REPUBLIC OF TURKEY
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APPROVAL

This thesis has been approved by the committee above in conformity to the regulations and by laws of Hacettepe University Graduate Programs and has been accepted by the Board of Directors of the Institute of Health Sciences.

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ÖZET

Dawlaty, B. Diyabetes Mellitus'lu Sıçan Vasküler Doku Protein Profilindeki Değişiklikler ve Beta Blokerlerin Koruyucu Etkisinin Araştırılması. Hacettepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Biyokimya Programı, Yüksek Lisans Tezi, Ankara, 2014. Kardiyovasküler komplikasyonlar diabetes mellitusta başlıca ölüm sebebidir. Beta blokerler diabette ortaya çıkan kalp hastalıklarını tedavi etmede kullanılan bir grup ilaçtır. Bu çalışmanın amacı, streptozotosinle indüklenmiş diyabetik sıçanlarda, kütle spektrometresi profilindeki değişmeleri ve beta bloker grubu ilaçlardan propranolol ve timololün bu profile etkilerini incelemektir. Kontrol, diyabetik, propranolol verilen diyabetik ve timolol verilen diyabetik olmak üzere altışar sıçandan oluşandört grubun vasküler dokuları sıvı azot muamelesi ve sonikasyonla homojenize edildi ve matriks-yardımlı lazer desorpsiyon/ iyonlaştırmalı - uçuş zamanlı - kütle spektrometrisi (MALDI-TOF-MS) spektrumları alındı. Bulguların analizi Mann-Whitney U testi ile, $\alpha = 0.05$ kabul edilerek yapıldı. Sonuçta, dört grubun her birinde 16 pik tespit edildi. Bu piklerden 12'sinin düzeyi kontrol ve diyabetik grup arasında değişim gösterdi. Diyabetik grupta bu piklerden ikisinin yoğunluğu kontrol grubuna göre daha yüksek, diğer on pikin yoğunluğu ise daha düşüktü. Bu 12 pike karşılık gelen protein(ler) diyabetik vasküler dokudaki değişikliklerle ilgili olabilir. Bu piklerden dokuzu propranolol ve /veya timolol ile muamele edilen grupta diyabetik gruptaki eşleniğine göre farklılık gösterdi. Altı pikin düzeyleri hem propranololle hem timololle muamele edilmiş grupta diyabetik gruba göre farklıydı. Bu altı pikten beşinin düzeyi istatistiksel olarak kontroldeki eşleniğinin düzeyinden farksız bulundu. Propranololle muamele edilmiş gruptaki diğer bir pikin ve timololle muamele edilmiş gruptaki diğer iki pikin düzeyleri kontroldeki eşleniklerinin düzeyinden farksızdı. Bu piklere karşılık gelen protein(ler) sıçan diyabetik vasküler dokusunda propranolol ve/veya timololün hedefleri olabilir ve bu ilaçlar verildiği zaman meydana gelen koruyucu olaylarda rol alabilirler. Bu protein(ler)in niteliğini araştırmak, diyabetik komplikasyonların mekanizmasının daha iyi anlaşılmasında ve tedavi için yeni ilaçların geliştirilmesinde önemli olabilir.

Anahtar Kelimeler: Diabetes mellitus, vasküler doku, sıçan, beta blokerler, MALDI-TOF-MS.

ABSTRACT

Dawlaty, B. Changes in the Vascular Tissue Protein Profile of the Diabetic Rats and the Protective Role of the Beta Blockers. Hacettepe University, Institute of Health Sciences Biochemistry Program, Master of Science Thesis, Ankara, 2014.
Cardiovascular complication is the main cause of mortality in diabetes mellitus. Beta blockers are groups of drugs that used in the treatment of heart disease in diabetes mellitus. The object of this study is to investigate the changes in the mass spectrometric profile of the vascular tissue of the streptozotocin induced diabetic rats and the role of beta-blockers propranolol and timolol on this profile. Vascular tissues of four groups of rats (6 in each group): control, diabetic, diabetic treated with propranolol and diabetic treated with timolol were homogenized by liquid nitrogen treatment and sonication and Matrix Assisted Laser Desorption Ionization - Time of Flight - Mass Spectrometry (MALDI-TOF-MS) spectra were obtained. The data was analyzed by Mann-Whitney U test with $\alpha = 0.05$. Results demonstrated 16 peaks in all groups. The intensities of 12 peaks were altered between control and diabetic group. The intensities of two were increased, while the intensities of the rest were decreased as compared to the control. The protein(s) corresponding to these 12 peaks may be involved in the changes that take place in the diabetic vascular tissue. Among these peaks, 9 of them were changed in propranolol and/or timolol treated groups as compared to diabetes group. The levels of the six peaks for both propranolol and timolol treated groups were changed as compared to the corresponding peaks of diabetic group, the levels of the five approaching to the levels of the control. The level of another peak in the propranolol treated and two other peaks in the timolol treated groups were statistically similar to the levels of the corresponding peaks of the control. The protein(s) corresponding to these peaks may be the targets of propranolol and/or timolol in the diabetic vascular tissue of the rat and they may be involved in the protective events that take place when these drugs are administered. It will be of value to investigate the identity of these protein(s) for better understanding of the mechanism of diabetic complications and for development of novel drugs for the treatment of the complications of the disease.

Key Words: Diabetes mellitus, vascular tissue, rat, beta blockers, MALDI-TOF-MS.

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LIST OF ABBREVIATIONS

AGEs	Advanced Glycation End Products
ACE	Angiotensin Converting Enzyme
ACN	Acetonitrile
AMPK	Adenosine Monophosphate Kinase
ARs	Adrenergic Receptors
BSA	Bovine Serum Albumin
DM	Diabetes Mellitus
DAG	Diacylglycerol
DIGE	Difference Gel Electrophoresis
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
ECs	Endothelial Cells
eNOS	Endothelial Nitric Oxide Synthase
ESI	Electron Spray Ionization
ERK	Extracellular Signal-Regulated Kinase
FOXO	Forkhead Box
FPG	Fasting Plasma Glucose
FT-ICR	Fourier Transform Ion Cyclotron Resonance
GLUT2	Glucose Transporter 2
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HDL	High Density Lipoproteins
HbA1C	Hemoglobin A1C (Glycated Hemoglobin)
IRS	Insulin Receptor Substrate
ICAT	Isotope Coded Affinity Tag
KE	Kinetic Energy
LDL	Low Density Lipoproteins
MALDI- TOF-MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MAPK	Mitogen-Activated Protein Kinase
MMP-2	Matrix Metalloproteinase
m/z	Mass to Charge Ratio

NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
OGTT	Oral Glucose Tolerance Test
PARP	Poly ADP - Ribose Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PEDF	Platelet Endothelial Derived Factor
PMSF	Phenylmethylsulfonly Flouride
PKC	Protein Kinase C
PKA	Protein Kinase A
PI3K	Phosphatidylinositol-3-Kinase
RBG	Random Blood Sugar
RAGE	Receptors for AGEs
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
Ryr2	Ryanodine Receptor 2
SNR	Signal to Noise Ratio
STZ	Streptozotocin
TFA	Trifluoroacetic Acid
TRIS	Tris(hydroxymethyl)aminomethane
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell

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1. INTRODUCTION

Diabetes mellitus (DM) is a chronic disease that primarily occurs as a result of disorder of carbohydrate metabolism. The cause of it is deficiency or diminished effectiveness of insulin resulting in hyperglycemia. Secondary changes occur in lipid and protein metabolisms (1).

Insulin is a protein hormone which is secreted by β cells of islets of Langerhans of pancreas. It effects on carbohydrate, protein and lipid metabolism through its receptors. Net effect of insulin is low blood glucose, elevated glycogen synthesis, low level of free fatty acid, high level of triglyceride storage and increased protein synthesis. Besides these, blood potassium and inorganic phosphate are lowered due to enhancement of glycogenesis and phosphorylation of glucose (1).

Mainly two types of DM, Type 1 and Type 2 exist which are manifested as a result of deficiency and resistance to insulin respectively.

There are many factors like genetic, diseases (exocrine and endocrine), drugs and chemicals that can cause DM.

As a result of DM, long term dysfunction and damage of different organs occur which is called complications of DM (2). Cardiovascular complication is the main cause of morbidity and mortality. Beta adrenergic blockers are one of the groups of drugs that lower the mortality rates in DM (3). Propranolol and timolol which are nonselective beta blockers have cardioprotective role in cardiac tissue of streptozotocin induced diabetic rat (4,5).

Streptozotocin (STZ) is a toxic chemical for β cells of pancreas. As illustrated in Figure 1.1 it is 2-Deoxy-2-([(methylnitrosoamino) carbonyl] amino) – D – glucopyranose.

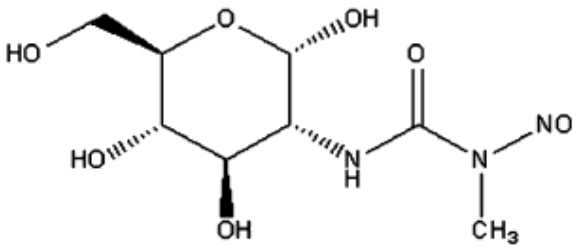


Figure 1.1. Chemical structure of streptozotocin (6).

In STZ, a cytotoxic moiety (N-methyl-N-nitrosourea) is attached to glucose. It is a hydrophylic, β cell toxic glucose analogue, stable for one hour at pH 7.4 and 37°C. It is a protein alkylating agent. STZ is selectively accumulated in pancreatic beta cells via glucose transporter 2 (GLUT2). It causes cell toxicity due to alkylation and as a result of necrosis of beta cells insulin dependent DM (Type I DM) is developed (6).

In this study vascular tissues of Wistar rats were investigated in four groups, namely the non-diabetic group or control group, STZ induced diabetic rats (DM), the group of diabetic rats that are treated with propranolol (DM+Propranolol) and the group of diabetic rats that are treated with Timolol (DM+Timolol). Proteomic profiles of these tissues were investigated by Matrix Assisted Laser Desorption Ionization-Time of Flight –Mass Spectrometry (MALDI-TOF-MS).

In this study, it is aimed to investigate the changes in the proteomic profile of the vascular tissue in type 1 DM with respect to normal control; and try to find out if there is any restoration in the profile in response to beta blockers propranolol and timolol. These findings may shed light to the roles of these drugs in prevention of the diabetic complications.

2. LITERATURE BACKGROUND

2.1. Diabetes Mellitus (DM)

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia (1,7). It is a pandemic disease that affects more than 170 million people worldwide (8). The pattern of DM varies according countries. In developing countries most people who are suffering from DM are between 40 and 60 years of age while in developed countries the majority of diabetic patients are over 60 years (9).

Chronic hyperglycemia of diabetes causes overproduction of advanced glycation end products (AGEs) which are heterogeneous group of compounds formed by the nonenzymatic glycation of proteins, lipids or nucleic acids. AGEs cause long term damage and dysfunction of different organs like eyes, kidneys, nerves, heart and blood vessels that is known as complication of DM (2,10). It plays a major role in the mechanism of vascular damage. DM affects both large and small vessels. The complications can be classified as microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (heart disease, stroke, peripheral arterial disease) complications (10,11). The cause of hyperglycemia and metabolic disorder in DM is inadequate secretion of insulin or diminished response of tissue to insulin (2,7).

2.2. Insulin and its Mechanism of Action

2.2.1. Insulin

Insulin is a protein hormone secreted by β-cells of Langerhans islets of pancreas. It is made up of two polypeptide chains, the polypeptide A and the polypeptide B which have 21 and 30 amino acids respectively. These two chains are linked to each other by two disulphide bonds (cystein 7 and cystein 20 of A chain are linked to cystein 7 and cystein 20 of B chain). There is also an intrachain disulphide bond between Cys-6 and Cys-11 in A chain (1).

Pancreatic β-cells secrete insulin in response to glucose which is taken up by GLUT2. Glucose uptake cause generation of ATP in these cells that result in closing of potassium channels (K-ATP channels). These events increase the resistance of the membrane which cause calcium and sodium channel opening and action potential firing. As a result, K-ATP channel dependent calcium influx cause the secretion of

insulin scretory granules. Release of glucagon like peptide 1 which is a gut hormone enhance secretion of insulin not only by closing K-ATP channels but also by stimulation of cyclic adenosine monophosphate which acts by protein kinase A (PKA) dependent and independent pathways. This is the reason of excessive stimulation of insulin secretion in response to oral glucose as compared to intravenous glucose (12).

2.2.2. Insulin Receptor

Insulin receptor belongs to the subfamily of tyrosine kinase that include insulin-like growth factor I and insulin related receptor. Both of these receptors have α and β subunits that show conformational changes while binding to insulin. As insulin receptor is a tyrosine kinase receptor it undergoes autophosphorylation and catalyzes phosphorylation of the cellular proteins such as insulin receptor substrates (IRS). Both insulin receptors and IRS not only undergo tyrosine phosphorylation but also undergo serine phosphorylation. Serine phosphorylation inhibits tyrosine phosphorylation and attenuates insulin signaling. IRS proteins after tyrosine phosphorylation can interact with signal transduction molecules. This results in a series of signaling like activation of phosphatidylinositol-3-kinase (PI3K) and Ras/mitogen-activated protein kinase (MAPK) pathways (13).

2.2.3. Mechanism of Action of Insulin

The protective effect of insulin on cell is mediated by PI3K pathway while injury mechanisms stimulated by insulin is mediated by Ras/MAPK pathway (14). Insulin through the pathway of PI3K has anabolic effect on the metabolism of proteins, carbohydrates and lipids. It promotes protein and lipid synthesis, increase the rate of glycogenesis and decrease the rates of gluconeogenesis and glycogenolysis (13). Insulin in vascular cells prevent apoptosis by many pathways like expression of vascular endothelial growth factor (VEGF), inhibition of forkhead box (FOXO) proteins (a nuclear factor that up regulation of it promote apoptosis), by activating protein kinase B (14). VEGF expression is normally limited and necessary for angiogenesis. It is required for oxygen supply in the cases of inadequate blood flow or hypoxia through nitric oxide (NO) production. When it is over expressed it causes disease (15,16).

2.3. Types of DM

2.3.1. Type 1 DM

This form of DM which accounts for the 5-10% of the affected individuals is known as juvenile onset diabetes and insulin dependent DM (2). It arises as a result of autoimmune destruction of insulin producing β cells, characterized histologically by insulitis and associated β cell damage. The inflammatory lesion within islets is typically characterized by decrease of β cells with a pancreatic islet cells infiltrate composed of T lymphocytes, B lymphocytes, macrophages and other cells representing the immune response. It is shown that Type 1 DM is not just related to genetic disorders but also environmental factors. Viruses and nutritional factors also play important role in pathogenesis of the disease (17).

2.3.2. Type 2 DM

This type of DM accounts for the 90-95% of the affected. Previously it was referred to as non-insulin dependent DM and adult onset DM. The cause is a combination of insulin resistance to insulin action and an insufficient compensatory insulin response. One of the main risk factors for this type of DM is obesity, but genetic predisposition, age and lack of physical activity are also important factors for development of this type (2).

2.4. Mechanisms of Vascular Damage in DM

As discussed above hyperglycemia is the main factor of vascular damage in diabetes, it affects normal function of vascular tissue by overproduction of AGEs, diacylglycerol (DAG) and oxidants (18).

2.4.1. Vascular Function

Endothelial and smooth muscle cells are the two main components of the vascular unit.

Endothelial cells (ECs) form the inner lining of blood vessels laid on a basement membrane and are surrounded by the smooth muscle cells. These cells play important roles in regulation of blood flow and pressure, permeability, blood fluidity,

thrombotic and fibrinolytic balance and leukocyte traffic. Endothelial cells encounter circulating glucose. Glucose transporter 1 facilitates the uptake of glucose by ECs but during changes in plasma glucose level the activity and expression of this transporter does not change. This describes that hyperglycemia may have profound detrimental effects on ECs. One of the earliest functional changes in DM is the imbalance of vasodilator (NO) and vasoconstrictor (endothelin-1) (19). Dysfunction of ECs is characterized by changes in proliferation, barrier function, adhesion of other circulating cells and sensitivity to apoptosis (7).

Vascular smooth muscle cells (VSMCs, Figure 2.1) are involved in regulation of vascular tone (18,19). During hyperglycemia, an imbalance between cell growth and cell death occurs in the small vessels (which are also called pericytes) and apoptosis in VSMCs decreases (18).

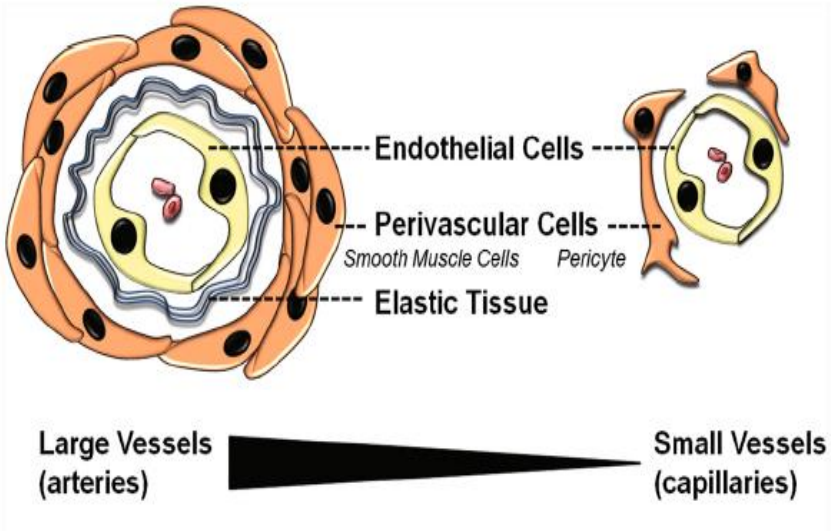


Figure 1.1. Diagram of large and small blood vessels (19).

