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EVALUATION OF CALCIUM, PHOSPHATE, IRON AND FERRITIN, IN TYPE 2 DIABETES MELLITUS SUBJECTS ATTENDING FEDERAL TEACHING HOSPITAL OWERRI, IMO STATE

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ABSTRACT
Diabetes mellitus is a metabolic disorder caused by either a lack of insulin secretion, impaired insulin action, or both. This study was aimed at the evaluation of serum levels of calcium, phosphate, iron, ferritin, and enzymatic antioxidants activities in type 2 diabetes mellitus patients attending Federal Teaching Hospital Owerri. The cross-sectional study population comprised 50 participants within the age range of 30 and 65years, with age matched control of 50 apparently healthy individuals. The diabetic subjects were further divided into 25 males and 25 females. The samples were centrifuged at 3,000rmp for 5 minutes after clotting to separate and obtain the serum. The sera was obtained using a pasteur pipette and put into appropriate container, and stored at -20^{0}C prior to use. Test with probability of $p<0.05$ was statistically significant. The result showed there was a significantly higher level of iron and ferritin ($p=0.004$ and $p=0.000$, respectively) in diabetes mellitus subjects (25.29±5.56 µmol/L and 200.56±60.68 ng/mL, respectively) compared to the control (21.90±5.88 µmol/L and 83.53±43.27 ng/mL, respectively).There was no significant difference in the mean values of calcium and phosphate ($p=0.429$ and $p=0.530$, respectively) in diabetes mellitus subjects (2.42±0.11 mmol/L and 1.20±0.14 mmol/L, respectively) compared to the control (2.43±0.11 mmol/L and 1.22±0.14 mmol/L, respectively). However, there was no significant difference in the mean values of calcium, phosphate, iron and ferritin ($p=0.170$, $p=0.697$, $p=0.899$ and $p=0.979$ respectively) between male (2.39±0.10 mmol/L, 1.21±0.15 mmol/L, 25.39±5.64 µmol/L, 200.79±59.42 ng/mL respectively) and female (2.45±0.11 mmol/L, 1.19±0.14 mmol/L, 25.19±5.60 µmol/L, 200.33±63.15 ng/mL respectively) diabetes mellitus subjects. In conclusion, the mean serum calcium and phosphate phosphate were not altered between the diabetic subjects and controls. The mean serum of Ferritin and iron were significantly higher than the control while the serum levels of the enzymatic antioxidants were significantly lower than the controls.

***KEYWORDS**: Calcium, Phosphate, Iron and Ferritin, in type 2 diabetes mellitus.*

INTRODUCTION
Diabetes mellitus is a metabolic disorder caused by either a lack of insulin secretion, impaired insulin action, or both. Notably, insulin plays an important role as an anabolic hormone, affecting the metabolism of carbohydrates, lipids, and proteins.[1-4] The American Diabetes Association (ADA) categorized diabetes into four main types based on different pathogenesis: type 1 diabetes, type 2 diabetes, specific types of diabetes caused by other reasons (e.g., monogenic diabetes syndromes, diseases of the exocrine pancreas, and drug- or chemically induced diabetes), and gestational diabetes, with type 2 diabetes accounting for 90–95% of all cases.[5] Type 2 diabetes mellitus (T2DM) is a heterogeneous metabolicdisorder characterized by chronic hyperglycemia, resulting ina deficiency in insulin secretion, insulin action, or both.[6-8] β-cell dysfunction and insulin resistance are potential mechanisms in the development of type 2 diabetes mellitus. Insulin or receptor defectsin target tissues lead to chronic hyperglycemia, increasedoxidative stress, and proinflammatory cytokine production,impairing insulin signaling pathways, lipid metabolism, protein synthesis and cell differentiation. In addition, chronic hyperglycemia leads to dehydration, urinary excretion of glucose, and damage to the endothelium and many tissues.[9-11] Dietaryhabits, a sedentary lifestyle, and environmental and geneticfactors play an important role

in the development of type 2 diabetes mellitus. A balanced diet with adequate nutrient content can reduce HbA1c by 0.3–2 % in people with type 2 diabetes mellitus.[12-15]

