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Pleiotropic Effect of Folate-Cobalamin Combinational Therapy on Diabetes Mellitus

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Abstract

Introduction: Diabetes Mellitus type 2 is a metabolic condition when insulin is produced by our body but, it is not used properly by us. The number of diabetic patients is increasing in the whole world. The problem of obesity is also very closely related to it, which itself is expanding. Diabetic patients have high chance of micro vascular problems (like nephropathy, retinopathy and neuropathy). Many micronutrients like vitamins are involved in combating the Diabetes Mellitus. This indicates that many antidiabetic agents should be administered in combination, to maintain normal sugar level in blood. Thus a therapy of Folic acid and Cobalamin can help to the individuals facing Diabetes Mellitus.

Method: Serum samples of 300 Diabetic patients were collected from the Health Centre, University of the Punjab, Lahore An equal and sex matched number of normal healthy control subjects were chosen from the Hostels and Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore. The physical and biochemical parameters of all the 600 individuals were noted. The level of Folic acid and Cobalamin was checked at Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC), Lahore, Pakistan.

Results: The different biochemical parameters like blood fasting sugar, HbA1c, lipids profile and cholesterol were highly raised in individuals facing Diabetes Mellitus. A statistically significant lower level of Folic acid and Cobalamin was noted in patients suffering from Diabetes Mellitus.

Conclusion: This research is done do give a therapy of vitamin B9 (Folic acid) along with vitamin B12 (Cobalamin) to the individuals facing Diabetes Mellitus to lower their sufferings.

Keywords: Diabetes mellitus; Insulin; Micronutrients; Folic acid; Cobalamin

Abbreviations

SDS: Sodium Dodecyl Sulphate; PAGE: Polyacrylamide Gel Electrophoresis; ALP: Alkaline Phosphatase; AST: Aspartate Aminotransferase; ALT: Alanine Transaminase; HbA1c: Hemoglobin A1c; RFT: Renal Function Tests; LFT: Liver Function Tests; HDL: High Density Lipoproteins; LDL: Low Density Lipoproteins; VLDL: Very Low Density Lipoproteins.

Introduction

Diabetes mellitus is estimated to come to be one of the world's leading disablers and killers within the next 25 years. The permanent harm, dysfunction, and failure of multiple human body parts, like eyes, kidneys, heart, nerves and blood vessels could be caused by persistent hyperglycemia [1]. Pakistan is at 7th position for having Diabetes Mellitus. According to report of International Diabetic Federation (IDF) published in 2015 [2], 415 million individuals are suffering from diabetes mellitus and the figure will exceed to 642 million by year 2040. Thirteen distinct kinds of vitamins are found that are ordered by their organic and substance action [3]. A few nutrients are necessary for the body cell development and improvement (for instance folate and vitamin B12). Folate is known as vitamin B9. We need folate for the repair, creation and methylation of DNA [4]. Vitamin B12 assumes a significant job in providing basic methyl bunches for protein and DNA amalgamation [5].

Pernicious anemia due to chronic autoimmune gastritis can be very much widespread amongst type 1 'diabetic people. Pernicious anemia and chronic autoimmune gastritis are present within almost 2% as well as up to 1% common people correspondingly. Amongst people having type 1 diabetes, incidence raises 3 to 5 times [6]. Vitamin B12 shortage because of pernicious anemia present repeatedly amongst individuals having type 1 diabetes. The type 2 Diabetes Mellitus is a heterogeneous malady, which is usually connected to starch and fat administration in the living being. The outcomes and problems of diabetes are the aftereffect of a disparity between free radical development and their control by common cancer prevention agents [7].

Diabetes Mellitus has a strong link with energy metabolism. The quantity of vitamin B12 (Cobalamin) and B9 (Folate) is significantly diminished in diabetics and these two vitamins are the center of my research. The main motive of the research is to give such a therapy to the patients suffering from Diabetes Mellitus which is cost effective, have no side effects and easy to administer.

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Materials and Methods

Study Area

We carried out our research from June, 2017 to June, 2018 at the Department of Microbiology and Molecular Genetics, University of the Punjab and its allied Health Centre situated in Lahore, Pakistan.

