Estimation of Na\K - ATPase Enzyme Activity and Endogenous Digitalis in Patients with Diabetic Neuropathy

Eman Hameed Al-Rikabi[1*], Mazin J. Mousa[2], Oda M. Yasser[3]

Abstract

Background: Among the most common complications of diabetes is diabetic neuropathy (DN). Diabetic neuropathy is a heterogeneous group of disorders, which involves a different part of somatic and autonomic nervous systems, with a gradual loss of neural conductivity. Some studies have shown that they reduce the activity of the Na/K ATPase, however, elevated levels of endogenous sodium pump inhibitor in diabetic individuals, including those with neuropathy. Changes in this transfer enzyme are believed to be due to several diabetes complications. **Objective:** The study had designed to evaluate the Na/K ATPase enzymatic activity in the erythrocyte-membrane among three groups. The first group had represented the patients with type 2 diabetes mellitus (DM2) and neuropathy. The second group is diabetics without neuropathy. The third group was a healthy subject. As well, the study had estimated the inhibitory activity of endogenous digitalis among patient groups. Furthermore, the aim of this research was to see whether there was a connection between red blood cell membrane Na-K ATPase activity and the medical facts of the analysis subjects. **Design and Methods:** One-hundred fifty subjects had enrolled in this case-control study; 80 patients complained of diabetic neuropathy of both sexes, the mean age 59.3 years with an age range of 40-81, 40 DM2 without neuropathy (53.9 years), (35 – 70), and 30 healthy controls (30 years, 25 to 45). Patients in the first group were selected carefully according to their clinical manifestations and the nerve conduction study results. The evaluations of both inhibitory activities of endogenous digitalis and Na/K ATPase had completed using a spectrophotometer. Enzyme activity had expressed in micrograms of phosphate concentration per grams of red cell ghost total protein concentration. **Results:** The mean enzyme activity of Na/K ATPase was significantly lower ($p<0.001$) in patients with diabetic neuropathy (381±17.9) compared with the diabetic group without neuropathy (498±22.9) and the normal controls (837±61.43). There was a significant inhibitory activity of endogenous digitals (17.87±2.15) in patients with DNP, compared with the diabetics without neuropathy (8.78±0.89) and healthy control (5.3±1.33). There was a significant association of enzyme activity with the following parameters: duration of diabetes, age, level of glycated hemoglobin and endogenous digitalis with the respective p-values (0.000, 0.000, 0.000 and 0.021). Gender showed no significant relationship with enzyme activity (p 0.43). **Conclusions:** In DM2 with neuropathy, hyperglycemia can much reduce the activity of erythrocyte Na/K ATPase. In addition, it may enhance the inhibitory activity of endogenous digitals. The time-dependent increase in diabetic complications can be due to a strong association between diabetes duration and erythrocyte Na/K-ATPase activities.

Key Words: Na/K ATPase, Neuropathy, Diabetic, Enzyme Activity, Endogenous Digital.

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Introduction

In diabetes mellitus the major reason for physical incapacity is a neuropathy. In contrast to retinopathy, neuropathy may occur in nearly 50% of diabetic patients (Young, Boulton et al. 1993).