Recent studies have confirmed the role ofminerals in the synthesis, secretion, and action of insulin.[16] Zinc, potassium (K), calcium (Ca), and magnesium (Mg) minerals are reported to be necessary for the regulation of glucose metabolism.[17] Several extensive cohort studies have associated higher serum Ca levels with an increased risk of type 2 diabetes mellitus. It is known that disturbances in calcium (Ca) levels can affect the secretion of insulin from glucose-dependent insulin secretion. Insulin secretion is regulated by Ca which depends upon its concentrations in pancreatic β cells.[18] It was known that the function of pancreatic β cells is associated with the serum Ca levels. Alterations in the Ca influx can result in the abnormal β-cell function and consequently can increase the risk of type 2 diabetes mellitus.[19] Also, the disturbed Ca levels can reduce the expression of GLUT 4 transporters; hence the uptake of glucose will be reduced causing high plasma glucose levels.[20]

MATERIALS AND METHODS

Study Area

The study was carried out at Federal Teaching Hospital in Imo State Owerri, Nigeria.

Advocacy, mobilisation and Pre-Survey Contacts

A letter of introduction from the Head of Department of Medical Laboratory Science, Imo State University, Owerri was collected and submitted to the gate keeper Federal Teaching Hospital (CMD) and thereafter to the ethics Committee. On request a research proposal was submitted to the ethics Committee. The ethical approval letter was obtained. After several meetings with the nurses clinic days were chosen for the collection of samples. Demographic data, informed consents and questionnaires were obtained from qualified study participants to obtain their medical and demographic characteristics. Thereafter the samples were collected from the qualified participants.

Study Population/Sample Size

The study population are subjects that were confirmed with diabetes mellitus through fasting blood sugar test with values above 126 mg/dL (7.0 mmol/L) and apparently healthy individuals as control. A total of fifty diabetic patients were recruited for the study. They were age matched with fifty apparently healthy individuals who served as control.

Selection Criteria

Inclusion

The inclusion criteria are as follows:
(i) Subjects between the age range of 30-65 years.
(ii) Subjects who gave consent to participate in the study.
(iii) Diabetes mellitus type 2 subjects who have been diagnosed according to WHO criteria.

Exclusion

i) Subjects who did not give consent to participate in the study.
ii) Subjects with major medical illnesses (e.g. cardiovascular disease, cancer, liver or renal failure).
iii) Type 2 Diabetes mellitus patients below 30 years and above 65 years.
iv) Pregnant women with diabetes.

Study Design

A cross-sectional study was carried out among type 2 diabetes mellitus patients.

Sample Collection

Blood samples were collected through venipuncture, to evaluate the calcium phosphate, iron, ferritin and enzymatic antioxidants using asceptic measures, using 5ml sterile disposable syringe and niddle from all the subjects and were then dispensed into a plain dry container. The samples were centrifuged at 3,000rmp for 5 minutes after clotting to separate and obtain the serum. The sera were obtained using a pasteur pipette and put into appropriate container, and stored at -20^{0}C prior to use.

Laboratory Procedures

All reagents were commercially purchased and the manufacturer's standard operating procedure (SOP) was strictly followed.

Determination of Serum glucose

The result was determined using Glucose oxidase (GOD)/peroxidase (POD) method[21] as modified by Atlas Medical reagent, Germany (2022). Catalogue number: 15827.

Procedure

Working reagent was prepared. Tubes were labelled Blank, Standard, Controls, Tests then 1.0 ml of working reagent was transferred into each tube after which 0.01 ml (10 µl) of sample was added to the respective tubes and mixed and incubated at 37°C for 5 min. the absorbance of the standard and test was then measured at 505nm using a spectrophotometer and recorded.

