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Analysis of Pyridoxine in the Male Diabetic Population of Lahore and Sheikhupura

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ABSTRACT

Pyridoxine is an important vitamin involved in metabolism, and its deficiency can lead to serious diseases among which diabetes is a common one. In the present research work, the aim is to compare pyridoxine level between diabetic patients of Punjab University Lahore premises and D.H.Q hospital Sheikhupura. 100 samples of diabetic patients and 50 samples of normal healthy controls are collected from PU Lahore premises and D.H.Q Sheikhupura. To assess the pyridoxine level, serum level of samples is estimated and analysed initially using different standard referred assays, protocols and then High Performance Liquid Chromatographic (HPLC) assay of all the samples is performed. Data obtained by performing all assays confirmed the low level of Pyridoxine among the diabetic community as compared to normal population. SDS Page analysis acted as the direct indicator of these results. The results may suggest Pyridoxine supplementation as an effective method for early diagnosis of risk for this disease.

Keywords: Diabetes Mellitus, Pyridoxine, Diabetic Female Patients, High Performance Liquid Chromatography (HPLC), Sodium Dodecyl Sulphate (SDS).

Introduction

Pyridoxine is a water soluble and colourless crystal. It is partially soluble in alcohol i.e. ethanol. Pyridoxine act as cofactor in several life sustaining processes mainly amino acid, glucose and fatty acid metabolism, therefore living organism must synthesis or take it up as nutrienten [1,2]. Around about 160 enzymatic reaction involves the Pyridoxine as a cofactor, reactions generally include: synthesis of neurotransmitters, the degradation of glycogen and synthesis, interconversion, and degradation of nucleic acids, lipids, amino acids and porphyrins [3,4]. In the 1-carbon metabolic pathway Pyridoxine carries out the DNA synthesis and methylation by transferring 1-carbon groups. In animals, impaired 1-carbon metabolism is linked with vitamin B6 deficiency [4,5]. Chemical formula of Pyridoxineis C8H11NO3. Naturally reciprocative forms of pyridoxine-pyridoxal 5-phosphate, pyridoxamine, pyridoxine and pyridoxal- are defined under the term Pyridoxine [6]. Pyridoxine consists of three compounds which are inter-related. These compounds are Pyridoxal, pyridoxine and pyridoxamine and their related phosphorylated forms respectively. Pyridoxal-5'-Phosphate is the active form of this vitamin, denoted as PLP. (PLP) is a biologically active phosphate ester derivative, that ensures the long term storage of Pyridoxine in body [1,7,8].

It is an aminotransferase in nature and helps in glucose metabolism by performing action of a coenzyme, utilizing glycogen in liver. About 100 chemical reactions are controlled by Pyridoxal 5- Phosphate abundantly present in plasma and used as status marker for vitamin B6 concentration in body [3-5,9-11]. Pyridoxal is able to translocate through plasma membrane and it act as major catabolite of Pyridoxic acid synthesis [10,11]. As an enzyme cofactor, Pyridoxine helps in the catalysis of reactions involving aldol cleavage, α-carboxylation and replacement reactions [12,13]. PLP dependent enzymes help in breakdown as well as the synthesis of amino acids attached to active sites of such enzymes [14]. Homocysteine to cysteine conversion is also an important role of this enzyme [15-17].

Fish, poultry, avocados, bananas, fortified cereals and starchy vegetables are the major natural food sources which provide Pyridoxine [3,4]. Meat, fish, nuts, banana, potatoes and egg yolk are rich sources of Pyridoxine.

Sideroblastic anemia or microcytic, dermatitis and neurologic symptoms are the adverse effects of deficiency of Pyridoxine. Pyridoxine deficiency is also linked with oxidative stress and inflammation [1,8,18,19] which can further lead to anemia, abnormal bone metabolism and CAD [1,19-23]. Above mentioned severities mostly prevail in patients going through haemodialysis at the last-stage of chronic renal disease [1,24-29]. Deaths of patients having haemodialysis are the mainly the due to abnormal bone and mineral metabolism and anemia [1,30,31]. 24%-56% of the patient getting hemodialysis are deficient in Pyridoxine but nutritional supplementation can overcome this deficiency proved by dietary intervention studies performed on hemodialysis patients undergoing treatment to anemia [32,33].

