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SERUM CALCIUM AND PHOSPHORUS LEVELS AND THEIR ROLE IN INFLAMMATION IN TYPE II DIABETES MELLITUS (T2DM)

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ABSTRACT: The purpose of the present study is to focus on comparative analysis between antidiabetic drugs and minerals like calcium and phosphorus to understand their role in inflammation in type 2 diabetes mellitus. The sample size is 500 blood samples of both genders which was divided into 3 groups: A, B, and C. Group A on metformin of daily dosage 500mg consisted of 200 subjects, Group B on other anti-diabetic drugs (Glimepiride) of daily dosage 2 mg consisted of 200 subjects. Both groups A and B had no other complications from diabetes. Group C included controls, consisting of 100 healthy individuals with no history of diabetes. When compared between two drug (metformin and other anti-diabetic drug) groups, the percentages of Serum calcium, phosphorus in both male and female groups were significantly decreased and consequently, inflammatory markers like leptin, IL-6, hsCRP, TNF-α were significantly increased in metformin group rather than who were on another anti-diabetic drug group. Decreased serum calcium and phosphorus levels in type 2 diabetes mellitus who are on metformin drug can be a mediator in inflammatory process which leads to micro and macrovascular complications when compared to another anti-diabetic drug group.

INTRODUCTION: India and China are the epicenters in Asia displaying a significantly rising prevalence of worldwide Type 2 Diabetes Mellitus epidemics [1, 2]. Diabetes is a multifactorial disease, and metformin is the primary line of drug suggested. Metformin is an extensively used drug that has many effects associated with glucose metabolism and diabetes-associated complications through enhancing peripheral insulin sensitivity [3, 4]. Physiologically, metformin reduces hepatic glucose production, but no longer all of its results may be defined through this mechanism, as there's growing proof of its crucial function with inside the gut.

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At the molecular level, the findings range depending at the dosage of metformin and the duration of treatment. Metformin has been proven to act through each AMP-activated protein kinase (AMPK)-structured and AMPK-independent mechanisms, through inhibition of mitochondrial respiration, however additionally possibly through inhibition of mitochondrial glycerophosphate dehydrogenase, and a mechanism related to the lysosome.

Minerals exert important implications for the risk of diabetes mellitus, as well as its progression and complications. The best recommendation should be to consume adequate amounts of foods that contain minerals in sufficient quantity to ensure an adequate nutritional status [5]. Minerals play an important role in glucose homeostasis, so understanding the effects of mineral deficiencies and the potential benefit of supplementation for the prevention and/or treatment of type 2 diabetes

mellitus (DM) is relevant. Micronutrient deficiency in diabetics has been linked to oxidative stress and resulting hyperhomocysteinemia. Calcium has also been studied to play an important role in insulin secretion, a calcium-dependent process [6].

T2DM is considered an oxidative stress disease, the burden of which is amplified by micronutrient deficiencies. Many studies point to the role of micronutrients in delaying the harmful effects of diabetes. However, studies on the relationship between drugs used to treat T2DM and their effects on micronutrients are lacking.

METHODOLOGY: This study was conducted through collaboration among the physiology department of a teaching medical institute, the medicine department, and the Genetics Lab. Ethical approval for this study was received (Ref: 007/02/2019/IEC/SMHC), and the study was conducted from March 2019 to February 2021. Based on WHO and ADA guidelines for the screening of type 2 diabetes mellitus, the plasma glucose criteria, the Fasting Plasma Glucose (FPG) value or the 2-h Plasma Glucose (2-h PG) value during a 75-g Oral Glucose Tolerance Test (OGTT), or A1C criteria. The present study recruited 500 participants between 30 and 65 years of age attending Medicine OPD and samples are collected randomly and prospectively. The patient pool consisted of individuals who had been diagnosed with type 2 diabetes for more than 12 to 18 months. The study design was divided into 3 groups: A, B, and C. Group A consisted of 200 subjects with type 2 diabetes only on metformin with a daily dosage of 500 mg/day, and Group B on other anti-diabetic drugs (Glimepiride) of daily dosage 2 mg consisted of 200 subjects. Group C included controls, consisting of 100 healthy individuals with no history of diabetes. The study included type 2 diabetics for more than a year as cases and age-matched non-diabetic healthy volunteers as controls. People who are on calcium supplements, proton pump inhibitors were excluded. No complications from liver diseases, renal diseases, gastrointestinal disorders, thyroid diseases, or parathyroid disorders, alcoholics, and smokers were excluded. A detailed clinical history, drug history, dosage of the drug, and Mineral supplementation if any were documented in a structured proforma. The blood samples were collected from the subjects *via* vein puncture for fasting plasma glucose, glycated haemoglobin (HbA1c), were determined and quantified. Whole blood samples were collected into EDTA-coated vacutainers for quantification of glycated haemoglobin (HbA1c) in the blood. Fasting plasma glucose and postprandial blood glucose were estimated using the glucose oxidase-peroxidase method [19, 20]. Serum calcium and phosphorus levels were measured using a Chemiluminescent Microparticle Immunoassay, which is a modified and advanced form of the Enzyme Linked Immuno Sorbent Assay (ELISA) technique. We ensured that the study complies with international ethical norms according to the Helsinki Declaration (World Medical Association *et al.* 1964).

