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A Modified Method for the Assessment of Na-K ATPase Specific Activity in the Red Blood Cell Membranes in Iraqi Patients with Pre-eclampsia

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Abstract.

Background: Pre-eclampsia is a human pregnancy-specific disorder characterized by the appearance of proteinuria-related hypertension that appears de novo after 20 weeks of pregnancy in a previously normotensive woman and is fully resolved by the sixth week of postpartum. This is the main reason for morbidity and pregnancy-related death. It is a multisystem disease, but its etiology is not well understood. Pre-eclampsia is associated with anomalies in many systems, including ion transport disorder in placental cell lines, maternal and neonatal. Objective: This study aimed to develop a modified method and measured the specific enzyme activity of Na-K ATPase in red blood cell ghosts in pre-eclampsia patients compared to normal pregnancies. Design and Methods: This was a case-control study involving 105 cases, divided into two groups, 60 normal pregnancy and 45 pre-eclampsia patients. The modified method for estimating specific activity included the determination of inorganic phosphate produced by enzymatic ATP hydrolysis and protein released through 30 minutes of incubation (Pi). The specific activity was expressed in milligrams of phosphate concentration per gram of total protein concentration. The analysis of the results was carried out using version 26 of the Social Sciences Statistical Package (SPSS®) and a p-value of ≤ 0.05 was considered to be significant. Results: Specific enzyme activity was significantly lower in pre-eclampsia patients compared to control groups, $P < 0.05$. The study also found an inverse correlation between specific enzyme activity, systolic pressure, and diastolic pressure. Conclusion: The modified method of determining the activity of the Na-K ATPase described in this work is cost-effective and accurate. Reduction in enzyme activity is significant in patients with pre-eclampsia compared to normal pregnancy.

Keywords: Na-K ATPase, Pre-eclampsia, Pregnancy.

1. Introduction

Pre-eclampsia (PE) is a pregnancy condition associated with more than 300 mg per day of proteinuria and hypertension. It is a severe disease that can lead to fetal and maternal morbidity and death. After 20 weeks of pregnancy, the disease begins [1]. Red blood cell breakdown, low blood platelet count, liver function damage, swelling, vision problems, or breathing deficiency can occur in serious illness due to fluid in the lungs [2]. Older age, diabetes mellitus, obesity, and past hypertension provide several risk factors for pre-eclampsia [2,3]. Primigravida is also more

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widespread and multiparty. In this disorder, the key pathological characteristic was irregular placental tissue vascularization. In most cases, signs occur during pregnancy and only a few cases are detected after birth [3,4]. In order to validate the diagnosis, both elevated blood pressure and proteinuria have traditionally been necessary. Ordinary hypertension is characterized as having a systolic pressure greater than 140 mmHg or a diastolic pressure greater than 90 mmHg at two separate readings more than four hours apart in a woman after 20 weeks of pregnancy[4]. Prenatal treatment is routinely monitored for pre-eclampsia to prevent complications and maternal and fetal fatalities[5]. 2 to 8 percent of births worldwide are affected by pre-eclampsia[3]. One of the most common causes for death is hypertensive pregnancy disorders [5]. In various structures, pre-eclampsia is associated with anomalies, including defects of ion transport in placental, maternal and neonatal cell lines. These include intracellular ion imbalances such as elevated sodium and calcium, frequently identified in cases of essential hypertension. [6]. [...] ATPases are integral enzymes in multiple pathways in all kingdoms of life. ATPase acts as molecular motors that use the energy of ATP hydrolysis to power various reactions such as protein trafficking and assembly, ion pumping, cell metabolism, muscle movement, cell motility, replication and transcription. Enzymes that generate inorganic phosphate (Pi) by breaking down the gamma phosphate of ATP are adenosine triphosphates (ATPase). Cation transport [7,8] is a crucial representative of this large family. In the erythrocyte membranes (Ca2+-ATPase (EC 3.6.3.8), Mg2+-ATPase (EC 3.6.3.1), and Na-K-ATPase), three ATPases can be present in different quantities (EC 3.6.3.9). The most significant of the three forms is considered to be Na-K-ATPase [9]. Na-K-ATPase is an integral membrane protein present in most higher eukaryotes that is found in the cells' plasma membrane. Skou first discovered it as a membrane-bound structure in 1957. This enzyme consists mainly of two subunit types: the catalytic alpha (alpha) subunit (110-115 kDa), which extends the plasma membrane ten times, and the beta (β) subunit (35 kDa) with a single transmembrane component, which changes the alpha subunit's conformational stability and action. A third small polypeptide, the γ subunit (10 kDa), has been found in combination with the alpha-β heterodimer[11]. For the Na-K ATPase to be functional, it does not appear to be essential and can play a regulatory role[12]. All three subunits carry 3 Na+ ions and 2 K+ ions into the cell, thus preserving a balanced transmembrane potential. The importance of this transporter mechanism is due to the fact that nearly all animal tissues have been identified[13]. The enzyme can, in two ways, transport Na+ and K+ through the membrane. In the enzyme, there is a pocket or gate that is open to the extracellular fluid. Alternatively, there is an open pocket or door in the cytoplasm. By alternating between the two states [14], the enzyme transfers Na+ and K+ through the membrane. The purpose of this research is to investigate the use of a modified method to measure Na-K-particular ATPase's activity as a complementary tool for the diagnosis of pre-eclampsia disease.