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This phenomenon is similar to that seen with diabetic nephropathy (Krolewski, Warram et al. 1987), that had previously been linked to genetic predisposition (Marre, Bernadet et al. 1994; Mousa M. 2020; Samer Majeed 2020). Several evidences included variability among diabetic patients and a wide diversity of severity despite the short duration of DM among some patients indicate that neuropathic changes might comprise genomic disposition. Though it had generally believed that duration of DM besides impaired metabolism (Maser, Steenkiste et al. 1989; Young, Boulton et al. 1993) are contributing issues. There is no clear-cut pathological explanation for the variation between individuals concerning the neuropathic progression. The impact of gender, age, stature, and cigarette smoke still debatable (Maser, Steenkiste et al. 1989; Harris, Eastman et al. 1993; Dhulfiqar A. 2020). A higher frequency and extent of neuropathy among patients with insulin-dependent diabetes mellitus (IDDM) or type1 DM or (DM1) of a North-African origin compared with the European diabetics had indicated a genetic involvement (Vague, Brunetti et al. 1988). In a study involving a noninsulin-dependent DM (NIDDM, DM2), a dissimilar incidence of neuropathic pain was identified among ''black vis Hispanic Americans'' relative to their corresponding white group (Harris, Eastman et al. 1993). Diabetic neuropathy (DN) is characterized by a decline in the activity of the Na/K ATPase in the nervous tissues (Greene, Lattimer et al. 1987; Scarpini, Bianchi et al. 1993). A ''Na/K ATPase (EC3.6.1.37)'' is a membrane-bound enzyme that hydrolyses an ATP molecule to pump 3-Na^+ outside the cell and 2-K^+ inside the cell. This transporting pathway is essential for many biological processes, including potential membrane generation and nerve conduction, as well as maintaining Na^+ and K^+ concentration gradients across the cell-membrane (Lingrel and Kuntzweiler 1994). Hence, it is in charge of maintaining Na/K homeostasis via neuron cell membrane. Loss of this balance results in depolarization of the nerve as Ca ions enters the cell. The latter has followed by the release of a neurotransmitter and neuronal swelling, which damage cell function. Na/K ATPase is a membrane-bound enzyme crucial for controlling membrane potential, cell size, and membrane flux in Ca. as well, it is vital in the ordinary cell-growth cycle and differentiating nervous tissues. The preservation of Na and K ions across cell membrane is a required for normal cell activities (Albers RW 2012). The activity of neural Na/K ATPase has concentrated in the membranes nearby the nerve terminal, where there are special receptors for usual neurotransmitters (Mains RE 2012) had introduced and may interact well in the central nervous system (CNS). In DM1, there is a lack of intracellular glucose due to absolute insulin deficiency. In DM2, a lack of intracellular glucose due to relative insulin deficiency, which in turn causes a reduction of intracellular energy - generated by the glycolytic pathway in the form of ATP normally- and culminating in reduced function of ATP-dependent enzymes.

In this study, we investigated the enzymatic activity of Na+/K+ ATPase in red cells of patients with diabetic neuropathy. Besides, the inhibitory role of endogenous digitalis had achieved spectrophotometrically. Endogenous digitalis had found to increase in sera of patients with heart failure and renal impairment (Devynck, Pernollet et al. 1985).

Experimental

Ethics Issues

Ethical approval had obtained from the scientific committee of Merjan Teaching Hospital and the center of diabetes in Babylon province. To gain verbal acceptance from participant patients, the goals of the study had explained to all participants.

Study Design

The study is a case-control, blood samples were collected from patients complaining of DM2 registered in the Diabetes-care center in Merjan Teaching Hospital in Babylon Governorate, Iraq. The age range of the participating patients ranged from (30-70) years. These cases had divided into three groups, (80) diabetic peripheral neuropathy (DPN) cases, (40) diabetic patients without neuropathy (positive control group), and (30) healthy control group (negative control group). All patient details had collected, including disease duration, degree of control of blood glucose, and the concomitant illnesses. The details of anti-diabetes and concomitant medications had collected. Body mass index (BMI) had estimated for all patients. Patients with coexisting cardiac or renal disorders had excluded.

Chemicals

All chemicals were of analytical grade and purchased from different manufacturers.

Collecting Blood Samples

Venous blood samples were collected in Na-EDTA tubes as an anticoagulant, immediately transferred to the lab, and centrifuged at 3000 X g. The plasma had separated into a different test tube and frozen for other estimation of endogenous digitalis inhibitory effect. Meanwhile, the lower cellular layer had immediately treated for the preparation of red cell membrane ghosts.

Determination of Na/K-ATPase Activity

A. Principle: The activity of Na-k ATPase in RBC (ghost) membranes was determined to be inorganic phosphate formed during enzymatic hydrolysis of ATP and expressed in milligrams of phosphate per gram of protein released during 30 minutes of incubation (Pi) (Kaššák 2006).

