THE EFFECTS OF STREPTOZOTOCIN INDUCED-DIABETES ON RAT TESTES AND THE RECOVERY ROLE OF VITAMIN C

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ABSTRACT

THE EFFECTS OF STREPTOZOTOCIN INDUCED-DIABETES ON RAT TESTES AND THE RECOVERY ROLE OF VITAMIN C

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Type I Diabetes is a multisystem disease having both biochemical and structural consequences. It causes alterations in carbohydrate, protein, and fat metabolisms due to hyperglycemia. Type I diabetes is also correlated with increased formation of free radicals and decreased levels of antioxidant potential. Lower endogeneous antioxidant amounts and elevated lipid peroxidation levels in diabetes constitute the basis of risk factors for the development of diabetic complications. These complications lead to irreversible damages in nearly all vital organs and systems. Since the antioxidant capacity lowered in diabetic conditions, it becomes important to be able to use some common antioxidants, as a complementary treatment strategy for diabetes.

The effect of type I diabetes and the recovery role of Vitamin C on the structure, composition and function of the macromolecular content of testicular tissue is still unknown. Therefore, in the current study, it was aimed to investigate the alterations in the macromolecules of rat testes due to Streptozotocin (STZ)-induced type I diabetes using Attenuated Total Reflectance (ATR)-Fourier Transform Infrared (FTIR) spectroscopic and FTIR microspectroscopic techniques. Furthermore it was

aimed to gain useful information about the recovery role of Vitamin C, as an antioxidant, against the diabetic complications.

The detailed spectral analysis revealed that, the macromolecular structure and composition of rat testes are highly affected due to the development of diabetes. The lipid and protein content of diabetic rat testes were shown to decrease considerably, indicating an increase in lipolysis and proteolysis processes. Diabetes was also shown to lead to a decrease in the content of fatty acids and nucleic acids. In addition to the compositional alterations, protein conformation, and protein secondary structural components were also found to alter in diabetic state. Besides, lipid peroxidation levels were found to increase, and the elevated levels of lipid peroxidation products end up with increased levels of unsaturation, and also end up with increased levels of disorderness in diabetic conditions. On the other hand, with the administration of Vitamin C, the diabetes-induced alterations were found to be partially recovered, indicating that after more confirmative researches, Vitamin C may have a chance to be used as a complementary therapy in the treatment of diabetes.

Keywords: Type I diabetes, Vitamin C, rat testes, ATR-FTIR spectroscopy, FTIR microspectroscopy.

ÖZ

STREPTOZOTOSİN'E BAĞLI OLARAK OLUŞTURULAN DİYABETİN SIÇAN TESTİS DOKUSUNDAKİ ETKİLERİ VE C VİTAMİNİ'NİN İYİLEŞTİRİCİ ROLÜ

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Tip I diyabet hem biyokimyasal hem de yapısal açıdan çeşitli sonuçlara neden olan ve birden çok sisteme etki eden bir hastalıktır. Bu hastalık, hiperglisemiye bağlı olarak karbonhidrat, protein ve yağ metabolizmalarında çeşitli değişiklere yol açmaktadır. Tip I diyabet aynı zamanda, serbest radikal üretimindeki artış ve antioksidan potansiyelindeki azalmalarla da ilişkilendirilmektedir. Diyabette gözlemlenen düşük endojen antioksidan miktarları ile yüksek lipit peroksidasyon seviyesi diyabetik komplikasyonların gelişmesindeki temel risk faktörlerini oluşturmaktadır. Bu komplikasyonlar, neredeyse tüm hayati organ ve sistemlerde geri dönüşü olmayan hasarlara yol açmaktadır. Diyabet durumunda antioksidan kapasitesi azaldığından, yaygın olan antioksidanların tamamlayıcı tedavi stratejisi olarak kullanılması büyük önem kazanmaktadır.

