

Study of Advanced Glycation End Products in Patients with Diabetic Neuropathy

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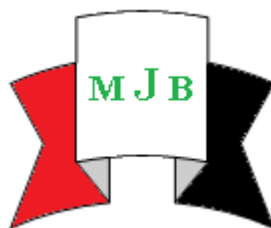
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Received 8 September 2014

Accepted 21 September 2014

Abstract

Diabetic peripheral neuropathy (DPN) is the most common microvascular complication of diabetes mellitus (DM). The study included 138 type 2 diabetic patients with DPN with age group 45-65 years matched to 50 diabetic without neuropathy and 50 normal healthy control. The study found that there is higher incidence of DPN in elderly age group than younger while the incidence is equal in male and female. Also its found that level of advanced glycation end products (AGE) is higher in patients with DPN compared to those without and healthy controls. Also the study found that AGE level is correlated with duration of DM and not correlate with glycosylated Hb level. Hyperglycemia and subsequent metabolic derangement results in accumulation of advanced glycation end products in peripheral nerves with subsequent nerve injury.

Key words: Diabetic peripheral neuropathy, advanced glycation end products, nerve conduction.

الخلاصة

يعتبر اعتلال الأعصاب المحيطية السكري من أكثر مضاعفات داء السكري التي تؤثر على الاوعية الدموية الدقيقة شيوعا. شمل البحث ١٣٨ مريضا بداء السكري من النوع الثاني يعانون من اعتلال الاعصاب المحيطية تتراوح اعمارهم بين ٤٥-٦٥ عاما قورنوا مع ٥٠ مريضا بداء السكري لا يعانون من اعتلال الاعصاب المحيطية و ٥٠ شخصا سليما كمجموعة السيطرة. وجد البحث ان اعتلال الاعصاب المحيطية السكري اكثر شيوعا في كبار السن مقارنة بالاقبل عمرا بينما يكون الشيوخ متساوي بين الذكور والاناث. ووجد البحث ايضا ان مستوى منتجات النهاية السكرية المتطورة اعلى لدى مرضى اعتلال الاعصاب السكري عند مقارنتهم بمرضى السكري بدون اعتلال الاعصاب والاشخاص الاصحاء. كما وجدت الدراسة ان مستوى منتجات النهاية السكرية المتطورة يرتبط مع مدة الاصابة بداء السكري و لا يرتبط مع مستوى الهيموغلوبين السكري. فرط سكر الدم يؤدي الى تراكم منتجات النهاية السكرية المتطورة في الأعصاب الطرفية مع إصابة العصب اللاحقة.

Introduction

Diabetic neuropathy is the most common microvascular complication of diabetes mellitus (DM) reaching 45-50% prevalence compared to 25-30% of retinopathy and 20% of nephropathy (1). The prevalence of diabetic neuropathy increases with age and duration of diabetes, and tends to be more common in patients with type 2

DM than in those with type 1 DM. The prevalence of neuropathy is estimated to be about 8% in newly diagnosed diabetics (at time of diagnosis of DM) and greater than 50% in patients who had diabetes longer than 25 years with average prevalence of 30% (2).

Glycation is the non-enzymatic reaction of glucose, α -oxoaldehydes, and other saccharide derivatives with proteins,

nucleotides, and lipids, with formation of early glycation adduct (fructosamines) and advanced glycation end-products (AGE) (3). Protein glycation is a spontaneous reaction and is dependent on the degree and duration of hyperglycaemia, the half-life of the protein and permeability of the tissue to free glucose. In the last several years, the role for glycation/glycoxidation in diabetic complications including diabetic neuropathy has extensively been reviewed (4).

Regarding the role of protein glycation in the pathogenesis of diabetic neuropathy, there is increased glycation of myelin in diabetes. Glycated myelin is susceptible to phagocytosis by macrophages in vitro and can also stimulate macrophages to secrete proteases and this might contribute toward nerve demyelination in diabetic neuropathy. Furthermore, the AGEs on myelin can trap plasma proteins such as IgG and IgM to elicit immunological reactions that contribute toward neuronal demyelination (5).