B. Preparation of ATPase Reagent: ATPase reagent had prepared by mixing the following materials:

1. Tris - HCl (100 mM), has set by weight of 1.2 g.
2. MgCl2 (10 mM) had prepared with a weight of 0.095 g.
3. KCl (15 mmol) prepared weight of 0.11 g.
4. Sodium chloride (85 mM), prepared by a weighting of 0.4 g.
5. Na2-EDTA (1 mM) with a prepared weight of 0.036 g.
6. ATP (2 mM), prepared with a weight of 0.1 g. All materials had dissolved in 100 ml of distilled water, then the pH of the reagent had adjusted to 7.4 by adding NaOH.

C. Preparing ghosts of red blood cells

1. Fresh blood was collected in EDTA-K3 tubes as an anticoagulant and centrifuged at 4000 rpm for 10 minutes.
2. RBCs were obtained by taking 250 µl of the erythrocyte sediment after centrifugation.
3. In 1 ml of a pharmaceutically available PBS (10Mm phosphate buffer, 150Mm NaCl, pH7.4), 250 µl of red cell sediment was added and washed three times with subsequent centrifugation and decanting the remaining normal saline supernatant.
4. The washed RBCs obtained through the above procedure were subjected to lysis by adding Tris-HCl buffer (5mM, pH7.4) of 500 ml and centrifuging at 4000 rpm for 10 minutes.

D. Measurements of Na-K ATPase Activity (Kaššák 2006)

1. Ten µl of red cell ghosts had added to 500 ml of ATPase reagent prepared in section B and incubated for 30 minutes exactly.
2. After incubation, samples had centrifuged at 4000 rpm for 10 minutes. Then withdraw 50 µl of the supernatant to identify the inorganic phosphate.
3. The inorganic phosphate was determined spectrophotometrically according to the method described by Baykov et al. (1988) (Baykov, Evtushenko et al. 1988).
4. For standardization, the protein concentration in phantoms of red cells had estimated according to the standard biuret method described of Lowry et al. (1951) (Lowry, Rosebrough et al. 1951).
5. The enzyme activity was expressed with the concentration of inorganic phosphorous to the ghost protein of red cells as follows:

$$Enzyme\ Activity\ (mg\backslash g) = \frac{Concentration\ of\ Inorganic\ Phosphorus}{Concentration\ of\ Total\ Protein}$$

Determination of Endogenous Digitals Modified Method (Lowry, Rosebrough et al. 1951)

1. Red cell membrane ghosts were prepared as mentioned above in section 2.5. C
2. Identification of Na-K ATPase of the red cell ghosts had accomplished.
3. In another tube, mix up 25µl plasma (patient) and 25µl ghosts (control), and then incubate in a water bath for 10 minutes.
4. After the incubation period is over, Na-K ATPase activity was determined as described above.

Calculation of Results

Inhibitory effect of endogenous digitalis evaluated as a percent of inhibition according to the following equation:

$$\% = \frac{activity\ of\ normal\ -activity\ of\ patient}{activity\ of\ normal} * 100$$

Statistical Analysis

The data had logged into a personal computer, and analyses of data had performed using SPSS statistical package version 26. T-test used for comparing means. *P*-value considered statistically significant if it was < 0.005.

Results

The erythrocyte Na/K ATPase activity in the diabetic neuropathy group is considerably lower (381±17.9) than in diabetic patients and normal healthy subjects (8.780.89), (5.31.33), respectively.

While plasma inhibitory of Na-K ATPase activity is significantly higher in the diabetic neuropathy group (17.872.15) than in the diabetic group without neuropathy and control individuals (8.78±0.89), (5.3±1.33) one by one as shown in (Table 1).

The duration of DM (16.075±6.6) and age (59.3±9.04) were higher important in the diabetic neuropathy group when compared to diabetic (4.150±2.09), (53.9±9.97) and normal healthy persons. Whereas the lower results suggest that Hemoglobin A1C levels in diabetic patients have a close relationship, there is also an indirect proportion between groups 1 and 2 (9.42±2.34), (10.48±2.4).

There was no significant difference in the gender profile for both sexes (F\M ,36/44, 18/22) between the neuropathic and no neuropathic individuals with study parameters as seen in the table 2.

Also, the study population had a significant relation with diabetes duration, age, HbA1C, endogenous digitals, and enzyme activity (r=-0.425, p=0.000), (r=-0.495, p=0.000), (r=-0.380, p=0.000), and (r=-0.167, p=0.021) respectively like seen within table 2. But, certainly not correspondence among levels of enzyme activity and gender for analysis classes.

