Abstract

Diabetic nephropathy is a leading cause of DM-related morbidity and mortality. Pathogenesis of diabetic nephropathy is related mostly to chronic hyperglycemia.

The study was designated to estimate some possible risk factors of diabetic nephropathy and assessed clinically, haematologically the patients with diabetic nephropathy, since the changes in these parameters are important in detecting, quantifying, identifying some possible risk factors and assessing patient’s response to treatment.

The patients and controls enrolled in this study had undergone full assessment that included clinical assessment by history and examination, haematological assessment (measurement of RBCs counts, blood Hb and PVC and RBCs indices), hormonal study (serum erythropoietin level).

The study revealed that diabetic nephropathy prevalence more in female with long duration of DM.

In regard to haematological parameters the results of study show that there is highly significant decreased (p< 0.01) in RBCs counts, blood Hb, PCV and RBCs indices, as well as serum erythropoietin in diabetic nephropathy patients in compare to control groups.

In conclusion, diabetic nephropathy associated with increased and decreased in some hematological parameters may be attributed to anemia that accompany diabetic nephropathy.
Introduction

Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria >300 milligram(mg) /day or >200 microgram(mcg)/minute that is confirmed on at least 2 occasions 3-6 months apart associated with decline in the glomerular filtration rate (GFR), and elevated arterial blood pressure [1].

Diabetic nephropathy is an important cause of morbidity and mortality, and is now among the most common causes of end – stage renal failure (ESRF) in developed countries[2]. About 30% of patients with type 1 diabetes mellitus (DM) have developed diabetic nephropathy 20 years after diagnosis, but the risk after this time falls to less than 1% per years, and from the outset the risk is not equal in all patients [3].

Microalbuminuria is defined as 30–300 mg/day in a 24-hours collection. Microalbuminuria is therefore most reliable as an indicator of incipient diabetic nephropathy within the first 10 years of type 1 DM, when the majority of patient with microalbuminuria will progress to overt nephropathy within a further 10 years. It is a less reliable predictor of nephropathy in older patients with type 2 DM, in whom it may be accounted for by other diseases, although it is a potentially useful marker of an increased risk of macrovascular disease [4].

The pathogenesis of diabetic nephropathy is related either to chronic hyperglycemia (poor control of blood glucose), or a family history of diabetic nephropathy, long duration of diabetes, presence of other microvascular complication, pre-existing hypertension and genetic factors also play a role [2]. As well as, smoking accelerates the decline in renal function. Additional susceptibility factors remain unidentified, because only 20–40% of patients with diabetes develop diabetic nephropathy [3].

Aim of the Study

This study aim to:
1- Identify some possible risk factors of diabetic nephropathy in Babylon province.
2- Assessment of patients with diabetic nephropathy that includes:
   a- Clinical assessment, that includes taking full history and physical examination.
   b- Haematological assessment, include complete blood count, also the effect of serum erythropoietin in haematological changes.

Materials and Methods

The study was conducted in general words in Marjan teaching hospital in AL-Hilla City. This study lasted from 12th / November/2010 to 31th / May / 2011. The total number of subjects involved in the study was 185 (105 patients & 80 controls). The study group consisted of 79 male and 66 female. The age distribution of study group ranged from 16-78 years. They classified into patients group & control groups.

Patients group included patients with diabetic nephropathy(whose not on dialysis or undergo dialysis before more than one month) were classified according to the type of DM into: patients with type 1 DM (22 male & 11 female, total 33), whose ages between 16-34 years & patients with type 2 DM (32 male & 40 female, total 72), whose ages between 45-78 years. They were asked if they had any symptoms of renal dysfunction, duration of nephropathy and others. Patients who experienced any of these symptoms had been assessed clinically by specialist doctors and sent for albumin: createnine ratio in urine and only those with high ratio were
included in patient group (including stage 4 & 5 chronic kidney disease patients).

The control group was subdivided into diabetic patients without nephropathy (control -1, 40 patients) and healthy subjects (control -2, 40 persons). The control groups (diabetic without nephropathy(control -1 and healthy subjects(control -2)) had matched sex and age group distribution to the patients. Diabetic patients without nephropathy (control -1) were classified into: patients with Type 1 DM (8 male & 6 female, total 14) & patients with Type 2 DM (17 male & 9 female, total 26).

