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Lactoferrin A Promising Sign for Developing Peripheral Neuropathy in Patientswith Type 2 Diabetes Mellitus

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Abstract

Aim: This work has been carried out to evaluate the levels of iron and lactoferrin (LF) as biochemical markers in Iraqi patients with type 2 diabetic peripheral neuropathy (DPN).

Methods: Eighty-one Iraqi people (aged range 40-65 years) were included in the study: 28 healthy control subjects (G1 group),26 Type 2 diabetes without any complication (G2 group), 27diabetic peripheral neuropathy (DPN, G3 group). Diagnosis was based upon clinical examination as well as dermo copy. Serum Lactoferrin, iron levels, TIBC, and transferrin were determined for each participant. **Results:** The results of lactoferrin showed highly significant increases (p= 0.000) in G2 as compared to G1 (0.62±0.15ng/ml vs. 0.36±0.53 ng/ml), while G3 showed a highly significant increase (p=0.000) as compared to G1 (0.77±0.79 ng/ml vs. 0.36±0.53 ng/ml), also G3 showed a highly significant increase (p=0.000) as compared to G2 (0.77±0.79 ng/ml vs. 0.62±0.15 ng/ml). Serum Fe showed that there were no significant differences (p=0.336) between G1 and G2 (88.53±7.42 µg/dl vs. 98.32±10.39 µg/dl), but G3 showed a highly significant increase (p=0.000) as compared to G1 (118.64±43.90 µg/dl vs. 88.53±7.42 µg/dl), and a significant increased (p=0.017) as compared to G2 (118.64±43.90 µg/dl vs. 98.32±10.39 µg/dl). The results of TIBC showed that G2 showed highly significant increase (p=0.000) as compared to G1 (400.48±49.13 µg/dl vs. 308.79 ± 36.05µg/dl) also G3 showed highly significant increase (p=0.005) as compared to G1 (342.89±28.88 µg/dl vs. 308.79 ± 36.05 µg/dl), while G2 (p=0.000) showed a highly significant increase as compared to G3 (400.48±49.13µg/dl vs. 342.89±28.88µg/dl).The Transferrin level showed that G2 has a high significant increase (p=0.000) as compared to G1 (291.07±47.39 µg/dl vs. 216.10 ± 25.26 µg/dl), also G3 showed a significant increase (p=0.013) as compared to G1 (242.23±22.14 µg/dl vs. 216.10 ± 25.26 µg/dl) while G2 showed a highly significant increase (p= 0.000) as compared to G3 (291.07±47.39 µg/dl vs. 242.23±22.14 µg/dl).

Conclusion: Lactoferrin, iron and transferrin showed highly elevated levels in patients with diabetic peripheral neuropathy. They can be used to diagnosis of DPN and suggest the best protocol to treat those patients.

Keywords: Type 2 diabetes, diabetic peripheral neuropathy, Lactoferrin, iron.

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Introduction

Diabetes is one of the top ten leading causes of death in the world as a consequence of the chronic hyperglycemia of diabetes that is associated with long-term microvascular complications affecting the eyes, kidneys and nerves, as well as an increased risk for cardiovascular disease (CVD) [1]. Type 2 diabetes mellitus (T2DM) is the most communal type of diabetes, which has been increasing very fast lately in some countries of the world, expected that people effected with to double in the next decade due to increase in ageing population [2]. The development of type 2diabetes is strongly associated with genetic factors related to impaired insulin secretion and insulin resistance as well as environmental factors such as obesity, lack of exercise, overeating and stress, insufficient energy consumption, alcohol drinking, smoking, as well as aging and certain drugs [3].

Diabetic neuropathy (DN) is one of the most important microvascular complication of diabetes can be defined as a neurological damage in patients with diabetes mellitus; it affects 30% - 50% of diabetic patients. It is the most common complication of diabetes that leads to the greatest disability and mortality [4].DN can affect the peripheral, autonomic, and central nervous systems, exhibiting, therefore, several clinical symptoms [5]. The method that used to diagnose the peripheral neuropathy disease is the questionnaire that were used to assess the presence of neuropathy which include asking several questions and answering them with yes or no: pain, burning, or aching legs or feet, unsteadiness on walking, numbness of the legs or feet and prickling sensations in the legs or feet [6]. The management of patients with type 2 diabetes is depending on the major control of glucose. Studies have showed that reducing hyperglycemia decreases the progression of microvascular complications [7]. Analysis of HbA1c in blood provides evidence about an individual's average blood glucose levels during the previous periods. Nowadays HbA1c become as a standard of care (SOC) for testing and monitoring diabetes, specifically type two diabetes [8].

