb concentration is significantly associated with a more rapid decline in the glomerular filtration rate (GFR). Many recent studies have incriminated chronic hypoxia as a possible final common pathway in end-stage kidney

injury [4], so treating anemia early in renal failure has been demonstrated to slow the development of diabetic nephropathy [2].

In animals, veterinarians commonly use blood urea nitrogen and serum creatinine concentrations, coupled with urine specific gravity, to assess the renal function. Unfortunately, these laboratory values only become abnormal when approximately 25 to 30% of renal function remains. By that time, renal disease is often too advanced for medical intervention to significantly help the condition [5].

In dogs with diabetes mellitus, the classic clinical course of diabetic nephropathy is described as the development of microalbuminuria, which eventually leads to progressive loss of glomerular filtration rate (GFR) [6].

Microalbuminuria is an early sign of diabetic nephropathy which defined as a urinary albumin concentration between 1.0 and 30.0 mg/dl. These concentrations are not detected by standard urine

dipstick assays. Microalbuminuria generally precedes overt proteinuria so; early screening for microalbuminuria is an excellent marker of early kidney disease and subclinical disease to prevent end-stage renal disease [7].

The aim of the present work is to discuss the different methods for early screening and diagnosis of diabetic nephropathy through microalbuminuria in correlation to clinicopathological examination and urine analysis. Furthermore, studying the effect of human recombinant erythropoietin (RHEPO) on the diabetic rats.

MATERIALS AND METHODS

Animals and Experimental groups: Thirty-six Sprague Dawley male Albino rats (Approx. 180 g) obtained from the animal house of Faculty of Veterinary Medicine, Cairo University, Egypt. The rats were housed in standard cages with free access to a standard commercial diet and water *ad libitum*.

The rats were randomly divided into 4 groups (n =9) as follows;

Group I: (Non-diabetic control animals), received a normal diet (Saline solution, i.p.).

Group II: (Diabetic control animals treated with alloxan monohydrate to induce diabetes in rats). The dose was 120 mg/K.g.B.W as a single dose Alloxan was dissolved in saline solution (0.9% sodium chloride PH 7) and injected intra-peritoneal.

Group III: [Treated with erythropoietin vial (Epoetin beta, human recombinant erythropoietin (RhEPO) by Amoun in a dose of 300U/kg i.p. once per week for six weeks].

Group IV: Rats treated with alloxan (120mg/kgBW i.p.) then treated with human recombinant erythropoietin (RhEPO) in a dose of 300U/kg i.p. once per week for six weeks.

The rats developed hyperglycemia after 7 days from alloxan injection. The rats with blood glucose above 200 mg/dl with signs of polyuria, polydypsia, polyphagia, weight loss, glucosuria and hyperglycemia were selected for the experiment. The experiment was conducted for six weeks.

Samples

Blood Samples: Blood sample was collected from each rat through venous plexuses once per week. Samples were divided into two portions, the first blood sample

(Whole blood) was anticoagulated by disodium ethylene diamine tetra-acetic acid (EDTA) and used for evaluating hemogram [Erythrocyte count (RBCs), packed cell volume (PCV), hemoglobin concentration (Hb), total leukocyte count (TLC) and differential leukocytic count (DLC)] [8].

The second portion was collected in a clean centrifuge tube for serum separation. The clear, non-hemolysed supernatant serum was harvested for biochemical studies {Blood glucose [9], blood urea nitrogen (BUN) [10] and serum creatinine [11]}.

Serum biochemical parameters were assayed using reagent kits supplied by Stan Bio-Laboratories incorporation, USA.

Urine Samples: Each rat was placed overnight in a metabolic cage for urine collection once per week. The urine analysis was assayed in all groups for presence of glucose and protein by urine dipstick (Multistix, Bayer Corporation, Elkhart, Ind, USA); urine protein / creatinine ratio(UP/ C) [12] and microalbuminuria (Albumin in urine) [13].

Statistical Analysis: Values were expressed as mean \pm SD. Statistical comparisons among the means of different experimental groups were made with completely randomized two ways ANOVA "Student-Newman-Keuls test" by COSTAT program version one. A probability "P" value of <0.05 was assumed for statistical significance.