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2. Experimental

2.1. Ethics Issues

Ethical approval was obtained from thescientific committee of maternity hospital and children hospital in Babylon governorate, Hilla city. To gain the verbal acceptance from participate women, the goal of this study were explained to all participants in the current study.

2.2. Study Design

This was a case-control study consisting of 105 women attending the maternity hospital and children and Hilla teaching hospital in the town of Hilla in the province of Babylon, and the participation of women ranged from (16 to 40) years of age. The exclusion was made for patients with heart failure, renal disease and diabetes mellitus. Blood samples were obtained as anticoagulated in the Na-EDTA tube and divided into 2 groups; 60 normal pregnancies and 45 patients with pre-eclampsia. Clinical and laboratory parameters from the hospital registry system were collected. According to the updated method described below, Na-K ATPase specific behavior was calculated. In order to compare the readings of the two classes, statistical analysis was conducted and a p-value < 0.05 was considered important.

2.3. Chemicals

All chemical substances were attained from standard commercial suppliers.

2.4. Collection of Blood Samples

Venous blood samples were gathered during the period of 11/2/2019 to 20/2/2020. The blood samples were drawn from all subjects by using a disposable syringe (3 mL) in the sitting position with a tourniquet. Three milliliters of blood were obtained from each individual by venipuncture and collected into the EDTA tube and mixed gently. Blood was centrifuged at 3000 RPM for approximately 10 minutes. After centrifuge, the plasma was transferred to a plain tube and stored at -20˚C until the analysis of endogenous digitalis. Whereas immediately, the red blood cell was used for the measurement of specificactivity of Na-K ATPase.

2.5. Determination of Na-k ATPase Specific Enzyme Activity

A. Principle

The specific enzyme activity of Na-K ATPasein RBCs membranes (ghosts) was determined as inorganic phosphate produced through enzymatic hydrolysis of ATP and expressed in milligrams of phosphate per gram of protein released through 30 minutes of incubation (Pi), according to a modification of the method described by [11].

B. Preparation of ATPase Reagent

The reagent of ATPase was prepared by mixing the following materials

1. Tris – HCl (100 mM, prepared by weighting 1.2 g).
2. $MgCl_2$ (10 mM, prepared by weighting 0.095 g).

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3. KCl (15 mM, prepared by weighting 0.11 g).
4. NaCl (85 mM, prepared by weighting 0.4 g).
5. Na_2-EDTA (1 mM, prepared by weighting 0.036 g).
6. ATP (2 mM, prepared by weighting 0.1 g).

All materials weredissolved in 100 mLof distilled waterthen adjusted the pHof reagent at 7.4 by adding sodium hydroxide.