2.4.2. Advanced Glycation End Products (AGEs)

Advanced glycation end products (AGEs) are a group of heterogeneous compounds formed by the nonenzymatic glycation of proteins, lipids or nucleic acids. This reaction is called “Maillard reaction” a tribute to the French scientist Louis Camille Maillard. This reaction consists of several steps. The first step is the reaction of carbonyl group of a sugar with an amino group of a protein, which is called “Schiff’s

base" (Figure 2.2). As a result of irreversible structural rearrangements, these products change to the stable keto-amines which are called as Amadori products that through oxidation, dehydration and degradation change into AGEs. In physiological conditions these products form slowly. Mechanisms involved in degradation of AGEs are extracellular proteolysis. AGE receptor 1 mediates intracellular uptake and degradation of AGEs. The degradation result in production of soluble peptides that leave the cells, appear in the blood and finally are excreted by kidneys. During hyperglycemia AGEs are overproduced so the vascular homeostasis would be affected in two main ways: receptor dependent and receptor independent. Receptors for AGEs (RAGE) are present on the surface of different cell types like macrophages, adipocytes, vascular smooth muscle cells and endothelial cells. RAGE is mostly involved in the biological effects of AGEs. It is a member of immunoglobulin multiligand receptor family involved in signal transduction. The interaction of AGEs with RAGE triggers the activation of signaling pathways like protein kinase C (PKC). The receptor independent effects include the glycation of low density lipoproteins (LDL), high density lipoproteins (HDL) and matrix proteins. Glycation of LDL increases its uptake by macrophages in the intima layer of vascular tissue. The susceptibility of glycated LDL and also HDL to oxidation is increased which enhance production of reactive oxygen species (ROS). Modification of matrix proteins by AGEs is also receptor independent that cause alteration of the signaling between matrix and cell and cause cellular dysfunction. AGEs reduce endothelial barrier function, increase permeability and subendothelial lipid entry by the receptor dependent pathway. In addition it was reported that endothelial cell processes depend upon intracellular calcium and calcium signaling, one of which is formation of vasodilator NO from arginine catalyzed by NO synthase (eNOS). AGEs cause depletion of intracellular calcium stores and therefore inhibit the formation of NO and promote the generation of ROS. Endothelial angiogenesis is also decreased by AGEs through degradation of VEGF 2. RAGE activation cause vascular smooth muscle cell proliferation and migration by reducing adenosine monophosphate kinase (AMPK) activation. Receptor dependent pathways of AGEs on the surface of monocytes induce formation of mediators of inflammation, and AGEs are chemotactic for blood monocytes. At the side of the injury in the

vascular tissue, AGEs cause migration of inflammatory cells, therefore vascular complication of diabetes is known as an inflammatory disorder (10,20).

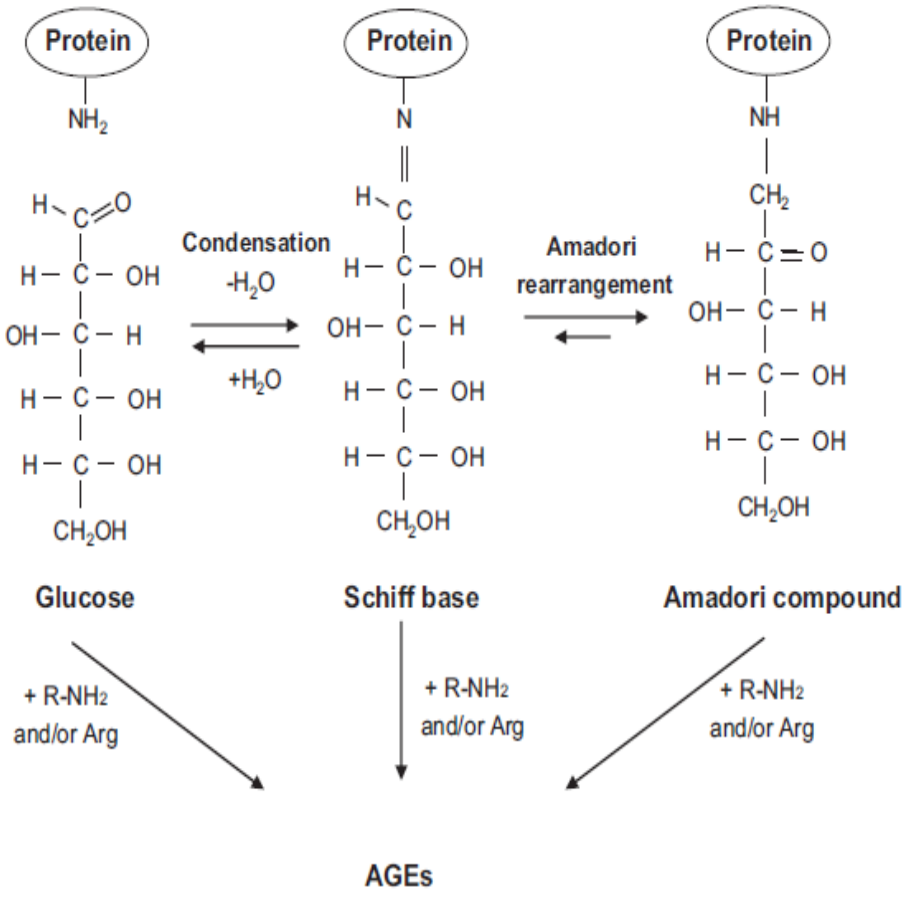


Figure 2.2. The synthesis of advanced glycation end products (10).

2.4.3. Diacylglycerol (DAG)

As described earlier, PKC is activated by AGEs during vascular injury. This enzyme participates in a wide range of cellular signaling. Isoforms of it is classified as classic, novel and atypical. PKCβ and PKCδ belong to the classic and novel groups respectively. Both of them can be activated by DAG which is increased by hyperglycemia induced inactivation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (14).

2.4.4. Oxidants

Hyperglycemia causes overproduction of ROS and reactive nitrogen species (RNS). Major ROS and RNS consist of paramagnetic free radicals like superoxide, NO free radicals, hydroxyl and dimagnetic molecules like peroxynitrite and hydrogen peroxide (21). Several enzymes produce ROS in the vessel wall. Among them, superoxide, is generated by an NADH using oxidase which is thought to resemble phagocytic NADPH oxidase. It is the major source of superoxide production in vascular tissue. This enzyme is activated by an increase in NADH/NAD ratio that is caused by activation of polyol pathway (the pathway that can reduce NAD in sorbitol pathway and consume NADPH in the aldose reductase reaction) during hyperglycemia (14).

NO is the endothelium derived relaxing factor and its role is very important in the normal activity of cells. It is synthesized by endothelial eNOS. NO has vasodilator effect and inhibit platelet aggregation and adhesion and inhibits proliferation of VSMCs and leukocyte adhesion. During oxidative stress that occurs as a result of hyperglycemia, NO reacts with superoxide and form peroxynitrite. In addition, eNOS, instead of forming NO, generate superoxide. This phenomenon is called eNOS uncoupling. Several mechanisms are involved in uncoupling the eNOS, such as oxidation of eNOS cofactor and depletion of arginine (21,22).

Hyperglycemia causes overproduction of NADH oxidase by enhancement of polyol pathway. NADH oxidase causes overproduction of ROS which increase the activity of poly ADP- ribose polymerase (PARP). PARP activity causes inactivation of GAPDH by addition of ADP ribose to it. This inactivation, in turn increases the level of glyceraldehyde 3-phosphate. Increased glyceraldehyde 3-phosphate causes overproduction of DAG which in return ends to activation of PKC (14).

As a result it can be concluded that AGEs, DAG and oxidants cause activation of PKC. PKC inhibits the protective (IRS/AKT/PI3K) pathway because this enzyme can inhibit the tyrosine phosphorylation by phosphorylation of serine and threonine sites on insulin receptor and IRS. Additionally PKC can phosphorylate and inhibit the PI3K regulatory site (P58). Therefore this enzyme inhibits the protective pathway through serine/threonine phosphorylation without affecting the extracellular signal-regulated kinase (ERK)/ MAPK pathway. It can be concluded that in DM, the

ERK/MAPK pathway remains active or even over activated and IRS/AKT/PI3K is inhibited (14,23).

Once PKC is activated it can influence the signaling proteins like ERK/MAPK, functional enzymes like NADPH oxidase, cytokine expression like VEGF and TGF-β and cell cycle factors and transcriptional factors like nuclear factor kappa B (NF-κB) which can cause pro-inflammatory gene expression. The most studied pathway of PKC is DAG/PKC pathway as illustrated in Figure 2.3 (18,24).

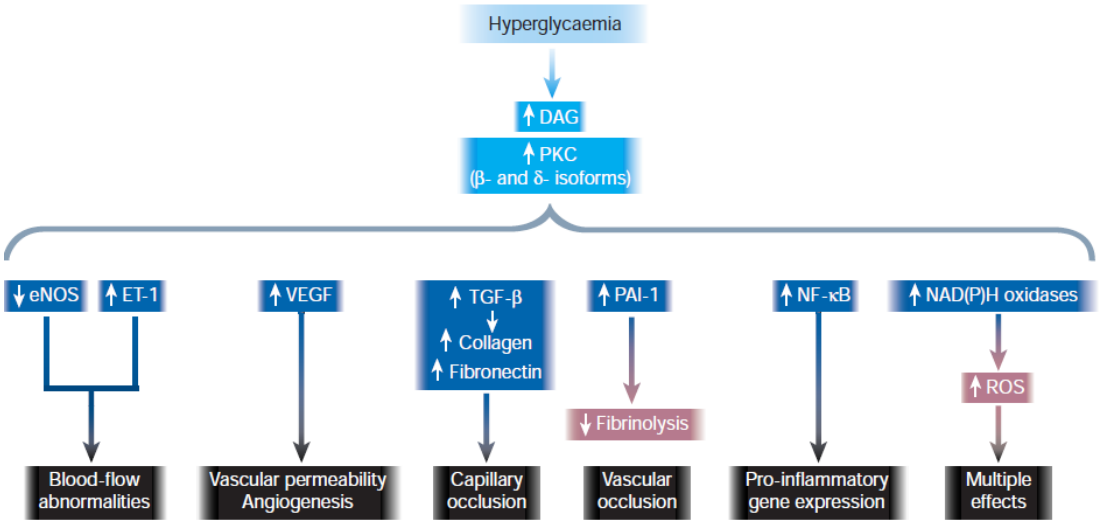


Figure 2.3. Consequences of hyperglycemia and DAG induced PKC pathway (24).

Moreover, hyperglycemia enhances the renin angiotensin system by activating components of this system, angiotensinogen and angiotensin converting enzyme. When this systems is activated the blood pressure increases and blood flow to pancreas decreases which in turn lowers the secretion of insulin (25).

As mentioned earlier, as a result of glycation and oxidants effect, there will be changes in signaling proteins, functional enzymes and transcriptional proteins (18). Hence, for better understanding of the pathogenesis of vascular complications of DM, the changes in proteins in aorta of normal and diabetic rats were investigated. The result was a reduction in glycolytic enzymes and mitochondrial proteins and a profound difference in proteins linked to oxidative stress and structure of myofibrils in diabetic group. Proteins implicated in inflammation were also up-regulated in DM group (26).

Further studies on human atherosclerotic coronary media layer demonstrated the dysregulation of cytoskeleton proteins in media layer. Some of the down regulated proteins are five cytoskeleton proteins (filamin A, gelsolin, beta-tubulin, vimentin and vinculin), heat shock protein 27 (Hsp27) which is linked to actin cytoskeleton organization and antithrombotic protein (platelet endothelial derived factor, PEDF). Down regulation of cytoskeleton proteins and Hsp27 explains the changes of VSMC from contractile to synthetic phenotype, and down regulation of PEDF explains the detachment of media layer from intima layer so that there is no need to synthesize this protein. Besides these, changes of oxidative stress related proteins (carbonyl reductase 3 and superoxide dismutase 3), glycolysis related proteins (phosphoglycerate mutase 1 and triphosphate isomerase) were detected, that imply the oxidative stress response in medial layer (27).

2.5. Diagnosis

As discussed before DM is characterized by hyperglycemia which is due to inadequate insulin secretion or diminished tissue response to insulin. Hence investigating the degree of hyperglycemia has a major role in diagnosis of DM.

Symptoms of marked hyperglycemia are polyuria, polydipsia, polyphagia and blurred vision. In majority of cases especially in type 2 DM a degree of hyperglycemia is enough to cause pathological changes in various target tissues without clinical symptoms. Therefore glycemic values should be investigated for diagnosis of DM. The glycemic markers used as a criteria for diagnosis are fasting plasma glucose (FPG), oral glucose tolerance test (OGTT), hemoglobin A1C (glycated hemoglobin, HbA1C) and for patients with classical symptoms of hyperglycemia random blood glucose (RBG). The level of FPG $\geq$ 126mg/dl, in OGTT 2 hour plasma glucose > 200mg/dl, HbA1C $\geq$ 6.5% and RBG $\geq$ 200mg/dl are accepted as diagnostic criteria for DM. Expert committee on diagnosis and classification of DM recognized the following parameters above normal but do not meet the criteria of diagnosis: HbA1C (5.5-6.4%), FPG (100-125mg/dl) and 2 hour plasma glucose level (140-199mg/dl). This group is classified as pre-diabetic indicating high risk for developing DM in the future (2).

2.6. Treatment

As hyperglycemia is the main cause of micro vascular disease in diabetes, improving glycemic control may improve the micro vascular outcome in diabetic patients (3).

In addition to glycemic control inhibition of AGEs, blockade of AGEs/RAGE system and restriction of food derived AGEs become the novel therapeutic strategies in vascular complications of DM. Heat processing of food containing sugar, lipid and proteins generate AGEs. Food processed by high heat such as over frying contains more AGEs than foods boiling for longer period (28). Several studies showed that restriction of dietary AGEs cause a decrease in circulating AGEs and decrease in endothelial dysfunction and insulin resistance and inflammation in DM. Therefore changing the cooking method (reducing temperature, increasing humidity and decreasing pH) is effective in treatment of DM. Some vitamins like thiamine and pyridoxamine also has an important role in prevention of AGE formation and inhibition of AGEs. Thiamine activates transketolase and enhance the pentose phosphate pathway decreasing the AGEs productive pathway. Pyridoxamine acts as an AGEs inhibitor (10).

PKC inhibitors also can prevent many pathways that are activated by this enzyme. The two PKC inhibitors calphostin C and staurosporine can improve NO production and inhibit the permeability of endothelial cells respectively (18).

Since hyperglycemia causes enhancement of renin-angiotensin system, blocking agents of this system like ACE inhibitors or angiotensin receptor blockers are accepted as drugs of choice for treatment of DM associated with hypertension and renal disease (3).

Cardiovascular disease is the main cause of mortality and morbidity in DM and a group of drugs that lowers the mortality rate and is used safely in heart failure are called beta receptor blockers (3). These drugs are the mostly used drugs for treatment of cardiovascular disorders as hypertension, cardiac arrhythmia and ischemic heart disease (29).

2.7. Beta Adrenergic Receptor Blockers

Adrenergic receptors (ARs) are transmembrane G protein coupled receptors which have a key role in the mediation between sympathetic stimulation and intracellular signal transduction. There are two types of receptors, α receptors and β receptors. The alpha receptors are of two types, α_1 ARs and α_2 ARs. These receptors in VSMCs act as vasoconstrictor and in endothelial cells associate with vasorelaxation.

There are three types of β ARs: β_1-ARs, β_2-ARs and β_3-ARs which are expressed mostly in the epicardial coronary arteries, arteriols of media and endothelial cells of arterioles respectively. The ARs mostly control the vascular tone (30). Stimulation of these receptors in cardiac tissue results in positive chronotropic and inotropic effect and vasodilatation of coronary artery which increase cardiac output and finally lead to high blood pressure which coexists with DM and control of it is the most effective means of reducing cardiovascular risk in diabetic patients (31,32). The β_2-ARs are the most abundant types expressed in vasculature, liver, skeletal muscle and adipocytes and play important role in cardiac function and body metabolism. This receptor not only play role in controlling vascular tone but also can induce insulin resistance through G protein coupled receptor kinase 2. It exerts inhibitory effect on insulin signaling by phosphorylation of insulin receptor and inhibit the tyrosine phosphorylation (33). In addition β_1-ARs via sympathic nerve stimulation cause renin secretion which in turn cause hypertension (34). Therefore blocking of these receptors by antagonists that are called beta receptor blockers have a major role in controlling hypertension associated with diabetic cardiovascular complication (32).

Beta receptor blockers are among the oldest and most widely used antihypertensive drugs. They are very heterogeneous in their pharmacologic properties, hemodynamic effects. Their lipophilicity, β1-ARs selectivity, intrinsic sympathomimetic activity and vasodilatory activity are also variable (35). Lipid solubility determines the degree to which beta blockers penetrate the blood brain barrier and leads to central nervous system side effects. According to selectivity to β_1-ARs these drugs are classified into three groups. The nonselective beta blockers that can block both β_1 and β_2 receptors like propranolol, timolol, pindolol and nadolol, the selective beta blockers that block only the β_1 receptors like esmolol, atenolol and metoprolol and the vasodilatory beta blockers that beside blocking beta receptors have

antagonist effects on α_1 receptors like carvediolol, or have effects on NO release like nebivolol (36). The nonselective, selective and vasodilatory beta blockers are also called first generation, second generation and third generation respectively (35).

According to the chemical structure, these drugs have at least one aromatic ring attached to the alkyl chain which possesses a secondary hydroxyl group and an amine functional group, all of the available beta blockers has one or more chiral carbons residing in alkyl side chain and directly attached to a hydroxyl group. The number of enantiomers differ with the number of chiral carbon. For example propranolol has one chiral carbon and has two (R and S) enantiomers, and is marketed as racemate consisting of two enantiomers. Among the beta blockers timolol is the exception because it is marketed just as S enantiomer (29).

Beta blockers reduce blood pressure by reducing cardiac output, reducing vasomotor tone, inhibiting renin release and generating NO (36). Vasodilating beta blockers show significant antioxidant and antiproliferative effect on vascular tissue. For example nebivolol increase the ratio of NO to peroxynitrite and inhibit proliferation by interfering with cell cycle regulatory signaling (35).