RESULTS AND DISCUSSION

Hematology: Mean values of the hemogram [Erythrocyte counts (RBCs), packed cell volume (PCV), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), total leukocyte (TLC) and differential leukocytic count (DLC)] were illustrated in Tables (1- 4).

In comparison to the mean values of group I (Non-diabetic control animals), normocytic normochromic anemia was observed in group II (Diabetic control group) manifested by significant decrease in total RBCs count, PCV and hemoglobin concentration with normal MCV and MCHC. Regarding to treated group with erythropoietin only (Group III) showed significant increase in RBCs count, PCV and hemoglobin concentration from the 4th week of experiment till the end of the experiment. Group IV (Rats treated with alloxan then treated with human recombinant erythropoietin) revealed insignificant change in erythrogram all over the experimental period.

Table 1: Erythrogram of different experimental groups (means \pm SD).

Weeks	RBCs count ($\times 10^9/\mu\text{l}$)				PCV (%)				Hb concentration (g/dl)			
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)
0	5.61 \pm 0.56	5.56 \pm 0.41	5.98 \pm 0.41	6.21 \pm 0.81	36.22 \pm 2.09	34.26 \pm 2.11	35.60 \pm 2.59	35.23 \pm 2.47	12.02 \pm 1.95	12.11 \pm 1.94	12.41 \pm 1.71	11.97 \pm 1.84
1	5.56 \pm 0.92	5.71 \pm 0.71	6.05 \pm 0.61	5.72 \pm 0.95	35.60 \pm 2.90	34.21 \pm 2.83	36.11 \pm 2.59	36.40 \pm 2.81	12.70 \pm 1.78	11.59 \pm 1.04	12.71 \pm 1.94	12.11 \pm 1.34
2	5.04 \pm 0.34	3.94 \pm 0.59	6.11 \pm 0.48	5.65 \pm 0.58	36.59 \pm 2.83	28.09 \pm 2.93	38.12 \pm 2.94	34.81 \pm 2.34	12.46 \pm 1.92	10.04 \pm 1.76	12.69 \pm 1.67	11.14 \pm 1.95
3	6.42 \pm 0.53	3.92 \pm 0.29	6.13 \pm 0.86	5.91 \pm 0.65	36.88 \pm 2.34	27.89 \pm 2.38	38.61 \pm 2.95	36.11 \pm 2.63	12.95 \pm 1.09	10.02 \pm 1.65	13.51 \pm 1.87	11.98 \pm 0.97
4	6.04 \pm 0.45	3.90 \pm 0.12	7.24 \pm 0.72	6.84 \pm 0.79	37.08 \pm 2.83	27.51 \pm 2.11	42.02 \pm 2.52	36.19 \pm 2.71	12.75 \pm 1.05	10.12 \pm 1.32	14.61 \pm 1.52	12.67 \pm 0.82
5	5.80 \pm 0.35	3.81 \pm 0.51	7.56 \pm 0.84	6.02 \pm 0.59	37.23 \pm 2.91	27.4 \pm 2.48	42.08 \pm 2.96	36.09 \pm 2.45	13.13 \pm 1.61	10.08 \pm 1.34	15.51 \pm 1.23	12.85 \pm 1.04
6	6.01 \pm 0.81	3.83 \pm 0.51	7.69 \pm 0.73	6.08 \pm 0.71	36.72 \pm 2.13	27.35 \pm 2.30	42.10 \pm 2.68	36.05 \pm 2.93	12.79 \pm 1.45	10.02 \pm 1.14	15.64 \pm 1.50	12.92 \pm 1.72
LSD	1.62				5.52				2.34			

Group (I) represents non-diabetic control rats.

Group (II) represents alloxan diabetic control rats.

Group (III) represents non-diabetic rats treated with erythropoietin.

LSD represents least significant difference between different groups at probability $P < 0.05$.

Group (IV) represents alloxan diabetic rats treated with erythropoietin.

Table 2: Values of MCV and MCHC of different experimental groups (Means \pm SD).