This study aims to evaluate the advanced glycation end products in patients with and without diabetic peripheral neuropathy and in normal healthy control and it's correlation with duration of diabetes and glycemic control.

Materials and Methods

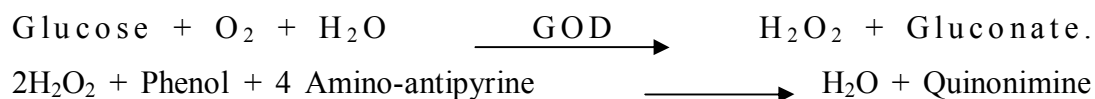
The study was conducted in Merjan medical city in Babylon and it included one hundred thirty eight patients (72 males and 66 females) with type 2 DM. These were selected to have a complaint of peripheral nerve dysfunction like pain, paresthesia and/or a sense of weakness at their extremities, especially lower limbs and documented by abnormal nerve conduction study. Those were considered as patients with diabetic peripheral neuropathy (DPN). Their age ranged from 40 to 65 years.

Additional 100 adults (42 males and 58 females) were also included as the control group whose their ages were consistent with those of the patient group (40-65 years old). This group was subdivided into two subgroups; half of them were diabetic patients without neuropathy (no signs and symptoms and their nerve conduction study were normal) and the rest were normal healthy adults.

All the patients and control undergo measurement of fasting blood, glycosylated hemoglobin, total antioxidant capacity and electrophysiological testing.

Fasting blood sugar

Glucose is oxidized by glucose-oxidase to gluconate and hydrogen peroxide according to the following equation.



The absorbance of standard and sample are measured against reagent blank at 546 nanometer (nm) according to a procedure recommended (Human, Germany) (6).

Glycosylated hemoglobin measurement

Whole blood preparation was mixed with a weakly binding cation-exchange resin and the non-glycosylated hemoglobin was bound to the resin, leaving HbA1c. The percent of HbA1c was determined by measuring the absorbance values at 415nm

of the HbA1c fraction to the total Hb fraction, according to the procedure explained by the company (Stanbio lab., USA)(7).

Advanced glycation end products (AGE) assay

The assay protocol included:

1- 100 microliter (μL) of the 10 microgram/milliter ($\mu\text{g/mL}$) protein samples or prepared BSA standards are added to the 96-well Protein Binding Plate, and then

incubated at 37°C for at least 2 hours or 4°C overnight.

2- 200 µL of assay diluent are added per well and incubated for 1-2 hours at room temperature on an orbital shaker.

3- The wells are washed 3 times with 250 µL of 1X wash buffer with thorough aspiration between each wash. After the last wash, the wells are emptied and tapped microwell strips on absorbent pad or paper towel to remove excess 1X wash buffer.

4- 100 µL of the diluted Anti-AGE antibody are added to all wells and incubated for 1 hour at room temperature on an orbital shaker. The strip wells are washed 3 times according to step 4 above.

5- 100 µL of the diluted secondary antibody-HRP conjugate are added to all wells and incubated for 1 hour at room temperature on an orbital shaker. Wash the strip wells 5 times according to step 4 above.

6- 100 µL of substrate solution are added to each well, including the blank wells and Incubated at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.

7- The enzyme reaction is stopped by adding 100 µL of Stop Solution to each well. Results should be read immediately (color will fade over time).

8- Absorbance is read for each well on a microplate reader using 450 nm as the primary wave length. Use the reduced BSA standard as absorbance blank (8).

Electrophysiological testing

Each participant had at least four motor nerves tested (median, ulnar, tibial and peroneal), and three sensory nerves (median, ulnar and sural nerves). Limb temperatures were measured using adhesive skin patch and were maintained between 33- 36°C° by exposing the patient to radiant

heater when needed, and the skin was prepared when necessary using abrasive skin cleanser. Maximal responses were obtained using electrical stimuli. Multiple parameters were assessed for each nerve including distal latency, conduction velocity and waveform amplitude, duration and shape were measured and recorded for each nerve at each stimulus site (9).

Statistical analysis

This case control study involves arithmetic mean and the standard deviation of distribution of each set of the data is calculated for each of the studied variables. Demographic, clinical, biochemical and electrophysiological data were compared between patients and control groups using a *t*-test. A P- value of 0.05 or less was considered significant (10). Also, correlation coefficient was used to test the significance of correlation between total antioxidant capacity and some biochemical parameters.