Pentosidine formation can also be stalled by Pyridoxine deficiency [1,34]. Deficiency of this vitamin can also cause nervous disorders, dermatitis, abnormal metabolism in bones and anemic conditions. Daily intake of 10-50 mg of pyridoxine hydrochloride can overcome Pyridoxine deficiency. However clinical efficacy regarding the recommendation is not yet proved [1,35-37]. Supplement of Pyridoxine can help treat diseases like asthma, diabetes, kidney stones and memory loss etc. [38]. It has been observed that PLP concentration in diabetic patients is less as compared to non-diabetics which is due to low level of Pyridoxine. The level of PLP in plasma plays an important role in Tryptophan Metabolism which is disturbed due to its low level. The abnormal `metabolism results in the production of Xanthuric acid which links with insulin forming XA-insulin complex. This leads to various complications which include cataracts, neuropathy, vascular damage and retinopathy.

Pyridoxine deficiency results in impaired gluconeogenesis and less glucose intolerance as well. Most of the carbohydrate metabolism aberrations are observed due to its deficiency. Its direct relation with type 2 DM has not been observed but once diabetes is present its deficiency result in the development of once present complications. Experiments have shown a decrease in retinal edema in diabetic patients when supplemented with Pyridoxine (Table 1).

Methods

1. Selection of patients:

Samples were collected from the Punjab University Health Care Centre and Sheikhupura Hospital.100 confirmed diabetic male patients were selected. Ample information of the patient's past and present history, socio-economical history was also obtained from both places.

All races/ethical groups should be included in the inclusion criteria. Population should be male. Patient should have the symptoms of diabetes. Target blood pressure should be ≤ 130/80

2. Estimation of physical parameters:

Age of all the 100 subjects that consists of 50 controls subjects and 50 diabetic patients were calculated from the history of the subjects and was calculated in years.

Weight of all the 100 subjects that consists of 50 diabetic patients and 50 control subjects were measured by weighing machine and then recorded in kilograms (Table 2).

Height of all subjects was measured and recorded in inches.

Body mass record (BMI) is a standout amongst the most exact approaches to gauge obesity in normal life. BMI is computed by dividing the weight of body with the square of the height of the person(kg/m2). As per the World Health Organization, the ordinary scope of BMI is in the vicinity of 18.5 and 24.9, and people with a BMI of 25–30 are thought to be overweight, while those with a BMI more than 30 are termed obese [39]. BMI is considerably correlated with total body fat content. It is usually used to categorize the underweight, overweight and obese persons.

$$\text{BMI} = \frac{\boldsymbol{weight\ in\ Kg}}{(\boldsymbol{height\ in\ metres})\boldsymbol{2}}$$

3. Estimation of biochemical parameters:

3.1. Protein estimation: The quantification of protein content is very important and accurate protein content estimation is a critical step in protein analysis. Bradford assay is used for the protein estimation. Coomassie brilliant blue G-250 (sigma Aldrich catalog number: 27815)2. Bovine serum albumin (BSA)3. Phosphoric acid4. Bradford reagent5. Methanol (Figure 1).

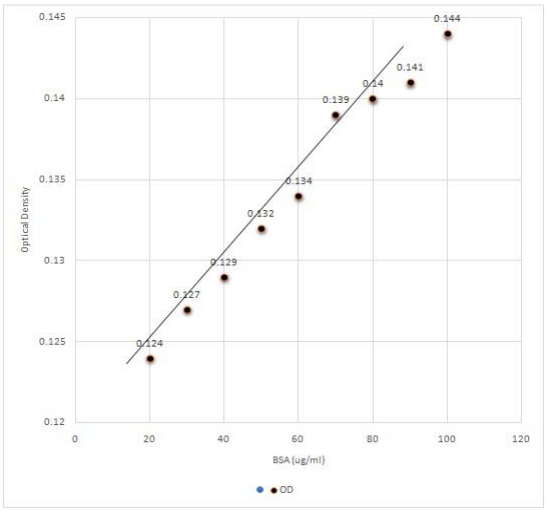

Figure 1: Graph shows the standard protein estimation by biuret method.