Tip 1 diyabetin sıçan testis dokusu makromoleküllerinin yapı, içerik ve fonksiyonları üzerindeki etkileri ile C Vitamini'nin koruyucu rolü henüz bilinmemektedir. Bu nedenle, bu çalışmamızda, Streptozotosin'e (STZ) bağlı olarak oluşturulan tip I diyabetin, sıçan testis makromolekülerinde meydana getirdiği değişimlerin ATR-FTIR spektroskopisi ve FTIR mikrospektroskopisi teknikleri kullanılarak

araştırılması amaçlanmıştır. Ayrıca, diyabetik komplikasyonlar karşısında bir antioksidan olarak C Vitamini'nin iyileştirici rolüyle ilgili yararlı bilgiler elde edilmesi amaçlanmıştır.

Detaylı spektral analizler sonucu, diyabet oluşumunun, sıçan testisinin makromoleküllerini yapı ve içerik açısından oldukça etkilediği tespit edilmiştir. Diyabetik sıçan testisinde, artan lipoliz ve proteoliz düzeylerinden kaynaklı olarak, lipit ve protein miktarlarında önemli ölçüde azalma gözlemlenmiştir. Diyabetin aynı zamanda, yağ asiti ve nükleik asit miktarlarında da azalmalara yol açtığı gösterilmiştir. İçerik açısından meydana gelen değişimlere ek olarak, diyabet durumunda protein konformasyonu ve protein ikincil yapılarında da değişimler olduğu saptanmıştır. Bu sonuçların yanı sıra, diyabete bağlı olarak, lipit peroksidasyon seviyesi ile lipit peroksidasyon son ürünlerinde artış gözlemlenmiş olup, bu artışların doymamış yağ miktarlarında ve lipit yapılarındaki düzensizlik seviyesinde fazlalaşmaya neden olduğu görülmüştür. Diğer bir taraftan da C Vitamini'nin diyabet kaynaklı değişimler üzerinde iyileştirici bir rolünün olduğu saptanmıştır. Bu sonuçlar doğrultusunda, destekleyici araştırmaların da gerçekleştirilmesiyle C Vitamini'nin diyabet tedavisinde tamamlayıcı bir yöntem olarak kullanılma şansı kazanması mümkün olabilecektir.

Anahtar Kelimeler: Tip I diyabet, C Vitamini, sıçan testisi, ATR-FTIR spektroskopisi, FTIR mikrospektroskopisi.

To my mother,

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CHAPTER I

INTRODUCTION

In this study, the effects of Streptozotocin (STZ) induced type I diabetes and the recovery role of Vitamin C (Ascorbic acid) against the complications of diabetes were investigated on rat testes, using biophysical techniques and chemometric tools. In this chapter, detailed information about the structural characteristics and function of testicular tissue, the basis of diabetes mellitus, the complications of diabetes mellitus, the effects of Vitamin C on oxidative stress and diabetes, and ultimately the basics of infrared spectroscopy are briefly explained.

1.1. Male Genital System

The male genital, namely reproductive, system is specialized for mainly three functions. First of these functions is to produce, maintain and transport sperm and semen, second one is to discharge sperm within the female reproductive tract, and the third one is to produce and secrete male sex hormones.

The male reproductive system consists of a certain number of sex organs located inside (internal genital organs) and outside (external genital organs) of the pelvic region. The internal structures, also called accessory organs, include Vas deferens, seminal vesicles, ejaculatory ducts, bulbourethral glands, urethra and prostate gland, and the external structures include the penis, the scrotum, epididymis and testes (testicles).

Among the internal structures, Vas deferens participates in transportation of mature sperm to the urethra in preparation for ejaculation, and seminal vesicles participate in production of fructose rich fluid which provides energy for the sperm produced. The content of the ejaculatory ducts, composed of the combination of Vas deferans and seminal vesicles, empties into the urethra. On the other hand, bulbourethral glands produce another clear fluid which also empties into the urethra in order to lubricate the urethra and neutralize any probable acidity in the urethra. Besides its role in the excretory system, urethra has a different role in the time of sexual intercourse, as it provides the ejaculation of the semen throughout the body. An additional fluid, that helps the sperm to be nourished, is produced by prostate gland which contributes to the ejaculate.