Beta receptor blockers effect on myofibrils of heart and show favorable changes in heart failure (31). Carvedilol which is a third generation beta blocker effects calcium binding proteins, antioxidant proteins and cytosckeletal proteins in cultured aortic smooth muscle cell. This drug especially the S enantiomer lowers calcium binding proteins and cytoskeletal proteins and upregulates the antioxidant proteins like thioredoxine. This way it lowers the intracellular calcium level, cause vasodilation and increase antioxidant activity. Studies reported that the antioxidant activity of this drug is ten times more than propranolol (37). As these drugs have vasodilatory, significant antioxidant and antiproliferative effect, they are used safely in cardiovascular disorders (35) . Studies revealed that not only these beta blockers but nonselective beta blockers like propranolol and timolol have cardioprotective (4,38) and antioxidant (31,39) effects . Therefore these drugs will be discussed in detail.

2.7.1. Propranolol

Propranolol is a nonselective and lipophylic beta blocker (29,40) . According to IUPAC nomenclature propranolol is (*RS*)-1-(1-methylethylamino)-3-(1-

naphthyloxy)propan-2-ol as illustrated in Figure 2.4 (40). As other beta receptor blockers it has a chiral carbon and has S and R enantiomers (29) .

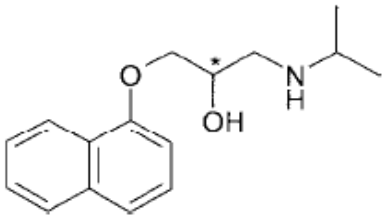


Figure 2.4. Chemical structure of propranolol (40). The chiral carbon is shown by an asterisk.

The beta adrenergic blocking effect resides mainly on S enantiomer while the R enantiomer has only membrane stabilizing effect (40).

Effect of propranolol on cultured peripheral blood mononuclear cell (PBMCs) was investigated. In this research propranolol inhibit the production of VEGF and reduce the viability of PBMCs, which confirm the usefulness of this drug in immunoproliferative and inflammatory disorders (41).

Studies on cultured human umbilical vein cells explained that propranolol can suppress angiogenesis by inducing G1 phase arrest that occurs by modulation of proteins of cell cycle progression. Propranolol decreases the expression of cyclin D1, cyclin D6 and cyclin dependent kinase 6. It also inhibits growth factor, induces ECs proliferation and ERK phosphorylation induced by VEGF. This drug also inhibits the matrix metalloproteinase 2 (MMP-2) secretion (42). Inhibition of VEGF and MMP-2 by propranolol were also reported in activated leukemic cells which support the antiangiogenic effect of this drug (43).

Cardioprotective effect of propranolol was studied in a STZ induced diabetic rat. In diabetic heart, hyper phosphorylation of ryanodine receptor 2 (RyR2) channels (intracellular calcium release channels) through PKA occur as a result of sympathic nerve hyperactivation. This causes a decrease in calcium loading of intracellular calcium stores (sarcoplasmic reticulum) and changes calcium signaling. In addition, FKBP12.6 protein which is a regulator of RyR2 channels is also down regulated in DM. This protein prevents the calcium leakage from RyR2 channels. Propranolol

prevents the hyperphosphorylation of channels and normalizes the level of FKPB12.6 protein. By improving the calcium signaling it impairs myocardial problem (4).

Changes in protein profile of vascular smooth muscle cells by the action of propranolol were observed in cell culture studies. Among 40 proteins that were expressed differentially between control and propranolol, 12 were metabolic enzymes, 9 were signaling molecules, 8 were involved in DNA synthesis/ translation of protein and 11 were cytoskeleton proteins (44) .

2.7.2. Timolol

Timolol is a nonselective beta blocker with chemical nomenclature of [(s)(-)-1-[tertbutylamino]-3-(4-morpholino-1,2,5-thiodiazol-3-yloxy)propan-2-ol] as illustrated in Figure 2.5 (45,46).

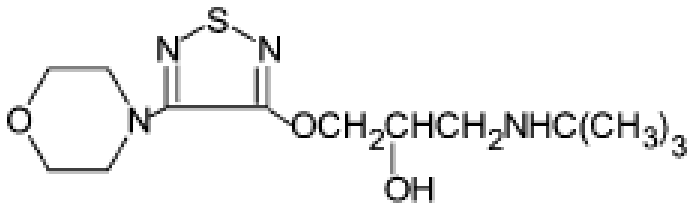


Figure 2.5. Chemical structure of timolol (45).

Findings on the effect of timolol on streptozotocin induced diabetic rat heart indicate that timolol not only prevent hyper phosphorylation of RyR2 channels and normalize the FKBP12 level but also shows a direct ROS scavenger action that is not seen with propranolol which has RNS scavenger action. It was also shown that dyshomeostasis of zinc level, which has a major role in redox signaling and its high level cause dysfunction of RyR2 channels, normalized by timolol (5).

Antioxidant role of timolol was investigated in three different age groups (3 month, 12month and 12 months old) of rats. It was shown that altered left ventricular function is due to the age related ROS production in 12 month age group. Timolol treatment decrease the activity of thioredoxin, GSH/GSSG ratio was regulated, in addition glucose 6-phosphate dehydrogenase activity was also reduced and reached

almost to normal level (38). Timolol not only effect the antioxidant proteins but also it was shown that timolol can change the total protein concentration of aqueous humor of glaucomatous rabbit eye (47).

Vasodilatory effect of timolol on rabbit ciliary artery was studied and it was concluded that this effect is due to reducing influx of excess calcium into VSMCs (48).

In the light of the effects of DM on vascular proteins discussed thus far and the changes of vasculature proteins by propranolol and timolol, it can be concluded that the effects of these drugs on diabetic vascular tissue can be revealed by proteomic analysis of the vascular tissue.

2.8. Proteomics

As proteins are responsible of the phenotype of cells, many types of information such as mechanism of disease and protein modifications cannot be revealed by studying the genes alone. Proteomics is important to characterize such changes (49). Proteomics describes the study and characterization of complete set of proteins present in a cell, organ or organism in a given time (50). The term "proteomics" was first described in 1995 and defined as large scale characterization of the entire protein complement of a cell line, tissue or organism (49). Generally proteomic approaches deal with the comparative analysis of two or more protein samples, proteome profiling, localization and identification of post translational modifications and protein- protein interactions (50).

2.8.1. Types of Proteomics

There are mainly three types of proteomics known as expressional, structural and functional proteomics. Expressional proteomics is the study of protein expression of samples that differ by some variables. By this type of proteomics protein expression between samples can be compared. The goal of structural proteomics is to map out the structure of proteins. It deals with identification of proteins in a sample, their location and protein-protein interactions. Functional proteomics provides information on the signaling, mechanism of disease and protein-drug interactions (49).

2.8.2. Technology of Proteomics

Proteomics deal with the study of protein identification and characterization (50). Several methods exist to resolve the mixture of proteins into individual components, thus proteins can be identified and characterized. A typical proteomics experiment is classified into three stages: the isolation and separation of the proteins, acquisition of the proteins for identification, and characterization of the proteins by utilization of databases (which provide accurate and quick identification of large number of proteins). The oldest and predominant technology for isolation is polyacrylamide gel electrophoresis. One and two dimensional gel electrophoresis is performed for simple and complex mixtures of proteins. Despite its advantages like quantitative and qualitative comparison of protein expression of samples and cell mapping, it has some limitations. It is a time consuming process; a complex of protein cannot be resolved on a single gel; too much acidic and too much basic proteins are not well represented; large and hydrophobic proteins do not enter the gel (49). Therefore alternative methods are developed such as difference gel electrophoresis (DIGE) that can identify protein differences between samples. Liquid based techniques also exist such as isotope coded affinity tag (ICAT) (50, 51). Edman degradation and mass spectrometry are methods used for sequencing proteins (49).

2.9. Mass Spectrometry

Mass spectrometry is an analytical technique in which a molecule can be converted to gaseous ion and separated according to its mass to charge (m/z) ratio (51). Mass spectrometric measurement is carried out on ionized sample (analyte) (52) so the essential features in all mass spectrometry are production of ions, movement and separation of them in mass analyzer and finally detection of ions in mass to charge ratio (51).

2.9.1. Ionization

There are different methods to ionize the sample and according to that different types of mass spectrometers can be used. In electron impact ionization which is used for pharmaceutical compounds, metabolites and pollutants analysis, the molecule of interest is vaporized by heater and ionized by electron beam. In chemical ionization a

reagent gas like methane or ammonia is ionized by electron impact and then this gaseous reagent transfers the ions to molecule (51).

As proteins are thermally unstable, they need an ionization method in which extensive degradation of sample is prevented. Two such techniques, matrix assisted laser desorption ionization (MALDI) and electron spray ionization (ESI) are used widely in proteomics studies.

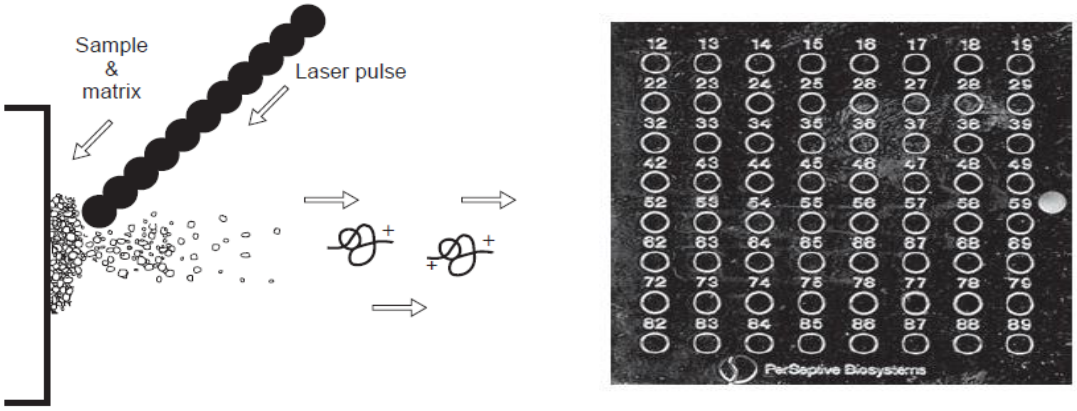
ESI ionize the sample out of a solution, therefore coupled with liquid based separation tools. In this method of ionization the sample is mixed with organic acid and then sprayed into an electrical field. By this way very small droplets of solvent containing sample are created, whereas in MALDI ionization, the sample is ionized out of a dry crystalline matrix (52).

MALDI is first described in 1988 by Hillenkamp and Karas as a nondestructive vaporization and ionization technique of small and large biomolecules (53). In this method the sample which is transferred by ultraviolet absorbing matrix is excited by laser energy (Figure 2.6a). For this reason first the sample is mixed with an organic acid such as derivatives of cinnamic acid and dehyrobenzoic acid which are ultraviolet absorbing matrix. Two derivatives of cinnamic acid which are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and cyano-4-hydroxycinnamic acid are applied for analyzing proteins with molecular mass greater than 10 kDa and peptides less than 10 kDa respectively (51). Matrix solution can be formed by dissolving matrix in acetonitrile (ACN): water (50:50) and adding 0.1% trifluoroacetic acid (TFA).

A mixture of protein and matrix solution is delivered to the plate of MALDI. Sample can be applied in plate of MALDI in different manner like dried drop method and stepwise manners that include thin layer, thick layer and sandwich methods. The mostly used method is dried drop method. In this method matrix solution is prepared in an organic acid as described before, then a mixture of protein sample and matrix solution is prepared and a spot (0.5µl-1µl) is transferred to the plate. After drying the spot in ambient temperature it becomes ready for analysis by MALDI-TOF-MS (53).

Different types of MALDI plates are available. 100 well stainless steel plates are used for multiple sample analyses as shown in Figure 2.6b. Four hundred spot Teflon coated plates are used for concentrating samples. Since the diameter of the spot

is very small, automatic sample spotting is preferred. The sample is concentrated in the center of each spot. Gold coated plates are used when the matrix is organic (51).



(a) (b)

Figure 2.6. (a) Process of ionization of sample by laser energy and movement of ions toward analyzer (53); (b) A hundred spot plate of MALDI (51).

2.9.2 Analysis

Once the sample is ionized it passes through the analyzer where the ions are separated and measured according to their mass at any given time (51). There are four basic types of mass analyzers currently used in proteomics researches: quadrupole, ion trap, time of flight (TOF) and Fourier transfer ion cyclotron (FT-ICR) (52). Quadrupole analyzer contains four rods that are imposed to direct current voltage and radio frequency so along the analyzer a continuous and varying electrical field is generated (51). In ion trap, the ions are first captured for a certain time and then subjected to analysis (52). In FT-ICR analyzer the ions are generated by MALDI or ESI orbiting in a magnetic field, and are excited by radio frequency signals (51). Concept of TOF is acceleration of produced ions toward the detector.

There are two types of TOF used with MALDI, linear TOF and TOF reflectron, as illustrated in Figure 2.7. In linear TOF the accelerated ions move toward detector, as all of the ions have the same energy so the movement toward the detector is according to the mass of ions, the smaller ions move faster than the larger ions hence

the ions reach the detector at different time. The mass is determined from ion's time of flight. In TOF the arrival time to detector is dependent to mass, charge and kinetic energy (KE). Since KE is equal to $1/2mv^2$ where v is the velocity, it can be concluded that $v = (2KE/m)^{1/2}$. As it is known that v=d/t where d is the distance that the ions travel, so t which stands for time is dependent on mass (m/z). In TOF reflectron, the reflectron increases the amount of time thus the kinetic energy is decreased and Δt (the difference between time of movement and time of reaching the detector) is also decreased and hence resolution which is defined as t/Δt is increased.

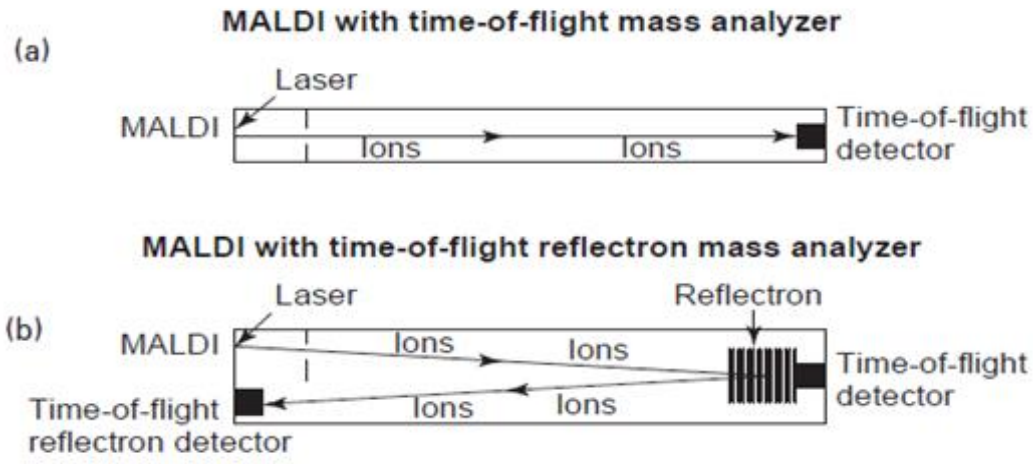


Figure 2.7. (a) MALDI with TOF; (b) MALDI with TOF reflectron (53).

In traditional MALDI-TOF-MS the sample after ionization is accelerated continuously. But before acceleration if the ions are cool, the kinetic energy is decreased so the resolution is increased (Figure 2.8). This cooling is called delay extraction period (53).

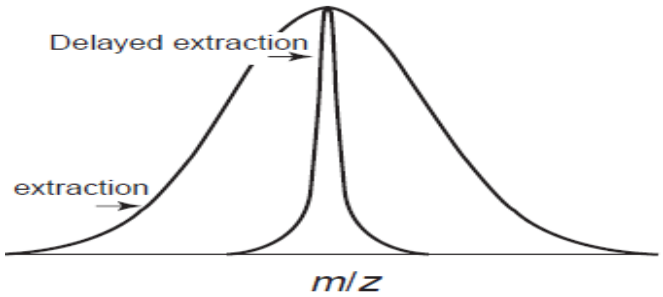


Figure 2.8. Resolution is dramatically improved with delay extraction (53).

2.9.3. Detection

When the ions pass the analyzer, they reach the detector. In most mass spectrometers the electron multiplier is used as detector which is combined with conversion dynode which convert ions to electrons. First the ions touch the conversion dynode. As a result the electrons are released and travel to the next dynode that itself emit secondary electrons. While the electrons travel from one dynode to the other, amplification occurs as shown in Figure 2.9. This cascade continues until a normal amplification is obtained (51).

After the detection of ions, it is reported in computer as a graph of percentage of abundance (intensity) of each protein according to mass to charge ratio. The mass of proteins are illustrated as signals in the graph.

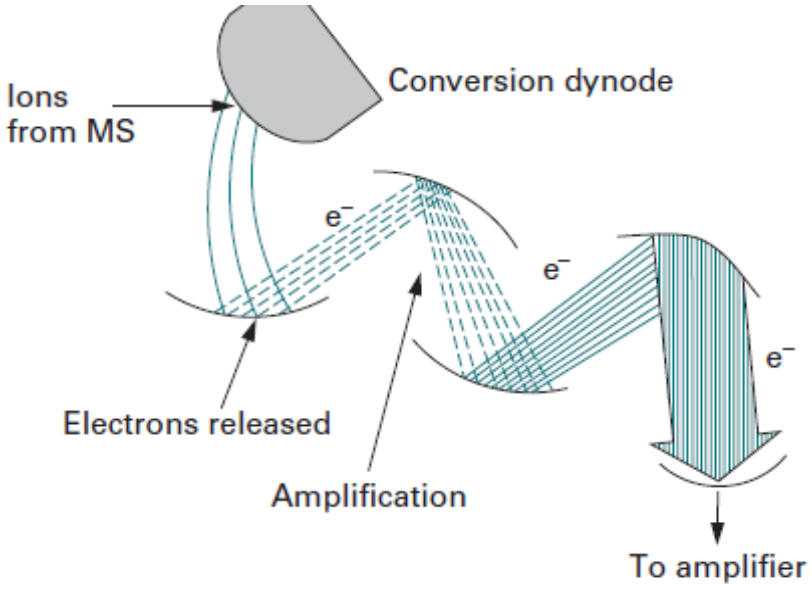


Figure 2.9. Amplification of electrons (51).

