

Full Length Research Paper

Obesity as risk factor and study of obesity related proteins in diabetes mellitus

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Obesity is one of the leading cause of diabetes mellitus in the world population due to the growing trend of sedentary lifestyle and increased food consumption of high caloric diet. A concomitant increase due to this has been observed in coronary heart diseases, hypertension and even diabetes. High lipid profile of the serum is one of the factors that may result in the development of obesity in diabetes mellitus and thus the progression of diabetes. This study was carried out to determine the effect of various physical and biochemical parameters of obesity to ascertain the link to presence and severity of diabetes. For this purpose physical parameters such as BMI, waist circumference, and biochemical parameters such as FBS, glycated HbA1c, albumin excretion rate, urine creatinine level, and lipid profile parameters such as cholesterol, triglyceride, HDL, LDL and VLDL were measured by standard referred protocols. Elevated serum leptin level was associated with components of the metabolic syndrome, including increased body-mass index, waist-to-hip ratio, serum triglyceride levels, fasting blood sugar, glycated HbA1c, total serum proteins and decreased high-density lipoprotein cholesterol levels in obese diabetes as compared to lean, non-obese and normal subjects. The results were found to be significant with $p < 0.05$. This study was also carried out to determine the risk factor of obesity in diabetes mellitus and found significant results in metabolic syndrome as physical and biochemical parameters are greater in obese diabetes as compared to non-obese diabetes group and also in lean. Proteins levels such as leptin are also greater in obese diabetes as compared to non-obese and lean objects.

Key words: Diabetes mellitus, obesity, leptin, SDS PAGE, MALDI TOF TOF.

INTRODUCTION

Diabetes mellitus is a world health problem and affects all human society at various stages of development. It is more common amongst developed countries where affluent and over weight individual lives longer than human being of under developed countries. The incidence of this disease in a society, whether in Pakistan or any developed country is difficult to judge but it is quiet obvious that the disease is multiplying geometrically more or less, because of genetic and environmental factors (Alberti and Zimmet, 1998). A worldwide epidemic exists with respect to diabetes mellitus, primarily because of

increased rates of obesity. Obesity has become widespread in developed countries along with a corresponding increase in the prevalence of diabetes. Epidemiological studies have shown that, compared to lean individuals, very obese men and women (body mass index >35) have a 60- and 90-fold increased probability of developing Type 2 diabetes, respectively (Thang et al., 2006). Although the precise underlying mechanisms in the development of diabetes are yet unknown, the initial path physiological event is usually insulin resistance, which involves a genetic component that is exacerbated by obesity and a sedentary lifestyle. There is a significant correlation between obesity and diabetes mellitus as obesity exacerbates insulin resistance in diabetic subjects (Afridi and Khan, 2004). The prevalence of diabetes

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mellitus is increasing all over the world particularly so in developing countries including Pakistan. According to World Health Organization (WHO), prevalence of diabetes mellitus in Pakistan for the year 2000 was 5.2 million and for 2030 it would be around 13.8 million. Epidemiological studies have shown that South Asians are more likely to have central obesity measured in terms of waist circumference and increased body mass index (BMI). Increased body fats are related to increase insulin resistance and may account for the increased prevalence of diabetes mellitus in South Asians (WHO, 1997). Adipose tissues in the abdominal region secrete a number of proteins that play a significant role in diabetes mellitus. Knowledge of adipocyte biology is crucial for understanding the patho-physiological basis of obesity and metabolic diseases such as diabetes. Adipose tissue produces a number of cytokines which have been associated with insulin resistance such as leptin and resistin (Bartness and Bamshad, 1998). The study was designed to examine the relationship of diabetes to physical and biochemical parameters of obesity in diabetes and determine the levels of proteins in diabetes as compared to normal individuals.

METHODOLOGY

Sample selection

Blood samples of diabetic ($n = 100$) and non diabetic ($n = 50$) were collected from diabetic Clinic of Sheikh Zayed Hospital, Lahore, Pakistan. Three study groups were made as control, diabetic obese and diabetic non-obese having equal number of individuals in each group.

Total protein estimation

Total serum protein content was estimated by carrying out Bradford microassay (Bradford, 1976).

Estimation of biochemical parameters of diabetes

Fasting blood glucose, glycated HbAc1, urine creatinine and albumin excretion rate was all determined by using Randox kit, following the standard protocol. Total cholesterol concentration was measured by CHOD-PAP method, triglyceride levels by GPO-POP method. HDL-cholesterol was measured by serial precipitation method using dextran sulphate and magnesium chloride. LDL-cholesterol in serum is measured by using Friedewald's equation (Friedewald et al., 1992).