Table 1. Correlation of Erythrocyte Na\K- ATPase activity (µg Pi\g protein) with other studied variables

Parameters	Diabetic neuropathy mean±S.E	Diabetes Mellitus mean±S.E	Control mean±S.E	Correlation coefficient (r)	p-value
Number	80	40	30	-	
Duration of DM (years)	16.075±6.6	4.150±2.09	-	-0.425	0.002
F/M ratio	36\44	18\22	15\15	-	>0.05
Hb A_1C (%)	9.42±2.34	10.48±2.4	5.2±0.04	-0.495	0.001
Age of subject	59.3±9.04	53.9±9.97	30.85±7.22	-0.380	0.003
Erythrocyte Na\K- ATPase activity (µg Pi\g protein)	381±17.9	498±22.9	837±61.43	-	-
Endogenous digitals (%)	17.87±2.15	8.78±0.89	5.3±1.33	-0.167	0.001

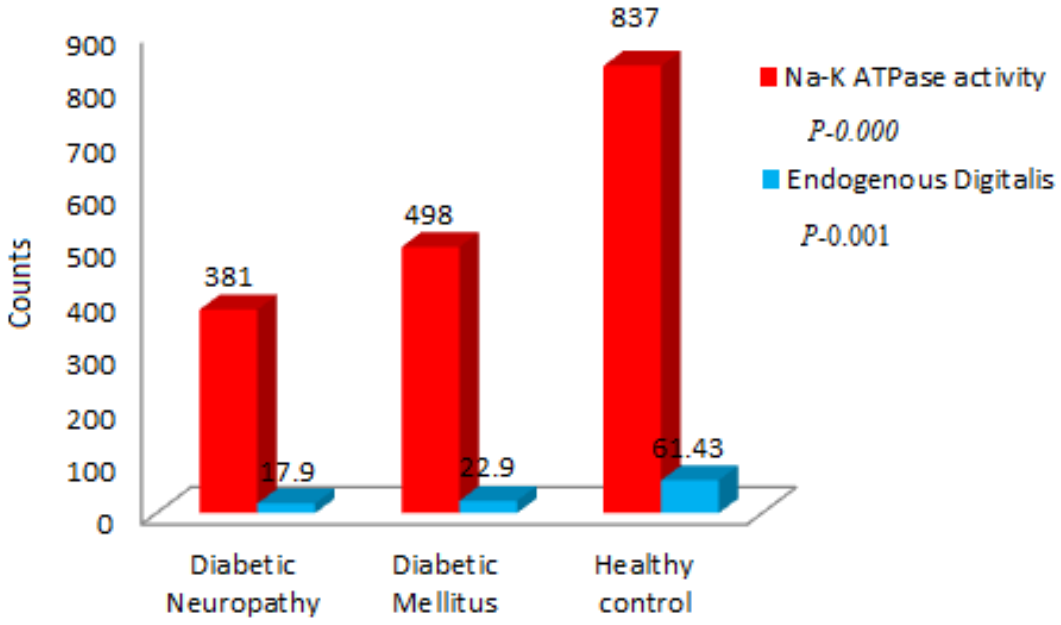

Fig. 1. Mean difference of Na-K ATPase activity(µg/g) and Endogenous digitalis (%)

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Table 2. Gender variation of the studied variables

	gender	Mean	Std. Error Mean	p-value
enzyme activity	F	513.23	34.732	>0.05
	M	495.97	27.674	
endogenous	F	14.1237	2.17375	>0.05
	M	11.9208	1.46619	
HbA1C	F	8.720	.3244	>0.05
	M	8.911	.3150	
duration	F	10.11	1.072	>0.05
	M	9.30	.870	
Age	F	51.66	1.665	>0.05
	M	52.70	1.550	