Estimation of Inflammatory Markers:

Interleukin 6 (IL6): Interleukin-6 (IL-6) assay specifies that all samples, standards, and controls were run in duplicate. It also states that intra-assay and inter-assay Coefficients of Variation (CV) were kept below 10% and 12% respectively. Any sample pair with a CV over 10% was re-assayed.

Leptin: Serum Leptin levels were determined using a [Insert Assay Type, e.g., ELISA] with results expressed in ng/mL. Analytical precision was confirmed with an intra-assay coefficient of variation (CV) of less than 10%.

Highly Sensitive C-reactive Protein Assay: hs-CRP were quantified using commercial ELISA kits (Calbiotech, USA; Catalog Nos. LE312C and CR120C, respectively). All assays were performed in duplicate to ensure reproducibility. Measured in mg/L. This ultra-sensitive assay is designed to detect low-grade inflammation, with a functional sensitivity of 0.1 mg/L.

Tumor Necrosis Factor α (TNF α): TNF α was measured in subjects' serum using a commercial kit (Diaclone SAS), and the protocol was the same as described in the section (IL-6). TNF-α ELISA kit with catalog number 950.090.096. All data were statistically analysed and expressed as mean standard deviation. The mean was analysed by one way ANOVA (with a student T-test for comparison with controls).

Sample size: The sample size was estimated for a 10% difference between the means of the

dependent variables of the control and experimental groups, with 40 % standard deviation, 90 % power and 5% significance level. The estimated sample size was 406 for three groups. Adding 20 % as dropout, the sample size was rounded to 500. For the two drug treatment groups (metformin and other antidiabetic drug) 200 participants each, and for the normal control 100 participants were taken. Sigma Plot 14.5 version (Sy stat software, USA) was used for the sample size calculation).

RESULTS AND DISCUSSION:

Biochemical Assays:

Distribution (%) of Biochemical Variables by Groups:

TABLE 1: A COMPARISON OF FBS, PLBS, HBA1C, CALCIUM, PHOSPHORUS, INFLAMMATORY MARKERS (LEPTIN, CRP), CYTOKINES (TNF, IL6) LEVELS AMONG METFORMIN GROUP, OTHER ANTIDIABETIC DRUG GROUP AND CONTROL GROUP

Variables	Cut Off Values	N	Groups			Total	X^2	P value
			Metformin	Metformin and Other Drug	Control			
FPG	<100	211	35.5	28.6	83.0	42.3	146.9	0.000
	100-126	136	42.0	17.6	17.0	27.3		
	≥126	152	22.5	53.8	0.0	30.5		
PPBS	<180	242	52.5	23.1	91.0	48.5	124.935	0.000
	≥180	257	47.5	76.9	9.0	51.5		
HBA1C	<5.7	61	18.0	0.0	25.0	12.2	219.248	0.000
	5.7-6.5	72	10.5	0.0	51.0	14.4		
	≥6.5	366	71.5	100.0	24.0	73.3		
Calcium	9.3 -9.9	52	9.0	2.5	29.0	10.4	50.743	0.000
	<9.3 ≥10	447	91.0	97.5	71.0	89.6		
Phosphorus	2.5-4.5	321	68.5	72.9	39.0	64.3	35.793	0.000
	<2.5 ≥4.6	178	31.5	27.1	61.0	35.7		
	<1.7 ≥2.3	394	97.5	100.0	0.0	79.0		
Leptin	3.6 -11.0	204	30.0	22.1	100.0	40.9	183.419	0.000
	<3.6 ≥11.1	295	70.0	77.9	0.0	59.1		
hsCRP	> 0.2	107	3.5	0.0	100.0	21.4	458.899	0.000
	<0.2	392	96.5	100.0	0.0	78.6		
IL-6	<5.15	97	3.5	2.5	85.0	19.4	343.323	0.000
	≥5.15	402	96.5	97.5	15.0	80.6		
TNF-α	<177	391	96.0	100.0	0.0	78.4	453.714	0.000
	≥177	108	4.0	0.0	100.0	21.6		

Table 1 represents the levels of different biomarkers in subjects on metformin alone and with metformin and other antidiabetic drugs. There is a significant increase in blood glucose levels and inflammatory biomarkers (leptin, hsCRP, TNF-, and IL6), but there is also a decline in calcium and phosphorus both metformin alone and metformin combined with other diabetic drugs when compared to control subjects.