Results

Demographic data and biochemical data

The study found significant differences between patients with DPN on one hand and patients without DPN and normal healthy control on the other hand regarding age, duration of DM, fasting blood sugar, glycated Hb and advanced glycation end products level. These findings are shown in table 1.

The correlations of advanced glycation end products with different parameters

There was a moderately direct correlation between AGE and duration of diabetes ($R=0.6$) & poor correlation was shown between AGE with HbA1c as shown in Figures 1 and 2

Table 1: shows the differences in demographic and biochemical data between study group

Variable		Patients with DPN	Patients without DPN	Healthy control
Age (years)		57.1±1.3	51.9±3.3*	55±4
Sex	Male	72	22	20
	Female	66	28	30
Duration of DM (years)		7.3±1	2.2±2*	
FBS (mmol/L)		12.6±1	6.4±2 *	5 ±1 †
HbA1c (%)		10.1±1.9	5.1±2 *	4.5±2 †
AGE (U/mL)		8.9±1.4	6.7±1 *	4.4±1 †

DPN= diabetic peripheral neuropathy, DM= Diabetes mellitus

FBS= fasting blood sugar, HbA1c= glycated hemoglobin, AGE= advanced glycation end products, mmol/L=millimol/liter, U/mL=unit/milliliter.

Values are expressed in mean ± standard deviation

* Significant differences between patient with DPN and those without DPN at P<0.05

† Significant differences between patient with DPN and control subjects at P<0.001

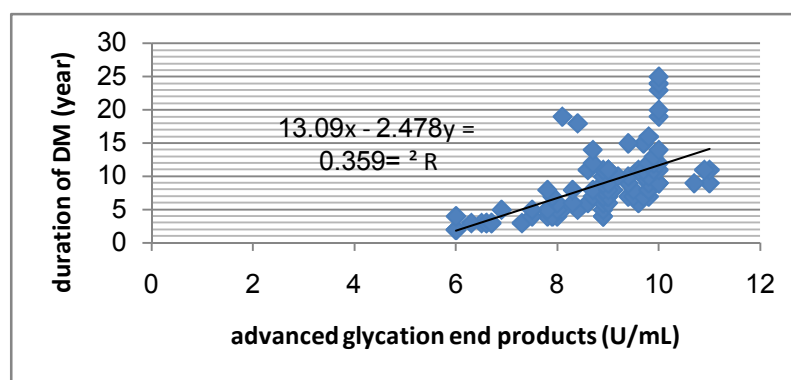


Figure 2: Correlation between advanced glycation end products and diabetes duration in patients with DPN.

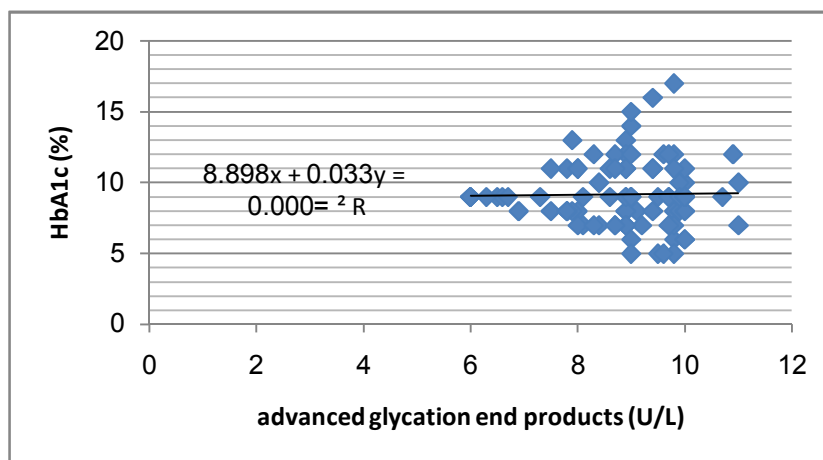


Figure 4.5: Correlation between advanced glycation end products and HbA1c in patients with DPN.

Electrophysiological assessment

The results of nerve conduction study (NCS) of motor and sensory nerves of upper and lower extremities are shown below.