Discussion

Diabetic neuropathy is a complex group of disorders marked by a deterioration of sensory and autonomic nerve sensation, and improvements in electrical function reflected in disrupted nerve conduction (Zhou, Yan et al. 2019). Several previous studies had demonstrated that diabetes causes a decline in the Na/K-ATPase enzyme action, which leads to a hemodynamic defect. These two changes had caused by altered microvascular flow and rheological abnormalities. The abnormalities might be due to both decreased deformability and increased fluidity of erythrocytes mutually, besides other complications including nephropathy, neuropathy, coronary diseases, in addition to peripheral arterial disease (Adewoye, Bolarinwa et al. 2000). The current analysis demonstrates that red blood cell membrane activity Na\K-ATPase was decreased in patients with DNP (Okegbile, Odusan et al. 1997). In the erythrocytes of IDDM patients and diabetic rats, there was even a drop in Na/K-ATPase level (Raccah, Fabreguetts et al. 1996). Furthermore, diabetic mice had impaired enzyme activity proportionate to their sciatic nerve and red blood corpuscles (RBCs) (Sundkvist, Dahlin et al. 2000). Na/K-ATPase action in RBCs differs according to ethnicity, being less among blacks and North Africans, prone to DN (Vague, Brunetti et al. 1988), compared to Scandinavians and Caucasians (Yorek, Dunlap et al. 1993). These results indicate that a decline in Na/K-ATPase activity may be a risk factor for DN. Another hypothesis is basing on a reversal hyperglycemic-induced. It had proposed that the defect in Myo-inositol metabolism resulted in altered lipid metabolism and decreased Na/K ATPase activity (Schultheis, Wallick et al. 1993). Notably, the direct effects of hyperglycemia on Na/K ATPase activity had never confirmed (Schultheis, Wallick et al. 1993).

Na/K ATPase inhibitors contain a set of composites attached to the Na/K-ATPase extracellular fragment and hinders its action. The amino-acids required for attachments of Na/K inhibitors occur in the extracellular areas in-between helices one/two and helices seven/eight of the alpha-subunit (Holland, Millett et al. 1991). The inhibitory activity of Na/K-ATPase assumed SPLs had been measured depend on its ability to promote G6PD in certain early experiments of digitalis-like natriuretic hormone (Muggeo, Moghetti et al. 1993). Hyperglycaemia seems to be common for produce the pentose-phosphate way of glucose breakdown. The catalysis of glucose-6-phosphate by G6PD is the first step in this pathway (Mosseri, Waner et al. 2000). G6PD insufficiency has historically known in DM (Zeng, Dohi et al. 1999), and affected subjects responded abnormally to oral glucose tolerance tests (Therien and Blostein 2000). In non-obese diabetic mice, the other hand, advantages of vanadate therapy were observed (Feschenko and Sweadner 1997). G6PD was shown to be amplified easily and reversibly by digitalis and endogenous digitalis-like substances (Gaskin, Estwick et al. 2001). The current roles of ''endogenous digitalis-like factors'' are to regulate glucose metabolism as seen in the Fig-2.

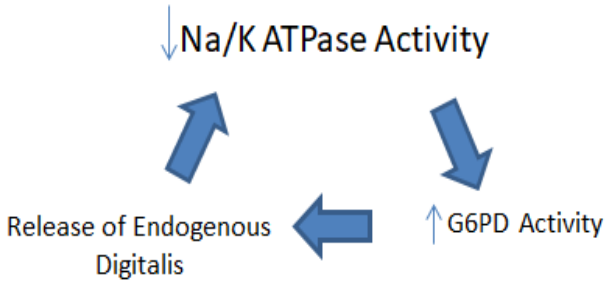

Fig. 2. The relationship between Na/K ATPase activity, G6PD activity, and endogenous digitalis

The current confirmation revealed that lower enzyme activity in DM based on either (1) a interruption of the normal-relations among the subunits of Na/K- ATPase molecule, owing to disturbed organization of proteins and lipids in the lipid bilayer of cell membrane or, (2) altered phosphorylation, causing changes in the enzymatic kinetic-property or its affinity to the ''endogenous ouabain-like factors'' (Scarpini, Bianchi et al. 1993). This study also revealed that the mean age of patients with diabetic neuropathy is higher than the

mean age of patients with diabetes only. The reported fraction of major lower-extremity amputation (Li, Burrows et al. 2012), myocardial infarction, vision dysfunction, and end-stage renal failure are discovered in older adults with diabetes. Many complications affect those over the age of 75 at a greater rate than those between the ages of 65 and 74. Deaths from hyperglycemic incidents are even more frequent in older people. Peripheral neuropathies are a dangerous disease in the elderly. In the United States in 1999–2000, a simple examination for decreased sensitivity at the foot showed that 28 percent of adults aged 70–79 years and 35 percent of adults aged 80 years had peripheral neuropathy (Gregg, Sorlie et al. 2004).