Statistical analysis

The data was analyzed by computer program special package for Social Sciences (SPSS). The mean values were calculated and the level of significance was put at $p < 0.05$. Study groups were compared with control group.

SDS PAGE

Proteins were separated and crude analysis of their expression was

done by using 10% polyacrylamide gel electrophoresis under denaturing conditions using Sambrook protocol. Gel was run at 40 V initially till the samples crossed stacking gel and later the voltage was increased to 100 V till the process was completed. 10 μg of denatured protein sample was loaded in gel for commassie stained while in gel for silver staining only 1 ng of sample was loaded.

Commassie staining

Gels were stained with commassie brilliant blue R-250 dye for 15 min. Later the distaining process was carried out using mixture of methanol and acetic acid

Silver staining

This type of staining is usually carried out to study the profile of low molecular proteins which are present in low concentration in the sample. The gel is first fixed in solution containing ethanol and acetic acid in 1:3. After subsequent washing steps the gel is allowed to stay in AgNO_3 and later developed by using developing solution (Sambrook and Russel, 2001).

Mass spectrometry analysis (MALDI TOF TOF)

Gel pieces showing bands of proteins were destained for 1 h at room temperature using 100 μL of 50% acetonitrile/50 mM NH_4HCO_3 . Gel pieces were dried and incubated with trypsin in 10 mM NH_4HCO_3 (Promega, Madison, WI) overnight at 37°C. Peptides were extracted from trypsinized gels by washing gel pieces for 2 h with 0.1% TFA and 60% ACN and were purified using ZipTip (Millipore Corporation). Zip Tip eluates were dried and resuspended in 12 μL of HPLC water with 0.1% formic acid prior to mass spectrometer analysis (Joshua et al., 2008). The molecular mass and charge ratio (m/z) of the proteins was determined by MALDI TOF TOF mass spectrometer (Bruker Daltonics, Germany).

RESULTS

BMI

The obesity is generally measured in terms of physical parameters. The physical parameters of obesity which we included in our study were BMI, waist circumference and waist to hip ratio. Body mass index (BMI) is measured in kg/m^2 by dividing weight in kilogram to square of height in meter. In the present study a significant difference of BMI between obese diabetic and non-obese diabetic is observed as shown in Figure 1. Body mass index (BMI) was calculated in kg/m^2 . The average BMI of diabetic obese, diabetic non-obese and normal subjects is 33.56 ± 0.44 , 25.1 ± 0.95 and $24.6 \pm 0.61 \text{ kg}/\text{m}^2$, respectively, as shown in Table 1. The diabetic groups, that is, diabetic obese and diabetic non-obese show 36 and 4.5% increase respectively in BMI when compared with control. The diabetic non-obese patients have average BMI 25% less than that in diabetic obese patients.

Waist circumference

Waist circumference is the second physical parameter of

Table 1. Effect of diabetes on various physical and biochemical parameters of obese and non obese subjects.

Categories	BMI (kg/m ²)	Waist circum. (cm)	Waist to hip ratio	Total proteins (ug/ul)	Blood glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	VLDL (mg/dl)	LDL (mg/dl)
Control (n=25)	24.6±0.61	89±9.8	0.82±0.08	75.8±4.3	91.4±2.74	163.56±9.3	108.64±13.91	51.24±2.05	46.12±1.84	87.12±5.24
Diabetic non-obese	25.1±0.95	96±8.0*	0.89±0.07	82.4±0.8	156.5±11.25*	172.68±6.29*	130.36±18.73	50.96±5.79	58.89±4.16*	98.0±6.51
Diabetic obese (n=25)	33.56±0.44*	104±12.1*	1.1±0.107	87.0±1.4*	200.68±10.98*	216.8±6.77*	250.26±19.4*	44.8±1.96*	70.59±5.06*	113.8±5.23

*Student t test was applied and values were found to be significant with $p < 0.05$.

obesity and is measured in centimeter. The individual having waist circumference greater than 100 cm is taken as obese and is at greater health risk of developing diabetes and those with waist circumference less than 100 were taken as non-obese subjects. Just as the case in BMI, waist circumference is the main criteria on which the grouping is done. We observe an increasing trend in the waist circumference from control to diabetic non-obese to diabetic obese as shown in Figure 1. An increase of 12.3% is seen in diabetic as compared to control. Average waist circumference of control, diabetic non-obese and diabetic obese is 89 ± 9.8 , 96 ± 8.0 and 104 ± 12.1 cm, respectively, as given in Table 1. An increase of 16.8 and 7.8% respectively is seen in case of diabetic obese and diabetic non-obese when compared to control. When comparing the two diabetic group an in-crease of 8.3% is seen in case of diabetic obese when compared with diabetic non-obese.