3.2. Procedure: Standard assay procedure (for sample with 5-100 µg ml-1 protein)

Prepare five to eight dilutions of a protein (usually BSA) standard with a range of 5 to 100 µg protein. Dilute unknown protein samples to obtain 5-100 µg protein/30 µl. Add 30 µl each of standard solution or unknown protein sample to an appropriately labelled test tube. Set two blank tubes. For the standard curve, add 30 µl H_2O instead of the standard solution. For the unknown protein samples, add 30 µl protein preparation buffer instead. Protein solutions are normally assayed in duplicate or triplicate. Add 1.5 ml of Bradford reagent to each tube and mix well. Incubate at Room Temperature (RT) for at least 5 min. Absorbance will increase over time; samples should incubate at RT for no more than 1h. Measure absorbance at 595 nm.

3.3. Biuret assay: 9gm Sodium Potassium Tartrate (f.w. 282.22), 3 gm Copper Sulfate x 5 H2O (f.w. 249.68), 5 gm Potassium Iodide (166.0). All dissolved in order in 400 ml 0.2 M NaOH (f.w. 40.0) before bringing to final volume. Volume of the sample and reagent can be scaled up/down and/or volume ratios can be varied, as per requirement in any assay. Warm up the spectrophotometer 15 minutes before use. Prepare standards from bovine serum albumin, preferably calibrated using absorbance at 280 nm and the extinction coefficient. Using 5 ml color reagent to 1 ml sample a recommended range is 0.5 to 20 mg protein. Prepare a reference tube with 1 ml buffer. If possible, dilute unknowns to an estimated 1 to 10 mg/ml with buffer; a range of dilutions should be used if the actual concentration cannot be estimated. Use 1 ml sample per assay tube. Add 9 ml Biuret reagent to each tube, vortex immediately, and let stand 20 min. Read at 550 nm.

3.4. Determination of glucose: Random and/or fasting blood glucose was measured using digital glucometer. Subject finger was sterilized using spirit, skin puncture was made by a sterile lancet, and blood drop was taken on the glucometer strip and the reading was noted. Standard curve was plotted using O.D of standards against the sample O.D and protein in samples (µg) was determined using the formulae (volume/sample, and dilution factor, if any).

3.5. Determination of HbA1c: The kit is sandwich enzyme immunoassay for invitro quantitative measurement of HbA1c in human plasma. Prepare all

reagents, samples and standards. Add 100ul standard or sample to each well. Incubate 2 hours at 37C.Aspirate and add 100ul prepared Detection Reagent A. incubates 1 hour at 37C. Aspirate and wash 3 times.Add 100ul of prepared Detection Reagent B. incubate 30 minutes at 37C. Aspirated and wash 5 times. Add 90ul substrate solution. Incubate 15-25 minutes at 37C. Add 50ul stop solution. Read at 45nm immediately.

3.6. Estimation of Blood Glucose: RANDOX kit method was used to estimate the bold glucose level. The GLU method used on the dimension® clinical chemistry system in an in vitro diagnostic test intended for the quantitative determination of glucose in serum, plasma, urine and cerebrospinal fluid.