Clinical Sample Collection

A recruitment of 300 individuals facing Diabetes Mellitus was done at the Health Centre, University of the Punjab, Lahore. An equal number of normal healthy control subjects were chosen from the Hostels and Department of Microbiology and Molecular Genetics of University of the Punjab, Lahore. We used same physical constraints like age, sex, height and weight to select the control group. Sample were collected from patients and healthy individuals according to the standard blood collection rule. Serum was then taken and kept at -80 degree centigrade for experimentation. The physical and biochemical parameters were noted. The level of Folic acid and Cobalamin was checked at Shaukat Khanum Memorial Cancer Hospital and Research Centre (SKMCH&RC), Lahore, Pakistan.

Approval from Ethical committee

The committee of ethics of School of Biological Sciences has approved the performance of this research work. The reference no. of the approval is SBS/158/14 on 17-6-2017.

Estimation of Biochemical Parameters

We took the blood samples from the Health Center, Hostels and MMG; University of the Punjab, Lahore. There were total six hundred samples. Half of the samples were diabetic patients whereas, half of them were normal healthy control subjects. As discussed, the serum was separated out of the samples and was in turn used for the sake of biochemical examination. We used VITLAB Selectra Junior' which is an automatic analyzer by a leading manufacturer i.e. 'Vital Scientific'. This analyzer was used to identify and assess the different chemical constraints.

Protein Assessment

The measurement of protein was done by method of Bradford by Kruger, 1994. The attachment of protein molecules to Coomassie dye under acidity is the principle of this method. These outcomes in a shade shift from the reddish/brown appearance of the dye with absorbance maximum of 465nm to the blue appearance of the dye with absorbance maximum of 610 nm. The amount of Coomassie dye ligands attach to each protein molecule is almost proportional to the amount of positive charges present on the protein.

Identification of Protein biomarker by Electrophoresis of Sodium Dodecyl Sulfate poly-acrylamide gel (SDS-page)

It work on the principal to separate the proteins according to their ability to transport when an electric field is applied. SDS was added to give the protein overall negative charge and acted as a detergent for purpose of removal of different structures of protein for their alignment in polypeptide chain form. The system were consisted of Casting frames that were set by clamping of two plates between casting frames, was done on the casting stands. We Prepared 2 gels, resolving, present at the bottom and stacking, that forms the top of the gel by adding different amount of acrylamide in it. Pipetting required quantity of resolving gel between the slit in the two plates of gel. The solution was then kept for about twenty to thirty minutes to allow it to polymerize properly. Then a well-forming comb was put in it. It was kept for twenty minutes to half hour to allow it to polymerize. Gel plates were unloaded from the casting frame and was placed in buffer tank. Then the samples were added in to the wells. The power supply was connected with the electrophoretic unit after covering its top. After finishing of run, the gel was stained for 30 minutes in Coomassie brilliant blue stain. Then the gel was de-stained overnight until the clear bands showed up.

Folate and Cobalamin (B12) Estimation by HPLC

Vitamin B6 and B12 assessment was made by HPLC (High Performance Liquid Chromatography). It was based on the adsorption principle. HPLC is used to ascertain the existence of tiny quantity of a compound in any solvent.

Statistical Assessment

We did the statistical analysis to have the final and confirmed results of research work. In this statistical analysis the main parameter of key interest was the reduction in Folate and Cobalamin levels in individuals with diabetes compared to the non-diabetic control subjects.

First, the mean of all the values were recorded. Then standard deviation was found out to further analyze the data. The next step was to measure the standard error. The final step is to apply a test to find out the final results of the data. The statistical assessment was completed using SPSS statistical software package of version 86.0. At the end the significant difference between the normal healthy controls and diabetics was found out.

Results

Arrangement of groups for study

The sample size was 600 having 300 patients suffering from Diabetes Mellitus and 300 normal healthy control subjects. The samples were assembled according to the following criteria.

1. All the patients suffering from Diabetes Mellitus Type 2.
2. Normal individuals serving as controls.

Normal standard values used in Pakistan

The normal values referred to as standards in Pakistan were given in Table 1.

Assessment of physical parameters

Physical parameters were assessed as age, weight, height, body mass index (BMI) of both diabetic patients and normal healthy control subjects. The results of these parameters were summarized and compared in tables 2 (comparison of total patients suffering from Diabetes Mellitus and Normal Healthy Control subjects).