Waist to hip ratio

Waist circumference is the third parameter of obesity and is taken by dividing waist circumference with hip circumference. The average waist to hip ratio of individuals in the study show an increasing trend from control to diabetic non-obese to diabetic obese as shown in Figure 1.

The average waist to hip ratio of diabetic and control is 0.82 and 0.94 respectively as given in Table 3. The average waist to hip ratio of control, diabetic non-obese and diabetic obese is 0.82, 0.94 and 1.10 respectively as shown in Table 1. An increase of 8.5 and 34% is seen in average waist to hip ratio of diabetic non-obese and diabetic obese when compared to control. When comparing the two diabetic groups we observe an in-crease of 23% in diabetic obese as compared to diabetic non-obese in the average waist to hip ratio.

Biochemical parameters

The biochemical tests for diabetes such as fasting blood glucose and total protein estimation was made. For obesity the complete lipid profile was performed including estimation of total cholesterol, triglycerides, HDL-cholesterol, LDL cholesterol and VLDL. Bradford assay was performed for the quantification of microgram quantities of protein. Optical densities were taken at 595 nm. Bovine serum albumin was used to plot the standard curve. Protein concentration was then calculated corresponding to optical density of each sample. We observed an increasing trend in the serum protein concentration from normal to diabetic non-obese to obese diabetic as shown in Figure 1. Average total protein content of diabetic obese,

diabetic non-obese and control is 87.0 ± 1.4 , 82.4 ± 0.8 and 75.8 ± 4.3 $\mu\text{g/ul}$, respectively, as shown in Table 1. The diabetic groups, diabetic obese and diabetic non-obese show increase in percentage, that is, 14.7 and 8.7% respectively of the average serum protein concentration when compared with the control. The diabetic non-obese patients in turn have average protein content 5.6% less than that of diabetic obese. Fasting blood sugar level in diabetic obese, diabetic non-obese and control are 200.68 ± 10.98 , 156.5 ± 11.25 and 91.4 ± 2.74 mg/dl, respectively, as shown in Table 1. Diabetic obese show about 119% and diabetic non-obese about 71% increases in blood glucose levels when compared with control. The comparison of two diabetic groups show approximately 22% decrease in average glucose level in diabetic non-obese when compared with diabetic obese. The average cholesterol concentration in diabetic obese, diabetic non-obese and control is 216.68 ± 6.7 , 172.68 ± 6.29 and 163.56 ± 9.3 mg/dl, respectively, as given in Table 1. In diabetic obese the average cholesterol concentration is approximately 32.5% more when compared with control where not significantly difference, that is, about 5.5%, in average concentration is seen between diabetic non-obese and control. Among the two diabetic groups, in diabetic non-obese the average cholesterol concentration is about 20.3% less than that in diabetic obese. The average TG concentration