Among the external structures, penis is the male organ for the sexual intercourse. The scrotum, in which the testicles are located and also many nerves and blood vessels are present, is mainly responsible for the regulation of temperature for the testes to be functional. Epididymis, located at the backside of each testicle, responsible for transportation and storage of the sperm cells produced in the testicles, and also at the time of sexual arousal it is responsible for the contractions which make the sperm to move into the Vas deferens. At last, the testes are in charge of production of the major male sex hormone, testosterone, and production of sperm.

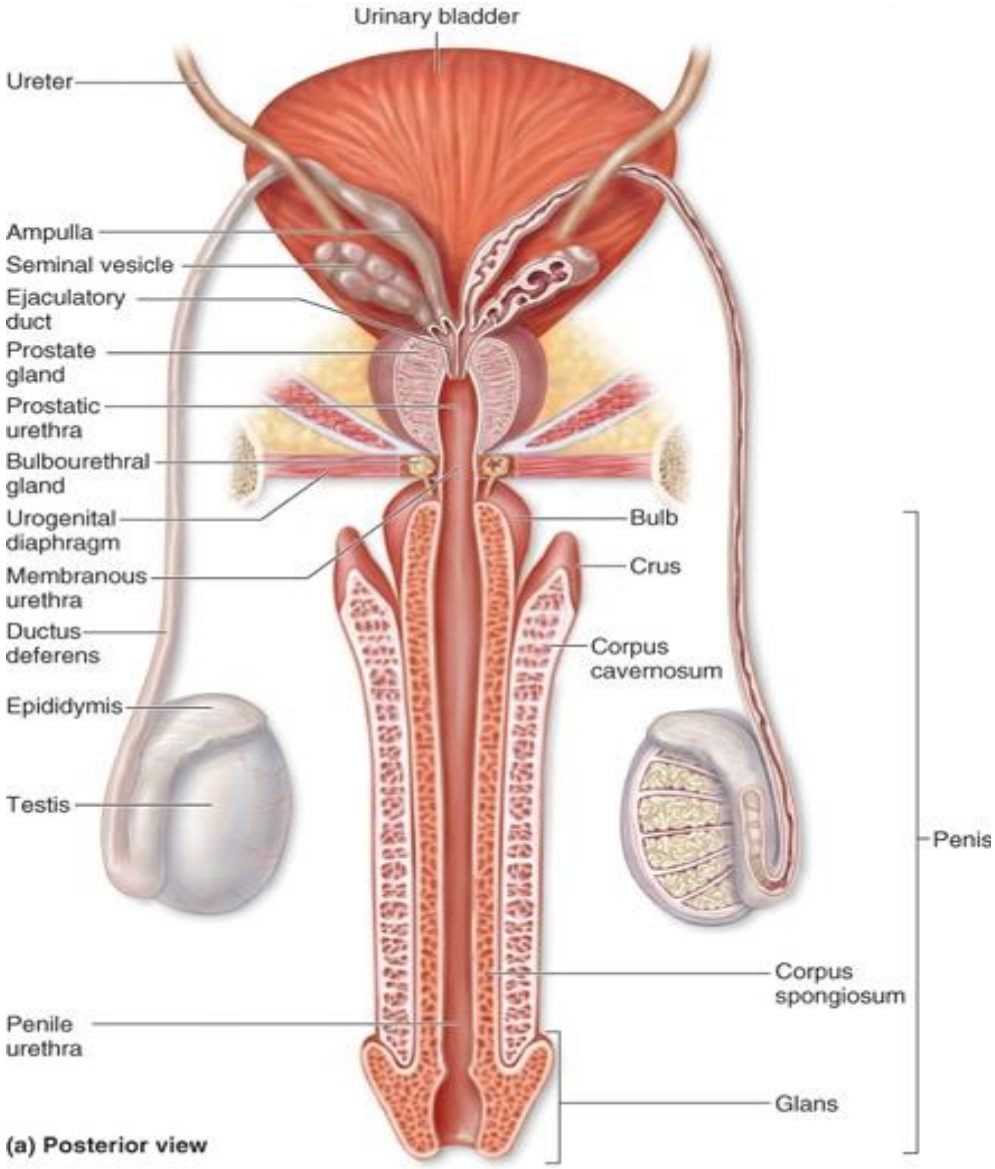

Figure 1. Organization of the main components of male reproductive system. (Widmaier *et al.*, 2007).