Physical Parameters

Mean age of the Normal Healthy Control subjects was calculated to be of 50.86±1.32 years and that patients' suffering from Diabetes Mellitus group was 61.58±0.44 years. The mean weight measured in all patients suffering from Diabetes Mellitus was 70.21±0.49 kg, and in Normal Healthy Control subjects was 63.56±1.20 kg. Mean height of total Diabetics was recorded as 5.38*±0.02 ft, and that of Controls was 5.41±0.02 ft. Body Mass Index is abbreviated as BMI. The units

Figure 1:

of the value of BMI are kg/m^2. The BMI of total patients suffering from Diabetes Mellitus was 28.22±0.27 kg/m^2 and the BMI of Normal Healthy Control subjects was calculated to be 23.52±0.54kg/m^2. In all the physical parameters, the difference of controls and Diabetics showed a noteworthy statistical P value that was <0.001 as given in figures 1(a).

Assessment of Biochemical Parameters

Glucose estimation

This section comprises of the results of HbA1c and blood sugar (fasting glucose). The results calculated as mean of fasting glucose of total patients suffering from Diabetes Mellitus and Normal Healthy Control subjects' group were 213.11±4.34mg/dl and 95.33±9.89mg/dl respectively, and of HbA1C were 9.98±0.138% and 6.5±0.11% correspondingly, given in figure 1(b) indicating that the results are highly significant (P<0.001).

Cholesterol estimation

The resulted values of Triglyceride, LDL, HDL and total cholesterol of total patients suffering from Diabetes Mellitus and Normal Healthy Control subjects were 223.213±4.90, 100.040±2.11, 37.74±0.158, 168.74±2.29 and 140.24±21.29, 94.08±2.15, 43.48±0.62, 155.21±10.11 respectively. The figure 1(c) show that the results of all these four parameters are highly significant (P<0.001).

Liver function tests

The mean of the Bilirubin test results in Normal Healthy Control subjects was 0.35±0.005mg/dl, of ALT was 30.70±5.57U/L and of ALP was 108.02+1.25U/L. Whereas, in patients suffering from Diabetes Mellitus, resultant mean of Bilirubin was 0.66±0.008mg/dl, of ALT was 39.79±1.12 U/L and of ALP was 208.61±5.05 U/L. The values of LFTs in patients suffering from Diabetes Mellitus stay meaningfully different (P<0.001) as related to the Normal Healthy Control subjects as showed in Figure 1(d) and 1(e) respectively.

Creatinine estimation

Kidney function is tested by measuring creatinine. The mean of the results total patients suffering from Diabetes Mellitus was 0.92±0.009 and Normal Healthy Control subjects was 0.89±0.03mg/dl. Giving a significant difference between Normal Healthy Control