3. MATERIAL AND METHOD

3.1. Material

3.1.1. Chemicals

Tris (hydroxymethyl) aminomethane (TRIS), sodium chloride (NaCl), Ethylene diaminetetracetic acid (EDTA) disodium salt dihydrate, dithithreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), octylphenoxypolyethoxyethanol (Igepal),Triton-X-100, isopropranolol, sodium hydroxide (NaOH), bovine serum albumin (BSA), ACN were from Sigma Aldrich, U.S.A.; protease inhibitor complete EDTA free mini tablets (Cat No.04693159001) were from Roche, Swiss; hydrochloric acid (HCl) was from Carlo-Erba, France; sinapinic acid from Acros, USA; TFA from Riedel-De-Haen, USA; Bradford Reagent was from Bio-Rad, Germany. All the chemicals were analytical grade.

3.1.2. Equipment

Freezer (-80°C, Sanyo, Japan), refrigerator (Bosch, Germany), balance (Mettler Toledo AG 204, Swiss), pH-meter (Ikamag, Belgium), magnetic stirrer (Daigger, U.S.A.), sonicator (Sonic,VCx750, U.S:A.), microcetrifuge (Eppendorf Centrifuge 5417R, rotor FA-45-24, Germany), spectrophotometer (UV-VIS-Spectrophotometer, Shimadzu; Japan), ELISA Reader (Spectra Max, M2, U.S.A.), MALDI Plate (100-well, stainless steel), MALDI-TOF-MS (Voyager-DE-Pro, U.S.A.) were used.

3.2. Method

3.2.1. Preparation of the Solutions

Stock Solutions

1- **100 mM stock buffer of TRIS-HCl, pH=7.5:** 3.028 g of TRIS was dissolved in 250 ml water and the pH is adjusted with 1N HCl.

2- **5 M NaCl:** 73 g of NaCl was dissolved in 250 ml of distilled water.

3- 0.5 M EDTA, pH=8: 46.5 g of EDTA was dissolved in distilled water. 10 N NaOH was added drop by drop and the pH was adjusted by pH paper. Volume was brought to 250 ml by distilled water.

4- 0.5 M DTT: Stock solution in 5 ml volume was prepared by dissolving 771.5 mg of DTT in distilled water. Immediately after preparation the aliquot of stock in Eppendorf tubes were stored in -20^{o}C freezer.

5- 100 mM PMSF: Stock solution was prepared by dissolving 87 mg of PMSF in 5 ml of isopropranolol. The aliquots of stock are kept in Eppendorf tubes and stored in -20^{o}C freezer.

6- EDTA free protease inhibitors: Stock solution was prepared by dissolving one tablet in 1430 µl of distilled water.

Extraction Buffer Solution

The protein extraction buffer was prepared with some modifications of the method described by Mourino-Alvarez et al (54). It is composed of TRIS/HCl, pH=7.5, NaCl (prevent nonspecific protein aggregation), Triton X-100 and Igepal, EDTA, pH=8, PMSF, DTT and complete EDTA free protease inhibitor cocktail. The concentrations of these chemicals in extraction buffer is shown in Table 3.1**.**

Table 3.1. Concentrations and volumes of the constituents in the extraction buffer.

Component of extraction buffer	Concentration in extraction buffer	Volume used (µl)
TRIS/HCl, pH=7.5	10 mM	1000
NaCl	250 mM	500
TritonX-100 (100%)	0.05 %	5
Igepal (100%)	0.05 %	5
EDTA, pH=8	1 mM	20
Distilled water	-	6930
PMSF	1 mM	100
DTT	1 mM	10
Complete EDTA free protease inhibitor	One tablet	1430

PMSF, DTT and EDTA free protease inhibitor are directly added to the homogenized sample because these chemicals are inactivated in a short period of time.

3.2.2. Sample Preparation

Tissue samples

Isolated vascular tissues were obtained from Prof. Dr. Belma Turan, Ankara University. Tissues were obtained by a protocol approved by the ethics committee of Ankara University with a reference number of 2007-11-38 (4). In summary, 24 male 3 month old Wistar rats, weighing 200-250 g were divided into 4 groups. The first group was kept as control. The other groups were treated with a single dose of intraperitoneal injection of STZ, 50 mg/kg dissolved in 0.1 M citrate buffer at PH= 4.5) following an overnight fast. The control group was treated with citrate buffer without STZ. Seven days and twelve weeks after the treatment, the onset of DM was proved by blood tests. The second group was the DM group. The third group was treated with propranolol (25mg/kg/day) (4) and the fourth group was treated with timolol (5mg/kg/day) for 12 weeks (5). These groups were DM+propranolol and DM+timolol groups respectively. The DM group received equal amounts of serum physiologic for the same duration. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996). The samples were kept in freezer (-80^{o}C) until used.

Homogenization

The samples were homogenized by modification of the method previously described (54). The sample was frozen inside the mortar with liquid nitrogen and simultaneously grinded with pestle until converted to powdered form. Then it was collected from mortar into Eppendorf tubes and weighted (Table 3.2).

10 µl of protein extraction buffer per mg of sample was used for preparation of the homogenate. The volume of buffer used for each sample is shown in Table 2.3. After adding the extraction buffer to the sample, the sample was kept in ice bath for 40 minutes. The homogenate was sonicated for 10 periods of 30 seconds with breaks of 20 seconds between each period. Sonication was performed (1 second pulse on and 1 second pulse off in order to prevent too much heat) with 40% amplitude. The 3 mm

diameter probe was selected and during sonication the homogenate was kept in ice bath.

The homogenates were centrifuged at 18000 *xg* for 40 minutes at 4^{o}C to remove the cell debris. After centrifugation the supernatants were carefully separated the volumes were measured and kept at -80^{o}C until use.

3.2.3. Measurement of Total Protein Concentration

Bio-Rad Bradford, microassay procedure was used for quantification of total protein (55). In this procedure the range of standard concentration is 8 -80 µg/ml. The following procedure was performed:

1. BSA was used as standard and concentration of 1mg/ml stock of this standard was prepared. For confirmation of concentration of the standard, absorbance of stock solution was measured at 280 nm by spectrophotomer and from the extinction coefficient of BSA ($\varepsilon_{g/dL}$= 6.3) concentration was determined as 1mg/ml .
2. Stock standard was ten times diluted and five standards with concentrations of 12.2 µg/ml, 25 µg/ml, 50 µg/ml, 62.5 µg/ml and 75 µg/ml were prepared from diluted standard (100 µg/ml).Volume of standards to be pipetted to plate was determined and transferred to wells of plates. As total volume of standard should be 160 µl, the volume was completed with 140 µl of distilled water. Triplicate of each standard were prepared. One triplicate without standard protein was prepared and used as a blank.
3. The volume of the Bradford reagent was 40 µl for every standard.
4. Triplicate of each sample were also prepared by adding 5 µl of sample and completing total volume to 160 µl with distilled water and pippeting 40 µl reagent in each well, hence the dilution factor for samples was 32.
5. After incubation for 10 minutes in room temperature, the absorbances were measured by microplate reader at 595 nm. Before reading the plate, in SoftMax software Bradford protein assay was selected, the area of plates were selected for blank, standards and samples. Concentration of standards and dilution factor of samples were introduced. Standard curve was plotted

(Figure 3.1). Concentrations of samples were determined from the standard curve.

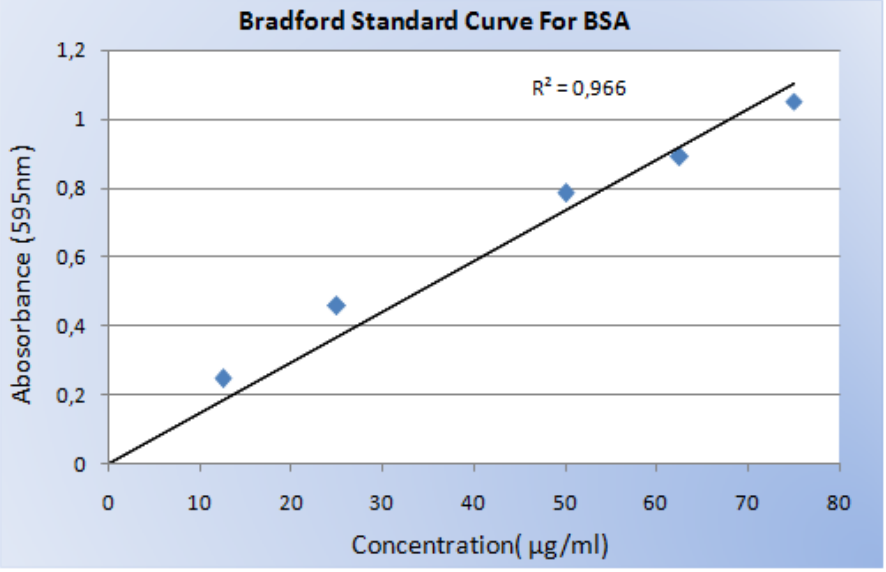


Figure 3.1. Bradford standard curve for BSA

3.2.4. MALDI-TOF-MS Analysis

MALDI-TOF-MS studies were performed at the Chemistry Department Laboratories of the Faculty of Science, Hacettepe University. The instrument was internally calibrated using known matrix ions and their cluster signals for better mass accuracy. For analyses, following procedures were performed (56).

1. Hundred well stainless steel MALDI plate was first cleaned with water followed by ACN. This procedure is repeated three times and followed by sonication in ACN and water (1:1) for 15 minutes, then kept to be dry.
2. Sinapinic acid was used as matrix. 10 mg of this matrix was dissolved in ACN:deionized water (1:1) and 1µl of 0.1% TFA was added.
3. Dried droplet method was applied. For every sample of proteins, in individual Eppendorf tubes, protein to matrix mixture with a ratio of 1:1 was prepared. From this mixture 0.5 µl spot was transferred to the spot places of MALDI plates.

4. After pipetting spots of all samples in plate of MALDI, it was dried by keeping the plate at room temperature for at least 10 minutes.

5. The plate was put in sample inlet of MALDI-TOF-MS and ionization procedure with laser nitrogen (337 nm) was applied. For ion extraction, acceleration potential of 25 kV was used in the source region. Analysis was performed in Data Explorer Software (Applied Biosystem). In order to receive the best specra for every sample, the beam of laser nitrogen was applied to many areas of spots. The best spectra for every sample were selected. Range of mass was considered between 10-25 kDa.

Ratio of amplitude of signal to amplitude of noise, in other words signal to noise ratio (SNR) was calculated for each signal of each sample. Signals with SNR>1 were accepted as significant signals.

3.2.5. Methods for Statistical Analysis

Non parametric Mann-Whitney U test was used for analyzing the results (57). First, differences of SNRs between control and DM group were calculated and then SNRs of DM group and two drug treated groups were compared. To investigate the effect of each drug on DM, SNRs of the drug treated groups were compared to each other and to corresponding SNRs of the control group. For analyzing the data, SPSS version 16 (SPSS Inc. Chicago) was used.

4. RESULTS

4.1. Results of Bradford Assay

Total volumes of the supernatants were measured and the protein concentrations were determined after centrifugation. The results for each sample are shown in Table 4.1.

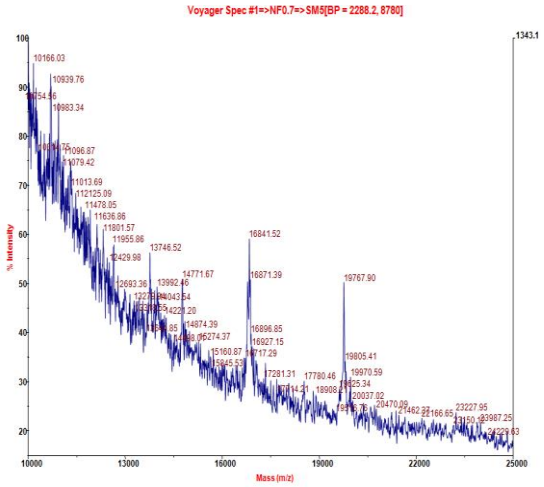
Table 4.1. The weight, total volume and protein concentrations of the samples.

No	Samples	Weight (mg)	Total volume of supernatant (μl)	Concentration of protein (mg/ml)	Concentration of protein (mg)
1	Control-1	7.6	65	0.5276	0.0343
2	Control-2	2.7	20	1.2741	0.0255
3	Control-3	36	270	2.0675	0.5582
4	Control-4	12.4	50	1.9921	0.0996
5	Control-5	16.8	60	1.2963	0.0778
6	Control-6	10.5	25	1.2804	0.0320
7	DM-1	18.5	100	1.8673	0.1867
8	DM-2	102.5	850	2.9162	2.4787
9	DM-3	90	530	2.3316	1.2358
10	DM-4	25.3	220	2.0674	0.4548
11	DM-5	91.8	500	2.4378	1.2189
12	DM-6	26.3	330	0.8219	0.2712
13	DM+Timolol-1	20	80	3.0751	0.2460
14	DM+Timolol-2	43.7	430	1.9967	0.8586
15	DM+Timolol-3	30	190	1.0671	0.2027
16	DM+Timolol-4	57.1	400	2.9922	1.1969
17	DM+Timolol-5	50.7	400	2.8530	1.1412
18	DM+Timolol-6	42.4	400	2.1949	0.8780
19	DM+Propranolol-1	41.3	400	1.9707	0.7883
20	DM+Propranolol-2	66.4	400	1.3923	0.5569
21	DM+Propranolol-3	20	110	1.7123	0.1884
22	DM+Propranolol-4	58.9	500	1.9837	0.9919
23	DM+Propranolol-5	17.1	100	3.5924	0.3592
24	DM+Propranolol-6	39	300	2.5214	0.7564

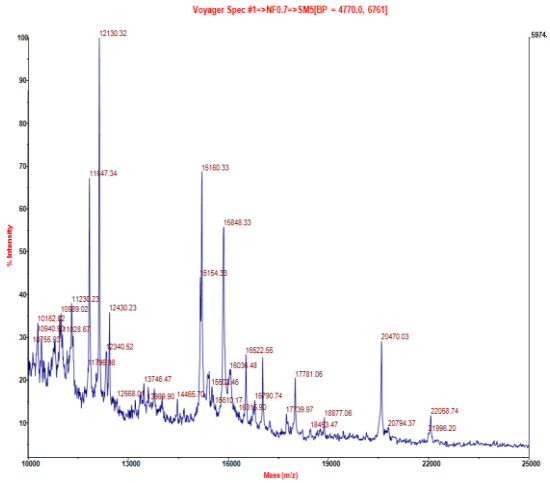
4.2. Results of MALDI-TOF-MS

MALDI-TOF-MS spectra of the samples are shown in Figures 4.1 – 4.4. Since the amplitude of the signals are affected by noise, for determination of the actual amplitude, signal to noise ratio (SNR) for each signal of each spectrum were calculated. Then the mean of the SNR were calculated for each peak of each group. 16 signals with SNR >1 were observed for each group. The results are listed in Table 4.2 - 4.5.

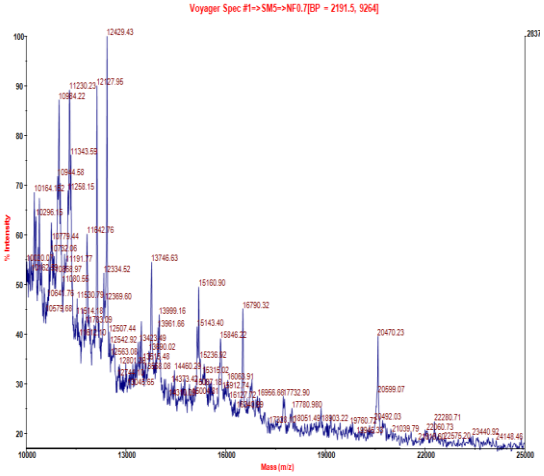
Control 1



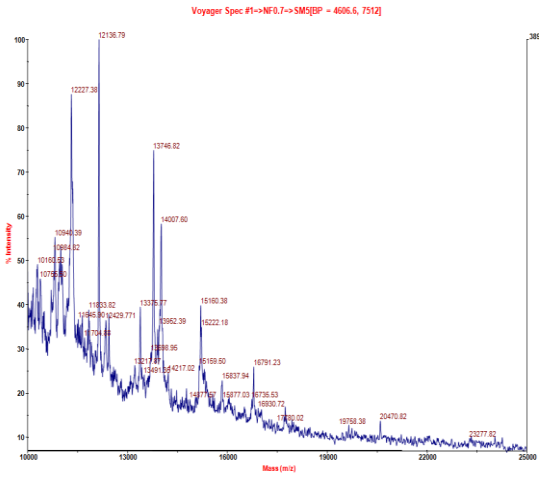
Control 2



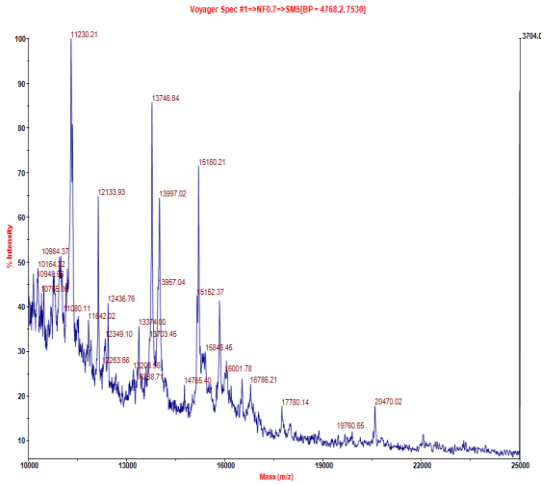
Control 3



Control 4



Control 5



Control 6

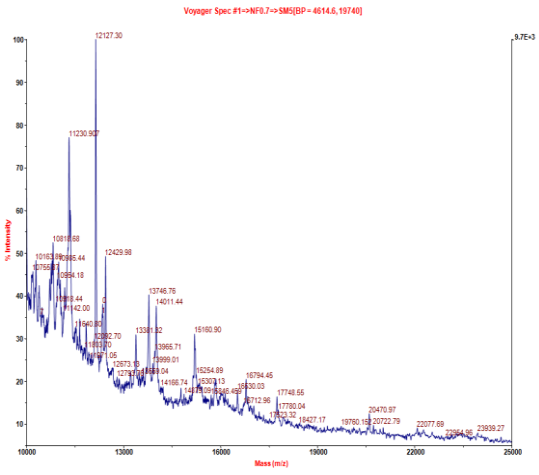
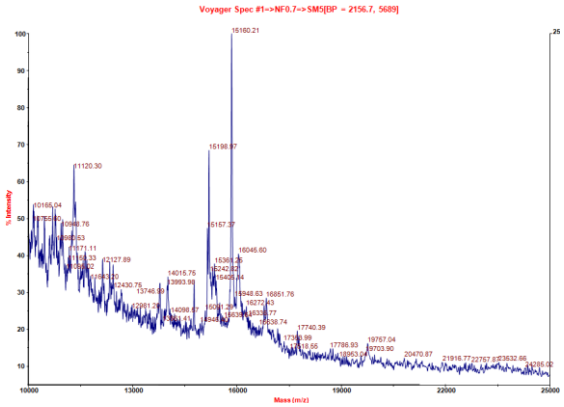
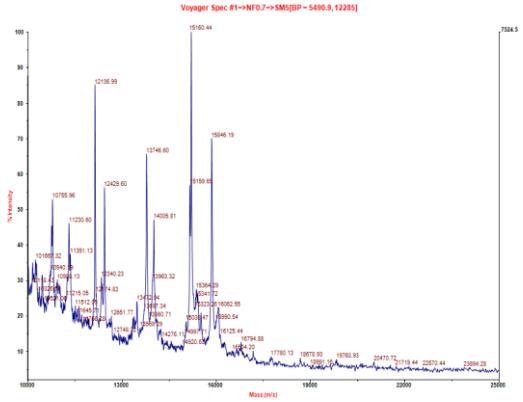


Figure 4.1. MALDI-TOF-MS spectra of control group.