C.Preparation of Red Blood Cell Ghosts

1. Fresh blood was collected in EDTA tubes as an anticoagulant and centrifuged at 4000 RPM for 10 min.
2. RBCs were obtained by taking 40 µl of red cell sediment after centrifugation.
3. In 1 ml of pharmaceutically available normal saline, the 40 µL red cell sediment was added and washed three times with subsequent centrifugation and decanting the residual normal saline supernatant.
4. RBCs was washed obtained by the above procedure were subjected to lysis by the addition of deionized distilled water by adding 500 mL DDW and centrifuged at 4000 RPM for 10 min.

D.Determination of Inorganic Phosphorus

1. Principle

Inorganic phosphate reacted with molybdic acid forming a phosphomolybdic complex as shown in equations 1 and 2. Its produced reduction in alkaline medium originates a blue molybdenum colour which intensity was proportional to the quantity of phosphorus existing in samples[15,16]

2. Preparation of Working Reagent

The working reagent was prepared by mixing equal volumes of R1 with R2, and this reagent was stable for 8 hours.

3. Procedure

Three tubes were prepared and the procedure carried out as in the following Table 1.

Table 1. Procedure Used for Determination of Inorganic Phosphorus According to Linear Kit

Tubes	Blank	Sample	Standard
Working Reagent	1 (mL)	1 (mL)	1 (mL)
CAL (Standard)			50 (µL)
Red cell membrane (ghost)		50 (µL)	
All tubes mixed and let to stand for 10 min. at room temperature			

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R3 Developer	0.5 (mL)	0.5 (mL)	0.5 (mL)
All tubes mixed and let to stand for 10 min. at room temperaturethen read the absorbance at 740 nm			

4. Calculation

The results were calculated as follows:

$$\textbf{Concentration of Inorganic Phosphorus (mg/L)} = \frac{\textbf{A. Sample}}{\textbf{A. Standard}} * \textbf{Concentration of Standard}$$

Concentration of Standard = 100 mg/L

E. Determination of Total Protein by Biuret Method

1. Principle

The Biuret approach focused on the complexation of cupric ions in the protein's peptide bonds to functional groups. To form a Cu^{2+}-protein complex and produce a violet-colored chelate product that was measured at 540 nm by absorption spectroscopy, two peptide bonds or longer were required[16].

2. Preparation of Working Reagent

By adding 3 ml of R2 to a container of R1, the working reagent prepared, and this reagent was stable for 6 months.

3. Procedure

Three tubes were prepared and the procedure carried out as in the following Table 2.

Table 2. Procedure Used for Determination of Total Protein According to Bio Maghreb Kit

Tubes	Blank	Sample	Standard
Working Reagent	1 (mL)	1 (mL)	1 (mL)
Reagent 3 (Standard)			20 (mL)
Red cell membrane (ghost)		20 (µL)	
All tubes mixed and incubation for 2 min. at room temperature then the absorbance was reading at 540 nm against the blank			

4. Calculation

The result was calculated as follows:

$$\textbf{Concentration of Total Protein (g/L)} = \frac{\textbf{Absorbance of Sample}}{\textbf{Absorbance of Standard}} * \textbf{Concentration of Standard}$$

Concentration of Standard = 50 g/L

F. Measurements of Na-K ATPase Specific Enzyme Activity

The modified method illustrated as below:

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1- Ten microliters of red cell ghosts was added to 500 mL of ATPase reagent which had been prepared in section B, and incubated for 30 min. exactly.
2- After incubation, the samples were centrifuged at 4000 RPM for 10 min. then 50 µL of the supernatant was pulled to determine inorganic phosphates.
3- The inorganic phosphates were determined spectrophotometrically according to the methodwhich had been illustrated in section D.
4- For the purpose of standardization, protein concentration in the red blood cell ghosts was estimated according to the standard biuret method which had been illustrated in section E.
5- Specific activity was expressed as the inorganic phosphorus to red cell ghost protein concentration as follow:

$$\textbf{Specific Activity of Na-K ATPase (mg/g. min)} = \frac{\textbf{Concentration of Inorganic Phosphorus}}{\textbf{Concentration of Total Protein}}$$

2.6. Statistical Analysis

The analysis of results was carried out using version 26 Statistical Package of the Social Sciences (SPSS®) to get on the variables as mean, standard error for mean (SEM), confidence interval, T-test, and correlation coefficient. A *p*-value of ≤ 0.05 was considered significant.