The results of compound sensory action potential (CSAP) amplitude (Amp) and conduction velocity (CV) in the three study groups along with their significance value are shown in Table (2).

Sensory nerve conduction study

Table 2: The comparison of sensory nerve conduction parameters of median, ulnar and sural nerves between patients and control subjects.

Variable	Amp (μ V)			CV (m/s)		
	median	Ulnar	Sural	Median	Ulnar	Sural
patients with DPN	7 $\pm$ 1	13 $\pm$ 3	2 $\pm$ 1	31 $\pm$ 5	35 $\pm$ 3	29 $\pm$ 7
patients without DPN	29 $\pm$ 13 *	41 $\pm$ 18 *	7 $\pm$ 3 *	39 $\pm$ 11 *	41 $\pm$ 5 *	40 $\pm$ 4 *
Control subject	69 $\pm$ 11 †	66 $\pm$ 10 †	14 $\pm$ 2 †	42 $\pm$ 4 †	49 $\pm$ 3 †	46 $\pm$ 2 †

DPN= diabetic peripheral neuropathy, Amp = Amplitude, CV= conduction velocity,
Values are expressed in mean $\pm$ standard deviation

* Significant differences between patient with DPN and those without DPN at P<0.05

† Significant differences between patient with DPN and control subjects at P<0.001

Motor nerve conduction study

The comparisons of the compound muscle action potential (CMAP) parameters of the median, ulnar, posterior tibial and common

peroneal nerves in the three study groups were shown in Tables (3and 4). All of the distal latency (DML), amplitude (Amp) and conduction velocity (CV) were considered.

Table 3 a: The comparison of the motor nerve conduction parameters of upper limbs between patients and control subjects.

Variable	DML (ms)		Amp (mV)		CV (m/s)	
	Median	Ulnar	Median	Ulnar	Median	ulnar
Patients with DPN	4.4 $\pm$ 0.3	3.3 $\pm$ 0.2	3 $\pm$ 1	5.6 $\pm$ 1	44 $\pm$ 2	46 $\pm$ 2
Patients without DPN	2.8 $\pm$ 1 *	2.6 $\pm$ 0.6 *	6 $\pm$ 2 *	7.2 $\pm$ 1 *	49 $\pm$ 1 *	50 $\pm$ 1 *
Control subject	2.3 $\pm$ 3 †	2 $\pm$ 0.1 †	12 $\pm$ 4 †	12 $\pm$ 1 †	56 $\pm$ 6 †	61 $\pm$ 7 †

Table 3 b: The comparison of the motor nerve conduction parameters of lower limbs between patients and control subjects.

Variable	DML (ms)		Amp (mv)		CV (m/s)	
	Peroneal	Tibial	Peroneal	Tibial	Peroneal	Tibial
Patients with DPN	7.3±0.5	6.5±3	1±1	4.2±4	32±3	32±11
Patients without DPN	4.7±1 *	4.3±2 *	3±2 *	8±12 *	40±2 *	44±5 *
Control subject	3.2±1 †	3.4±1 †	6±70 †	18±19 †	47±11 †	45±2 †

DPN= diabetic peripheral neuropathy, DML= Distal motor latency, Amp= Amplitude, CV= conduction velocity, Values are expressed in mean ± standard error

* Significant differences between patient with DPN and those without DPN at P<0.05

† Significant differences between patient with DPN and control subjects at P<0.001

Discussion

The results of this study showed a significant effect of age on the function of peripheral nerves and on disease duration within the patient group (Table 1).

Although it is not so clear why advancing age has such impact, these findings could be explained by the association of progressing patients' age with longer duration of the DM that usually resulted in accumulation of deleterious effects on their peripheral nerves' function (11). Also, with increasing age the possibility of macrovascular disease (even if subclinical) increases which might further augment microvascular complications (12). Furthermore, older age is usually associated with a deficiency of some neuroprotecting hormones and neuroactive steroids (sex steroids) which play important role in maintenance and regeneration of nervous system. This deficiency will be aggravated during DM (13).