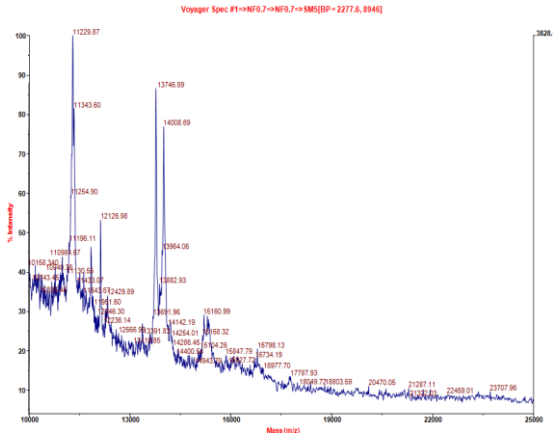
Diabetes 1



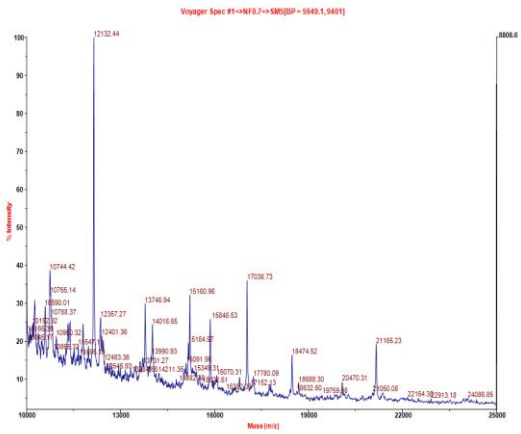
Diabetes 2



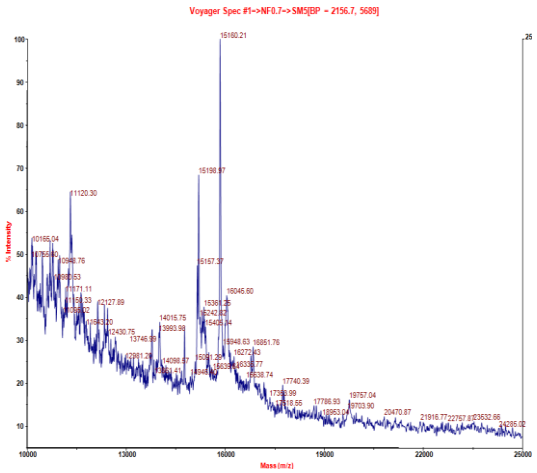
Diabetes 3



Diabetes 4



Diabetes 5



Diabetes 6

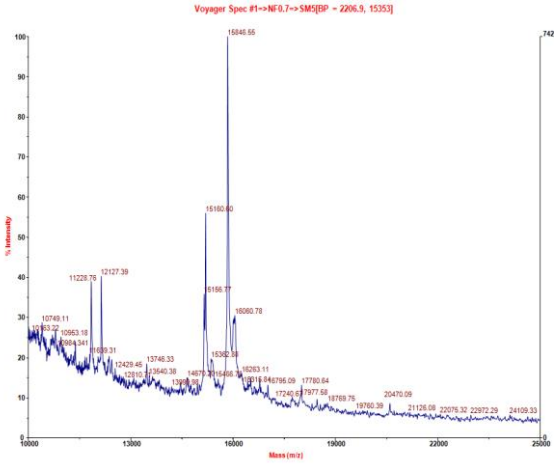
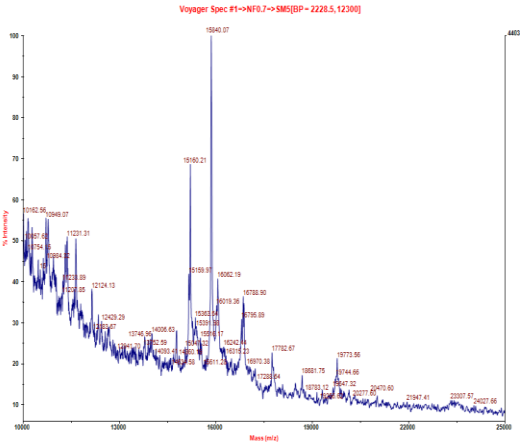
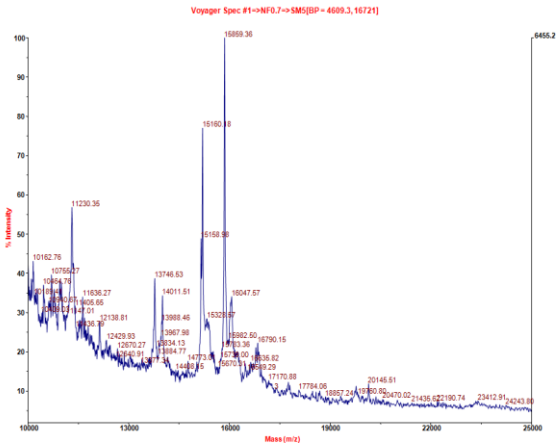


Figure 4.2. MALDI-TOF-MS spectra of DM group

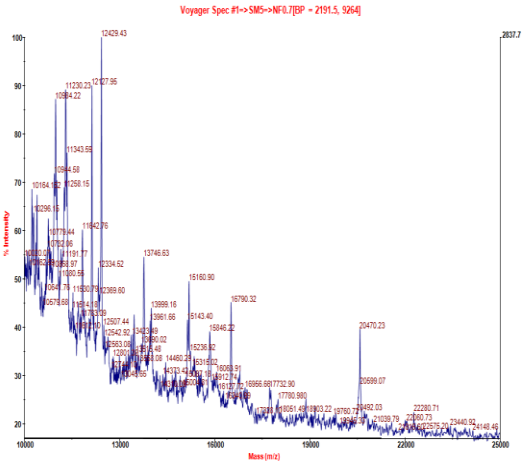
DM+propranolol 1



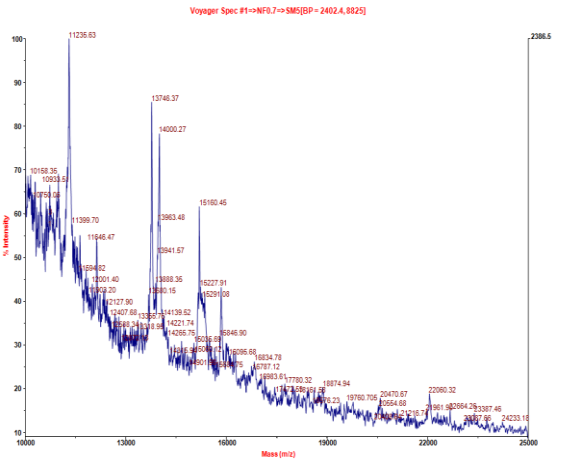
DM+propranolol 2



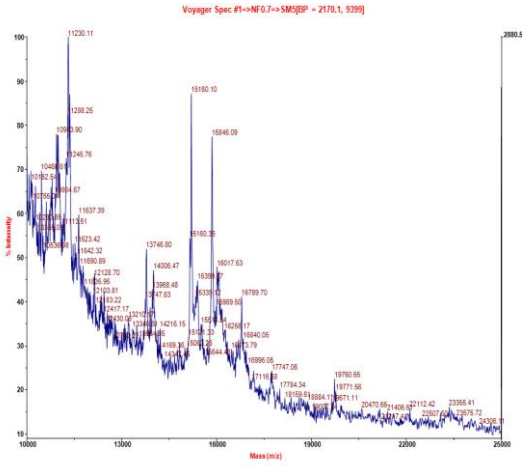
DM+propranolol 3



DM+propranolol 4



DM+propranolol 5



DM+propranolol 6

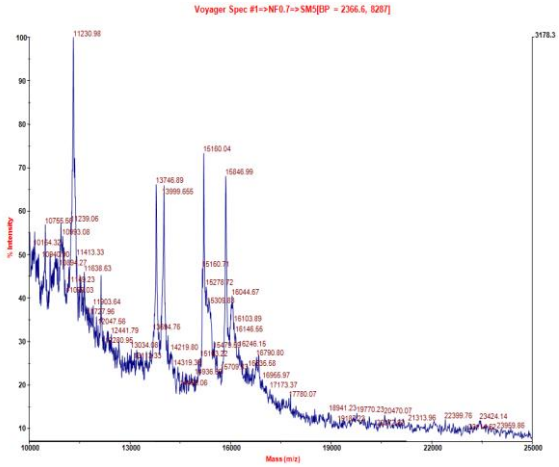
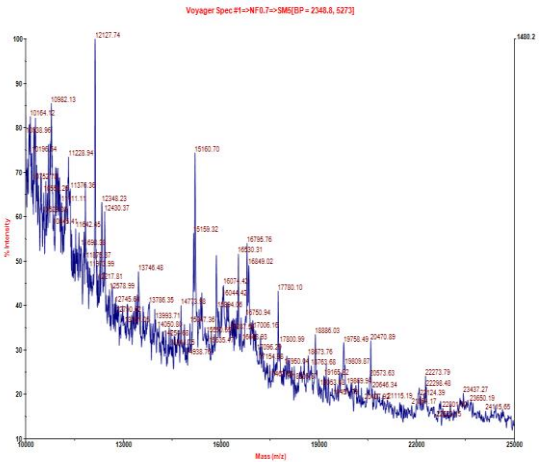
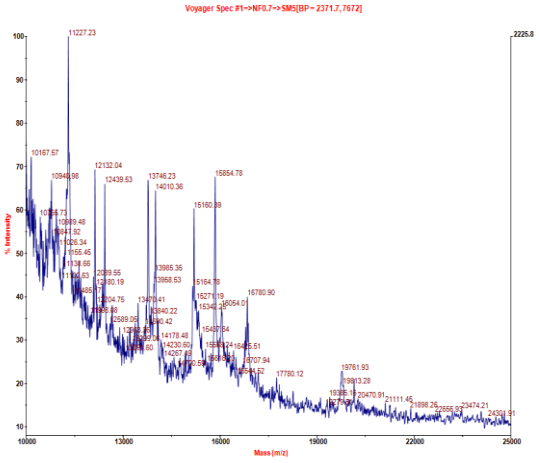


Figure 4.3. MALDI-TOF-MS spectra of DM+Propranolol group

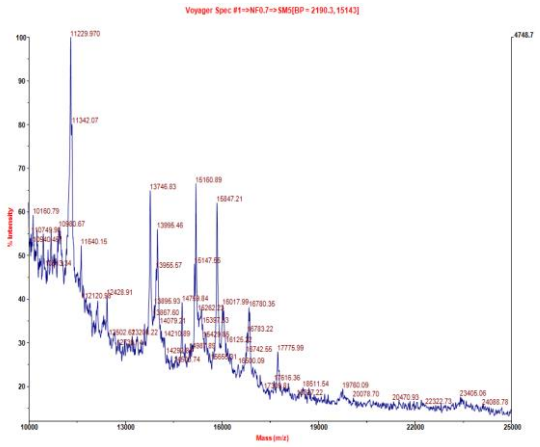
DM+Timolol 1



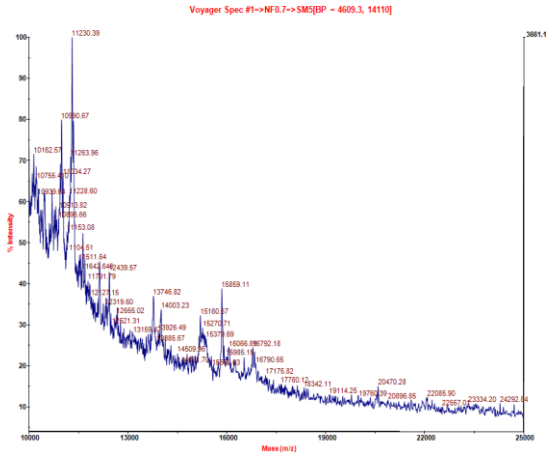
DM+Timolol 2



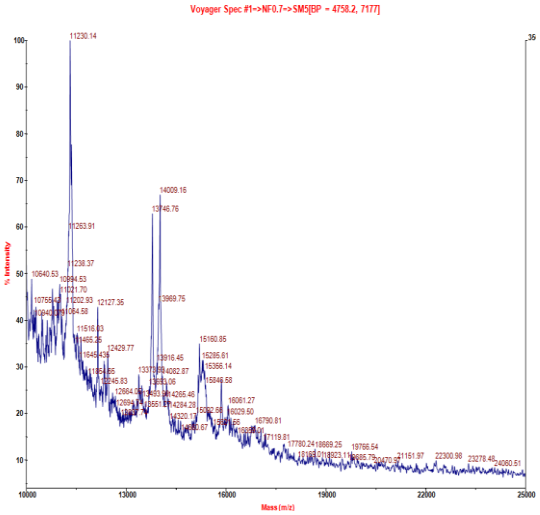
DM+Timolol 3



DM+Timolol 4



DM+Timolol 5



DM+Timolol 6

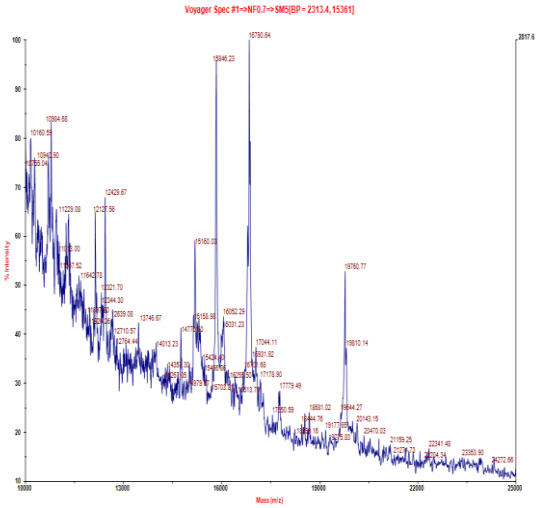


Figure 4.4. MALDI-TOF-MS spectra of DM+Timolol group

Table 4.2. SNR of individual samples of control group

Samples	Signal to noise ratio of the peaks for control group															
	Peak 1 (10164 KDa)	Peak 2 (10755 KDa)	Peak 3 (10940 KDa)	Peak 4 (10984 KDa)	Peak 5 (11230 KDa)	Peak 6 (11642 KDa)	Peak 7 (12127 KDa)	Peak 8 (12429 KDa)	Peak 9 (13746 KDa)	Peak10 (13999 KDa)	Peak 11 (15160 KDa)	Peak 12 (15846 KDa)	Peak 13 (16790 KDa)	Peak 14 (17780 KDa)	Peak 15 (19760 KDa)	Peak 16 (20470 KDa)
Control-1	16.70	6.40	16.50	13.00	42.00	5.40	141.00	37.00	11.10	10.50	17.70	7.90	11.40	7.30	14.60	3.50
Control-2	10.00	11.70	26.90	21.00	26.30	6.20	173.20	47.20	9.00	7.00	61.70	14.60	12.80	11.30	4.40	43.10
Control-3	6.00	8.00	19.70	31.10	34.70	8.00	55.70	72.50	27.00	12.60	17.70	10.70	9.80	7.90	4.60	21.70
Control-4	16.30	5.00	18.90	15.90	47.50	12.90	102.30	14.10	62.70	36.20	23.50	9.30	15.90	7.70	5.20	8.20
Control-5	18.00	11.50	24.40	14.60	47.80	10.00	54.70	20.70	60.00	35.00	61.70	30.00	9.50	11.00	4.40	14.40
Control-6	20.60	7.00	18.00	16.70	57.20	17.40	147.50	51.90	37.90	28.60	25.70	13.40	14.40	13.40	5.00	11.30
Mean	14.60	8.27	20.73	18.72	42.58	9.98	112.40	40.57	34.62	21.65	34.67	14.32	12.30	9.77	6.37	17.03
Median	16.50	7.50	19.30	16.30	44.75	9.00	121.65	42.10	32.45	20.60	24.60	12.05	12.10	9.45	4.80	12.85
Standard deviation	5.48	2.76	4.03	6.64	10.89	4.53	49.79	21.45	23.29	13.11	21.18	8.08	2.55	2.49	4.05	14.16
Maximum	20.60	11.70	26.90	31.10	57.20	17.40	173.20	72.50	62.70	36.20	61.70	30.00	15.90	13.40	14.60	43.10
Minimum	6.00	5.00	16.50	13.00	26.30	5.40	54.70	14.10	9.00	7.00	17.70	7.90	9.50	7.30	4.40	3.50