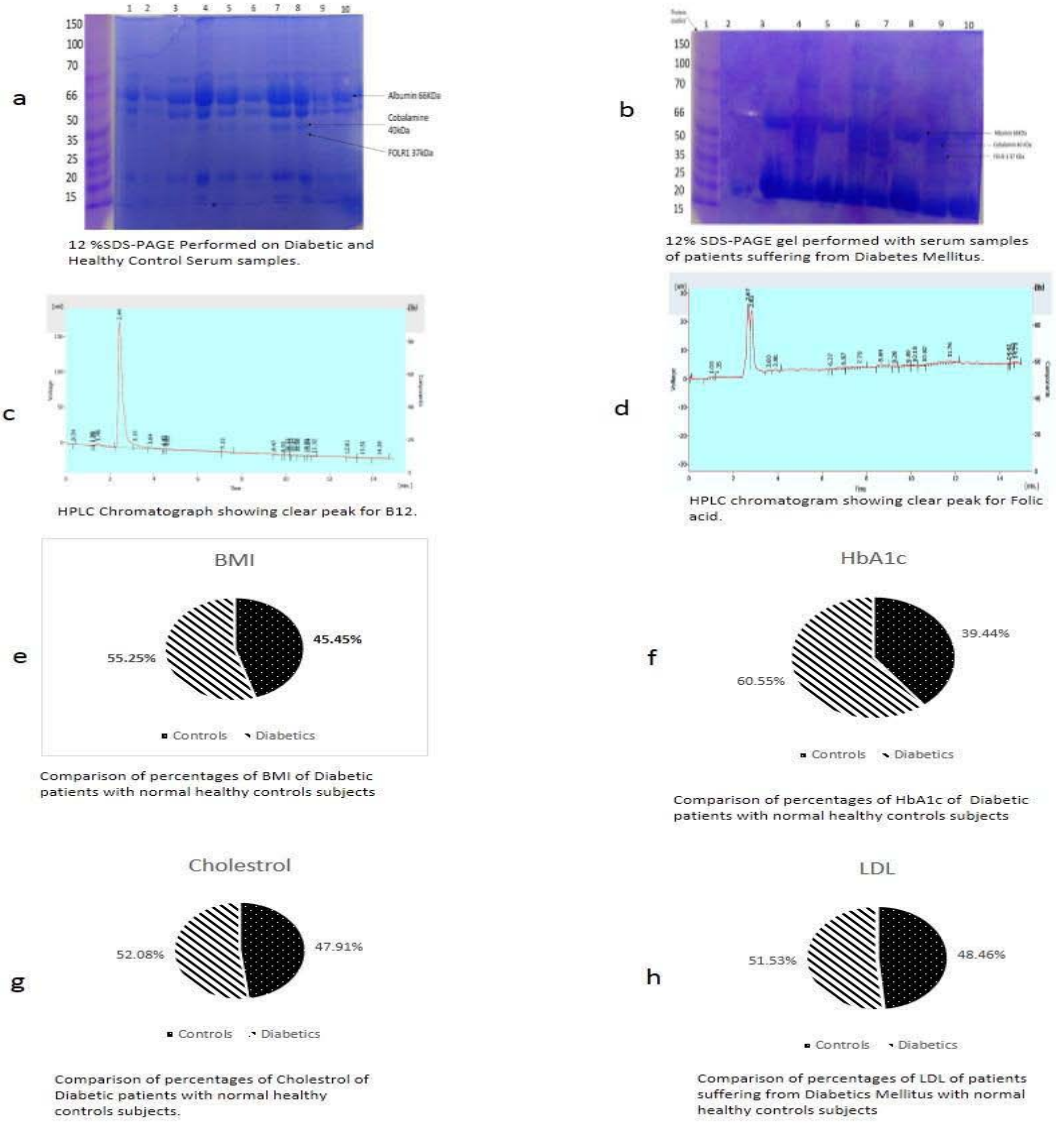

Figure 2:

subjects and patients suffering from Diabetes Mellitus (P<0.001) in Figure 1(f).

Vitamin B9 and B12 Monitoring

The value of Folate in total diabetics and control groups are 8.34±0.31ng/ml and 9.33±0.49 ng/ml correspondingly. A highly noteworthy picture (P<0.001) was obtained by comparing of the results as in Figure 1(g).

Assessment of proteins by Bradford

The estimation of proteins is done by finding the amount of the "Coomassie brilliant blue stain" as, the amount of the dye bounded will be as much as the protein's concentration in that sample. BSA standard was prepared with a concentration having range of 0µg/ml to 90µg/ml giving an OD range from 0.000 to 0.099 as shown in Figure 1(h). The mean of Normal Healthy Control subjects was 75µg/µl whereas of the patients suffering from Diabetes Mellitus was 60µg/µl.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (Sds-Page) Analysis

Standard SDS-PAGE gels showing the bands of FOLR-1 (Folate Receptor Alpha Protein) and Cobalamin are at 37kDa and 40kDa respectively. Large Bands of Albumin Are Clearly Visible in all Samples and Particularly Diabetic Samples. 1, 2, 6 and 9 are healthy control serum samples while 3, 4,5,7,8 and 10 are having Diabetic serum samples as shown in Figure (2a and b). This gel was performed at Lab 1 of the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore.