1.1.1. Structural organization of a testicle

In the early developmental stages, the testes are located in the abdominal cavity, near the kidneys, while in the later stages, they go beyond the abdomen and gain a final position as posterior to the penis. This positioning provides the temperature about 3-5^{o}C lower than the normal body temperature, which is essential in order for viable and healthy sperm to be produced. Most of the males have two oval shaped testes having typically similar sizes, about 5 cm long and 3 cm in diameter.

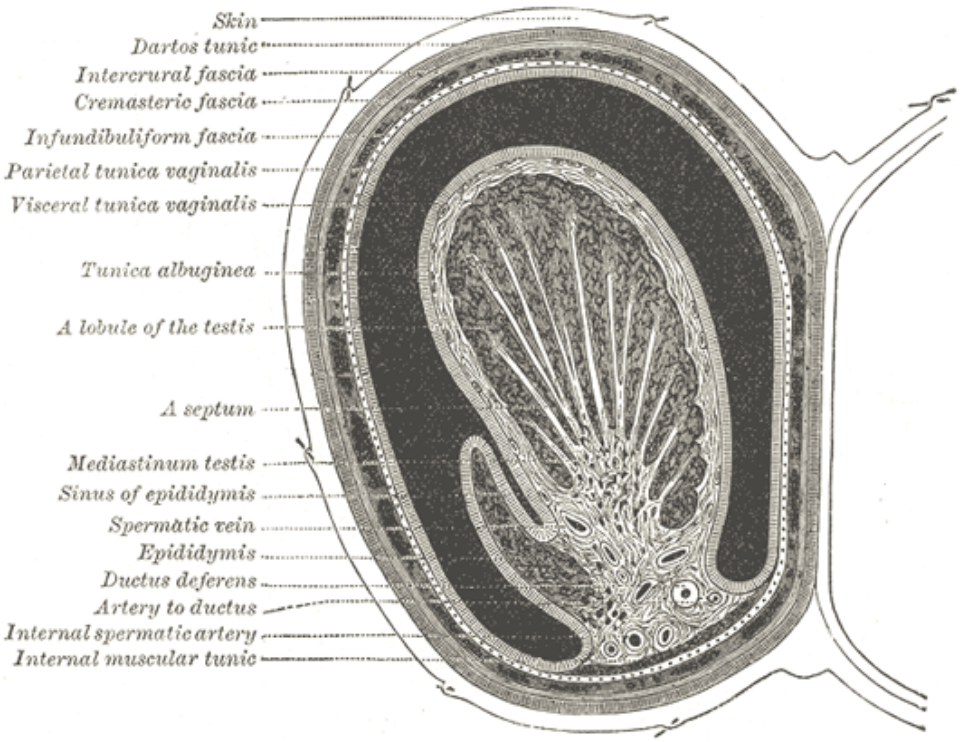

Figure 2. Transverse section through the left side of the scrotum and the left testicle.

1.1.2. Function of the testes

The outermost layer of a testicle, which surrounds the testicular tissue, is named as tunica albuginea. Tunica albuginea seems as a tough shell composed of white connective tissue capsule. This structure not only provides the protection of the testes and also provides partitioning of the testes into numerous lobules. Each testicle consists of about 250 lobules, and each lobule contains much more coiled tubular structures, called seminiferous tubules (Figure 3). Within the seminiferous tubules, two major types of cells are found, germ cells and Sertoli cells. Through spermatogenesis, the germ cells developed into spermatozoa and carried through the seminiferous tubules to the rete testis where the seminiferous tubules converged into a single tubule. In the spermatogenesis process, Sertoli cells are critical for the support of germ cell development into spermatozoa.