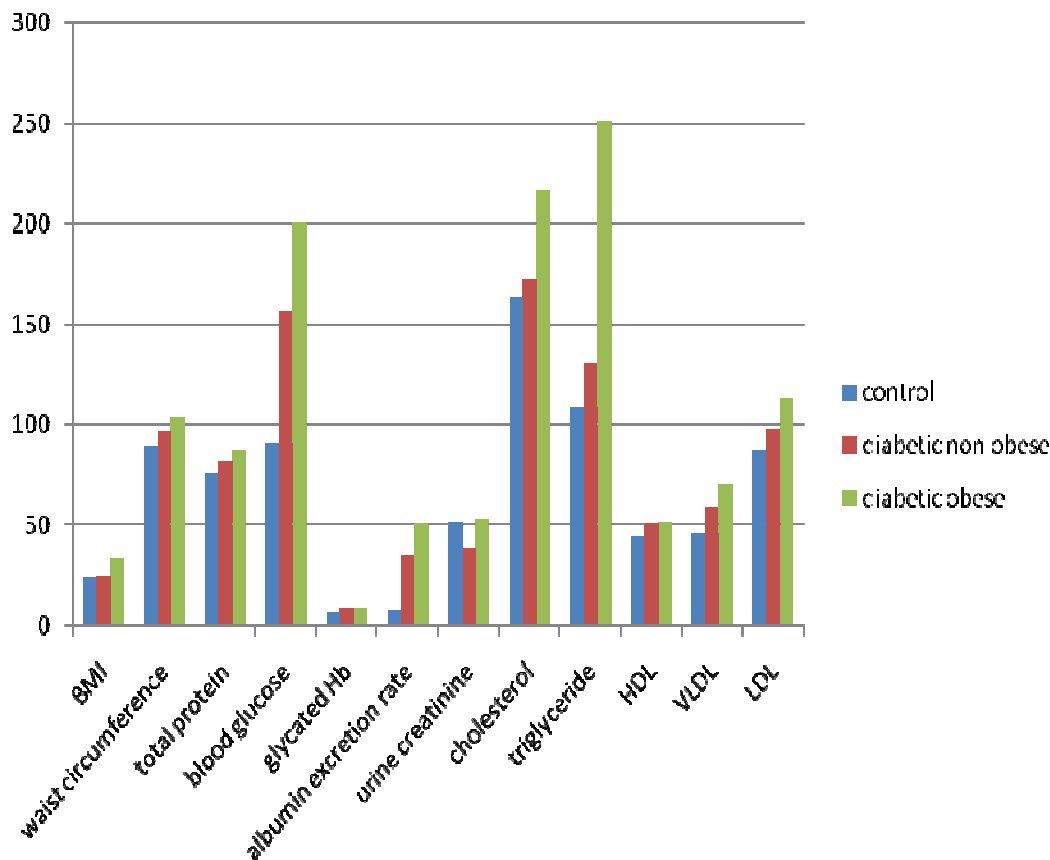


Figure 1. Comparison of physical and biochemical parameters of obesity and diabetes mellitus (obese and non-obese diabetic with normal control).

in diabetic obese, diabetic non-obese and control subjects is 250.26 ± 19.4 , 130.36 ± 3.6 and 108.64 ± 13.9 mg/dl, respectively, as seen in Table 1. An increase of 130 and 20% is seen in diabetic obese and diabetic non-obese respectively when compared with the normal control. Diabetic non-obese on other hand show 48% decreased average TG concentration when compared with diabetic obese. The average concentration of HDL in diabetic obese, diabetic non-obese and control subjects is 44.8 ± 1.96 , 47.0 ± 2.05 and 50.96 ± 5.79 mg/dl, respectively, as shown in Table 1. Diabetic obese show 12% decrease in HDL level as compared to control while the decrease in diabetic non-obese is about 7.7% compared to control. The difference between the two diabetic groups is about 5%. The average VLDL concentration in diabetic obese, diabetic non-obese and control subjects is 70.59 ± 5.06 , 58.89 ± 4.16 and 46.12 ± 1.84 mg/dl, respectively, as given in Table 1. An increase of 53 and 27.6% is seen in diabetic obese and diabetic non-obese when compared with control. The average LDL level in diabetic obese, diabetic non-obese and control is 113.8 ± 5.23 , 98 ± 6.51 and 87.12 ± 5.24 mg/dl, respectively, as seen in Table 1. Diabetic obese show 30.6% increase in LDL level as compared to control while

the increase is 12.4% in diabetic non-obese. When comparing the two diabetic groups, diabetic non-obese show 13.8% decrease as compared to diabetic obese.

Gel electrophoresis and mass spectrometry analysis

The 10% SDS polyacrylamide gel electrophoresis (PAGE) was performed for all the samples (15 ug/10 ul proteins was loaded in the gel) in the entire three groups, that is, diabetic obese, diabetic non-obese and control. The protein profile obtained on the gel was visualized by staining the gel with coomassie R-250 dye and with silver salts. The representative gel shown in Figure 2 is stained with coomassie R-250 dye, while the gel shown in Figure 3 is stained with silver nitrate. In all the gels a high molecular weight protein marker (10 - 200 KD) is loaded in the first lane. The first gel showed in Figure 2 carry the samples of diabetic obese and non-obese subjects along with control sample. Leptin a low molecular weight (18 KD) protein is more expressed in diabetic obese (lanes 2 - 9) as compared to diabetic non-obese (lanes 10 - 19) as shown in Figure 2 stained by commassie stain. In Figure 3, the gel stained by silver staining showed clearly pro-