HPLC analysis of Folate and Cobalamin

The folic acid and B12 were eluted from the SDS-PAGE gel for HPLC. Certain specific samples, after dilution, were run on HPLC for confirming the results of SDS-PAGE. The values of Folate and vitamin B12 were compared with the standards run along with. Some little peaks are also observed errors of handling, small impurities or

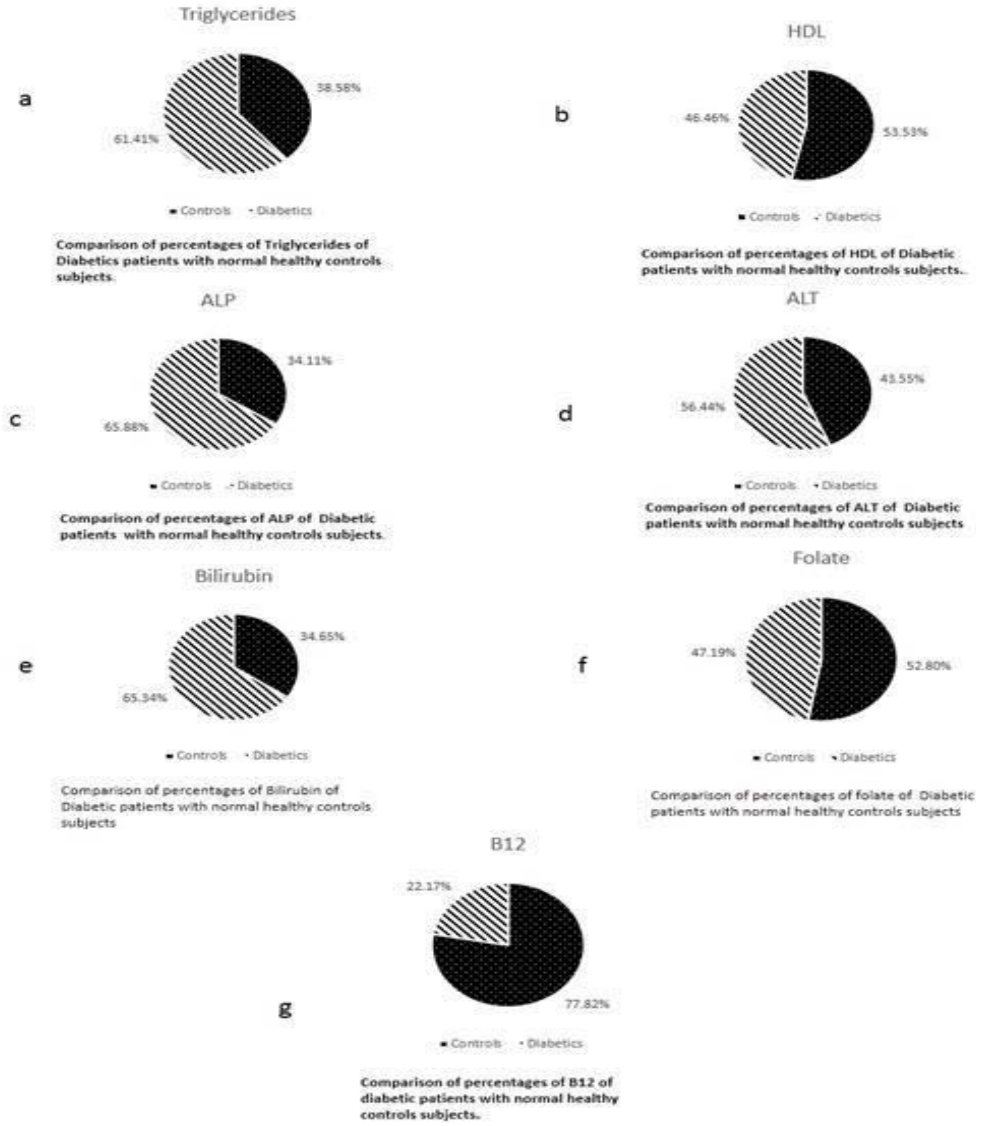

Figure 3:

any fluctuation in voltage of light.

The HPLC chromatogram results of B12 and folic acid are given Figure 2(c) and 2 (d) respectively.

Statistical Analysis

All the data was compiled in Excel and then in SPSS for analysis and arrangement of all the data was done. The presentation of the data was made more understandable using the following software applications:

- IBM SPSS Statistics (version 86)
- Microsoft office Excel 2016

Collectively 300 patients suffering from Diabetes Mellitus were included in the research. The data was first analyzed in Microsoft Excel for finding the means, standard deviation and standard error. Graphs were also generated using Microsoft Excel. One sample T- test was performed using IBM SPSS. The different parameters of Normal Healthy Control subjects and patients suffering from Diabetes Mellitus were compared by applying paired T-test. Folate and B12 were diminished in patients suffering from Diabetes Mellitus.