Lactoferrin (Lf) is an iron-binding glycoprotein in human and bovine milk which is presented in high concentrations, as well as in lower amounts in exocrine secretions and cells [9]. Lf is a glycosylated protein with a molecular mass of approximately 80 kDa includes single peptides of 700 amino acids with high homology among species that is considered a multifunctional glycoprotein[10]. Lactoferrin have a non-specific protection against infections and also have anti-inflammatory, antimicrobial and anticancer activities [11]. Lf have three isoforms: Iron binding isoform mentioned as LF-α, the other two isoforms LF- β and LF-g do not bind iron and have ribonuclease activity [12]. When lactoferrin is rich with iron it referred as Hololactoferrin, while it called apo lactoferrin when it irons free [13]. The levels of Lf are raised in the body during an infection or an inflammatory condition that makes Lf as biomarker for this reason [14]. There are several attempts to isolate Lf from different sources since it found to have a therapeutic potential against infection and extreme inflammation[15, 16]. A twofold significant elevate of Lf content was evident in the diabetic pediatrics compared to their control counterparts [17]. Iron, one of the essential trace mineral elements, is involved in regulating the differentiation and

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growth of living cells. The total amount of iron is about 60% in an adult but once its increase above the normal may cause severe damage to the pancreatic cells through excessive oxidative stress [18]. Iron is intracellular and deposed within ferritin in healthy individuals. It found as a co-factor of cytochromes, iron sulpher proteins, and as haem complexed to hemoglobin within erythrocytes. Circulating iron is rapidly bound by transferrin [19, 20]When hemoglobin or haem and erythrocytes lyse is released into the circulation, their hemoglobin is captured by haptoglobin and haem by hemopexin[21]. The serum ferroxidase circulating ceruloplasmin is important, as LF can bind to ceruloplasmin, so the direct transfer of ferric iron between the two proteins is possible [22]the importance of the direct transfer of ferric iron to lactoferrin can prevents both the formation of potentially toxic hydroxyl radicals [23].

Materials and Methods

Study Design and Population

This study was carried out in National Diabetes Center/ Al-Mustansiriya University under the supervision of Dr. Firas younus Muhsin during the period from December 2019 to march 2020. Individuals enrolled in the present study were divided into healthy, patients with type two diabetes mellitus without complication and type two diabetes mellitus with peripheral neuropathy complication (DPN).

The study included 81 Iraqi people (40-65 years old), 28 were healthy control (G1 group), 26 with type two diabetes mellitus (G2) (the duration of diabetic 2-8 years) treated with oral hypoglycemic drugs for example: Metformin, Sulfonylureas, Daonil glibenclamide and 27 with diabetic peripheral neuropathy (G3) (with a duration 2-10 years) treated also with oral hypoglycemic drugs(with same drugs above) besides drugs patients that take for neuropathic pain for example: Carbamazepine (Tegretol) and Lyrica (pregabalin).All groups included in this study were matched in age, BMI and gender. Full clinical investigation was carried out by consultant physicians in the hospitals. A questionnaire list was filled for each subject.The diagnosis of T2DM was done on the basis of the American diabetes association (ADA) **[24]**

Exclusion Criteria for Cases and Control

Some clinical conditions were excluded in our study, e.g. type one diabetic patients, patients with cardiovascular disease, and other known diseases which are associated with glucose metabolism disordered. As well as, type two diabetic patients who are taking insulin as a hypoglycemic therapy, retinopathy and nephropathy patients and those having liver diseases.

Blood Samples Collection

Five ml of blood was obtained by venipuncture from each individual, using a 5 ml disposable syringe between 08.00 and 11.00 a.m. after (10-12) hours of fasting. The blood sample was divided into two portions; 2 and 3 ml. The first portion was dispensed in tube containing ethylene diamine tetra acetic acid (EDTA) which used for the estimation of HbA1C. While the

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second portion was dispensed in a gel tube and left to clot at room temperature. The gel tube was centrifuged at (3000 r.p.m) for 10 minutes to collect serum which is used for estimation of FBG, Lactoferrin, iron, and TIBC. The serum was divided into portions (200µl) in Eppendorff tubes, and stored in the freezer (-20ºC) until use.