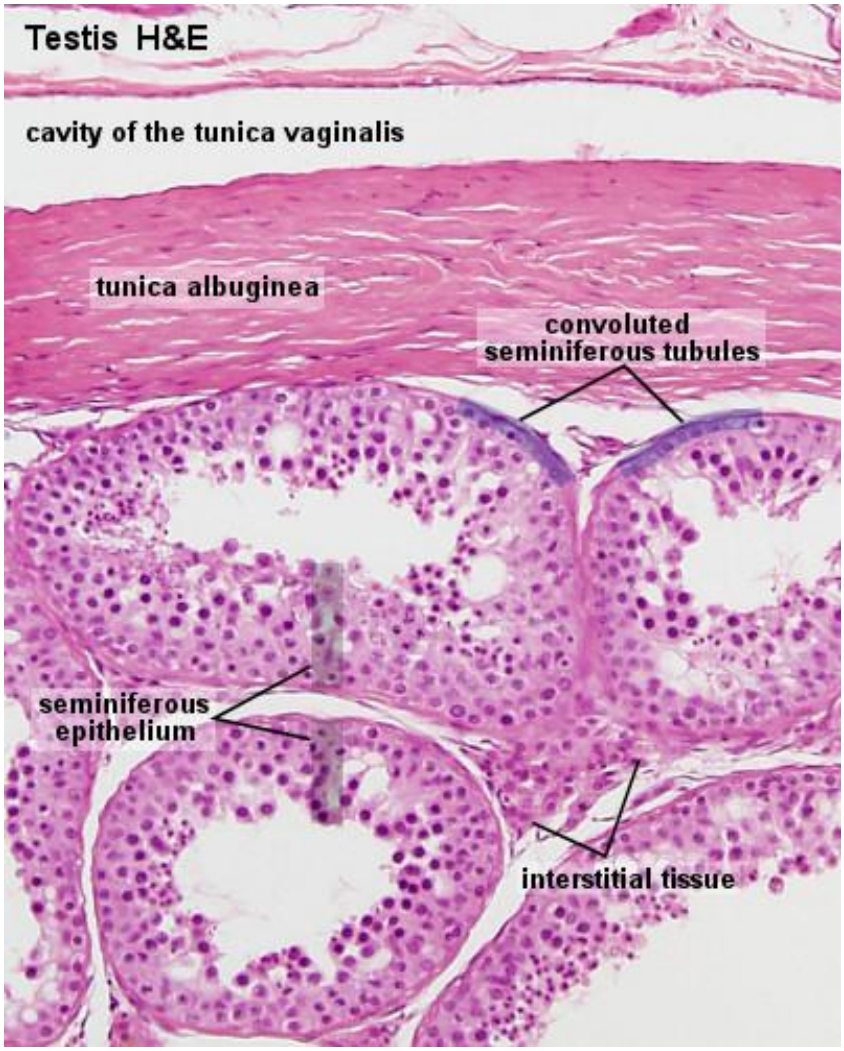

Figure 3. Structure of seminiferous tubule. (UWA Blue Histology).

In the lobules of testes, between the seminiferous tubules, special kinds of cells are located, named as Leydig cells (interstitial cells) (Figure 3). Testosterone and other androgens are formed in the Leydig cells which are important in the sexual development and improvement of the secondary sexual characteristics such as, body hair, facial hair and sexual behavior.

1.1.3. Sperm production

The germ cells reside in the lining of the seminiferous tubules of testis, divide to make sperm production to happen. Sperm production begins with the differentiation of a germ cell into a spermatogonium. Spermatogonium is a diploid cell, with 2n= 46 chromosomes, which stays dormant till puberty. With the beginning of the puberty, the levels of hormones secreted are altered, and these hormonal alterations lead to stimulation of division of these cells through mitosis. In spermatogenesis process, the spermatogonium goes under mitotic division and forms two diploid (2n) primary spermatocytes. It should be emphasized that, not all the spermatogonia in the testicular tissue divide in order to produce spermatocytes. Some of the spermatogonia only divide mitotically, in order to increase their number to supply sufficient energy, because high amount of energy is needed in order for spermatogenesis to proceed.