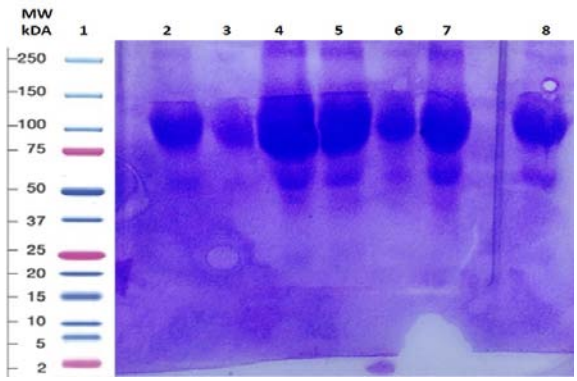

Figure 2: Pyridoxine receptor protein in control and diabetic patients shown on the gel (lane 2,3 & 6 show the serum sample of diabetics) while (lane 4, 5, 7, and 8 shows the serum sample of controls). SDS page was performed in MMG Research Lab I.
The figure demonstrates that the lanes 2,3 & 6 show the receptor protein in diabetic patients which is in very low concentration while lane 4,5,7 & 8 show serum sample of control in which the receptor protein of Pyridoxine is present in very high concentration. Protein marker is used to link the Mwt of samples by the control. Receptor Protein of Pyridoxine have Mwt of 55 kDa.

3.7. Protein Profiling, SDS-PAGE: Clean glass plates by washing them with detergent and rinse them with water. After rinsing, dry the plates. Assemble the glass plates with spacers Seal the bottom of plates with sealing tape. Prepare and pour the gel solutions. Resolving gel was poured inside the gel assembly leaving 0.5" vacant space at the top. After the resolving gel was polymerized, stacking gel was poured at the top of the gel assembly. Comb was inserted in the gel for the formation of wells for sample loading. Remove comb and fill with electrophoresis buffer and load the samples. Staining of gel: Coomassie brilliant blue staining. The gel is immersed for several hours in a concentrated methanol: acetic acid solution for destaining (Figure 2).

3.8. High performance liquid chromatography (HPLC): For the precipitation of the proteins present in the serum sample add 150ul of serum sample in 1.5ml of micro-centrifuge tube and mixed it with 150ul of cold (4°C) 5% (w/v) meta-phosphoric acid. Mixed the sample on a vortex at 2000rpm for 5min and then centrifuged at 4°C at 18000g for 15min. then take the supernatant of each sample and mixed it with 150ul of dichloromethane in another 1.5ml micro-centrifuge tube. Again, vortex the samples at 2000 rpm fi2min and centrifuged at 18000g for 15min. teh top layer from the samples was then filtered with the help of 0.22 um polyvinyl difluoride syringe filter (Billerica, Millipore, MA, USA) into an HPLC auto-sampler vial with self-centering 200ul conical vial insert.

For the preparation the standard and sample solutions, the HPLC-grade solvent and 0.45um filtered distilled aqueous deionized water were used. Pyridoxal pyrophosphate and 4 pyridoxic acid was obtained from the Sigma, single-compound stock solution was prepared of each sample by serial dilution in water. These stock solutions were stored in 1 ml aliquots at 80°C before use (Figure 3).

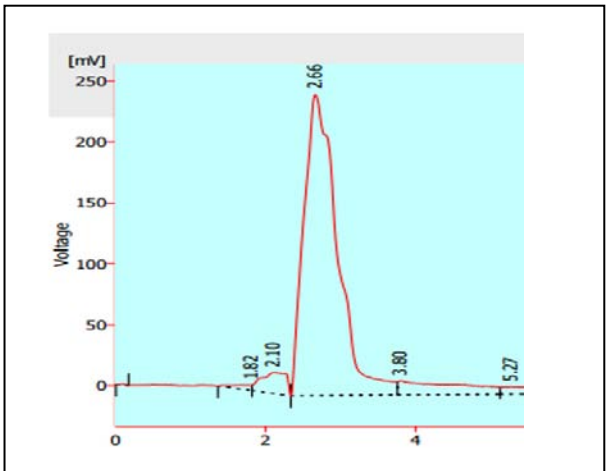

Figure 3: HPLC chromatograph representing clear peak of PYR1 with standard peak run alongside of the Pyridoxine (Sigma Aldrich ≥98%).