We observed that there is no link between Na-K ATPase levels and gender in diabetics with neuropathy and diabetics without neuropathy. The gender variations in erythrocyte Na/K-ATPase levels are of no concern. While there was little difference between the sample classes, female control subjects had slightly higher levels than the males in other studies.

Many studies had shown that the levels of Na/K-ATPase will vary due to gender variation (Persky, Ostrow et al. 1990). The hormonal variations between both sexes were criticized for the differences in erythrocyte Na/K ATPase levels (Persky, Ostrow et al. 1990). According to the previous study, only control subjects exhibited variations in Na/K-ATPase levels, while women's erythrocyte Na/K-ATPase levels were likely more vulnerable to reductions in diabetic complications (Tsong and Chang 2003).

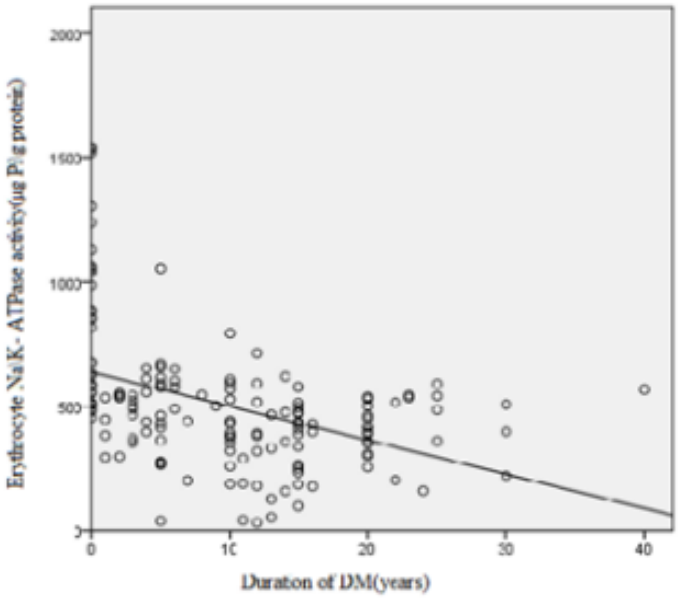

Fig. 3A. Relationship between erythrocyte Na\k-ATphase activity (ug Pi\g protein) and duration of (years) among study groups

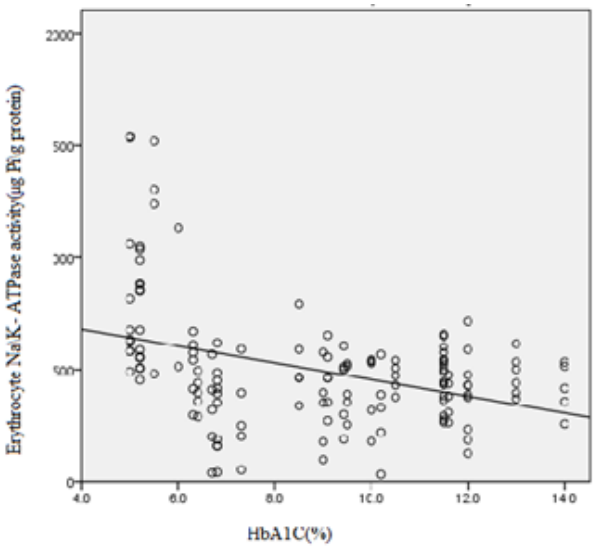

Fig. 3B. Relationship between Erythrocyte Na\K-AtPhase activity (ug Pi\g protein) and HbA1C(%) among study groups