These were in agreement with the findings of other studies that showed that older diabetics are at higher risk for developing peripheral neuropathy (14; 15 and 2). On

contrary, other studies denied any effect of age on the development of DPN (16 and 17). This discrepancy in assessing the effect of advancing age in diabetic patients on peripheral nerves can be accounted for patients sampling and the choice of diagnostic test used for DPN (18).

On another hand, the duration of DM by itself is related to the prevalence of the DPN (Table 1). This could be elucidated by the hypothesis that longer duration of DM is associated with poorer control of blood glucose level and the cumulative effect of the consequent injurious factors of poor metabolic control along with the atherosclerosis usually lead to and accelerates neuropathy (15 and 19). A finding that was proved by many other studies (20 and 21).

Fasting blood sugar (FBS) and glycated hemoglobin (HbA1c) (Table 1)

Chronic hyperglycemia represents the main causative factor involved in the pathogenesis of diabetic neuropathy. It has been assumed that nerve damage may be directly induced by accumulation of intracellular glucose with its consequences

like the generation of glycation sugars and advanced glycation end-products (AGE), enhanced oxidative damage and protein kinase C activation and others (15 and 21). These finding agrees with that of other studies (21; 22 and 23) that came up with same findings although it disagreed with others (24 and 25).

The inconsistency of these studies' results indicates that the pathogenesis of DPN in type 2 DM is much more complex and not simply related to the hyperglycemia as in type 1 (2 and 11). That is why the UK Prospective Diabetes Study (UKPDS) Group (1998) (26) could not demonstrate a lower progression of neuropathy in intensively treated type 2 diabetic subjects. Additionally, Damci and his co-workers (1999) (27) examined the changes in vibration perception threshold before and after modification of blood glucose levels within the same patients and found no significant difference in vibration perception threshold with instantaneous change in blood glucose levels.

On contrary, the Diabetes Control and Complications Trial (DCCT) in 1995 (28) have established that lowering of glycated hemoglobin (HbA1c) in patients with type 1 diabetes was associated with a reduction in subsequent development of clinical neuropathy.

Advanced glycation end products (AGE)

The elevated levels of AGE in diabetic patients can be due to the chronic hyperglycemia that could lead to the formation of heterogenous moieties called AGE via non-enzymatic glycation and glycoxidation processes. It has been demonstrated that these compounds increase in the cells and tissues of patients with DM and are associated with the development of microvascular and macrovascular complications (29 and 4). The accumulation of AGEs on myelin can quench immunoglobulins and elicit immunological responses that may lead to

demyelination. Many studies have demonstrated that pronounced AGE immunoreactivity is found on axons and myelin sheaths in 90% of type 2 diabetic patients with both distal symmetric as well as proximal neuropathy. Many previous studies had showed results similar to that of this study (30; 31 and 32)

A research accomplished by Sekido and his co-workers (2004) (33) on diabetic rats had demonstrated that AGEs were directly perturbed the function of Schwann cells (cell viability, cell replication and production of pro-inflammatory cytokines). Taking this finding in consideration, such defect may in-turn be responsible for impaired survival and regeneration of peripheral nerves in diabetic patients.

Furthermore, several trials were attempted to counteract the effect of AGE in diabetic patients and animals. Glyoxalase 1 had been used to detoxify AGE precursors, Pyridoxamine to inhibit formation of AGE, Atorvastatin to inhibit receptor of AGE and many others agents have shown to be effective in reducing some diabetic complication in animals and human trials (34).

Advanced glycation end products correlations with different parameters

The results of this study showed perfect indirect correlation between AGE levels with duration of diabetes (Figures 4.2 and 3). These finding can be illustrated by the association of the longer duration of DM with cumulative damaging effect of chronic hyperglycemia and the high AGE were parts of its reflections (4).

On contrary, a no correlation between AGE was seen with HbA1c measurement as shown in figures 4.4 and 5. These can be explained by the presence of patients with fair glycemic control although they had a long history of bad control that resulted in their complications. Furthermore, this finding may reflect that the pathogenesis of type 2 DM is complex and in addition to

hyperglycemia, oxidative/nitrosative stress, neurovascular injury, deficiency of neuroprotective factors, accumulation of AGE and more others have important role in the pathogenesis of DPN (35).

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