Statistical Analysis

The study objectives were analyzed in all participants. The variable of primary interest was the decrease in plasma PLP levels in patients with diabetes compared to non-diabetic control subjects. The study group size was determined as N=50. All other variables were considered secondary and were analyzed in a strictly exploratory manner.

All statistical analysis was done with SPSS statistical software package (version 22.0). Significance of difference between mean analysis of control and diabetic groups was determined by applying ANOVA. Significance of difference between control group, diabetic males of Lahore and diabetic males of Sheikhupura was determined using ANOVA.

ANOVA also known as Analysis of Variance is a statistical technique that assesses potential differences in a scale-level dependent variable by a nominal-level variable having 2 or more categories. Analysis of variance (ANOVA) is a method for testing the hypothesis that there is no difference between two or more population means (usually at least three).

One-Way ANOVA is applied when there is only one qualitative variable which denotes the groups and only one measurement variable (quantitative), a one-way ANOVA is carried out. It tests the equality of population means when classification is by one variable. The classification variable, or factor, usually has three or more levels (one-way ANOVA with two levels is equivalent to a t-test), where the level represents the treatment applied.

Two-Way ANOVA performs an analysis of variance for testing the equality of populations means when classification of treatments is by two variables or factors. In two-way ANOVA, the data must be balanced (all cells must have the same number of observations) and factors must be fixed.

Results

Of the 150 participants, 50 were diabetic males from Lahore, 50 were diabetic males from Sheikhupura and 50 were control healthy individuals. Among the physical parameters, age, weight and height showed results that were not statistically significant. BMI ratio, HbA1c, total protein, serum total albumin, creatinine, urea and bilirubin of the diabetics from both cities was greater as compared to healthy individuals. The results showed that value of fasting blood glucose contents in diabetic patients is increased as compared to the healthy individuals. But the value was much higher in diabetic males from Sheikhupura as compared to those from Lahore (Figure 4). The level of pyridoxine of all the three groups is less as compared to the normal people. The mean value of pyridoxine in diabetic cases of Lahore was 25.2***±1.52 nmol/L, in diabetic cases of Sheikhupura it was 20.5***±1.36 nmol/L and in controls the mean value of Pyridoxine was 50±2.02 nmol/L. The results show statistically high decrease in the level of pyridoxine in diabetics from Lahore and Sheikhupura as compared to the control group with $p<0.001$.The results of Bradford and Biuret test also represented low pyridoxine level in the diabetic males as compared to the healthy male group.

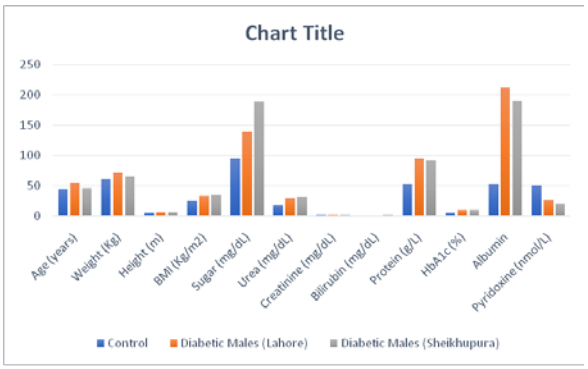

Figure 4: Graph between average of physical parameters (Age, weight, height, BMI) and biochemical parameters (Sugar, urea, creatinine, bilirubin, protein, HbA1c, albumin pyridoxine) of control and diabetic patients of Lahore and Sheikhupura.