3. Results and Discussion

Pre-eclampsia is a multi-system and multifactorial condition that affects both the mother and the fetus through intrauterine growth and vascular dysfunction restrictions[17]. Figure (1) indicates the age distribution for pre-eclampsia cases, mean and SEM in pre-eclampsia (26,978 ± 0,862), respectively. In order to model the age distribution of the patient groups, samples from the control groups were randomly selected and clustered. This age matching helps to remove variations in the outcomes of parameters that could occur due to the large variance in age.

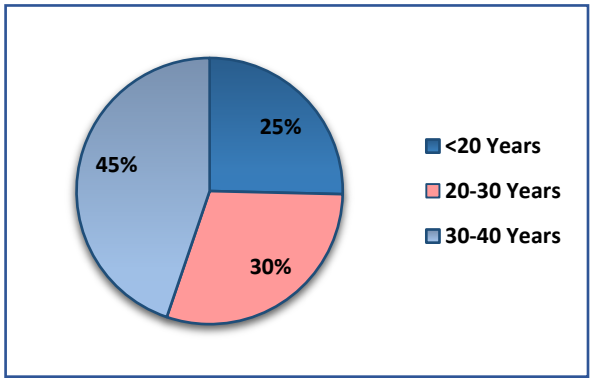

Figure 1. Age Distribution in Patients of Pre-eclampsia

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As well, the mean and SEM values of the clinical characteristics of the control and patient groups are shown inTable (3).

Table 3. The Demographic Characteristics of Studied Groups

Variables	Control Mean ± SEM	Case Mean ± SEM	T- Test	P- Value
Albumin in Urine	0.225 ± 0.100	2.806 ± 0.242	-9.817	0.000*
Diastolic BP (mmHg)	77.105 ± 1.219	98.205 ± 1.936	- 9.167	0.000*
Systolic BP (mmHg)	117.948 ± 1.690	149.512 ± 1.708	-13.132	0.000*
Gestational Age (Weeks)	36.105 ± 0.321	34.846 ± 0.518	2.062	0.043*
Weight of Baby (gm)	3104.800 ± 74.416	2726.300 ± 144.044	2.335	0.025*

*p value < 0.05 was significant

The Demographic results for mean systolic and mean diastolic pressures were shown significantly higher in pre-eclampsia than in normal pregnant women. Also, the mean of pre-eclampsia albumin in the urine is significantly higher than that of normal pregnant women. However, this was expected because of the criteria used for pre-eclampsia diagnosis.

Pre-eclampsia is clinically present in the form of hypertension, proteinuria, with or without edema during pregnancy. These are primarily found in the interstitial with an appropriate electrolyte and water content during pre-eclampsia, with a corresponding decrease in intravascular circulating volume. This drop in the amount of intravascular circulation results in the activation of baroreceptors and the release of water retention and natriuretic reasons for antidiuretic hormone[18]. Other clinical features include gestational age, which showed that the results of pre-eclampsia were significantly lower than normotensive pregnant women. Baby weight outcomes in pre-eclampsia also appeared substantially lower than in normotensive pregnant women. In this research, the updated approach was used to evaluate the basic activity of Na-K ATPase by using ATP as an enzymatic reaction substrate, MgCl2 to provide the Mg2+ ion as a cofactor, and Na-EDTA to inhibit Ca+2 ATPase. This result indicates that pre-eclampsia is associated with low fetal weight and premature birth. The findings of Na-K ATPase specific activity in a pre-eclampsia patient were significantly lower than in a typical pregnant woman, as shown in Table 4 and 'Figure 2.' The findings were in line with the research conducted by Adair et al.,[20].