The healthy subjects (control-2) group with negative medical history, no smoking, free from any illness, those control for type 1 DM (11 males and 11 females, total 22) & those control of type 2 DM (10 males and 8 females, total 18).

All the patients and control groups were assessed clinically, haematologically as following:

Full history were taken from each patient regarding personal data like name, age, sex, smoking history, any symptoms of renal dysfunction as fatigue, nausea and vomiting, poor appetite and swelling of the legs... etc., also asked about the duration of DM. Exclusion other causes of chronic kidney diseases. (e.g., recurrent UTI, renal stone, history of polycystic kidney PCK or family history of PCK , family history of renal diseases or systemic diseases (i.e. SLE) [5].

Generally, diabetic nephropathy is considered after a routine urinalysis and screening for microalbuminuria in the setting of diabetes. Patients usually have physical findings associated with long-standing diabetes mellitus, so we measured the blood pressure and do ophthalmoscope examination (for evidence of retinopathy).

Venous blood samples were aspirated at about 9 a.m. from anticubital fossa. From each person, 10 milliliter (ml) of blood aspirated, 4 ml of blood (anticoagulated with EDTA) used for haematological measurements, and other part placed in centrifuge (12000g) for 10 minutes after waiting for 45 minutes to separate serum from whole blood. Serum samples stored in deep freeze (-20c°). Serum samples were used for measurement of blood sugar, lipid profile, erythropoietin level [6].

Blood was diluted with formal citrate solution (1 ml formalin ,3.8 gram(gm) tri-sodium citrate, 99 ml distilled water). Blood is drawn up to mark 0.5 in the RBCs pipette, without letting any bubble as into the pipette by holding the pipette at a angle 45 degree and touching the space between the coverslip and the chamber by the point of the pipette, an appropriate drop of the mixture is allowed to run under the coverslip by capillary action. The counting chamber (neubaur hemocytometer ) was filled by holding the pipette at an angle 45 degree and touching the space between the coverslip and the chamber by the point of the pipette, an appropriate drop of the mixture is allowed to run under the coverslip by capillary action. The chamber is examined under 40X objective lens of the microscope to count in RBCs counting area of the chamber (in the four corners and center tertiary squares of the RBCs), and the depth of the field is 0.1 millimeter (mm) [7].

A cyanomethemoglobin method was used to estimate the hemoglobin contents of the blood. The method was based on Drabkin's cyanide- ferric cyanide solution. Twenty micro liter...
(µl) of blood was added for 5 ml of Drabkin's solution mixing, and incubated for at least 5 minutes at 37°C and then the results were estimated by using Hb meter at 540 nanometer (nm) wave length [8].

Microhematocrit method was used to determine PCV. Heparinized capillary tubes used, and blood was filled to approximately three quarters of their lengths then the unmarked end is closed with modeling clay and put in the microhematocrit centrifuge. After centrifugation (12000 g) for 5 minutes, the red blood cells were separated from plasma and remain a band of buffy coat at the interface between them consisting of leukocytes and blood platelets [6]. The mean corpuscular volume (MCV) was calculated as the following:

\[
\text{MCV} = \frac{\text{Packed cell volume}}{\text{Red corpuscles count} \times 10^{12}} \text{Femtoliters (fl)}
\]

The mean corpuscular hemoglobin (MCH) was calculated as the following:

\[
\text{MCH} = \frac{\text{Hemoglobin in g/dL}}{\text{RBCs count} \times 10^{12}} = \text{picogram (pg)}
\]

The mean corpuscular hemoglobin concentration (MCHC) was calculated as following:

\[
\text{MCHC} = \frac{\text{Hemoglobin gm/dL}}{\text{PCV}} = \text{(in percent)}
\]

The demeditec EPO immunoassay is a two-site ELISA(Enzyme-Linked ImmunoSorbent Assay) for the measurement of the biologically active 165 amino acid chain of EPO. It utilized two different mouse monoclonal antibodies to human EPO specific for well-defined regions on the EPO molecule. One mouse monoclonal antibody to human EPO is biotinylated and the other mouse monoclonal antibody to human EPO is labeled with horseradish peroxidase (HRP) for detection. In this assay, calibrators, controls, or patient samples are simultaneously incubated with the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well. At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine.
(TMB). An acid stopping solution is then added to stop the reaction and converts the color to yellow. The intensity of the yellow color is directly proportional to the concentration of EPO in the sample (according to procedure recommended by the serum EPO, demeditec, Germany)[9].