Weeks	MCV (fl)				MCHC (%)			
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)
0	64.59 \pm 0.14	61.19 \pm 0.87	58.58 \pm 1.75	58.56 \pm 1.64	33.10 \pm 1.87	35.34 \pm 2.90	34.01 \pm 2.13	33.97 \pm 2.09
1	64.61 \pm 0.97	61.15 \pm 0.97	58.53 \pm 0.97	58.30 \pm 0.24	35.12 \pm 2.34	33.90 \pm 1.98	35.19 \pm 1.76	33.26 \pm 2.16
2	65.52 \pm 1.23	59.64 \pm 1.32	60.52 \pm 1.85	63.80 \pm 1.80	34.30 \pm 1.87	36.09 \pm 1.95	33.28 \pm 1.08	33.19 \pm 2.05
3	68.25 \pm 1.42	70.12 \pm 1.62	62.90 \pm 1.93	62.10 \pm 0.85	35.81 \pm 0.85	35.92 \pm 0.83	34.97 \pm 2.06	33.17 \pm 2.34
4	66.64 \pm 0.96	70.53 \pm 0.59	63.30 \pm 0.83	61.20 \pm 0.99	35.52 \pm 2.61	36.08 \pm 2.92	35.95 \pm 2.76	35.02 \pm 2.91
5	64.05 \pm 1.64	70.07 \pm 1.87	64.02 \pm 0.87	60.71 \pm 1.45	35.41 \pm 1.98	36.70 \pm 2.14	36.91 \pm 2.93	34.91 \pm 2.13
6	60.68 \pm 1.23	71.30 \pm 0.97	62.90 \pm 1.23	59.82 \pm 1.06	34.82 \pm 2.07	36.66 \pm 2/15	36.92 \pm 2.54	35.83 \pm 1.98
LSD	11.38				3.82			

Group (I) represents non-diabetic control rats.

Group (II) represents alloxan diabetic control rats.

Group (IV) represents alloxan diabetic rats treated with erythropoietin.

LSD represents least significant difference between different groups at probability $P < 0.05$. _Group (III) represents non-diabetic rats treated with erythropoietin.Table 3: Total leukocyte (TLC), neutrophil and eosinophil count of different experimental groups (Means \pm SD).

Weeks	TLC ($\times 10^3/\mu\text{l}$)				Neutrophil count ($\times 10^3/\mu\text{l}$)				Eosinophil count ($\times 10^3/\mu\text{l}$)			
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)
0	6.91 \pm 2.03	6.12 \pm 2.01	6.05 \pm 1.31	6.92 \pm 1.41	3.42 \pm 0.64	3.45 \pm 0.74	3.96 \pm 0.23	3.09 \pm 0.76	0.32 \pm 0.05	0.26 \pm 0.12	0.15 \pm 0.04	0.23 \pm 0.15
1	6.94 \pm 1.98	6.06 \pm 1.56	5.71 \pm 1.63	6.84 \pm 1.94	3.44 \pm 0.89	3.78 \pm 0.34	3.21 \pm 0.91	3.61 \pm 0.86	0.06 \pm 0.03	0.10 \pm 0.02	0.51 \pm 0.05	0.32 \pm 0.05
2	6.86 \pm 2.09	10.47 \pm 1.65	6.32 \pm 1.82	7.81 \pm 2.04	3.50 \pm 1.09	5.88 \pm 0.62	3.53 \pm 0.83	5.10 \pm 0.54	0.08 \pm 0.02	0.25 \pm 0.12	0.37 \pm 0.11	0.21 \pm 0.08
3	5.78 \pm 0.87	10.52 \pm 3.02	6.26 \pm 1.92	11.23 \pm 2.63	2.19 \pm 0.67	8.91 \pm 1.69	3.45 \pm 0.45	8.83 \pm 2.95	0.20 \pm 0.05	0.19 \pm 0.01	0.24 \pm 0.09	0.19 \pm 0.04
4	7.40 \pm 1.65	12.89 \pm 3.01	6.81 \pm 1.11	12.48 \pm 2.34	3.61 \pm 0.65	10.21 \pm 1.87	3.51 \pm 0.83	7.98 \pm 2.04	0.25 \pm 0.13	0.31 \pm 0.14	0.22 \pm 0.14	0.22 \pm 0.13
5	7.21 \pm 2.43	12.60 \pm 2.63	6.91 \pm 2.05	10.02 \pm 2.83	3.87 \pm 0.64	10.19 \pm 2.08	3.23 \pm 0.64	7.42 \pm 2.63	0.23 \pm 0.14	0.29 \pm 0.11	0.37 \pm 0.18	0.30 \pm 0.11
6	6.29 \pm 2.05	10.59 \pm 2.05	6.61 \pm 1.69	9.56 \pm 2.13	3.23 \pm 0.75	8.42 \pm 2.12	3.22 \pm 0.86	7.34 \pm 2.81	0.35 \pm 0.16	0.23 \pm 0.16	0.30 \pm 0.15	0.24 \pm 0.16
LSD	3.51				2.20				NS			