Table 4.3. SNR of individual samples of DM group

Samples	Signal to noise ratio of peaks for DM group															
	Peak 1 (10164 KDa)	Peak 2 (10755 KDa)	Peak 3 (10940 KDa)	Peak 4 (10984 KDa)	Peak 5 (11230 KDa)	Peak 6 (11642 KDa)	Peak 7 (12127 KDa)	Peak 8 (12429 KDa)	Peak 9 (13746 KDa)	Peak10 (13999 KDa)	Peak 11 (15160 KDa)	Peak 12 (15846 KDa)	Peak 13 (16790 KDa)	Peak 14 (17780 KDa)	Peak 15 (19760 KDa)	Peak 16 (20470 KDa)
Diabetes-1	3.600	15.50	13.00	13.60	44.00	3.00	36.00	10.80	67.60	42.90	11.80	15.00	8.90	5.20	2.90	3.70
Diabetes-2	15.60	63.40	10.00	5.90	40.00	1.75	30.00	18.00	104.10	42.90	90.00	119.60	8.80	5.10	2.30	3.10
Diabetes-3	10.00	14.40	12.00	8.20	26.00	1.50	33.70	8.00	2.00	3.60	6.40	129.00	4.00	4.00	1.50	2.00
Diabetes-4	9.80	52.50	16.20	10.00	23.10	6.00	11.00	19.30	48.70	26.20	25.50	49.70	11.50	8.90	3.30	3.00
Diabetes-5	10.30	10.00	7.20	12.70	8.00	3.00	40.00	9.70	5.00	2.00	87.70	187.60	9.50	4.90	2.70	3.10
Diabetes-6	12.50	13.30	14.50	14.20	18.00	2.70	12.20	13.00	11.80	11.10	41.00	81.80	7.50	7.50	4.40	2.60
Mean	10.30	28.18	12.15	10.77	26.52	2.99	27.15	13.13	39.87	21.45	43.73	97.12	8.37	5.93	2.85	2.92
Median	10.15	14.95	1.50	11.35	24.55	2.85	31.85	11.90	30.25	18.65	33.25	100.70	8.85	5.15	2.80	3.05
Standard deviation	3.95	23.39	3.22	3.30	13.53	1.61	12.48	4.59	41.01	18.69	36.95	61.54	2.51	1.86	0.98	0.57
Maximum	15.60	63.40	1.20	14.20	44.00	6.00	40.00	19.30	104.10	42.90	90.00	187.60	11.50	8.90	4.40	3.70
Minimum	3.60	10.00	7.20	5.90	8.00	1.50	11.00	8.00	2.00	2.00	6.40	15.00	4.00	4.00	1.50	2.00

Table 4.4. SNR of individual samples of DM + Propranolol group

Samples	Signal to noise ratio of peaks for DM + Propranolol group															
	Peak 1 (10164 KDa)	Peak 2 (10755 KDa)	Peak 3 (10940 KDa)	Peak 4 (10984 KDa)	Peak 5 (11230 KDa)	Peak 6 (11642 KDa)	Peak 7 (12127 KDa)	Peak 8 (12429 KDa)	Peak 9 (13746 KDa)	Peak10 (13999 KDa)	Peak 11 (15160 KDa)	Peak 12 (15846 KDa)	Peak 13 (16790 KDa)	Peak 14 (17780 KDa)	Peak 15 (19760 KDa)	Peak 16 (20470 KDa)
DM+propranolol-1	16.50	10.70	16.30	12.70	13.20	5.00	39.00	12.20	6.20	7.50	29.20	28.00	14.00	10.00	10.50	4.50
DM+propranolol-2	18.70	10.20	20.10	18.20	36.10	6.00	38.00	9.50	4.90	13.20	92.70	9.00	12.50	6.20	7.20	3.50
DM+propranolol-3	11.00	2.80	12.00	15.00	4.00	6.80	40.00	7.00	8.40	1.70	2.20	10.00	3.80	2.90	1.60	1.80
DM+propranolol-4	9.00	6.50	17.80	13.30	34.90	7.00	47.00	7.60	42.00	23.90	26.60	38.00	6.00	5.90	6.00	6.20
DM+propranolol-5	14.50	9.80	19.70	16.70	33.60	5.50	36.40	11.60	21.10	12.20	48.60	20.00	15.60	5.60	6.20	4.20
DM+propranolol-6	12.10	6.50	16.00	17.00	39.30	9.20	50.00	7.40	38.30	34.40	39.20	29.00	4.90	5.20	4.40	2.50
Mean	13.63	7.75	16.98	15.48	26.85	6.58	41.73	9.22	20.15	15.48	39.75	22.33	9.47	5.97	5.98	3.78
Median	13.30	8.15	17.05	15.85	34.25	6.40	39.50	8.55	14.75	12.70	34.20	24.00	9.25	5.75	6.10	3.85
Standard deviation	3.62	3.05	2.97	2.19	14.56	1.49	5.46	2.26	16.57	11.82	30.25	11.47	5.14	2.30	2.95	1.56
Maximum	18.70	10.70	20.10	18.20	39.30	9.20	50.00	12.20	42.00	34.40	92.70	38.00	15.60	10.00	10.50	6.20
Minimum	9.00	2.80	12.00	12.70	4.00	5.00	36.40	7.00	4.90	1.70	2.20	9.00	3.80	2.90	1.60	1.80

Table 4.5. SNR of individual samples of DM + Timolol group

Sample	Signal to noise ratio of peaks for DM + Timolol group															
	Peak 1 (10164 KDa)	Peak 2 (10755 KDa)	Peak 3 (10940 KDa)	Peak 4 (10984 KDa)	Peak 5 (11230 KDa)	Peak 6 (11642 KDa)	Peak 7 (12127 KDa)	Peak 8 (12429 KDa)	Peak 9 (13746 KDa)	Peak10 (13999 KDa)	Peak 11 (15160 KDa)	Peak 12 (15846 KDa)	Peak 13 (16790 KDa)	Peak 14 (17780 KDa)	Peak 15 (19760 KDa)	Peak 16 (20470 KDa)
DM+timolol-1	10.80	11.00	15.00	17.30	10.90	7.30	55.00	21.50	2.50	5.10	26.40	15.10	14.90	2.20	6.30	11.30
DM+timolol-2	12.70	11.60	10.00	10.20	34.60	12.10	62.00	35.60	29.20	7.80	22.90	41.00	13.10	5.10	5.50	3.20
DM+timolol-3	10.00	13.00	17.00	19.90	13.10	14.00	42.00	18.00	13.50	9.00	14.50	14.40	3.10	3.60	2.10	3.20
DM+timolol-4	19.00	8.00	18.00	32.80	46.70	19.60	30.00	16.70	13.00	20.00	12.30	24.80	8.50	6.40	5.60	8.40
DM+timolol-5	18.30	16.10	16.90	18.30	57.60	11.30	46.00	16.10	51.90	38.10	18.70	18.00	7.00	5.50	6.60	3.80
DM+timolol-6	14.90	19.10	21.00	13.40	17.10	10.00	44.00	26.00	7.50	5.20	21.00	49.00	43.00	7.90	25.00	3.80
Mean	14.28	13.13	16.32	18.65	30.00	12.38	46.50	22.32	19.60	14.20	19.30	27.05	14.93	5,12	8.52	5.62
Medain	13.80	12.30	16.95	17.80	25.85	11.70	45.00	19.75	13.25	8.40	19.85	21.40	10.80	5.30	5.95	3.80
Standard deviation	3.21	3.34	3.11	6.58	16.45	3.54	9,36	6.38	15.54	11.12	4.45	12.50	12.25	1.71	7.02	2.96
Maximum	19.00	19.10	21.00	32.80	57.60	19.60	62.00	35.60	51.90	38.10	26.40	49.00	43.00	7.90	25.00	11.30
Minimum	10.00	8.00	10.00	10.20	10.90	7.30	30.00	16.10	2.50	5.10	12.30	14.40	3.10	2.20	2.10	3.20

4.3. Results of Statistical Analysis

Differences of SNR of individual peaks between groups were investigated by Mann-Whitney U test. For difference between groups, $p < 0.05$ was accepted as significant. Values of p for different groups are shown in Table 4-6.

These 16 peaks can be categorized into two main groups. The first group consists of four peaks which do not change in DM (Peak 1, Peak 9, Peak 10 and Peak 11), $p > 0.05$. Statistical analysis of these peaks is shown in Figure 4.5.

The second group of 12 peaks is changed in DM (Peak 2, Peak 3, Peak 4, Peak 5, Peak 6, Peak 7, Peak 8, Peak 12, Peak 13, Peak 14, Peak 15, Peak 16), $p<0.05$. This second group can further be divided into two subgroups with respect to their response to propranolol and/or timolol.

The intensities of the first subgroup of three peaks were decreased in DM but did not change significantly in response to neither propranolol nor timolol treatment (Peak 5, Peak 13 and Peak 14), $p>0.05$. Statistical analyses of these peaks are shown in Table 4.7 and Figure 4.6.

The nine peaks in the second subgroup were Peak 2, Peak 3, Peak 4, Peak 6, Peak 7, Peak 8, Peak 12, Peak 15, and Peak 16. SNR of peak 2 and 12 were increased while the SNR of the rest were decreased in DM. SNR of all were changed in response to propranolol and/or timolol, ($p < 0.05$).

Among these nine peaks, six of them (Peak 3, Peak 4, Peak 6, Peak 7, Peak 12, Peak 15) were changed by both propranolol and timolol ($p < 0.05$, Figure 4.7).

One peak (Peak 2) was affected only by propranolol and two peaks (Peak 8, Peak 16) were affected only by timolol ($p < 0.05$, Figure 4.8). Analyses of these peaks are shown in Table 4.8 and Table 4.9.

Peak 7 which was affected by both propranolol and timolol was not restored to control level by the action of these drugs, ($p<0.05$); and Peak 12 which was influenced by both drugs attained the control level by propranolol ($p >0.05$) but influenced only partially by the action of timolol ($p<0.05$). The remaining 7 peaks which were affected by propranolol and/or timolol did not differ significantly from the control values ($p>0.05$). Figure 4.9 shows the SNR of all 16 peaks in all four groups of treatment.

Table 4.6. Values of p for detected signals among different groups

Peaks	Values of p					
	Control vs DM	DM vs DM+Propranolol	DM vs Timolol	Control vs DM+Timolol	Control vs DM+Propranolol	DM+Timolol vs DM+Propranolol
1 (10164 KDa)	0.128	0.200	0.092	0.936	0.631	0.749
2 (10755 KDa)	**0.010**	**0.010**	0.262	**0.045**	0.748	**0.016**
3 (10940 KDa)	**0.010**	**0.030**	**0.037**	0.873	0.575	0.873
4 (10984 KDa)	**0.004**	**0.030**	**0.045**	0.092	0.128	0.873
5 (11230 KDa)	**0.037**	0.873	0.873	0.200	0.078	0.262
6 (11642 KDa)	**0.006**	**0.013**	**0.004**	0.297	0.150	**0.006**
7 (12127 KDa)	**0.004**	**0.020**	**0.020**	**0.016**	**0.004**	0.337
8 (12429 KDa)	**0.010**	0.078	**0.045**	0.150	**0.004**	**0.004**
9 (13746 KDa)	1.000	0.522	0.749	0.262	0.200	1.000
10 (13999 KDa)	0.873	0.630	0.630	0.148	0.423	0.749
11 (15160 KDa)	0.872	0.873	0.423	0.199	0.520	0.055
12 (15846 KDa)	**0.006**	**0.025**	**0.037**	**0.037**	0.337	0.631
13 (16790 KDa)	**0.020**	0.749	0.631	0.631	0.337	0.631
14 (17780 KDa)	**0.025**	0.575	0.575	**0.013**	**0.016**	0.522
15 (19760 KDa)	**0.006**	**0.045**	**0.037**	0.199	0.629	0.873
16 (20470 KDa)	**0.006**	0.336	**0.010**	0.064	**0.020**	0.520

Table 4.7. Peaks that are not affected by propranolol and timolol

Peaks	Values of p for peaks that are changed in DM but not affected by propranolol and timolol				
	Control vs DM	DM vs DM+Propranolol	DM vs Timolol	Control vs DM+Timolol	Control vs DM+Propranolol
5 (11230 KDa)	**0.037**	0.873	0.873	0.200	0.078
13 (16790 KDa)	**0.020**	0.749	0.631	0.631	0.337
14 (17780 KDa)	**0.025**	0.575	0.575	0.013	0.016

Table 4.8. Peaks that are restored to control level by propranolol

Peaks	Values of p for peaks that are changed to control level by propranolol		
	Control vs DM	DM vs DM+Propranolol	Control vs DM+propranolol
2 (10755 KDa)	**0.010**	**0.010**	0.748
3 (10940 KDa)	**0.010**	**0.030**	0.575
4 (10984 KDa)	**0.004**	**0.030**	0.128
6 (11642 KDa)	**0.006**	**0.013**	0.150
12 (15846 KDa)	**0.006**	**0.025**	0.337
15 (19760 KDa)	**0.006**	**0.045**	0.629

Table 4.9. Peaks that are restored to control level by timolol

Peaks	Values of p for peaks that are changed control level by timolol		
	Control vs DM	DM vs DM+timolol	Control vs DM+timolol
3 (10940 KDa)	**0.010**	**0.037**	0.873
4 (10984 KDa)	**0.004**	**0.045**	0.092
6 (11642 KDa)	**0.006**	**0.004**	0.297
8 (12429 KDa)	**0.010**	**0.045**	0.150
15 (19760 KDa)	**0.006**	**0.037**	0.199
16 (20470 KDa)	**0.006**	**0.010**	0.064

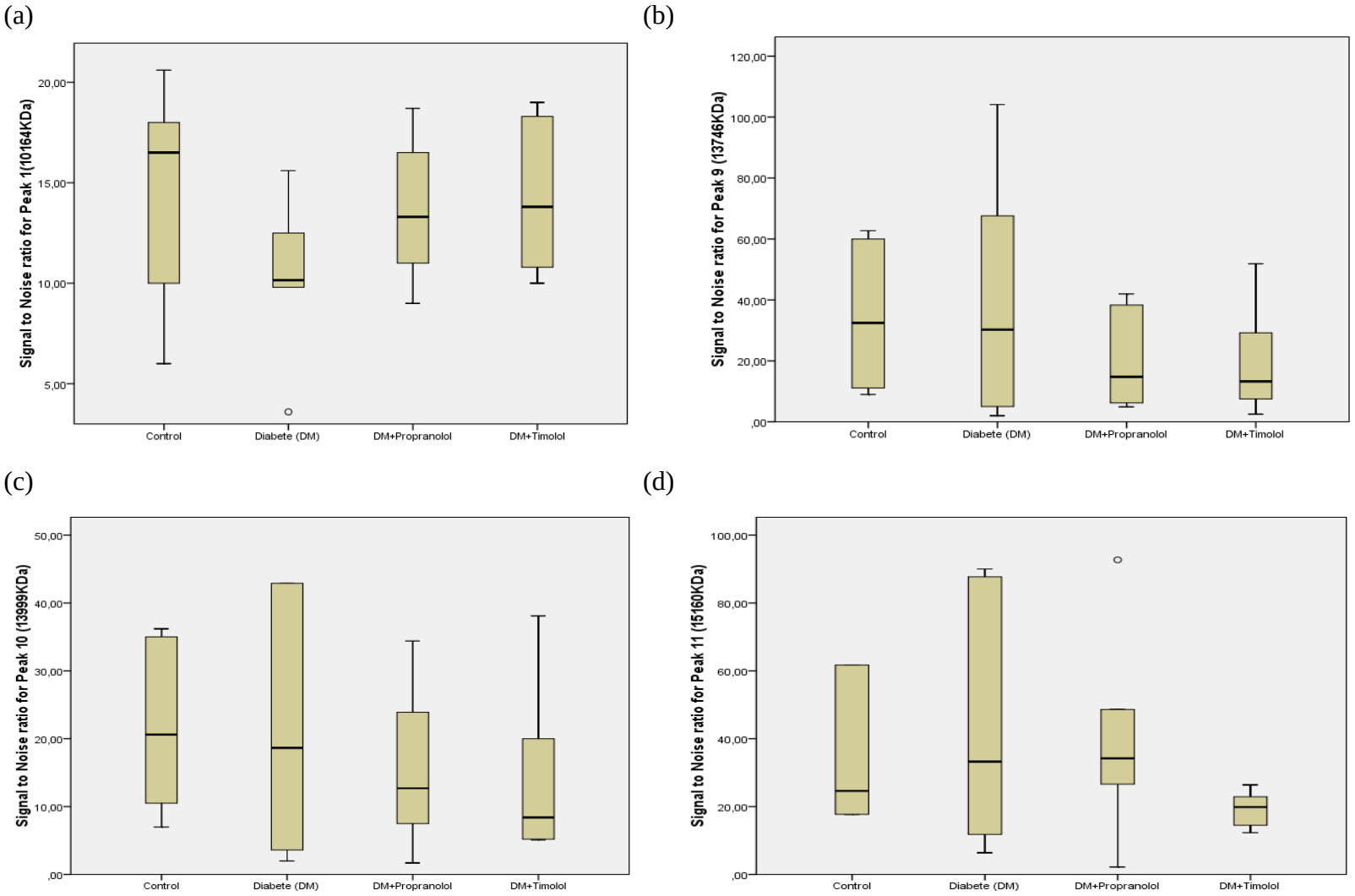


Figure 4.5. SNR for (a) Peak 1, (b) Peak 9, (c) Peak 10, (d) Peak 11 in each group ($p>0.05$).