Discussion

Diabetes Mellitus is a very old malady and it's increasing like an epidemic globally. The efforts are at peak in order to get rid of this disease. Bodily activities, medications and mineral treatment is the emphasis of most of these preemptive and therapeutic approaches [8]. The emphasis in this study was to give the sufferers a therapy, which is easy to administer, cost effective and has no side effects.

The basic two micronutrients focused in the underlying study Folate (Vitamin B9) and Cobalamin (Vitamin B12). This deficiency may cause several other complications increasing the miseries of diabetics. A combinational therapy of Folate and Cobalamin can save the patients from a number of further complications. It is also

Table 1: Physical and Biochemical Parameters Used as Standard Normal in Pakistan.

S. NO.	Parameters	Standard Values
1	BMI (kg/m^2)	19-25
2	Fasting blood sugar (mg/dl)	65-110
3	HbA1c (%)	4-6
4	Triglyceride (mg/dL)	50-250
5	LDL (mg/dL)	<150
6	HDL (mg/dL)	41-58
7	Total cholesterol (mg/dL)	20-200
8	Bilirubin (mg/dL)	0.2-1.3
9	ALT (U/L)	7-56
10	ALP (U/L)	38-126
11	MAU (mg/24 Hours)	3.5-4.8
12	Creatinine	0.5-1.4
13	Folate (Vitamin B6)	2-20 ng/ml
14	Cobalamin (Vitamin B12)	180-914 ng/l

Table 2: Physical Parameters of Control Healthy Individuals and Diabetic Patients.

Parameters	Control (N=300)	Diabetic (N=300)
Age (years)	50.86±1.32	61.58***±0.44
Weight (kg)	63.56±1.20	70.21***±0.49
Height (feet)	5.41±0.02	5.38**±0.02
BMI (kg/m^2)	23.52±0.54	28.22***±0.27

+ sign indicate the stand error; (P>0.05) = non-significant; (P<0.001)*= Significantly different; Number of "*" asterisks show the level of significant difference.

effective in preventing the pre-diabetics from becoming diabetics.

A diminished level of Folate and B12 not an issue in developed countries or in those areas where fortified food is used, instead, it is a problem of developing areas like Pakistan where this deficiency is highly ubiquitous [9].

A comparison regarding physical parameters with normal healthy control subjects revealed a noteworthy increase in the mean value of the patients suffering from Diabetes Mellitus. The BMI recorded for patients suffering from Diabetes Mellitus was 10% higher from normal healthy control subjects (Figure 2e). The BMI of normal controls was 25% less as compared to diabetics. It was also revealed that a raised BMI value is related to a higher chance of Diabetes Mellitus [10].

By performing the Biochemical analysis we assessed that the HbA1c level of patients suffering from Diabetes Mellitus was 21% higher from normal healthy control subjects (Figure 2f). The patients of diabetes having the HbA1c level more than 7% are more prone to develop micro vascular and macro vascular disorders. Likewise the risk of cardiac diseases, neuropathy, and nephron related disorders and untimely death are higher in them [11].

It was stated in a study by Riaz et al., 2009, that the diabetic showed an elevated value of cholesterol (32.5%) in contrast to the normal healthy individuals. The similar relation is found in this study where the cholesterol level of patients suffering from Diabetes Mellitus was 4% higher when related to the normal healthy control subjects (Figure 2g).

Global relation of diabetic kidney disease with increased level of triglycerides and reduced level of HDL was found in a research finding the effect of these two parameter on micro vascular ailments in patients suffering from Diabetes Mellitus [12]. In this underlaying research work, the bad fats (i.e. LDL) were 4% elevated in patients suffering from Diabetes Mellitus as compared to the normal healthy control subjects (Figure 2h).

Triglycerides were considerably raised i.e. 23% high in patients suffering from Diabetes Mellitus, (Figure 3a). A significantly higher value of triglyceride (P<0.011) reported in patients suffering from Diabetes Mellitus [13].