**Statistical analysis:**
SPSS program was used in this study. All values were expressed as mean ± standard error (SE). One way ANOVA was used to estimate differences between groups. The differences were considered significant when the probability (P) was less than 0.05 (P < 0.05) highly significant when the probability (P) was less than 0.01 (P < 0.01) [10].

**Results**

**History:**

**Different of gender:**
The differences of gender of persons were enrolled in the study as follow:
From 79 male diabetic patients enrolled in the study there were 54 (68%) affected by diabetic nephropathy, while of 66 female diabetic enrolled in the study there were 51 (77%) affected by diabetic nephropathy. These results indicated prevalent of diabetic nephropathy in females is more than males as show in figure (1).

![Figure 1](image)

**Figure 1** Gender differences between diabetic nephropathy patients and diabetic without nephropathy.

**Duration of diabetes mellitus:**
The duration of diabetes mellitus in patients with diabetic nephropathy was longer (mostly 10-20 years and > 20 years) than in diabetic without nephropathy as show in figure (2).
Figure 2 The duration of DM in patients with diabetic nephropathy and diabetic without nephropathy patients according to type of diabetic mellitus and sex.

Red blood cells (RBCs) counts:
Regarding type 1 DM male and female, there is a highly significant decreased (p < 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups as shown in table (3a).

Table 3a The values of RBCs (*10^6 / µl) in patients with diabetic nephropathy and control groups of type 1 of diabetes mellitus according to the sex.

<table>
<thead>
<tr>
<th></th>
<th>RBCs(*10^6/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Type1 Diabetics</td>
<td></td>
</tr>
<tr>
<td>Diabetic nephropathy patients</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Diabetics without nephropathy (control -1)</td>
<td>4.9±0.08</td>
</tr>
<tr>
<td>Healthy subjects(control -2)</td>
<td>5.9±0.09</td>
</tr>
</tbody>
</table>

-Values are mean ± SE
-The values with different capital letter mean significant at 0.01 level.
Table 3b: The values of RBCs (*10⁶/μl) in patients with diabetic nephropathy and control groups of type 2 diabetes mellitus according to the sex.

<table>
<thead>
<tr>
<th>Type2 Diabetics</th>
<th>RBCs(*10⁶/μl)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic nephropathy patients</td>
<td>3.2±0.1 (A)</td>
<td>3.5±0.2 (A)</td>
<td></td>
</tr>
<tr>
<td>Diabetics without nephropathy(control-1)</td>
<td>5.2±0.3 (B)</td>
<td>4.9±0.03 (B)</td>
<td></td>
</tr>
<tr>
<td>Healthy subjects (control- 2)</td>
<td>5.9±0.06 (C)</td>
<td>5.8±0.01 (C)</td>
<td></td>
</tr>
</tbody>
</table>

-Values are mean ± SE
-The values with different capital letter mean significant at 0.01 level.

Blood haemoglobin level(Hb) and packed cell volume(PCV):

**Hb:**
Regarding type 1DM male, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, but no significant decreased at 0.05 level between the two control groups, as shown in table (4a).

Regarding type 1DM female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups, as shown in table (4a).

Regarding type 2 DM male, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups as shown in table (4b).

Regarding type 1DM female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, but no significant decreased at 0.05 level between the two control groups, as shown in table (4b).

PCV:

Regarding type 1DM male and female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups as shown in table (4a).

Regarding type 2 DM male and female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups as shown in table (4b).

Table (4a): The values of blood haemoglobin (g/dl) and PCV (%) in diabetic nephropathy patients and control groups of type1 diabetes mellitus according to the sex.

<table>
<thead>
<tr>
<th>Type 1 DM</th>
<th>Hb g/dl</th>
<th>PCV %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Diabetic nephropathy patients</td>
<td>10±0.3 (A)</td>
<td>10±0.3 (A)</td>
</tr>
</tbody>
</table>
Table 4b The values of blood haemoglobin (g/dl) and PCV (%) in diabetic nephropathy patients and control groups of type1 diabetes mellitus according to the sex.