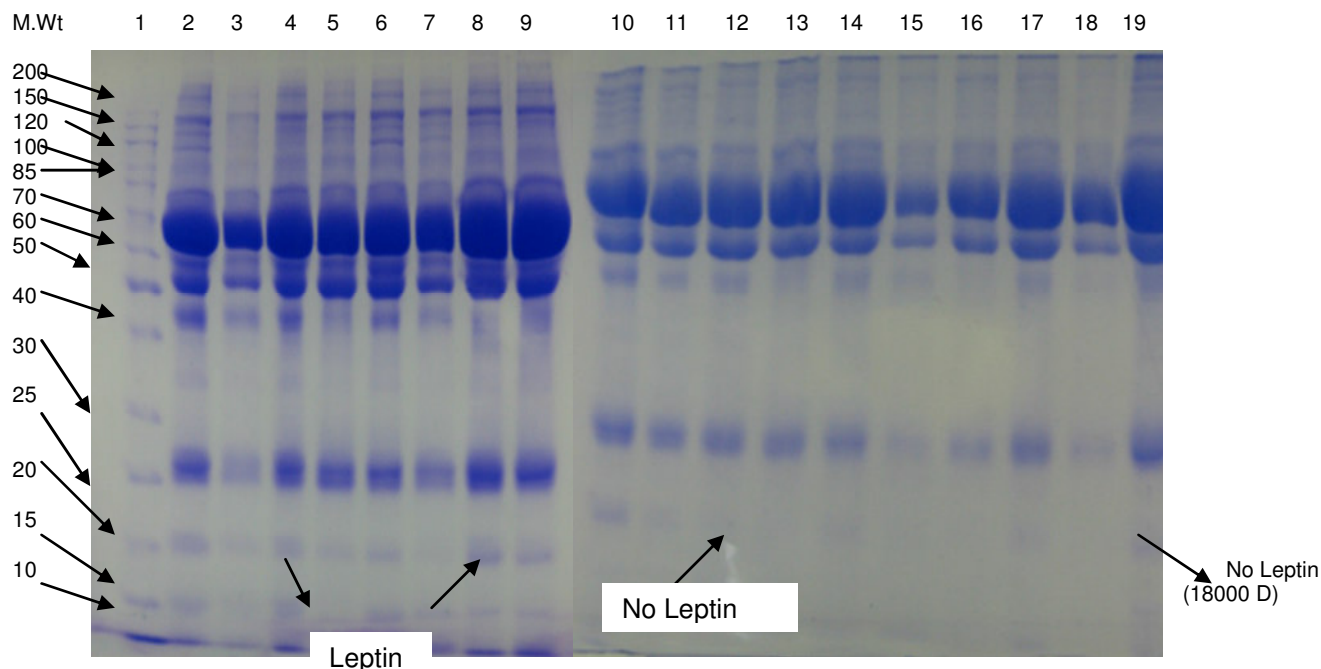


Figure 2. Protein profile of serum sample of diabetic obese and non-obese diabetic subjects. 10% SDS PAGE stained with commassie stain. Protein marker 10 - 200 kd (lane 1), lanes 2 - 9 are diabetic obese and lanes 10 - 19 are diabetic non-obese.

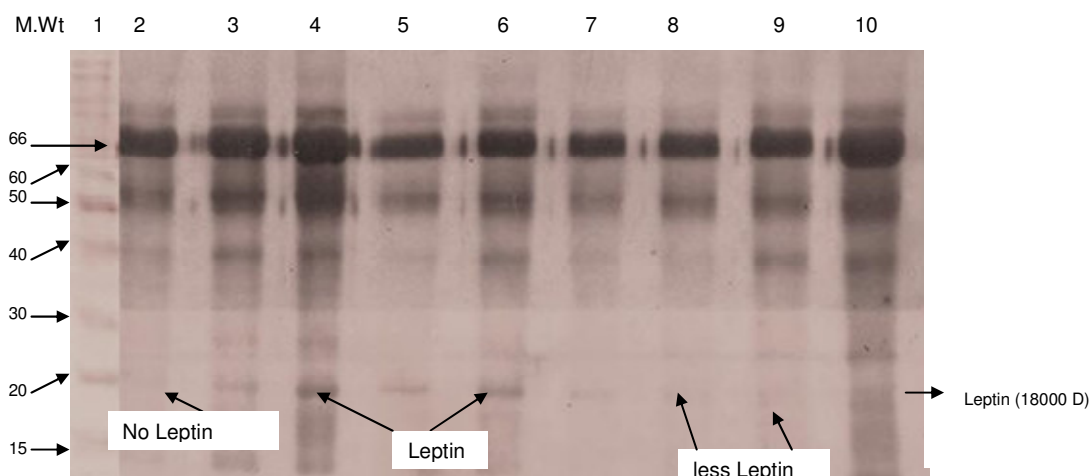


Figure 3. Protein profile of serum sample of diabetic and non diabetic subjects. 10% SDS PAGE stained with silver stain. Protein marker 10 – 200 kd (lane 1) normal person non diabetic (lane 2), diabetic obese (lanes 3 - 6), diabetic non-obese (lanes 7 - 9).