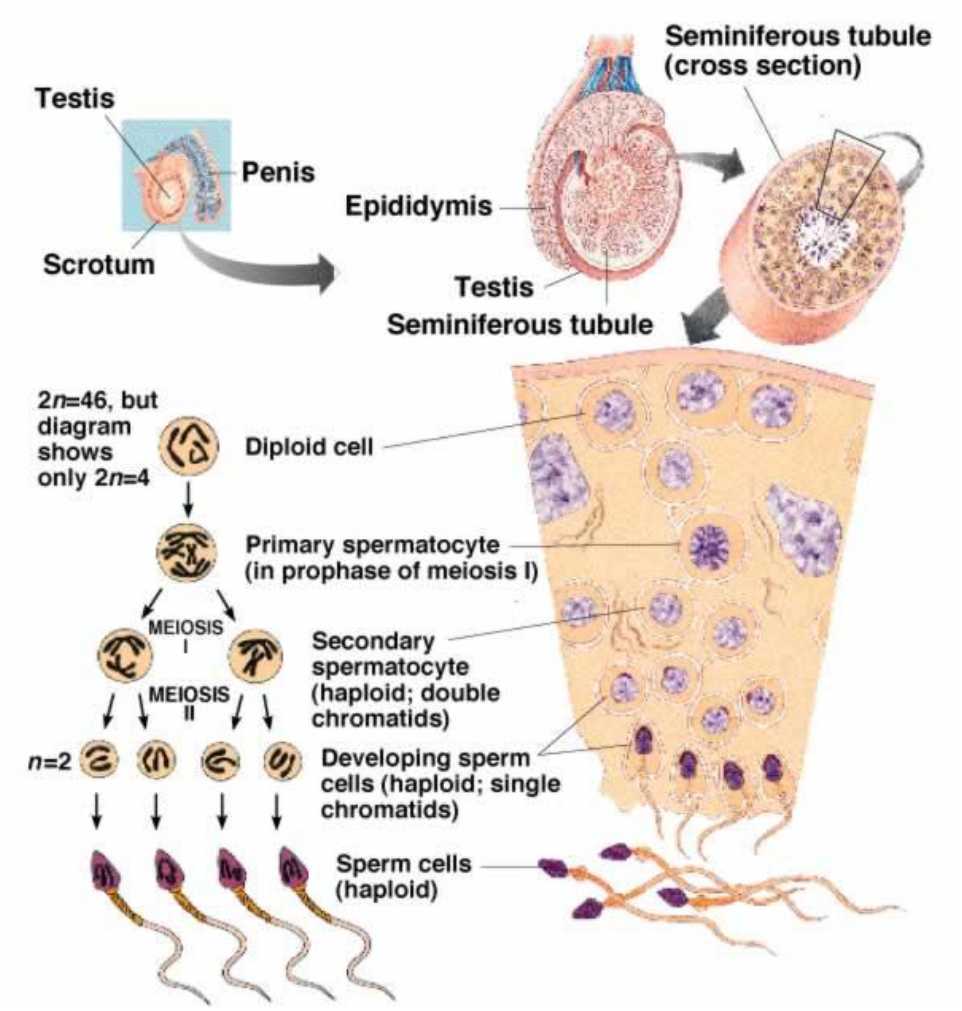

Figure 4. Stages of sperm development in the lining of the wall of seminiferous tubule**.** (Marieb and Mitchell, 2010).