Table4. The Specific Activity of Na-K ATPase in Control Group Compared with Pre-eclampsia Groups

Specific Enzyme Activity	Groups	Mean ± SEM	Mean Difference	T-Test	95% Confidence Interval for Mean Lower	Upper	P-value

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mg/g. min	Patient	0.239 ± 0.043	0.160	3.298	0.151	0.326	0.002*
	Control	0.399 ± 0.021			0.356	0.442	

*p value < 0.05 was significant

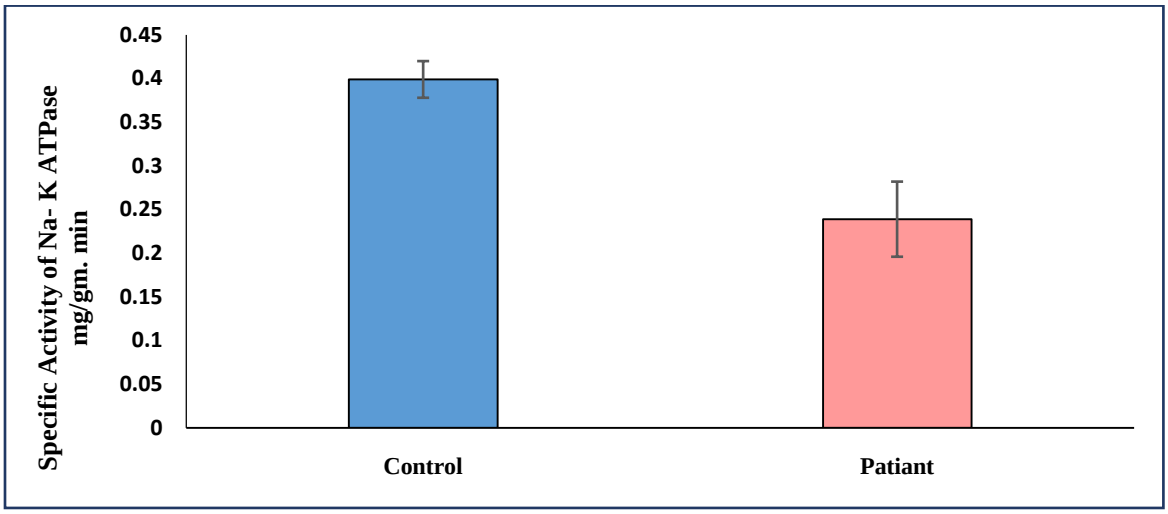

Figure 2. Specific Activity of Na-K ATPase Mean ± SEM for Patient and Control Groups

While the result of the correlation between specific enzyme activity and criteria for digenesis pre-eclampsia are shown in the Table 5 and this result are shown an inverse relationship between enzyme activity, systolic pressure, and diastolic pressure.This finding is particularly remarkable since the more was Na^+, K^+-ATPase reduced, the more individual presented rises diastolic blood pressure, appeared by the significant negative correlation between these variables, which propose a possible role for Na^+, K^+-ATPase lowering in blood pressure increase[21].

Table 5. The Correlation between Specific Enzyme Activity and Criteria for Digenesis Pre-Eclampsia

		Specific Enzyme Activity (mg/g. min)	Diastolic BP (mmHg)	Systolic BP (mmHg)	Albumin in Urine
Specific Enzyme Activity (mg/g. min)	Correlation Coefficient	1.000	-0.313**	-0.290*	-0.163
	P- Value	.	0.007	0.012	0.206
Diastolic BP (mmHg)	Correlation Coefficient	-0.313**	1.000	0.807**	0.581**
	P- Value	0.007	.	0.000	0.000
Systolic BP (mmHg)	Correlation Coefficient	-0.290*	0.807**	1.000	0.594**

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	P- Value	0.012	0.000	.	0.000
Albumin in Urine	Correlation Coefficient	-0.163	0.581**	0.594**	1.000
	P- Value	0.206	0.000	0.000	.

**. Correlation is significant at the 0.01

*. Correlation is significant at the 0.05

4. Conclusion

The modified method of determining the Na-K ATPase activity described in this work is cost-effective and accurate. The reduction of enzyme activity is significant in patients with pre-eclampsia compared to normal pregnancies.

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