Discussion

By comparing all physical and biochemical parameters it is concluded that in diabetic male patients of Sheikhupura Pyridoxine level is significantly very lower than in Lahore male patients. The results show statistically high decrease in the level of pyridoxine in diabetics from Lahore and Sheikhupura as compared to the control group with $p<0.001$. This occurred due the

increased production of homocysteine that disturb the level of vitamin b6. The increased level of homocysteine results in the decreased level of pyridoxine that is in turn related directly to the degree of neuropathy particularly in type 2 diabetes. That's why providing the patients supplements of pyridoxine help saving them from complications like incipient neuropathy. While, their HbA1c level and fasting blood sugar is very high as compared to Lahore diabetic patients. As well as their protein level, albumin, creatinine, bilirubin level as increased much as compared to Lahore patients. Reason can be that Sheikhupura population is not very well educated and has much knowledge about diabetes and its consequences. In contrast, Lahore population is very aware of diabetes and know how to control it and how to lower the chances of complications that are caused by diabetes. SDS page analysis also revealed the lower concentration of pyridoxine in diabetic males as compared to healthy individuals. The results suggest usage of pyridoxine supplements in early stages of diabetes to decrease the risk of disease and its complications.

In this study total 75 subjects were selected and they were nominated on the basis of diabetes. Basically in this research work the first group was control second was diabetic males from Lahore and third group included male patients from Sheikhupura. Various physical and biochemical parameters are accomplished to assess the difference between the two populations and the controls. Physical parameters include age, weight, height and BMI whereas biochemical factors include the total serum proteins, Fasting Blood Sugar FBS, serum vitamin b6 level, creatinine, urea, bilirubin etc. Since the physical parameters such as age was intended by patients past and average weight was estimated or measured by measuring balance.

BMI was calculated using the formula kg/m^2. Age, weight, height was not statistically significant, but the BMI results show that BMI is amplified in the diabetic patients as compared to the normal group. The upsurge in BMI is related with the insulin resistance. The increased BMI in diabetic patients is allied with the obesity and may be by insulin resistance.

Table 1: Supplementation of vitamin B6 [40].

Age in Years	Aim for an intake of milligrams/day	Stay Below*mg/day
Men and Women (19-50)	1.3	100
Women 51 and older	1.5	100
Men 51 and older	1.7	100
Pregnant women 19 and older	1.9	100
Breastfeeding women 19 and older	2.0	100

Table 2: Average values of different parameters of controls as compared to diabetic patients.

Parameters	Control	Diabetic Males (Lahore)	Diabetic Males (Sheikhupura)
Age (Years)	44.2±2.62	55.06*±3.4	45.9*±3.2
Weight (Kg)	61.03±2.3	71.13*±3.5	65.9*±3.4
Height (m)	5.36±1.46	5.78*±1.8	5.4*±1.5
BMI (Kg/m2)	24.06±1.80	32.62**±2.58	34.2**±2.6
Sugar (mg/dL)	94.3±9.82	138.85***±10.4	188.3***±16.85
Urea (mg/dL)	18±1.20	29.5**±2.18	31.8**±2.45
Creatinine (mg/dL)	0.789±0.028	0.99*±0.034	0.92*±0.029
Bilirubin (mg/dL)	0.485±0.008	0.53*±0.012	0.725**±0.026
Protein (g/L)	52±0.1	94.6**±1.98	90.6**±1.54
Albumin (g/L)	52±2.04	212.5***±18.4	190***±16.99
HbA1c (%)	5.262±0.39	9.8**±0.562	9.02**±0.67
Pyridoxine (nmol/L)	50±2.02	25.2***±1.52	20.5***±1.36

±sign indicates the standard error. *=P>0.05 (statistically not significant), ** =p<0.01 (statistically significant), ***=P<0.001 (statistically highly significant) comparison of control with diabetic baseline. Group 1 is control, group 2 is diabetic males of Lahore and group 3 is male diabetic population of Sheikhupura.Number of asterisks '*' show the level of significant difference.

Age of all study groups comprised of 75 subjects of whom 25 were diabetic males from Lahore, 25 diabetic males from Sheikhupura and 25 were controls was measured. The mean age of diabetic males from Lahore was 55.06±3.4 years, that of diabetic males from Sheikhupura was 45.9±3.2 years and of controls was 44.2±2.62. The difference in the mean age was not statistically significant ($p<0.05$). This result shows that diabetes is not related to the age, it can occur at any age.