Each primary spermatocyte produced, duplicates their own DNA and undergoes meiotic division. At the end of meiosis I, two diploid (2n) secondary spermatocytes are generated from a primary spermatocyte. The secondary spermatocytes go readily into meiosis II, and produce haploid (n) spermatids. As a result of the two successive meiotic divisions, each primary spermatocyte leads to production of four spermatids (Figure 4). The most prominent advantage of this meiotic division is to provide genetic variation through chromosomal crossovers. The spermatids are the immature and infertile structures, which have to become mature in order to be functionally

active. The maturation of a spermatid takes place with the trigger of testosterone. The spermatid begins to grow a tail, to develop a thickened mid-piece and thus it obtains a tadpole like structure. During maturation, the unnecessary cytoplasm and organelles in the cells are removed. The mature spermatozoan is released from the Sertoli cells into the lumen of the seminiferous tubule. This spermatozoan is mature, however still lacks motility. At this stage, the spermatozoan moves to epididymis with the help of testicular fluid released by Sertoli cells, through peristaltic contraction. In the epididymis, the spermatozoan gains movement ability, and then it becomes fertile. Till the ejaculation process, all the mature sperms are stored in epididymis. When ejaculation starts, the sperms pass through Vas deferans, to the urethra and finally to the penis with the help of fluids produced and secreted by prostate gland and seminal vesicles.

1.1.4. Hormonal Regulation

In males, there are number of hormones that control the testes and sperm production processes. The major hormones having important roles in functioning of the male reproductive system are follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone. FSH and LH are produced and secreted by the pituitary gland in brain, while testosterone is produced and secreted by the testes.

FSH and LH levels in the blood increase at the very beginning stages of puberty. This rise in the hormone levels provides growth and maturation of testes. Particularly, FSH is essential for sperm production, and LH stimulation is essential for the testosterone production which is necessary for spermatogenesis to occur. In addition, testosterone is also required for the development of male characteristics, such as muscle mass and strength, bone mass and sex drive.

1.2. Diabetes Mellitus

Diabetes mellitus (DM) is a heterogeneous, metabolic disorder characterized by elevated blood glucose levels (hyperglycemia). In diabetic patients, either the absence or insufficient production of insulin by pancreas or the defective usage of insulin by body, results with hyperglycemia. This chronic disease leads to destructions in carbohydrate, fat and protein metabolisms and these destructions end up with irreversible damages in all vital organs and systems of the body.

The most common symptoms of diabetes are frequent urination, increased thirst, extreme hunger, sudden weight loss and blurring of vision. In more severe stages of the disease, ketoacidosis may develop and lead to unconscious state, then lead to coma and, if not treated effectively, it will eventually lead to death. Diabetes causes about 5% of all deaths globally each year.

DM can be divided into three major groups as type I diabetes (insulin-dependent diabetes mellitus- (IDDM)), type II diabetes (noninsulin-dependent diabetes mellitus- (NIDDM)) and gestational diabetes mellitus (GDM). DM is one of the most common chronic diseases in children and adolescents. Children are assumed to have type I, or juvenile-onset diabetes whose incidence increases especially in young children. Over the past few years, metabolic syndrome, characterized by diseases such as type II diabetes which was formerly known as adult-onset diabetes, has also found increased prevalence, even among children and adolescents (Wellen and Hotamisligil, 2005).

1.2.1. Type I Diabetes - Insulin-Dependent Diabetes Mellitus (IDDM)

Type I diabetes is the insulin dependent type of diabetes mellitus, which is resulted from the absence or insufficient production of insulin by the beta cells of the islets of Langerhans, in pancreas. Insulin is a natural hormone that has a role in controlling the blood glucose levels in the body. If insulin is absent, or present in insufficient levels, then glucose in the blood can not be transported to the cells efficiently, and

blood glucose levels remain excessively high. Since glucose can not be used by the cells in this situation, it spills over into the urine and is lost. Loss of excessive levels of glucose leads to the appearance of first diabetic complications, such as weakness, weight loss, frequent urination, and excessive hunger and thirst. Daily insulin has to be provided in order for a patient to survive and keep the complications under control. Otherwise, the complications replace with more severe ones which can result in coma and death.

The main reason underlying the development of type I diabetes is the autoimmune destruction of insulin producing pancreatic beta cells. The rate of beta cell destruction is quite variable, it can be rapid in some individuals and slow in others (Zimmet *et al.*, 1994). While also can be seen in adults, the rapidly progressive form of type I diabetes is most commonly observed in children (Humphrey *et al.*, 1998).