A tendency towards the development of Diabetes Mellitus can be forecasted from the proportion of Triglycerides and HDL as a higher ratio is related to a higher casualty rate [14]. There was a 7% increased level of HDL regarding normal healthy control subjects in contrast to the patients suffering from Diabetes Mellitus (Figure 3b).

The pointedly raised values of Alkaline Phosphatase (ALP), Alanine Transaminase (ALT) and Bilirubin were noted regarding patients suffering from Diabetes Mellitus. ALP was 32% higher, ALT was 13% higher and Bilirubin was 31% higher in the group of patients suffering from Diabetes Mellitus in contrast to the normal healthy control subjects (Figures 2c, 2d and 2e respectively). Bilirubin, ALP and ALT, all showed a prominent elevation (P<0.001) during ours research work. Riaz et al. reported a considerable higher level of Bilirubin (P<0.001) in patients suffering from Diabetes Mellitus [15]. Comparing to another study, there was a 12% raise in Bilirubin, 15% raise in ALT and 46% raise in ALP [16]. The intake of a diet rich in proteins as compared to carbohydrates and fat increases the chance of Diabetes Mellitus. Whereas the habit of taking cereals and fiber rich food can converse the situation [17].

A significant difference was experienced on SDS PAGE gel in Figure 2a and 2b. The quantity of albumin was more in patients suffering from Diabetes Mellitus. The band resulted from the serum of normal healthy control subjects were sharper as compared to the bands observed from patient's serum. The bands resulting from the serum of patients suffering from Diabetes Mellitus were large, huge and clearer, particularly, albumin protein band of 65KDa and of α-1-glycoprotein at 54KDa.

Main target protein FOLR1 (Folic acid receptor alpha protein, 37KDa) was observed in the diabetic patients and rarely in normal healthy individuals. As, it was confirmed later that folate levels were lower in diabetic than the normal individuals. FOLR1 was observed at 37KDa on SDS gel is actually a 26KDA protein, which migrate to 37KDa in SDS-PAGE due to reducing condition caused by glycosylation [18].

Albumin proteins found in Human Serum (HAS) make up almost 60% of total proteins found in the body. It has a molecular weight of 66.7KDA [19]. A raised value of albumin protein in serum can be considered to be related to the final stage of kidney disease. It can be healed by giving an amino acid supplementation [20]. The values of HbA1c and Glycated albumin are elevated in the patients of diabetes with the increasing age. Glycated albumin level may help in lowering

the sugar level [21]. Bradford results also confirmed the quantity to be high in diabetic patients.

The level of Vitamin B9 and B12 is significantly lessened due to the continued administration of metformin. That's why the said vitamins are found to be at a lower level in diabetics and require daily intake by the patients [22].

In ours research work, Folate was found to be 5% and B12 was 56% lower in patients suffering from Diabetes Mellitus (Figures 3f and 3g respectively). A case-control study disclosed that low levels of vitamins B9 and B12 regarding the diabetic were associated to hyperhomocysteinemia [23]. DNA damage as measured by the presence of micronuclei can be reverted by folic acid supplementation, thus reducing the effect of stress produced by oxidation reactions in patients suffering from Diabetes Mellitus [24]. A study found lower plasma levels of Cobalamin in patients suffering from Diabetes Mellitus type 2 who were using metformin, while a retrospective review of medical records also showed this association [25]. After reviewing the information, we were concluded that diabetic patients who were using metformin had a greater chance of emerging the deficiency of vitamin B12 and its consequent hyperohomysteinemia that may lead to neuropathy and other complications, thus, it seems wise to recommend cobalamin supplementation in these subjects [26-36].

Conclusion

Patients suffering from Diabetes Mellitus can develop many other complications, which increase their miseries. It's important to take preventive measures in order to lower their miseries. These measures include body mass index, sugar level, HbA1c, Cholesterol, Lipid profile, serum proteins and the level of micronutrients (Vitamins). All the parameter studied should be maintained to a normal range. Our main focus in this research work were Vitamins B9 (Folate) and B12 (Cobalamin). These two parameters were decreased in patients suffering from Diabetes Mellitus. The maintained level of these vitamins will control the main problems in patients suffering from Diabetes Mellitus like neuropathy, anemia and many others.

For this purpose, a therapy of Folate (Vitamin B9) and Cobalamin (Vitamin B12) was administered to the diabetic patients enrolled in our study.

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