Height of all the groups under study that included 75 subjects, of whom 25 were diabetic males from Lahore, 25 diabetic males from Sheikhupura and 25 were controls, was measured. The mean height of diabetic males from Lahore was 5.78**±1.8m, that of diabetic males from Sheikhupura was 5.4*±1.5m and of that of controls was 5.36±0.046. The difference in the mean height was not statistically significant ($p<0.05$). This result shows that diabetes is not related to the height.

Weight of all study groups that comprised of 75 subjects of whom 25 were diabetic males from Lahore, 25 diabetic males from Sheikhupura and 25 were controls was measured. The mean weight of diabetic males from Lahore was71.13**±3.5kg, that of diabetic males from Sheikhupura was 65.9**±3.4kg and of that of controls was 61.03±2.3kg. The difference in the mean weight was not statistically significant ($p<0.05$).

BMI was calculated using the formula kg/m2 for all three groups. The average BMI of diabetic males from Lahore was 32.62**±0.58, that of diabetic males from Sheikhupura was 34.2**±0.6 and of that of controls was 24.06±0.6. The results show an increased BMI of diabetics as compared to the controls that is directly related to obesity in them.

HbA1c was determined by the ELISA kit method. It is evaluated in %. HbA1c value of all three study groups was estimated. The mean value of HbA1c of diabetic males from Lahore was 9.8**±0.562, that of diabetic males from Sheikhupura was 9**±0.67 and of that of controls was 5.262±0.39. The results show statistically significant increase in the level of HbA1c in diabetic patients as compared with the controls. The reference value of HbA1c in normal people is in the range of 4-6%.

Total protein value of all study groups that comprised of 75 subjects of whom 25 were diabetic males from Lahore, 25 diabetic males from Sheikhupura and 25 were controls was measured. The mean value of Total protein of diabetic males from Lahore was 94.6**±1.98, that of diabetic males from Sheikhupura was 90.6**±1.54 and of that of controls was 52±0.1. The difference in the mean Total protein was statistically significant. This shows that in diabetic patients the value of total serum protein increases as compared to the normal people. The reference value of total proteins is in the range of 60-80mg/dL, more precisely 63-89mg/dL.

Fasting Blood glucose values of al study groups that comprised of 75 subjects of whom 25 were diabetic males from Lahore, 25 diabetic males from Sheikhupura and 25 controls was measured. The mean value of Total protein of diabetic males from Lahore was 188.3***±16.85, that of diabetic males from Sheikhupura was 138.85***±10.4 and of that of controls was 94.3±25.52. The results show that value of fasting blood glucose contents in diabetic patients is increased as compared to the normal person. As the breakdown of glucose doesn't occur in diabetic patients due to the disturbance of carbohydrate metabolism that's why the level of fasting blood glucose is upsurge in diabetic patients.

The level of pyridoxine of all the three groups that included 75 subjects of whom 25 were diabetic males from Lahore, 25 diabetic males from Sheikhupura and 25 controls was evaluated by using High Performance Lipid Chromatography Technique (HPLC). The results show that in diabetic patients the value of vitamin b6 is less as compared to the normal people. The mean value of pyridoxine in diabetic cases of Lahore was 25.2±1.52 nmol/L, in diabetic cases of Sheikhupura it was 20.5±1.36 nmol/L and in controls the mean value of Pyridoxine was 50±2.02 nmol/L. the results show statistically high decrease in the level of pyridoxine in diabetics from Lahore and Sheikhupura as compared to the control group with $p<0.001$. This occurred due the increased production of homocysteine that disturb the

level of vitamin b6. The increased level of homocysteine results in the decreased level of pyridoxine that is in turn related directly to the degree of neuropathy particularly in type 2 diabetes. That's why providing the patients supplements of pyridoxine help saving them from complications like incipient neuropathy.

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