Group (I) represents non-diabetic control rats.

LSD represents least significant difference between different groups at probability $P < 0.05$.

Group (III) represents non-diabetic rats treated with erythropoietin.

Group (IV) represents alloxan diabetic rats treated with erythropoietin.

Group (II) represents alloxan diabetic control rats.

It has been suggested that the reason for the early onset of normocytic normochromic anemia in diabetic control group is due to diabetic neuropathy, causing denervation of the kidney and loss of appropriate erythropoietin (EPO) production; damage to the renal interstitial and inhibition of EPO release [3]. The anemia characteristically responds to exogenous EPO administration in the form of recombinant human EPO which stimulate erythroid progenitor cells and

differentiation of normoblasts to increase the red cell mass as noticed in the fourth group or may lead to dramatic increase of RBCs mass as seen in the third group in absence of anemia [4, 14].

Concerning the leukogram, the results of group II (Control diabetic) showed leukocytosis with absolute neutrophilia and lymphopenia from the 2nd week till the end of experiment. The treated group with human recombinant erythropoietin only (Group III) showed

Table 4: Lymphocyte and monocyte counts of different experimental groups (Means \pm SD).

Weeks	Lymphocyte count ($\times 10^3/\mu\text{l}$)				Monocyte count ($\times 10^3/\mu\text{l}$)			
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)
0	2.15 \pm 1.23	2.10 \pm 2.03	2.14 \pm 1.34	2.09 \pm 1.32	0.21 \pm 0.02	0.14 \pm 0.11	0.45 \pm 0.11	0.51 \pm 0.08
1	2.25 \pm 1.24	2.06 \pm 1.87	2.13 \pm 1.86	2.12 \pm 1.01	0.19 \pm 0.11	0.17 \pm 0.11	0.51 \pm 0.13	0.34 \pm 0.09
2	2.26 \pm 1.09	1.75 \pm 1.08	2.11 \pm 1.04	2.01 \pm 1.21	0.18 \pm 0.10	0.18 \pm 0.12	0.40 \pm 0.16	0.23 \pm 0.14
3	2.07 \pm 1.81	1.72 \pm 0.97	1.94 \pm 1.80	1.87 \pm 0.97	0.45 \pm 0.12	0.24 \pm 0.17	0.37 \pm 0.17	0.42 \pm 0.17
4	2.11 \pm 1.42	1.65 \pm 0.78	2.92 \pm 1.01	1.09 \pm 0.65	0.60 \pm 0.13	0.41 \pm 0.24	0.35 \pm 0.15	0.34 \pm 0.08
5	3.02 \pm 1.62	1.89 \pm 0.88	2.12 \pm 1.20	1.19 \pm 0.79	0.50 \pm 0.14	0.35 \pm 0.19	0.37 \pm 0.16	0.29 \pm 0.04
6	2.82 \pm 1.43	1.67 \pm 0.96	2.10 \pm 1.11	1.14 \pm 0.54	0.65 \pm 0.21	0.39 \pm 0.18	0.98 \pm 0.45	0.58 \pm 0.05
LSD	0.45				NS			

Group (I) represents non-diabetic control rats.

Group (II) represents alloxan diabetic control rats.

Group (III) represents non-diabetic rats treated with erythropoietin.

LSD represents least significant difference between different groups at probability $P < 0.05$.