Laboratory Tests

Lactoferrin measured by using enzyme linked immunosorbent assay (ELISA) using the commercially available ELISA kit (Mybiosource, U.S.A). All procedures were carried out according to the manufacturer's instructions, fasting blood sugar (FBS) (HUMAN, Germany), HbA1c (HUMAN, Germany), iron (HUMAN, Germany), TIBC (HUMAN, Germany).UIBC was calculated from this equation: UIBC = [TIBC- Serum Iron Concentration].Transferrin was calculated from this equation: [Transferrin (mg/dl) = 0.7 × TIBC (µg/dl)],while saturation transferrin was calculated from this equation: Saturation of [Transferrin (%) = Serum iron / Serum TIBC *100]

Statistical Analysis

Data was statistically analyzed by SPSS software version 22. The variables were reported as means ± standard deviation. The groups were compared by using one-way ANOVA and post hoc Tukey test, with a P value of <0.05 indicating the statistically significant difference.

Results

In our study, 81 Iraqi people (aged range 40-65) were divided into three groups: healthy control group (n=28, G1), Type two diabetes mellitus (T2DM) group (n=26, G2), and Diabetic peripheral neuropathy (DPN) group (n=27, G3). The results of our study showed in Table (1) revealed that fasting blood glucose (FBG) showed a high significant increase in G2 (p=0.000) as compared to G1 (154.38±50.61mg/dl vs. 86.61±5.12mg/dl), also G3 showed a highly significant increase (p=0.000) as compared to G1 (220.88±91.58mg/dl vs. 86.61±5.12mg/dl),while G2 (p=0.000) showed a highly significant decrease as compared to G3(154.38±50.61mg/dl vs. 220.88±91.58mg/dl).For HbA1c the results showed that G2 has a high significant increase (p=0.000) as compared to G1 (8.09±1.29 % vs. 4.82±0.56 %), also G3 showed a highly significant increase (p=0.000) as compared to G1 (8.72±1.86 % vs. 4.82±0.56 %) while there was no significant different (p=0.210) between G2 and G3 (8.09±1.29 % vs. 8.72±1.86 %). The results of lactoferrin showed highly significant increases (p= 0.000) in G2 as compared to G1 (0.62±0.15ng/ml vs. 0.36±0.53 ng/ml), also G3 showed a highly significant increase (p=0.000) as compared to G1 (0.77±0.79 ng/ml vs. 0.36±0.53 ng/ml), and to G2 (p=0.000) (0.77±0.79 ng/ml vs. 0.62±0.15 ng/ml). Serum iron (Fe) showed that there was no significant differences (p=0.336) between G1 and G2 (88.53±7.42 µg/dl vs. 98.32±10.39 µg/dl), but G3 showed a highly significant increase (p=0.000) as compared to G1 (118.64±43.90 µg/dl vs. 88.53±7.42 µg/dl), and significant increase (p=0.017) as compared to G2 (118.64±43.90 µg/dl vs. 98.32±10.39 µg/dl). The results of TIBC showed that G2

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showed highly significant increase (p=0.000) as compared to G1 (400.48±49.13 µg/dl vs. 308.79 ± 36.05µg/dl) also G3 showed highly significant increase (p=0.005) as compared to G1 (342.89±28.88 µg/dl vs. 308.79 ± 36.05µg/dl) , while G2 (p=0.000)showed a highly significant increase as compared to G3 (400.48±49.13µg/dl vs. 342.89±28.88µg/dl).The results of UIBC showed that G2 (p=0.000) showed a highly significant increase as compared to G1 (304.24±53.09 vs. 220.21±36.32) ,while there was no significant differences (p=0.936) between G1 and G3(220.21±36.32 vs. 224.69±52.71) ,but there was a high significant increase in G2 (p=0.000) as compared to G3(304.24±53.09 vs. 224.69±52.71). The level of Transferrin results showed that G2 has a high significant increase (p=0.000) as compared to G1 (291.07±47.39 µg/dl vs. 216.10 ± 25.26 µg/dl),also G3 showed a significant increase (p=0.013) as compared to G1 (242.23±22.14 µg/dl vs.
216.10 ± 25.26 µg/dl) while G2 showed a highly significant increase (p=0.000) as compared to G3 (291.07±47.39 vs. 242.23±22.14 µg/dl). The result of saturation transferrin showed that there was no significant difference(p=0.317)between G1 and G2 (86.94±11.92% vs. 74.80±12.63%),also between G1and G3 (p=0.052) (86.94±11.92 % vs. 106.49±50.08%),while G2showed a highly significant decrease (p=0.001) as compared to G3(74.80±12.63% vs. 106.49±50.08).