minent band of low mol wt. protein as leptin that is more expressed in diabetic obese (lanes 2 - 6) and almost little expression was observed in diabetic non-obese as in lanes 7 - 10. Confirmation of these proteins was done by mass spectrometric analysis as MALDI TOF TOF before and after tryptic digestion of these proteins (Figures 4 and 5). In Figure 4 the intact mass of protein has been shown by the MALDI TOF TOF (Bruker Daltonics, Germany) that is about 18641.304 dalton that is mol. Wt.

of leptin precursor protein in human. In Figure 5, the different small peptides were produced from the protein in the sample after treatment of trypsin (TCPK treated). The data of these fragments or peptides were than subjected to MASCOT software and analysis is shown in Figure 4 and 5. It has confirmed the presence of leptin precursor protein in the human being serum sample of diabetic obese persons and absence in the normal healthy control.

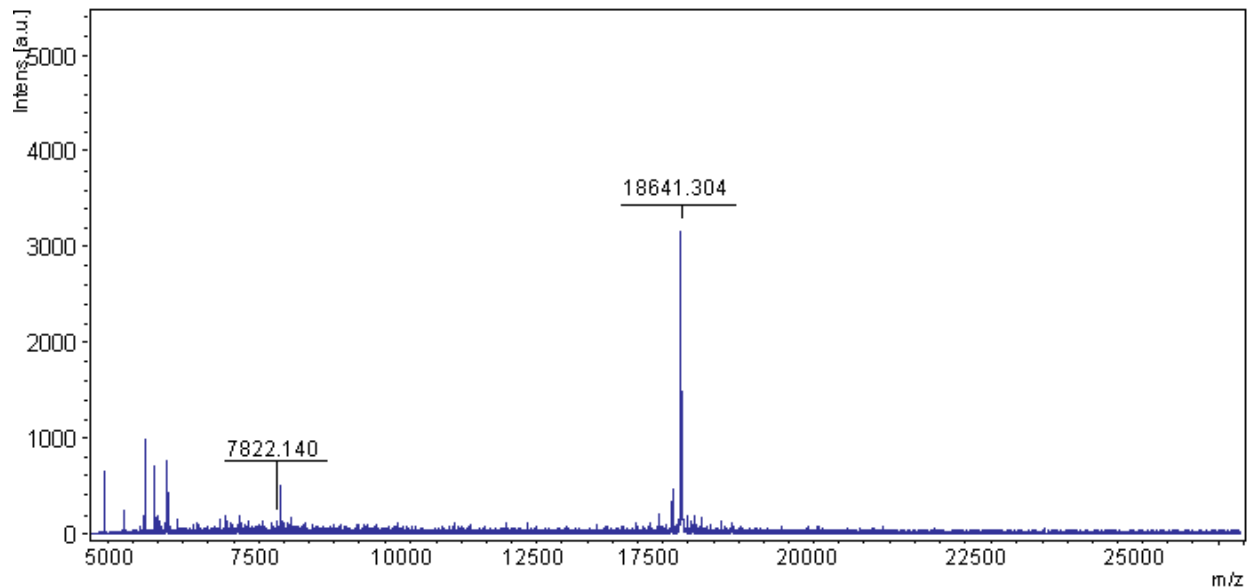


Figure 4. Mass spectrometry analysis MALDI TOF TOF showed the mass (m/z) of intact protein leptin (18 KD).