Group (IV) represents alloxan diabetic rats treated with erythropoietin

insignificant alterations in leukogram all over the experimental period when compared with Group I (Non-diabetic control animals). In Group IV (Rats treated with alloxan then treated with human recombinant erythropoietin) significant leucocytosis was denoted from the 3rd week with neutrophilia and lymphopenia (At 3rd and 4th week respectively) till the end of experiment. Significant changes were recorded in absolute values of eosinophils and monocytes in all treated groups.

Recorded leukocytosis in both Group II and Group IV (May contributed to the stressful condition or it may be associated with diabetic nephropathy due to changing of plasma cortisol and insulin levels. Both factors are known to increase WBC counts by increasing neutrophile influx from marrow storage and decreasing efflux from the blood stream [15].

The increasing of neutrophile number may be due to the engagement of these cells in the phagocytic process against different antigens or due to increase in hematopoietic activity after releasing the granules of neutrophile by exocytosis to lyses the antigens extra-cellular [16]. Moreover, it has been suggested that the body's defense mechanism against infections was disturbed due to the disturbed neutrophile function in diabetes [17].

The peripheral lymphocyte count was inversely related to the severity of diabetic nephropathy in the present study especially at the 4th week till the end of experiment in both second and fourth groups. This persistence lymphopenia may be as a response to stressful condition after antigen (Alloxan) injection which was in agreement with Soveny *et al.* [18], or it may be due to the production of specific or non specific

antibodies against different antigens, since lymphocytes are responsible for achieving the defense mechanism in the body [19].

Biochemical Examination

Blood Glucose: Blood glucose levels of experimental rats were shown in Table (5). Group II (Control diabetic) as well as Group IV (Rats treated with alloxan then treated with human recombinant erythropoietin) revealed significant increase in glucose levels from the first week till the end of experiment. Alloxan is widely used to induce experimental diabetes in animals. The mechanism of action of alloxan in B cells of the pancreas has been intensively investigated and now is quite well understood. The cytotoxic action of this diabetogenic agents is mediated by ROS. Alloxan and the product of its reduction, dialuric acid, establish a redox cycle with the formation of superoxide radicals. These radicals undergo dismutation to hydrogen peroxide. Thereafter highly reactive hydroxyl radicals are formed by the Fenton reaction. The action of ROS with a simultaneous massive increase in cytosolic calcium concentration causes rapid destruction of B cells [20- 22]. No significant alteration was observed in Group I and Group III.

Serum Creatinine and Blood Urea Nitrogen:

Serum creatinine and blood urea nitrogen levels of experimental rats were shown in Table (6). The diabetic group showed significant increase in both serum creatinine and BUN from the 2nd week till the end of experiment while Group IV showed significant increase in serum creatinine and BUN from the 5th and 3rd week till

Table 5: Levels of serum glucose concentrations of different experimental groups (Means \pm SD).

Weeks (p.i)	Serum glucose (mg/dl)			
	Group (I)	Group (II)	Group (III)	Group (IV)
0	76 \pm 5.73	74 \pm 11.63	72 \pm 9.59	75 \pm 10.34
1	79 \pm 10.7	251 \pm 9.62	75 \pm 9.72	199 \pm 6.62
2	78 \pm 10.81	262 \pm 15.69	79 \pm 7.92	187 \pm 6.32
3	84 \pm 9.51	273 \pm 13.54	84 \pm 8.66	197 \pm 5.83
4	88 \pm 9.91	288 \pm 10.19	86 \pm 8.10	188 \pm 7.55
5	89 \pm 9.81	269 \pm 10.42	87 \pm 8.80	207 \pm 5.94
6	87 \pm 5.96	289 \pm 13.42	88 \pm 8.65	205 \pm 5.33
LSD	13.50			

Group (I) represents non-diabetic control rats.

Group (II) represents alloxan diabetic control rats.

Group (III) represents non-diabetic rats treated with erythropoietin.

LSD represents least significant difference between different groups at probability $P < 0.05$.

Group (IV) represents alloxan diabetic rats treated with erythropoietin

Table 6: Levels of serum creatinine and blood urea nitrogen concentrations of different experimental groups (Means \pm SD).