<table>
<thead>
<tr>
<th>Type 2 DM</th>
<th>Hb g/dl</th>
<th>PCV %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Diabetic nephropathy patients</td>
<td>9.3±0.3 (A)</td>
<td>10±0.3 (A)</td>
</tr>
<tr>
<td>Diabetics without nephropathy (control -1)</td>
<td>10.9±0.7 (B)</td>
<td>13.3±0.1 (B)</td>
</tr>
<tr>
<td>Healthy subjects (control -2)</td>
<td>13.2±0.2 (C)</td>
<td>14±0.1 (Ba)</td>
</tr>
</tbody>
</table>

-Values are mean ± SE
-The values with different capital letter mean significant at 0.01 level.
- The values with same letters are no significant at 0.05 level

Red blood cells indices (MCV, MCH, MCHC):

MCV:
Regarding type 1 DM male, there is a highly significant decreased (p< 0.01) between diabetic nephropathy patients and the two control groups, while there is no significant decreased at 0.05 level between the two control groups, as show in table (5a).

Regarding type 1 DM female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups, as show in table (5a).

MCV:
Regarding type 2 DM male and female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and control groups, as well as between the two control groups, as show in table (5b).
Regarding type 2 DM male and female, there is a highly significant decreased (p< 0.01) between patients with diabetic nephropathy and the two control groups, as well as between the two control groups as show in table (5b).

**MCHC:**

Regarding type 1 DM male and female, there is significant decreased at 0.05 level between patients with diabetic nephropathy and control groups, as well as between the two control groups as show in table (5a).

Regarding type 2 DM male and female, there is significant decreased at 0.05 level between patients with diabetic and control groups, as well as between the two control groups as show in table (5b).

**Table 5 a** The values of red blood cells indices in diabetic nephropathy patients and control groups of type 1 diabetes mellitus according to the sex.

<table>
<thead>
<tr>
<th>Type 1 DM</th>
<th>MCV(fl)</th>
<th>MCH(pg)</th>
<th>MCHC(g/ dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>82.8±1.6</td>
<td>76±0.1</td>
<td>26.3±0.6</td>
</tr>
<tr>
<td>patients</td>
<td>(A)</td>
<td>(A)</td>
<td>(A)</td>
</tr>
<tr>
<td>Diabetic without</td>
<td>87.5±1.4</td>
<td>82.3±0.8</td>
<td>29.9±0.4</td>
</tr>
<tr>
<td>nephropathy (control-1)</td>
<td>(Bb)</td>
<td>(B)</td>
<td>(B)</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>92.5±0.3</td>
<td>92.2±0.4</td>
<td>31.2±0.3</td>
</tr>
<tr>
<td>(control-2)</td>
<td>(Bb)</td>
<td>(B)</td>
<td>(C)</td>
</tr>
</tbody>
</table>

Values are mean ± SE
- The values with different capital letter mean significant at 0.01 level.
- The values with different small letters are significant at 0.05 level.

**Table 5b** The values of red blood cells indices in diabetic nephropathy patients and control groups of type 2 diabetes mellitus according to the sex.

<table>
<thead>
<tr>
<th>Type 2 DM</th>
<th>MCV(fl)</th>
<th>MCH(pg)</th>
<th>MCHC(g/ dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Diabetic nephropathy</td>
<td>81± 1.1</td>
<td>76± 0.8</td>
<td>29± 0.3</td>
</tr>
<tr>
<td>patients</td>
<td>(A)</td>
<td>(A)</td>
<td>(A)</td>
</tr>
<tr>
<td>Diabetic without</td>
<td>74± 2.6</td>
<td>79.3± 1.2</td>
<td>24± 1.2</td>
</tr>
<tr>
<td>nephropathy (control-1)</td>
<td>(B)</td>
<td>(B)</td>
<td>(B)</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>92.4± 0.3</td>
<td>93± 0.01</td>
<td>31± 0.01</td>
</tr>
<tr>
<td>(control-2)</td>
<td>(C)</td>
<td>(C)</td>
<td>(C)</td>
</tr>
</tbody>
</table>

-Values are mean ± SE
- The values with different capital letter mean significant at 0.01 level.
The values with different small letters are significant at 0.05 level.