Determination of Serum Calcium

The test was determined by spectrophotometeric method[22] as modified by Teco Diagnostic reagent USA (2022l. Catalogue number: 92807.

Procedure

Working reagent was prepared. Tubes were labelled Blank, Standard, Controls, Tests then 1.0 ml of working reagent was transferred into each tube after which 0.02 ml (20 µl) of sample was added to the respective tubes and mixed. The tubes was let to stand for at least sixty seconds (60) at room temperature. The

spectrophotometer was zeroed with blank at 570 nm. (Wavelength range: 550 - 600 nm) Absorbances of all tubes was read and recorded.

Determination of Serum inorganic phosphate

The test was determined by spectrophotometeric method[23] as modified by Teco Diagnostic reagent USA,(2022). Catalogue number: 92807.

Procedure

Test tubes were labelled Blank, Standard, Control, Tests. 1.0 ml of reagent was pipetted into each tube, and allowed to come to room temperature (25°C). 0.02 ml (20ul) sample was added to the respective ttubes, mixed and allowed to stand for five (5) minutes at room temperature. Spectrophotometer was zeroed with distilled water at 340 nm. Absorbences of all tubes was read and recorded.

Determination of serum ferritin

Test was determined by Immunoenzymometric sequential assay(TYPE 4) method[24] as modified by Monobind Diagnostic reagent USA(2022). Catalogue number: 92630.

Procedure

Before proceeding with the assay, all reagents, serum references and controls was brought to to room temperature (20 - 27° C). The microplates' wells were formated for each serum reference, control and patient specimen to be assayed in duplicate, any unused microwell was removed before it was striped back into the aluminum bag, sealed and stored at 2-8°C. 0.025 ml (25µl) of the appropriate serum reference, control or specimen was added into the assigned well. 0.100 ml (100µl) of the Ferritin Biotin Reagent was added to each well. It is very important to dispense all reagents close to the bottom of the coated well. The microplate was swirled gently for 20-30 seconds then mixed and covered. It was incubated for 30 minutes at room temperature. The contents of the microplate were discarded by decantation or aspiration. While decanting, the plate was tapped and blotted dry with absorbent paper. 350µl of wash buffer (see Reagent Preparation Section) was decanted (tapped and blotted). It was repeated two (2) additional times for a total of three (3) washes. A manual plate washer was used. 0.100 ml (100µl) of the Ferritin Enzyme Conjugate will be added to each well. It was incubated for 30 minutes at room temperature. The contents of the microplate were discarded by decantation or aspiration. When decanting, the plate was blotted dry with absorbent paper. 300µl of wash buffer (see Reagent Preparation Section) was added. It was repeated two (2) additional times for a total of three (3) washes. 0.100 ml (100µl) of working substrate solution was added to all wells It will be incubated at room temperature for fifteen (15) minutes. 0.050ml (50µl) of stop solution was added to each well and mixed gently for 15-20 seconds. The absorbance in each well was read at 450nm (using a reference wavelength of 620-630nm to minimize well imperfections) in a microplate reader. The results were read within thirty (30) minutes of adding the stop solution.

Estimation of Iron

The test was determined using the method of Rotruck *et al*. (1973). as modified by Teco Diagnostic reagent USA, (2022). catalogue number: 92807.

Procedure

Test tubes were labelled Blank, Standard, Control, sample then 2.5 ml Iron Buffer reagent was added, after which 0.5ml of the sample was added to each tube respectively and mixed. The spectrophotometer was zeroed at 560nm with the reagent blank. A1 Absorbances were read and recorded. O.5ml of iron colour reagent was then added to all the test tubes, they were mixed and placed in the water bath at 37^0C for 10minutes. The spectrophotometer was zeroed at 560nm with the reagent blank. A2 Absorbances were read and recorded.

Statistical Analysis

Data obtained in this study was analysed using Statistical Package for Social Science (SPSS) version 23.0. The student independent T-test was used to determine the mean differences. Values were expressed as Mean ± standard deviation. Test with probability of $p<0.05$ was statistically significant. Results were presented in tables.