Table 1: The characteristics of participants of lactoferrin, FBG, HbA1c, Fe, TIBC, UIBC, transferrin and saturation transferrin among different groups (n=81).

Variables	G1 (Mean±SD) Control N=28	G2 (Mean±SD) T2DM N=26	G2 (Mean±SD) DPN N=27	*p-value*	
FBG(mg/dl)	86.61±5.12	154.38±50.61	220.88±91.58	G1*G2	0.000**
				G1*G3	0.000**
				G2*G3	0.000**
HbA1c (%)	4.82±0.56	8.09±1.29	8.72±1.86	G1*G2	0.000**
				G1*G3	0.000**
				G2*G3	0.210
Lactoferrin(ng/ml)	0.36±0.53	0.62±0.15	0.77±0.79	G1*G2	0.000**
				G1*G3	0.000**
				G2*G3	0.000**
Fe(µg/dl)	88.53±7.42	98.32±10.39	118.64±43.90	G1*G2	0.336
				G1*G3	0.000**
				G2*G3	0.017*
TIBC(µg/dl)	308.79 ± 36.05	400.48±49.13	342.89±28.88	G1*G2	0.000**
				G1*G3	0.005**
				G2*G3	0.000**
UIBC	220.21±36.32	304.24±53.09	224.69±52.71	G1*G2	0.000**
				G1*G3	0.936
				G2*G3	0.000**
				G1*G2	0.000**

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Transferrin(µg/dl)	**216.10 ± 25.26**	**291.07±47.39**	**242.23±22.14**	**G1*G3**	**0.013***
				G2*G3	**0.000****
Saturation transferrin (%)	**86.94±11.92**	**74.80±12.63**	**106.49±50.08**	**G1*G2**	**0.317**
				G1*G3	**0.052**
				G2*G3	**0.001****

G1: Control group, G2: T2DM group, G3: DPN group. Significant: *P < 0.05; highly significant: **P < 0.001; no significant: P > 0.05.

The correlations of serum lactoferrin with other biochemical parameters in Type 2 diabetes mellitus (G2) and diabetic peripheral neuropathy (G3) were summarized in Table (2). The results showed that lactoferrin was correlated positively with HbA1c in G3(r=0.769, p=0.020) and negatively with Fe in G3 (r= -0.399, p= 0.039), while there was no correlation relationship between lactoferrin and other parameters in G2 and G3 respectively.

Table 2: Pearson correlation of lactoferrin in type 2 diabetes mellitus (G2) and in diabetic peripheral neuropathy (G3)

parameters	**Lactoferrin(ng/ml)**			
	G2		**G3**	
	r	***p***	***r***	***p***
FBS (mg/dl)	**-0.199**	**0.329**	**0.024**	**0.904**
HbA1c (%)	**-0.061**	**0.769**	**0.445**	**0.020***
Fe(µg/dl)	**0.004**	**0.984**	**-0.399**	**0.039***
TIBC(µg/dl)	**-0.040**	**0.848**	**-0.098**	**0.628**
UIBC	**-0.053**	**0.798**	**-0.248**	**0.213**
Transferrin(µg/dl)	**0.166**	**0.417**	**0.131**	**0.516**
Sat. transferrin (%)	**-0.033**	**0.872**	**-0.090**	**0.655**

R, Pearson coefficient
***Statistically significant at $p \leqslant 0.05$**

Discussion

The present study may consider a link between the effect of serum iron and serum lactoferrin concentration in patients with diabetes mellitus. We found in our study an inverse relation

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between iron and lactoferrin concentration in DPN patients, where elevation of serum iron in type 2 diabetes causing vascular dysfunction as a result of decreasing insulin level and elevation of ROS. At the same time serum lactoferrin concentrations start to rise as immune response to counteract the inflammation that result from elevation of iron and ROS. High blood glucose level is referred to hyperglycemia; it develops when there is too much sugar in the blood and it is happening when the body has too little insulin or when the body cannot use insulin properly [25].