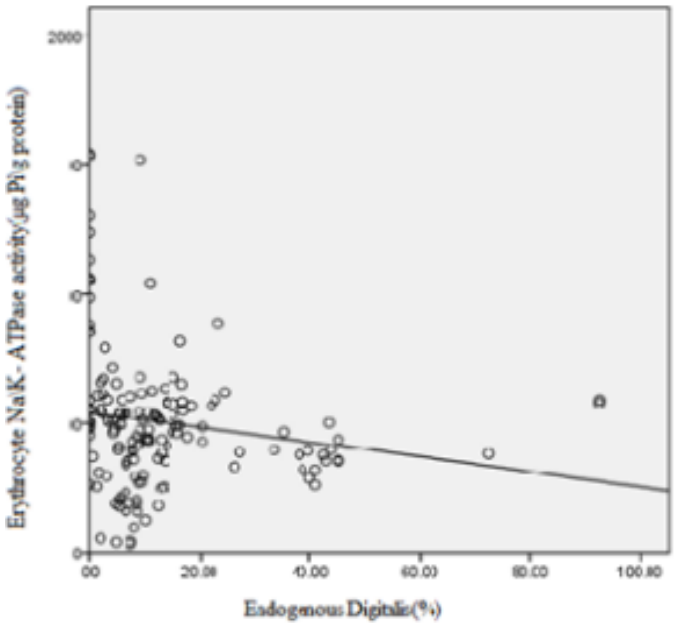

Fig. 3C. Relationship between Erythrocyte Na\K ATphase activity (ug protein and Endogenous digitalis (%)

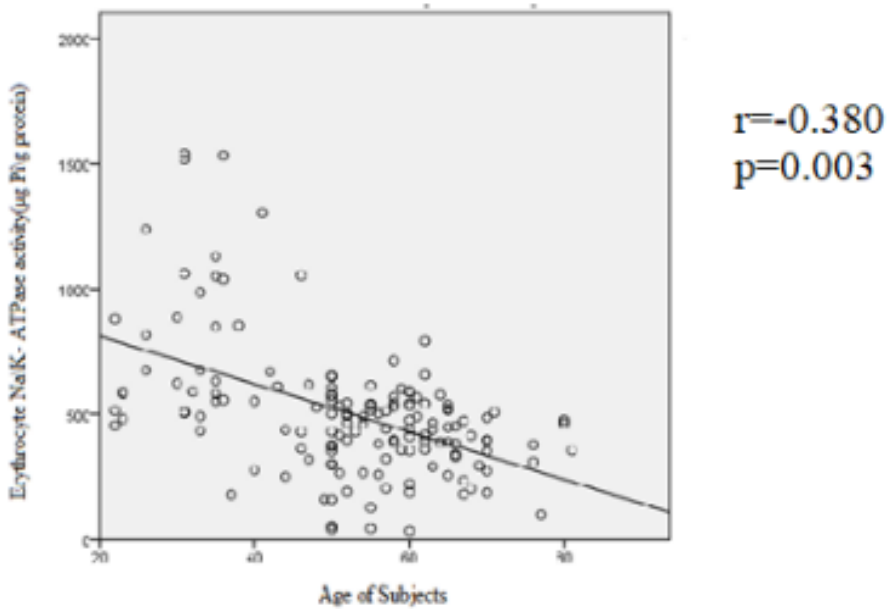

Fig. 3D. Relationship between Erthrocyte Na\K ATPhase (ug Pi\g protein) and age of subject among study groups

Fig. 3. Strong positive correlation of erythrocyte Na\K-ATPase activity (µg Pi\g protein) and A- period of diabetes (years), B- HbA1C (%), C- plasma of endogenous digitalis (%), D- age of subjects, among study groups

Additionally, in our work, we study the relationship between erythrocyte Na/K ATPase activity and plasma level of the inhibitory presence of Na\K-ATPase activity. We found an inverse-correlation between Na/K-ATPase activity and plasma inhibitory of Na/K ATPase activity (r=-0.167, p=0.000).

Conclusions

Hyperglycemia leads to a decrease in erythrocyte Na+/K+ ATPase activity in diabetic neuropathy subjects in DM2. The Na/K-ATPase inhibitory activity of endogenous digitals present in the plasma of neuropathy patients is the most likely cause of decreased enzyme activity.

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