**Hormonal study:**

**Serum erythropoietin (EPO) level:**
The value of serum EPO level in type 1 DM in patients with diabetic nephropathy for male is 2.2±0.1 and for female is 2.1±0.08, while for those control-1 male is 7.7±1.6 and for female is 4.3±0.2, and for control-2 male is 8.7±1.3 and for female is 9.6±1.1.

The value of serum EPO level in type 2 DM in patients with diabetic nephropathy for male is 1.2±0.2 and for female is 2±0.1, while for those control-1 male is 5.7±0.9 and for female is 7.2±1.1, and for control-2 male is 10.0±1.5 and for female is 7.7±1.04.

Regarding type 1 DM male and female, there is a highly significant decreased (p< 0.01) in serum EPO between the diabetic nephropathy patients and two control groups, as well as between the two control groups as shown in figure (6).

Regarding type 2 DM male and female, there is a highly significant decreased (p< 0.01) in serum EPO between the diabetic nephropathy patients and the two control groups, as well as between the two control groups as shown in figure (6).

**Figure 6** The values of erythropoietin level (mlU / ml) in study groups in type 1 diabetes mellitus according to sex.

**Discussion**

Regarding the history, the study shows that females are more prone to develop diabetic nephropathy than males (figure 1). This result consist with results of other studies [11] in that incidence of diabetic nephropathy is more in females than males. This results because of pregnancy and used of oral contraceptive pills which is associated with worsening of diabetic complication as nephropathy [12].

The present study found diabetic nephropathy patients have longer duration of diabetes when compared with diabetics without nephropathy.
The same findings are reported by other studies [5, 13-21] in that longer duration of DM is a risk factor for the development of diabetic nephropathy.

[22] found out that there is a positive correlation between the prevalence of diabetic nephropathy and diabetes duration. Also, the results of the study shows a higher incidence of diabetic nephropathy in patients with duration of diabetes from 10 to 20 years when compared with those with duration of diabetes from 5-10 years and in patients with duration of diabetes from 5 to 10 years when compared to those with duration of diabetes below 5 years (figure 2). These results is assumed to be caused by:- longer duration of diabetes is a risk factor for development and progression of microvascular complications and it is nearly universal finding in previous studies [5, 18, 19, 22, 20].

Regarding haematological parameters, the study found there is statistically significant decreased in RBC counts in diabetic nephropathy patients in compare to the control groups (table 3a & b). This results matched the results of other studies [23 and 24]. The decreased in RBCs counts due to inadequate erythropoietin production and accumulation of uremic toxins suppresses red cell production in the bone marrow of uremic patients and shorten their life span[25].

The results of study shows statistically significant decreased in both PCV and Hb concentration in diabetic nephropathy patients compare to the control groups (table 4 a & b). This result matches with the results obtained by [26-28]. The result explained as anemia first appears when the GFR falls below 40 ml/minute, and is present in most patients with ESRD because in renal failure, erythropoietin production usually is insufficient to stimulate adequate red blood cell production by the bone marrow[29].

It is revealed in this study, there are statistically significant decreased in MCV, MCH and MCHC concentration in diabetic nephropathy patients in compare to control groups, (table 5 a & b). This consist with other studies[26, 30]. These results may related to decrease erythropoietin production in CKD, and iron deficient as a result of anorexia and dietary restrictions that limit intake, impaired erythropoiesis secondary to inhibitors or toxic metabolites[26].

Regarding to hormonal study, it is shown in this study there is statistically significant decreased in serum erythropoietin in diabetic nephropathy patients in compare to control groups (figure 6). This result matches with the results obtained by [31-36]. The explained of this result in that a reduction of renal erythropoietin production in chronic kidney disease due to tubulointerstitial lesion known to be present in diabetic nephropathy ,which may lead to damage or dysfunction of the EPO producing fibroblasts in the renal interstitium and inadequate extrarenal supply of EPO[37]). Other explanation in that EPO deficiency in these patients may be caused at least in part by renal denervation secondary to diabetic autonomic neuropathy in the presence of damaged EPO-producing fibroblasts in the renal cortex [36].

Other possible explanations for the failure of adequate EPO production include cytokine inhibition of EPO production or a failure of the oxygen-sensing mechanism, which triggers the production of EPO synthesis[32]. As well as low EPO in renal disease is due to immunomodulatory cytokine inhibition of EPO formation and Type
1 diabetes is closely associated with other autoimmune diseases in which cytokine release might be elevated.[36].

References