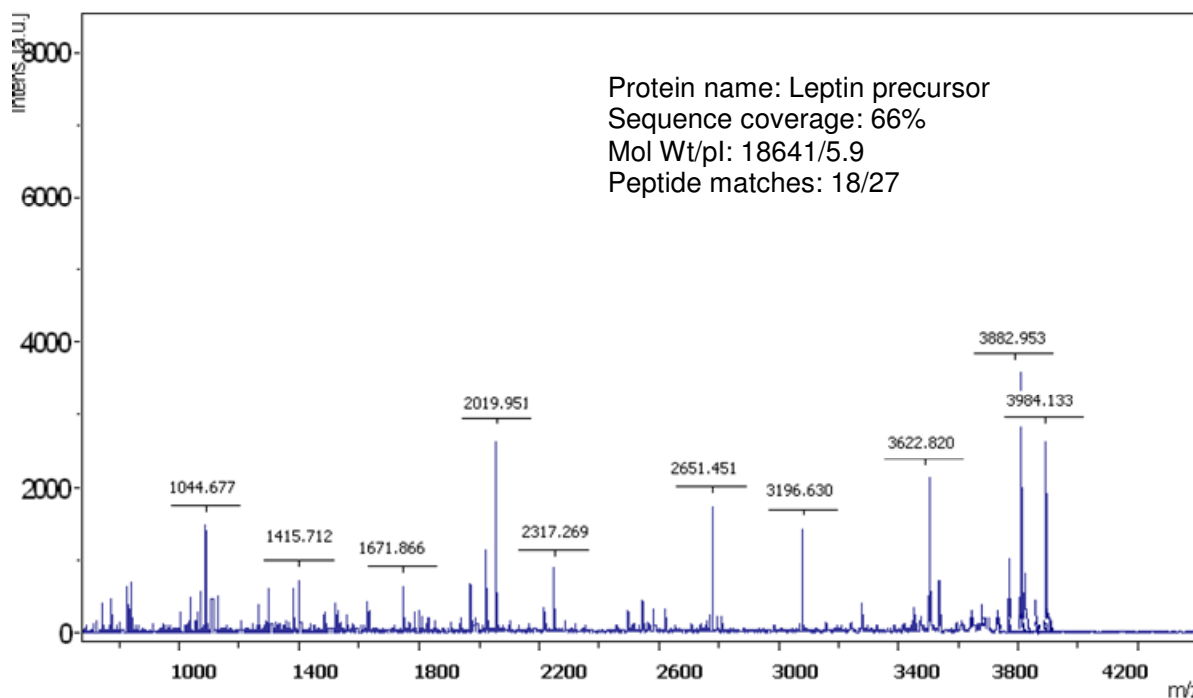


Figure 5. Mass spectrometry MALDI TOF TOF followed by MASCOT analysis confirmed the presence of peptides from protein human leptin precursor (18 KD) after the tryptic digestion.

DISCUSSION

Contributing to the current epidemic levels of obesity in the population are our permissive eating habits and declining physical activity levels. It has been speculated that these environmental factors unmask a genetic susceptibility to obesity, particularly in those individuals with

thrifty metabolic genotype. Regardless of the cause, obesity is an independent risk factor for the development of insulin resistance and the eventual onset of diabetes mellitus (Moran and Phillip, 2003). In this study the effect of obesity on various biochemical parameters of diabetes is studied. The samples were collected locally from Sheikh Zayed Diabetic Clinic. Firstly weight and height of

subjects were taken to calculate body mass index (BMI). BMI is one of the physical parameter of obesity and it is measured in kilogram per meter square (kg/m^2). Another physical parameter of obesity taken into account was waist circumference which was measured in centimeters (cm) with the help of measuring tape. Waist to hip ratio was taken by dividing waist circumference, taken just around the belly button with hip circumference (National Heart Lung Blood Institute, 1998).

On the basis of BMI and waist circumference, 100 diabetics were grouped into two separate categories, that is, diabetic obese with BMI, waist circumference and waist to hip ratio equal to or greater than $30 \text{ kg}/\text{m}^2$, 100 cm and 1.0 respectively and diabetic non-obese with BMI, waist circumference and waist to hip ratio less than $30 \text{ kg}/\text{m}^2$, 100 cm and 1.0 respectively. The 50 control samples were taken randomly but with most individuals having BMI and waist circumference lower than the risk limits. The value of BMI and waist circumference, in case of both diabetic obese and diabetic non-obese, was found to be significant with $p < 0.05$. Similar type of approach was also taken by Thang et al. (2006) in which he made the assessment of obesity following the criteria. The Bradford assay (Bradford, 1976) was performed to estimate total protein content of the serum. We observed an increased protein content in the diabetics as compared to the control and the results were found to be significant with $p < 0.05$. The results are similar to that shown by Carroll et al. (2000) who have done work on plasma viscosity by estimating the total protein and lipoprotein content of the serum. Fasting blood glucose level of subject was determined by enzymatic oxidation method in which red quinoneimine dye is formed which is used as indicator. Much higher level of FBS is seen in diabetic obese group as compare to diabetic non-obese and control, and the results were found to be significant with $p < 0.05$. This signifies the fact that insulin resistance is much more severe and common in obese subjects as compare to non-obese subjects. This means that obesity exacerbates insulin resistance in diabetic subject. Insulin resistance that corresponds to increase FBS increases with increasing BMI, waist circumference and waist hip ratio. Thus the outcome is similar to that obtained by Aronne et al. (2002) that have also shown increased FBS correlated with increased obesity in diabetics. In this study, in diabetic individual a 13% increase in the serum cholesterol level is seen as compare to normal subjects. Both diabetic obese and diabetic non-obese groups show high risk level of cholesterol as compared to control. Results of both the groups, that is, diabetic non-obese and diabetic obese were significant with $p < 0.05$ such as shown by Abbott et al. (1988) who have estimated high serum cholesterol level associated with diabetes and increased risk of myocardial infection in such individuals. Triglycerides (TG) level in serum is very common measure of obesity. It is also one of the factors that lead to insulin resistance in subjects. Muscle insulin resistance is associated with increased intramyocellular triglycerides,