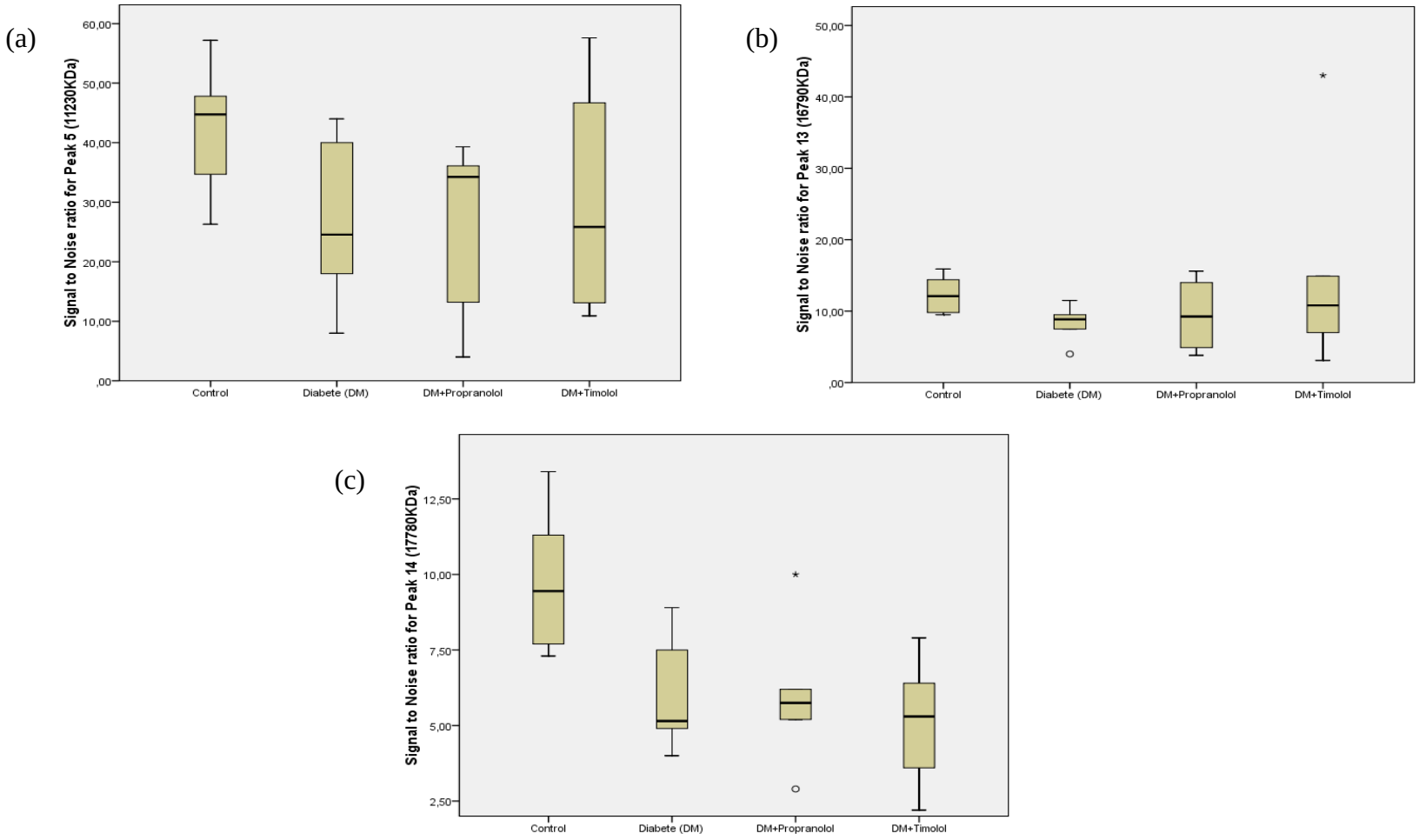
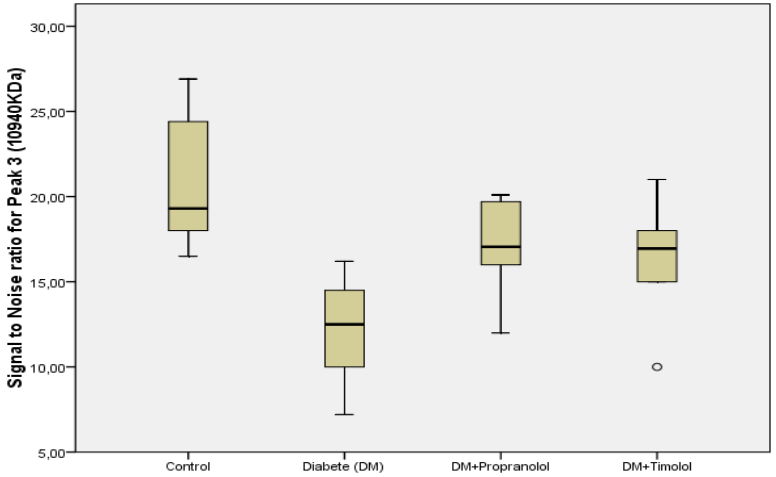
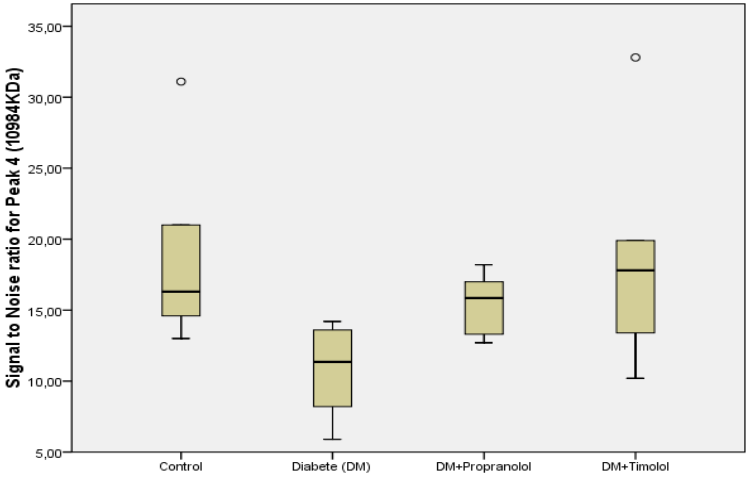


Figure 4.6. SNR for (a) Peak 5, (b) Peak 13, (c) Peak 14 in each group. For control and DM groups: $p<0.05$, and for other groups: $p>0.05$.

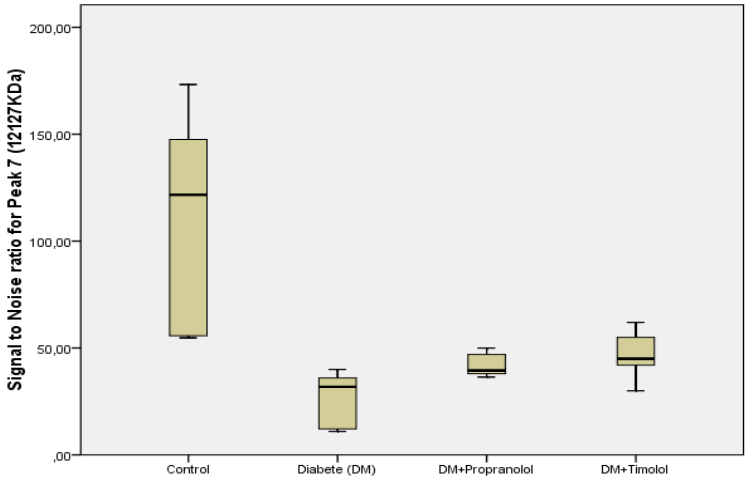
(a)


Signal to Noise ratio for Peak 3 (10940KDa)
30,00
25,00
20,00
15,00
10,00
5,00
Control
Diabete (DM)
DM+Propranolol
DM+Timolol

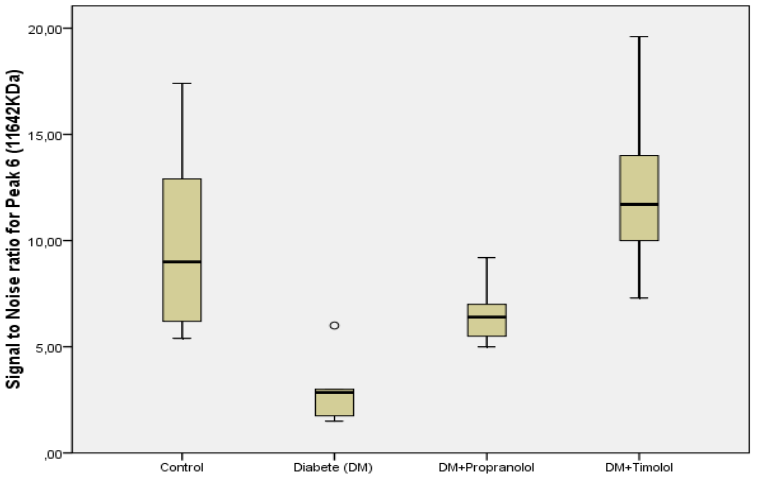
(b)


Signal to Noise ratio for Peak 4 (10984KDa)
35,00
30,00
25,00
20,00
15,00
10,00
5,00
Control
Diabete (DM)
DM+Propranolol
DM+Timolol

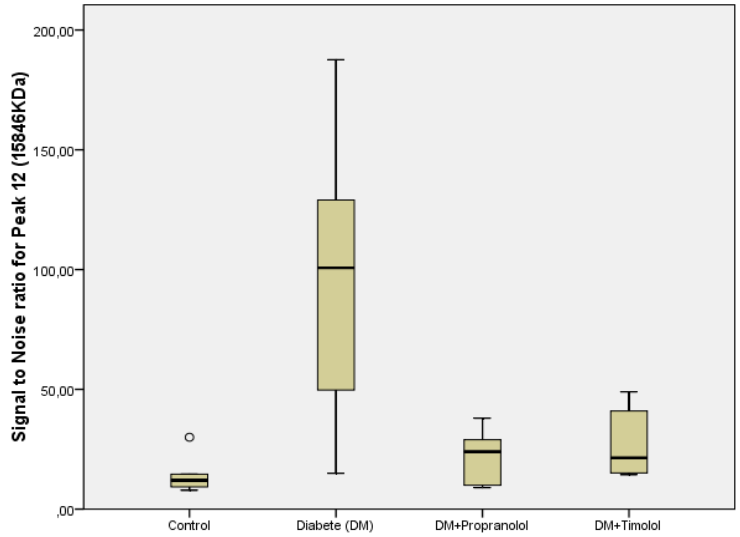
(c)


Signal to Noise ratio for Peak 7 (12127KDa)
200,00
150,00
100,00
50,00
,00
Control
Diabete (DM)
DM+Propranolol
DM+Timolol

(d)


Signal to Noise ratio for Peak 6 (11642KDa)
20,00
15,00
10,00
5,00
,00
Control
Diabete (DM)
DM+Propranolol
DM+Timolol

(e)



(f)

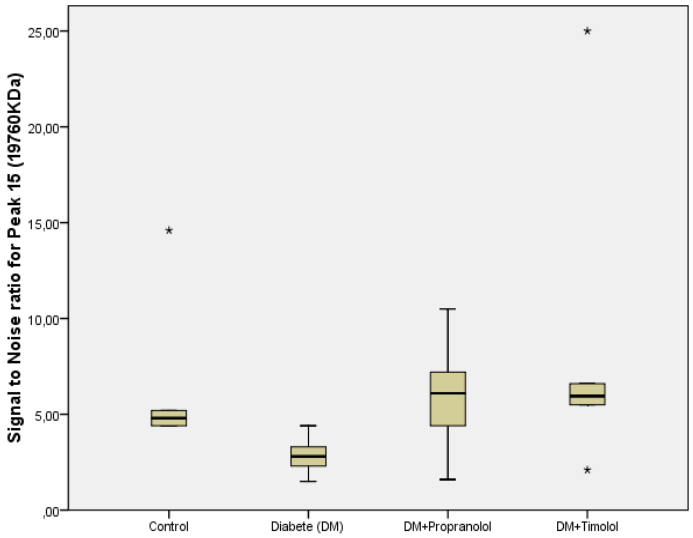


Figure 4.7. SNR for (a) Peak 3, (b) Peak 4, (c) Peak 7, (d) Peak 6, (e) Peak 12, (f) Peak 15. Between Control and DM and between DM and DM+propranolol or timolol, $p<0.05$; Between Control and DM+propranolol or timolol, $p>0.05$.

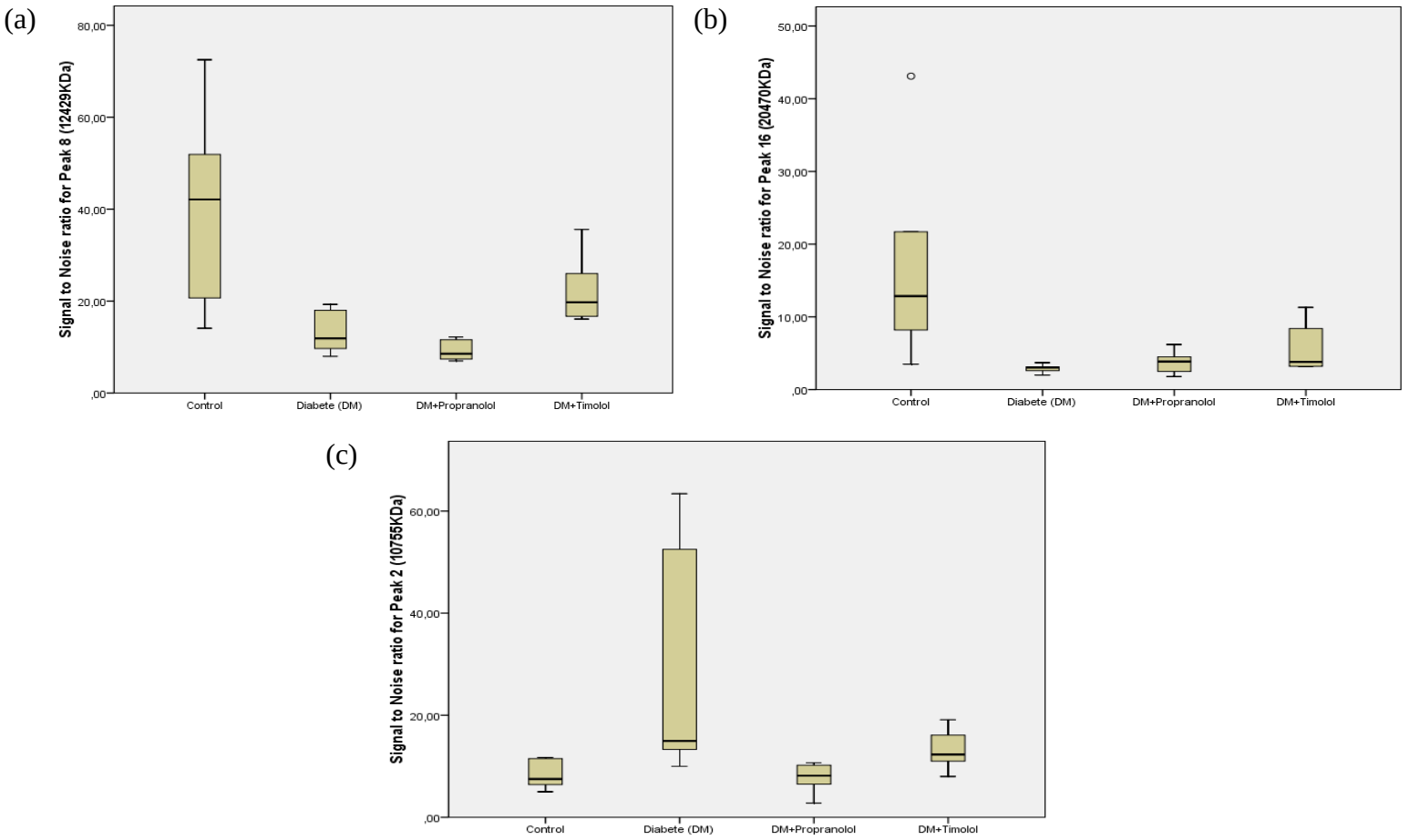


Figure 4.8. SNR for (a) Peak 8, (b) Peak 16, (c) Peak 2 in each group. In all three $p<0.05$ between Control and DM. In (a) and (b) $p<0.05$ between DM and DM+Timolol groups, in (c) $p<0.05$ between DM and DM+ Propranolol groups.