Weeks (p.i)	Creatinine (mg/dl)				Blood urea nitrogen (mg/dl)			
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)
0	2.13 \pm 0.83	2.12 \pm 0.73	2.11 \pm 0.62	2.03 \pm 0.28	24.43 \pm 1.76	24.78 \pm 1.83	22.43 \pm 1.83	23.74 \pm 1.84
1	2.06 \pm 0.72	2.15 \pm 0.59	2.03 \pm 0.43	1.99 \pm 0.73	24.85 \pm 1.87	24.94 \pm 1.45	24.23 \pm 1.94	24.87 \pm 1.45
2	1.98 \pm 0.84	2.89 \pm 0.52	2.05 \pm 0.51	2.09 \pm 0.81	22.40 \pm 1.65	29.64 \pm 1.87	25.13 \pm 1.84	25.65 \pm 1.87
3	1.99 \pm 0.95	2.95 \pm 0.13	1.98 \pm 0.54	2.38 \pm 0.53	23.86 \pm 1.04	32.32 \pm 1.64	24.98 \pm 1.75	31.54 \pm 1.94
4	2.05 \pm 0.83	2.97 \pm 0.51	1.99 \pm 0.81	2.96 \pm 0.62	25.31 \pm 1.41	35.19 \pm 1.61	24.87 \pm 1.43	39.94 \pm 1.54
5	2.01 \pm 0.57	2.98 \pm 0.84	2.01 \pm 0.34	2.94 \pm 0.64	22.43 \pm 1.83	45.87 \pm 1.84	25.09 \pm 1.81	42.52 \pm 1.63
6	2.02 \pm 0.79	2.98 \pm 0.64	1.97 \pm 0.62	2.99 \pm 0.41	23.43 \pm 1.73	50.13 \pm 1.54	25.23 \pm 1.93	58.98 \pm 1.86
LSD	0.90				6.00			

Group (I) represents non-diabetic control rats.

Group (II) represents alloxan diabetic control rats.

LSD represents least significant difference between different groups at probability $P < 0.05$.

Group (IV) represents alloxan diabetic rats treated with erythropoietin.

Group (III) represents non-diabetic rats treated with erythropoietin.

the end of experiment respectively in comparison to the control negative group non significant changes was observed in Group I and Group III.

The diabetic hyperglycemia induces elevation of serum levels of BUN and creatinine which is considered as significant markers of renal dysfunction [23]. These results indicated that diabetes could lead to renal dysfunction in the diabetic group earlier than Group IV (diabetic and treated with EPO) as recombinant human EPO (RHEPO) can delay occurrence of diabetic nephropathy [23]. Moore and Bellomo [24] suggested that EPO has additional organ protective effects, which may be useful in the prevention or treatment of acute kidney injury. These protective mechanisms are multifactorial in nature and include inhibition apoptotic cell death, stimulation of cellular regeneration and inhibition of deleterious pathways. Another study [25] had claimed that reversal

of anemia by RHEPO treatment retard the progression of chronic kidney disease specially in non diabetic patient.

Urine Analysis

Urine Dipstick and Urine Protein Creatinine Ratio: Urine protein creatinine ratio levels of experimental rats were shown in Table (7). Urine dipstick for Group II (Diabetic control) revealed presence of protein in urine with significant increase in Urine protein creatinine ratio from the 4th week till the end of experiment. On the other hand, Group IV (Diabetic and treated with EPO) showed proteinuria by dipstick at the last week only with significant increase of Urine protein creatinine ratio in the same period. Glucosuria was detected all over the experimental period in both Group II and group IV with no significant alteration observed in Group I and Group III.

Table 7: Levels of Urine Protein Creatinine ratio and Microalbuminuria concentrations of different experimental groups (Means \pm SD).