derived from adipose tissues lipolysis (Giorgino et al., 2005). TG level in diabetics is almost doubles when compared with control. This is because diabetic subject sub categorized as diabetic obese, all were obese with BMI and waist circumference greater than $30 \text{ kg}/\text{m}^2$ and 100 cm respectively. When individually taken both diabetic groups, that is, diabetic obese and diabetic non-obese show risk level concentration of triglyceride in their serum but the results were significant only for diabetic obese. This was also the experience of Abbot et al. (1988), who studied high TG levels in diabetic obese. This lipoprotein profile is characterized by increased concentrations of serum triglycerides and low concentration of high-density lipoprotein (HDL) cholesterol. In addition a low-density lipoprotein (LDL) subfraction profile of elevated concentrations of small, dense LDL particles has been shown to be prevalent in diabetic obese patients as proven by Elby et al. (1993). Total lipid concentration in serum is also more in diabetic than in control. Diabetic lipid profile in our study is characterized by high LDL and VLDL concentration and low HDL concentration in the serum while that of control is characterized by high HDL level and low LDL and VLDL level in the serum. The results were significant for HDL and VLDL with $p < 0.05$ but insignificant for LDL results in case of both diabetic obese and diabetic non-obese when compared to control. The results are same as that obtained by Barakat et al. (1992) who have studied the influence of obesity on impaired diabetes mellitus on serum LDL, VLDL and HDL level. Adipose tissues have endocrinal properties and secrete certain proteins, adipokines that have an important role in glucose homeostasis. Some of these adipokines are up regulated in diabetes and obesity while others are down regulated. In our study we have focused on proteins whose levels are increased in people with obesity impaired diabetes mellitus. We have determined the expression level of proteins secreted by adipose tissues with the help of protein profile obtained on SDS PAGE followed by mass spectrometry analysis. One of the protein which is up regulated in diabetes and obesity is leptin a low molecular weight protein (18000 dalton) produced by adipocytes which has an important role in glucose homeostasis, particularly in obese subjects. It serves to produce insulin resistance in obese subjects by binding with insulin receptors and rendering it unable to perform its function. Leptin also directly affects insulin sensitivity by regulating the efficiency of insulin-mediated glucose metabolism by the skeletal muscle (Cohen et al., 1996). Leptin level is increased in diabetic obese subjects in our study and almost negligible in control. An extremely light band of leptin is observed in our sample because the protein is present in very low concentration in serum. Friedman (2002) in his work performed on increased blood glucose level and obesity observed similar results. Expression of this protein is observed in the diabetic obese samples because the biochemical tests have proven most of our diabetic obese subjects having high TG and lipid concentration in blood. Skowron et al. (1996) in their work

on association of α -2-macroglobulin and serum triglyceride level with diabetes mellitus have observed similar results.

Conclusion

In the end it is concluded from this study that adipocytes secreted proteins like leptin not only affect the glucose homeostasis in the blood but also serve as important marker to study the progression of diabetes mellitus in obese individuals. The proteins secreted by adipose tissues in the visceral region play a very important role in glucose homeostasis. Also, obesity itself is a factor that can increase the severity of the disease, especially in the world population where the obesity is the major factor that contributes to the development of diabetes mellitus in elderly type 2 patients. In our results, the adipose tissue related proteins are highly expressed in the diabetic persons who are obese as compared to those which have no obesity and normal healthy control. One of the most important protein is leptin, whose level and expression has been determined and observed for their presence in diabetic obese and not in the control.

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