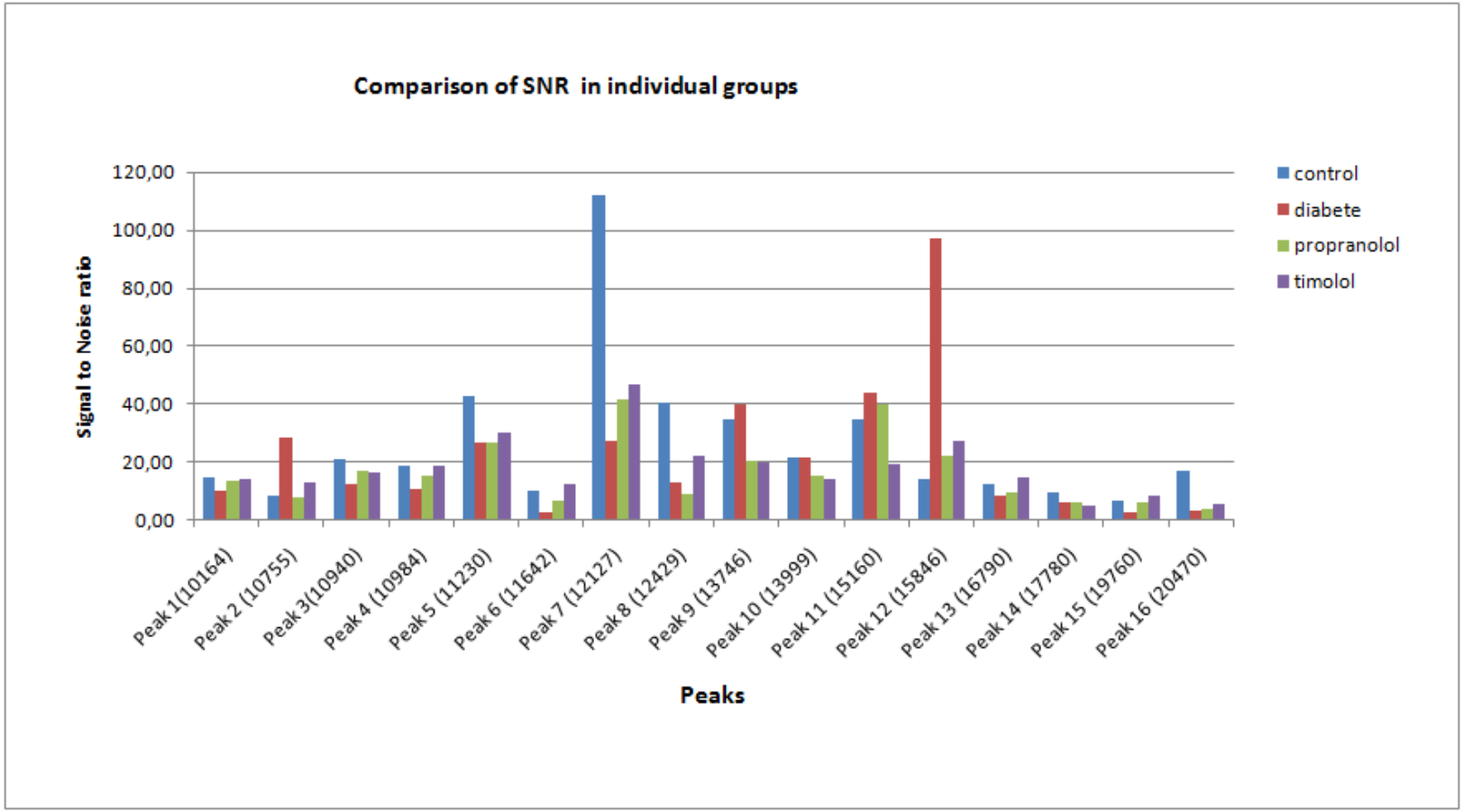


Figure 4.9. SNR of detected peaks for the four groups.

5. DISCUSSION

In this study, we tried to show the protein profile difference between normal and diabetic vascular tissue and the protein profile change of diabetic tissue when using beta blockers propranolol and timolol. For this purpose, Wistar rat vascular tissues were used. The four groups of rats (control, DM, DM+ propranolol, DM+timolol) were prepared elsewhere previously. The tissues were taken and kept at -85^{o}C (4, 5). In order to show the protein profile change, the tissues were homogenized and MALDI-TOF-MS spectra were taken.

As vascular tissue is generally formed of three layers, rich in collagen and elastin, it is not easy to homogenize it. Harsh disruption methods should be applied to homogenize these tissues (58, 59). Sonication is one of the mechanical distruption methods for hard tissues to disrupt cells efficiently. In sonication the sample is disrupted by pressure. A crystal such as quartz is attached to the probe of the sonicator. When the electric current is applied in high and low frequencies, these crystals contract and expand to cause rapid oscillation on the probe and cause shock waves. Meanwhile heat is generated during sonication. In order to prevent denaturation of the sample during sonication it should be kept in ice bath and a time break between every period of sonication should be applied. Also it is better to sonicate the sample with pulse off and pulse on periods (60, 61).

In our preliminary experiments we tried to homogenize the samples in the extraction buffer by directly sonicating, and centrifugation; but we observed that the cells were not ruptured and the supernatants of the samples did not show any significant signal at MALDI-TOF-MS. Later, we homogenized the samples by grinding them in a mortar with liquid nitrogen prior to sonication as described by Mourino-Alvarez et al (54). During homogenization, extraction buffer mixture was used with some modifications.

It is known that the results of MALDI-TOF-MS are affected by the existence of high levels of detergents, especially SDS in the extraction buffer (62). Therefore, we omitted SDS in the buffer and used nonionic detergents Triton X-100 and Igepal instead at tolerable concentration ranges.

Protein concentrations of the homogenates were determined before performing MALDI-TOF-MS measurements. Generally there are two methods for quantification

of total protein, the spectrophotometric assays such as measurement at 205 nm or 280 nm; and the colorimetric assays which are classified as copper based assays such as bisinchoninic acid, Lowry and Biuret assays; and dye binding assays such as Coomassie Brilliant Blue dye binding assay described by Bradford. In copper based assays, at alkaline pH the copper present in the reagent is reduced by proteins and a colored complex is formed. In bisinchoninic acid assay for example, as a result of the reaction a purple colored complex is formed. In Bradford assay, the dye (Coomasie Brilliant Blue G-250 which is dissolved in ethanol and phosphoric acid) binds to the protein at acidic medium. Upon binding of dye to protein, a metachromatic shift from 465nm (reddish form of the dye) to 595 nm (blue form) occurs due to the stabilization of anionic form of the dye (63). For method selection the level of the protein in the samples and the presence of the interfering substances are considered. Bisinchoninic acid shows interference with EDTA and DTT while Bradford method tolerates them at the concentrations we used. Bradford method is also compatible with high concentrations of salts, buffers, and detergents at low concentration (55).

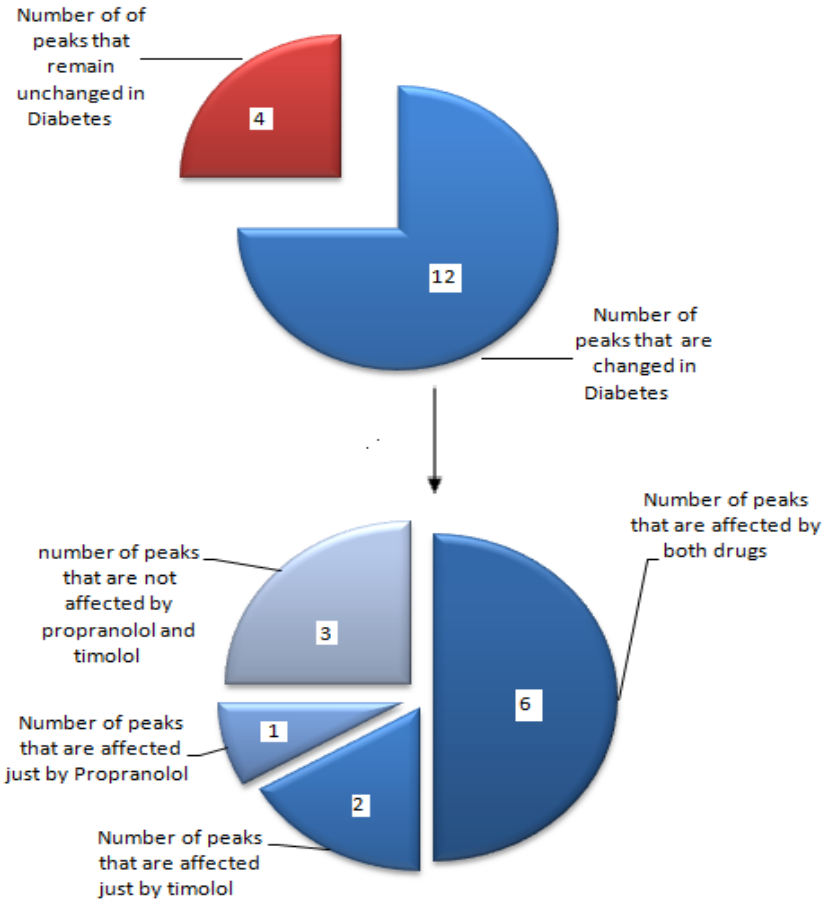
In Bradford method the protein samples can be transformed either to a microplate or individual cuvettes and then the Bradford Reagent is added. After incubation for 10 minutes at room temperature the absorbances of the samples are measured. If sample volume is too small, microplate assay is recommended which shows improved sensitivity up to 10 fold as compared to cuvette assays. For this reason, microplate assay was preferred in our studies.

MALDI-TOF-MS studies were performed at the Chemistry Department Laboratories of the Faculty of Science, Hacettepe University. Since the noise affects the actual amplitude, for determining the actual amplitude, SNR's were calculated for each signal of the spectra. SNR <1 shows that the corresponding amplitude is mostly because of noise, so not accepted as actual signal of a protein. Signals with SNR>1 are accepted as significant signals.

As a result of these calculations 16 significant signals in each sample were detected in the study range of 10-25 kDa. These signals were numbered as Peak 1 to Peak 16. The statistical analyses of these peaks showed that there was a significant profile change in the vascular tissue of diabetic rat as compared to normal, and in response to treatment with beta blockers, some of these peaks were converted back to

control level. The findings of our experiments are summarized in Figure 5.1 (a) and (b).

a



b

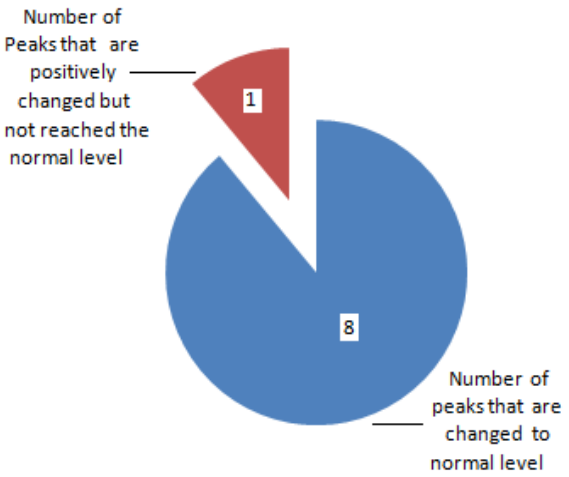


Figure 5.1. Summary of the findings of MALDI-TOF-MS experiments (a) Number of peaks that changed in DM and affected by timolol and/or propranolol; (b) Number of peaks that as a result of treatment with propranolol and/or timolol reached to normal level.

Several other investigators also worked on the protein profiles of vascular tissue in diabetes and/or cardiovascular disorders and found out that expression of several proteins were changed in these disease states. A proteomics comparison of aortic tissue from diabetic and healthy rats was done by Jüllig et al (26). They observed "significant changes or trends towards changes in relative abundance of 51 proteins (25 increased, 26 decreased). The most prominent diabetes associated changes were in groups of proteins linked to oxidative stress responses and the structure/function of myofibrils and microfilaments". Sui et al. (44) incubated vascular smooth muscle cells with enantiomers of propranolol and analyzed the protein profile by 2D LS-MS/MS. They observed that "four types of cellular proteins including metabolic enzymes, signaling molecules, cytoskeletal proteins, and those involved in DNA synthesis/protein translation were increased in the cells incubated with the S enantiomer." Proteomic profiling of cellular responses to carvedilol enantiomers, in vascular smooth muscle cell cultures were also investigated by Wang et al. (37). They identified thirteen proteins with statistically significant changes in cells incubated with S-Carvedilol. "Among these proteins, actin in aortic smooth muscle (ACTA2), calmodulin, S100-A6, S100-A10, S100-A11, thioredoxin, lactadherin and heat-shock protein 105 kDa were found to be closely relevant with the clinical effects of Carvedilol." On the other hand, Sui et al. (64) investigated the proteomics of vascular smooth muscle cells incubated with enantiomers of atenolol which is a beta-1 drug and found out that "some calcium-binding proteins such as calmodulin, protein S100-A11, protein S100-A4, and annexin A6 were downregulated" and "enzymes such as aspartate aminotransferase, glutathione *S*-transferase P, NADH-cytochrome *b*5 reductase, and alpha-*N*-acetylgalactosaminidase precursor were up-regulated." They also found out that S enantiomer was more efficient in up and down-regulation of these proteins. La Cuesta et al. (27) investigated the proteomic alterations in atherosclerotic and preatherosclerotic human coronary arteries by 2D electrophoresis. They found out that, 12 proteins were altered in atherosclerosis, 5 of which were cytoskeleton proteins (including filamin A, gelsolin, vinculin and vimentin). These proteins were found to be decreased in the atherosclerotic coronary media.

Tuncay et al (4) found out that when diabetic rats were treated with propranolol, level of FKBP12.6 protein was decreased and the high phosphorylated levels of PKA

and CaMKII were prevented. Long et al. (65) demonstrated that in hypertension, "displacement of FKBP12/12.6 from ryanodine receptors induces an endothelial intracellular Ca^{2+} leak and increases conventional PKC–mediated endothelial eNOS Thr495 phosphorylation leading to decreased NO production and endothelial dysfunction". Tuncay et al. (5) also showed that "timolol antagonized hyperphosphorylation of cardiac ryanodine receptor (RyR2), and significantly restored depleted protein levels of both RyR2 and calstabin2". Zamora et al. (66) who worked on the ocular vascular endothelium proteomics profile, compared the proteomics profile of human retinal and choroidal endothelial cells and attracted attention to the roles of several differentially expressed proteins such as calreticulin, cathepsin B, glutathione peroxidase 1, superoxide dismutase and UCH-L1, in regulation of angiogenesis which is a component of diabetic retinopathy. Kilic and Mandal (67) points to the relationship between heat shock proteins and atherosclerosis in their review article. They claim that under physiological conditions, heat shock proteins have a protective role but in disease states, they "can act as targets for detrimental autoimmunity". Advani et al (68) showed that "induction of diabetes in rats increased expression of thioredoxin inhibitory protein but not thioredoxin mRNA". They also showed that "kidneys from patients with diabetic nephropathy had significantly higher levels of thioredoxin inhibitory protein than control kidneys, but thioredoxin expression did not differ." They concluded that "impaired thiol reductive capacity contributes to the generation of reactive oxygen species in diabetes in a site- and cell-specific manner". Doyon et al. (69) investigated the reason of arterial stiffness in type 1 DM in rats. They focused on matrix gamma-carboxyglutamic acid protein and showed that mRNA for this protein is reduced in stiffed vessels. S100 family of calcium receptor proteins also attract interest related to the diabetic complications. In several tissues of rats with type 1 DM, expression of S100 family proteins were observed (70). It is proposed that S100A10 protein has an important role in fibrinolysis and angiogenesis (71). Bao et al. (72) proposed that S100A6 has an important role in regulating endothelial cell cycle progression and senescence. Jouve et al. (73) on the other hand showed that CD146 which is a highly glycosylated adhesion molecule and its ligand galectin-1 are involved in apoptotic regulation of endothelial cells.

There are also some publications on the protein profile of the extracellular matrix of vascular tissue. Didangelos et al (74) investigated the extracellular matrix proteins of human aorta and identified 103 proteins, some of which were novel glycoproteins. Extracellular matrix proteins may be important in remodeling and degradation of the extracellular matrix that yield to pathological conditions.

Our findings also give evidence for understanding the mechanism of diabetic complications of vascular tissue and mechanism of protective effects of beta blockers.

By investigating the previously published proteins related to DM and cardiovascular diseases, and searching the protein databases for the molecular weights of mouse endothelial tissue proteins, we may speculate that the proteins related to the signal peaks might be calmodulin, S100 proteins, FKBP12.6, galectin-1, heat shock proteins, thioredoxin, glutaredoxin, glutathione-S-transferase or similar molecules.

One of the drawbacks of our study was its being limited to the molecular weight range of 10-25 kDa. We have to investigate a wider range of molecular weights to obtain more reliable results. The protein(s) corresponding to the peaks which are changed in DM and converted to control level by beta blockers may be sequenced by mass spectroscopy. By investigating the protein databases, candidate proteins can be detected. By Western blot analyses, the identity of the proteins can be confirmed. The protein(s) detected most probably might be involved in the vascular protection mechanism of the beta blockers, and may be the targets for development of the novel drugs.

6. CONCLUSION AND FUTURE PLANS

In this study,

1- Protein profile changes in rat vascular tissue in type 1 DM was investigated by MALDI-TOF-MS. In the study range (10-25 kDa), sixteen significant peaks were obtained. There were significant changes in 12 of the peaks in DM. These were: Peak 2, Peak 3, Peak 4, Peak 5, Peak 6, Peak 7, Peak 8, Peak 12, Peak 13, Peak 14, Peak 15, and Peak 16. The remaining peaks did not change significantly.

2- Intensities of two of the peaks were increased in DM: Peak 2 and Peak 12. The intensities of the remaining peaks decreased.

3- Protective effects of propranolol and timolol in diabetic rats were investigated. Nine of the peaks out of twelve were responded to propranolol and/or timolol treatment: Peak 2, Peak 3, Peak 4, Peak 6, Peak 7, Peak 8, Peak 12, Peak 15, and Peak 16. The remaining did not show any significant change.

4- Six of the peaks were affected by both propranolol and timolol. These were: Peak 3, Peak 4, Peak 6, Peak 7, Peak 12, and Peak 15. Intensity of Peak 7 remained lower as compared to the control, in response to both propranolol and timolol treatment. Intensity of the peak 12, in response to timolol but not in response to propranolol treatment, remained lower as compared to the intensity of the control. Intensities of the remaining peaks reached the intensities of the control level by the action of beta blockers.

5- Peak 2 was affected only by propranolol and Peak 8 and 16 were affected only by timolol. Intensities of the peaks reached to the level of the control after treatment.

6- Protein(s) corresponding to these peaks must be investigated and identified. Molecular weight ranges beyond 10-25 kDa must also be investigated. These studies may shed light to the better understanding of the mechanism of vascular complications of DM and protective effects of beta blockers.

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