TABLE 2: A COMPARISON OF FBS, PLBS, HBA1C, CALCIUM, PHOSPHORUS, INFLAMMATORY MARKERS (LEPTIN, CRP), CYTOKINES (TNF, IL6), AND ITS CORRELATION WITH AGE

Variables	Groups			F Value	P Value
	Metformin (199)	Metformin and Other Drug (200)	Control (100)		
Age	49.3±10.5	46.4±10.70	48.5±9.97	3.911	0.021
FBS	124.3±61.65	127.0±34.80	84.0±11.635	34.297	0.000
PLBS	162.9±78.09	173.4±43.86	105.8±20.02	48.826	0.000
HBA1C	22.0±83.72	159.0±45.36	6.0±0.52	335.577	0.000
Calcium	8.2±1.36	7.6 ± 1.75	9.0 ±1.60	27.656	0.000
Phosphorus	4.1±1.45	3.4 ± 1.75	4.9 ±1.27	32.756	0.000
Leptin	152.7±729.22	14.1± 3.35	6.0±1.90	5.620	0.004
hsCRP	14.4±2.85	15.2±2.23	5.2±1.45	649.558	0.000
IL-6	29.1±23.54	24.7±13.40	6.7±9.30	55.620	0.000
TNF-α	365.5±133.52	566.8±2571.34	154.6±16.64	2.228	0.109

Observations made from **Table 2** clearly indicate the long-term side effects of metformin alone and with metformin-other antidiabetic drugs in subjects. There is a significant rise in the blood glucose levels and inflammatory biomarkers (leptin, hsCRP, TNF-a and IL6), but there is decrease calcium and phosphorus levels among other diabetic drugs in comparison to metformin group and control subjects.

TABLE 3: THE STANDARD REFERENCE RANGE

I	FBS	< 100 mg/dl
II	PPBS	< 180 mg/dl
III	HbA1c	< 5.7% normal
	Prediabetic	-5.7- 6.4%
	Diabetic	-6.5%
IV	Calcium	- 9.3 - 9.9 mg/dl
V	Phosphorous	- 2.5 - 4.5 mg/dl
VI	TNF-alpha	- 177 pg/ml
VII	hsCRP	- 0.2 to 10mg/ml
VIII	IL-6	- 5.15 pg/ml
IX	Leptin	- 7.3 ± 3.7ng/ml

DISCUSSION: An escalating proportion of research on animals and clinical trials suggests that the key function of metformin is to decrease hepatic glucose production, mainly by suppressing gluconeogenesis [7, 8]. The inhibitory effects on hepatic gluconeogenesis can be due to changes in enzyme activities or suppressed hepatic uptake of gluconeogenic substrates supported by research evidence. Cellular uptake of metformin is facilitated by the chief expression of OCT1 (organic cation transporter 1) in the hepatocytes. A rational accumulation of metformin in the liver could be higher compared to other tissues, leading to high micromolar concentrations in the per portal area. Research over a certain time period indicates metformin's action is targeted around the intestines, by reducing the net glucose uptake and enhancing anaerobic glycolysis in enterocytes, causing an increased release of lactic acid in the liver [9, 10, 11].

The molecular level findings vary depending on the dosage of metformin and the duration of treatment, with some differences between acute and chronic administration. Metformin has been shown to act *via* both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms; by inhibition of mitochondrial respiration and also perhaps by inhibition of mitochondrial glycerol phosphate dehydrogenase. In our study, we included screening for calcium levels in all the groups as it has a major role in the signalling cascade where it is shown to have 38% decreased levels of calcium in the metformin group, 58% in the associated antidiabetic drug group, and it was normal in the control group. In our present study, we could find a widespread association between phosphorus levels and different groups. A study done by Ellen *et al.* suggested that there was no effect on phosphorus levels in the subjects who were on metformin. Anwar et al. subjects with T2DM showed higher levels of fasting blood sugars, postprandial blood sugars, and glycated haemoglobin levels, which are correlated with a reduction in serum levels of calcium and phosphorus, showed a significant correlation with glycaemic control when compared to controls [12, 13].

In our study, we included screening for both serum levels of calcium and phosphorus in all the groups and we tried to associate the serum levels of calcium and phosphorus in the metformin group, other antidiabetic drug groups, and control group. We have also witnessed that there is an association between pro inflammatory markers with the levels of FBS, HbA1C, and micronutrients in subjects with metformin alone and also with other diabetic drugs [14, 15]. There is upregulation of pro inflammatory markers in the metformin group when compared with the control group. Further research is required to find the association between anti diabetic drugs and levels of pro inflammatory markers in T2DM [16, 17].

CONCLUSION: Finally, the study has led to an important proof against the antidiabetic drugs commonly advised to Type 2 Diabetes Mellitus patients for controlling their blood glucose levels, but instead they are facing the consequence of being micronutrient deficient and its associated manifestations. Conservatively potential confounding factors like glycaemic control, diet, renal handling, and concomitant medications must be acknowledged. As diabetes itself is a chronic inflammatory disease and diabetics on metformin have decreased serum micronutrients as a major side effect. Which itself is a potent stimulator for inflammation which leads to microvascular and macrovascular complications like retinopathy, nephropathy, microvascular neuropathy, ischemic heart disease, peripheral vascular disease, and cerebrovascular disease due to decreased

scavenging of reactive oxygen species, by increasing homocysteine induced oxidative stress when compared between both the drug groups, serum levels of calcium and phosphorus has significantly decreased in metformin group than sulfonylurea group. Future large and well-designed studies on screening for serum levels of calcium and phosphorus deficiency, and optimal supplementation dose among type 2 diabetic patients are warranted to help guide formulation of guidelines in diabetes clinical care.

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