Weeks (p.i)	Urine Protein Creatinine ratio				Microalbuminuria (mg/dl)			
	Group (I)	Group (II)	Group (III)	Group (IV)	Group (I)	Group (II)	Group (III)	Group (IV)
0	0.83 \pm 0.13	0.73 \pm 0.12	0.62 \pm 0.11	0.62 \pm 0.03	17 \pm 0.09	20 \pm 0.13	19 \pm 0.04	20 \pm 1.15
1	0.72 \pm 0.06	0.59 \pm 0.15	0.63 \pm 0.03	0.73 \pm 0.14	19 \pm 0.13	29 \pm 0.08	22 \pm 0.12	25 \pm 1.76
2	0.84 \pm 0.18	0.62 \pm 0.09	0.71 \pm 0.05	0.81 \pm 0.09	22 \pm 0.15	89 \pm 1.09	28 \pm 0.15	27 \pm 1.45
3	0.62 \pm 0.09	0.83 \pm 0.05	0.84 \pm 0.18	0.83 \pm 0.18	16 \pm 0.10	108 \pm 2.98	18 \pm 0.08	29 \pm 1.72
4	0.83 \pm 0.05	1.57 \pm 0.17	0.81 \pm 0.09	0.92 \pm 0.19	23 \pm 0.08	512 \pm 3.91	20 \pm 1.13	119 \pm 2.89
5	0.67 \pm 0.12	1.84 \pm 0.08	0.74 \pm 0.01	0.84 \pm 0.14	20 \pm 0.16	669 \pm 3.94	23 \pm 1.43	384 \pm 3.98
6	0.79 \pm 0.12	1.64 \pm 0.03	0.62 \pm 0.17	1.81 \pm 0.11	21 \pm 0.17	698 \pm 2.54	25 \pm 1.98	679 \pm 3.91
LSD	0.67				69			

LSD represents least significant difference between different groups at probability $P < 0.05$.

Group (II) represents alloxan diabetic control rats.

Group (III) represents non-diabetic rats treated with erythropoietin.

Group (IV) represents alloxan diabetic rats treated with erythropoietin.

Group (I) represents non-diabetic control rats.

In animals, urine dipstick testing is the most commonly used test for proteinuria. Urine dipstick testing is usually highly specific, although it can give false-positive results in some situations. On the other hand, it is not as sensitive as quantitative methods. Therefore, dipstick testing is useful only when urinary protein exceeds 300 to 500 mg/day which appear at 4th week till the end of experiment in the second group and at 6th week of experiment in the fourth group [26]. Furthermore, urine protein creatinine ratio (UPC), a quantitative test measures total protein only when urinary protein levels greater than 500 mg/dL [26].

UPC is also useful for trending proteinuria quantity as the excretion of creatinine is constant for healthy individuals or once in stable renal failure. Trending the level of proteinuria allows the veterinarian to monitor the progression of the disease. Although UPC is a good test for quantification of proteinuria, large variations in urine creatinine values exist between individuals making other detection methods more suited for initial screening for abnormal levels of urine protein [27].

Microalbuminuria: Microalbuminuria levels of experimental rats were shown in Table (7). The second group (Diabetic control) revealed presence of microalbuminuria at the 2nd and 3rd week of the experiment then macroalbuminuria appear till the end of experiment. Microalbuminuria detected in the fourth group (Diabetic and treated with EPO) at the 4th week then macroalbuminuria appear. Group II and Group IV (Control non diabetic group and group treated with EPO respectively) revealed absence of microalbuminuria.

Concerning of microalbuminuria, the detection of microalbuminuria in the second group and in the fourth group was earlier by two weeks than urine dipstick and UPC. Microalbuminuria is generally defined as subclinical albuminuria, ie, an albumin excretion rate that is abnormally elevated but not detectable by standard laboratory procedures such as the routine dipstick method [28]. This means the albumin excretion rate is above the normal level but below the usual proteinuric level. This result suggested that microalbuminuria is a good predictor of proteinuria and is used to predict early renal disease associated with diabetes mellitus [29, 30].

In humans, microalbuminuria has long been recognized as a predictive factor of late onset clinical proteinuria and end-stage renal disease as well as of the development of diabetic nephropathy. Similarly, microalbuminuria may precede proteinuria in dogs with hereditary nephropathy, in dogs infected with *Dirofilaria immitis* and in soft-coated Wheaten Terriers genetically predisposed to the development of glomerular disease [31].

In conclusion, the reason for the earlier onset of normocytic normochromic anemia in diabetic control group is due to diabetic nephropathy and anemia characteristically responds to exogenous EPO administration in the form of recombinant human EPO. UPC is not a reliable method for detecting albuminuria in the range of 0-300mg/dl. The result suggested that microalbuminuria is a good predictor of proteinuria and is used to predict early renal disease associated with diabetes mellitus.

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