RESULTS

Table 1: Mean±SD Values of Serum Calcium, Phosphate, Iron, and Ferritin in type 2 Diabetes Mellitus Subjects of the Study Population.

Parameter	Diabetics (n=50)	Control (n=50)	t-value	p-value (0.05)
Calcium (mmol/L)	2.42 ± 0.11	2.43 ± 0.11	-0.79	0.429
Phosphate (mmol/L)	1.20 ± 0.14	1.22 ± 0.14	-0.63	0.530
Iron (µmol/L)	25.29 ± 5.56	21.90 ± 5.88	2.95	0.004
Ferritin (ng/mL)	200.56 ± 60.68	83.53 ± 43.27	11.10	0.000

KEY

n: population size

***: Statistically significant (P<0.05)**

Table 1 indicates the serum levels of calcium, phosphate, iron, and ferritin in type 2 diabetes mellitus subjects of the study population. There was a significantly higher level of iron and ferritin (p=0.004 and p=0.000, respectively) in diabetes mellitus subjects (25.29±5.56 µmol/L and 200.56±60.68 ng/mL, respectively) compared to the control (21.90±5.88 µmol/L and

83.53±43.27 ng/mL, respectively). There was no significant difference in the mean values of calcium and phosphate (p=0.429 and p=0.530, respectively) in diabetes mellitus subjects (2.42±0.11 mmol/L and 1.20±0.14 mmol/L, respectively) compared to the control (2.43±0.11 mmol/L and 1.22±0.14 mmol/L, respectively).

DISCUSSION

The results from table 1 show that there was no significant difference in the mean level of calcium and phosphate between case and control participants. This is in agreement with the results of the previous study, which statistically (P value≥0.05) found that there was no significant difference in the mean serum calcium level between patients and non-diabetic subjects.[25] Phosphate plays an essential role in the body. It is involved in several metabolic processes with Ca stored in the bone.[26] In the present study, the level of serum phosphate was not altered between diabetic and non-diabetics individuals, thus is in agreement with the study conducted in 2021, by Maryam Barghi and colleagues at Kermanshah University of Medical Sciences' Imam Reza Hospital in Kermanshah, Iran. 40 type 2 diabetes mellitus patients and 40 healthy people (Controls) between the ages of 20 and 60 were included. The serum levels of ca and PO4 in the diabetic and non-diabetic groups did not differ noticeably.[27] In 2014, Revathi and Amaldas reported a study about clinical study of serum phosphate and magnesium in type II diabetes mellitus, the analyzed data showed that the serum phosphate level was significantly lower in diabetic patients than the level in control subjects. It is in disagreement with the present cross-sectional study.[28]

Furthermore, the result on table 1 also shows that the serum ferritin of the diabetic subjects was significantly higher than the control. This is consistent with the study conducted by Moirangthem and Vidyabati.[29] The relationship between elevated serum ferritin levels and type 2 diabetes involves an elevation in oxidative stress through the increased formation of free radicals catalysed by iron, which may lead to insulin resistance and hyperglycaemia.[29]

This study also showed that serum iron was significantly increased in diabetic subjects than the control which also consistent with the study done by Renuka.[30] This increase in iron levels may be explained in two different ways. Firstly, iron stores in the pancreas may lead to defective synthesis and secretion of insulin.[31] Secondly, excess iron deposition culminates in hyperinsulinemia due to obstruction in the insulin withdrawing ability of the liver.[31] Such deposits hinder insulin action, resulting in insulin resistance, which suppresses the yield of glucose in the liver.[32]

CONCLUSION

In conclusion, the mean serum calcium and phosphate phosphate were not altered between the diabetic subjects and controls. The mean serum of Ferritin and iron were significantly higher than the control. This findings reveal that measurement of ferritin and iron might be beneficial in type 2 diabetes mellitus management and prevention of prognostic complications.

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