The highly significant increase in FBG in G2 and G3 groups as compared to G1 group were in agreement with the results found by previous researchers[26, 27]. Glycated hemoglobin (HbA1c) is a form of hemoglobin primarily used to identify the average plasma glucose concentration over prolonged periods of time. The HbA1c level is proportional to average blood glucose concentration over the previous four weeks to three months. Higher levels of HbA1c are found in people with diabetes mellitus those they persistently with elevated blood sugar [28]. The results of HbA1c in our study was in agreement with that obtained by Dimitrios et al [29], Terekeci et al [30] and Daousi et al [31] who revealed that HbA1c levels showed a statistically significant increase in both T2DM and DPN patients in comparison to healthy controls. Lactoferrin increases during infection and inflammation processes, which leads to neutrophil degranulation and the activation of microglial cells [10].

The results of lactoferrin obtained from our study revealed that the concentration of Lf was two-fold increase in G3 (DPN) group than G2 and G1 groups. Our study was in agreement with El-Desouky et al [32] who showed that the level of LF in T2DM patients was higher as compared to control subjects. Also Waleed et al [17] reported that Lf increased during young obese patients with type2 diabetes, while Moreno-Navarrete et al [33]showed decreased circulating lactoferrin in association with insulin resistance and type 2diabetes. Inga et al [34] revealed that there was no significant difference between Lf levels in patients with T2DM and control subjects.

Some evidence suggests that there is a strong link between elevated body iron levels and risk of T2DM and that's why excessive levels of iron can causes pathology that have association with hypertension, dyslipidemia, metabolic syndrome, and increased risk of cardiovascular disease [35].The macrovascular and microvascular complications(like neuropathy , nephropathy and retinopathy) are produced by reactive oxygen species leads to oxidative damage which is generated by free radicals like free iron, various studies proved that iron overload or reactive free iron was responsible for diabetes [36]. Serum Fe in our results showed an agreement with Sayantaann and Roopa [37], Gajendra et al [38], Mandal [39], Gunjan et al [40], Renuka and Vasantha [41].who revealed that serum iron levels showed a statistically significant increase in T2DM and DPN patients in comparison to healthy controls. The results of Transferrin were in agreement with the results of study by Anand and Sharmila [36] who revealed that levels of serum transferrin showed a statistically significant increase in T2DM. In contrast to our results, Sayantaann and Roopa [37] concluded that serum transferrin was slightly lower in the T2DM patients as compared to controls. Also Ramazan and Ebubekir [42] showed that level of serum transferrin was lower in T2DM and DPN patients as compared to controls and that was in contrast with our findings.

The total iron-binding capacity (TIBC) is the measurement of the maximum concentration of

iron that can be bind. The TIBC test serves as a good indirect measurement of transferrin. The increasing of TIBC is often occurs in iron deficiency and decreased in malnutrition, malignancies, acute inflammation as well as chronic inflammatory disorders. [43]. Our TIBC results showed a disagreement with a studies by Sayantaann and Roopa [37] , Gunjan et al [40]who revealed that serum TIBC was decreased in T2DM Patients as compared to healthy control subjects and that a contrast to our study. The level of UIBC results was in agreement with Viswan and Amar [44] who revealed that UIBC levels in T2DM group were higher as compared to the healthy control subjects.

Transferrin saturation is the ratio of serum iron and TIBC. This parameter provides an estimate of how much serum iron is actually bound to transferrin and is expressed as a percentage. It can also be directly estimated from the TIBC value for convenience but this procedure overestimates the transferrin concentration by approximately 16%–20% because it assumes that all plasma iron is bound to transferrin as opposed to other serum proteins [43]. Our result was in agreement with Ibrahim et al [45] who showed that there was no significant difference between saturation transferrin level in T2DM patients and control subjects. In contrast with Sayantaann and Roopa [37] and Gunjan et al [40] who revealed that serum saturation transferrin was higher in T2DM as compared to healthy control subjects.

Conclusion

Both lactoferrin and iron are considered as good biomarkers for diagnosis of diabetic peripheral neuropathy and for detection of the best and most effective method for the treatment.

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