The autoimmune destruction of pancreatic beta cells due to attack of T-cells of the body's own immune system (Rother, 2007). Even the exact cause is still unknown, it is thought to arise from combination of two factors: genetic predispositions and environmental effects. The most prominent genetic signal for type I diabetes is located in the human leukocyte antigen (HLA) region on the short arm of chromosome 6 (6p21.3) (Nerup *et al.*, 1974). The major susceptibility for type I diabetes has been mapped to the HLA class II genes HLA-DQB1, -DQA1 and -DRB1, and it was shown that, approximately 95% of type I diabetics carry either HLA-DR3 or HLA-DR4 (Brorsson *et al.*, 2010). On the other hand, considering environmental factors, a possible viral infection may also lead to development of type I diabetes, in which a part of a viral protein highly resembles to a protein in pancreatic tissue that takes role in T-cell recognition. In this case, the host cell proteins can be encountered as viral proteins by antibodies which result in destruction of the body cells by T-cells of immune system (Akerblom *et al.*, 1998; Jun *et al.*, 2002).

Different pro-inflammatory cytokines such as, interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor-α (TNF-α) were also shown to have a critical role in the pathogenesis of type I diabetes (Rabinovitch, 1998). Even the underlying molecular mechanisms responsible for the cytotoxic effects of these cytokines remains unclear, it is thought to be resulted from the generation of free radicals due to oxidative stress in diabetic state. Free radicals, particularly reactive oxygen species (ROS), have been implicated in the cytokine-mediated islet cell injury in different models of type I diabetes. ROS generation, due to elevated levels lipid peroxidation products, is believed to be the ultimate cause of cytokine-mediated death of β-cells in isolated islets (Rabinovitch *et al.*, 1996).

1.2.2. Type II Diabetes - NonInsulin-Dependent Diabetes Mellitus (NIDDM)

Type II diabetes is the non-insulin dependent type of diabetes mellitus, which is resulted from insulin resistance, can also be accompanied by inadequate insulin secretion by pancreatic beta cells. This defective responsiveness of body tissues to insulin is thought to be related with the possible impairments in any step of insulin signal transduction pathway (Surampudi *et al.*, 2009), especially it can be related with some defects in the insulin receptor. Alterations in the insulin receptor tyrosine kinase activity (Slieker *et al.*, 1990; Block *et al.*, 1991) or alterations in the insulin sensitive glucose transporter (Garvey *et al.*, 1989; Strout *et al.*, 1990) can be resulted in insensitivities to insulin for target cells.

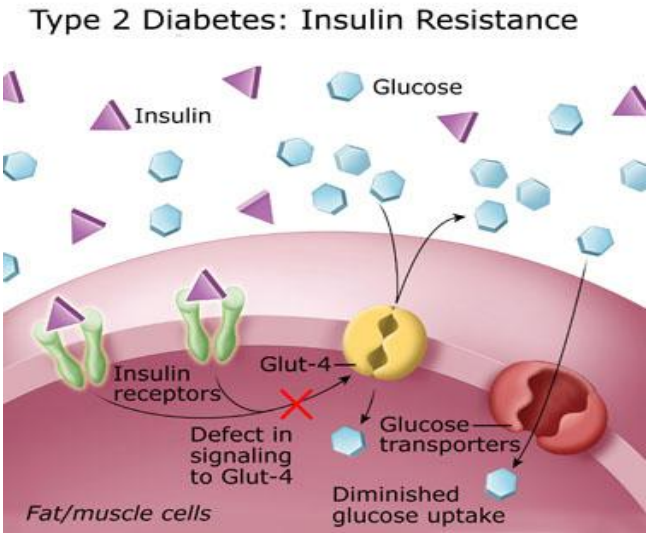

Figure 5. Model for insulin resistance, due to type II diabetes.

In type II diabetic patients, insulin can be produced and secreted by pancreas, but the body can only partially, or even completely unable to use the insulin produced. Thus, the body secretes more and more insulin in order to overcome this trouble. After a certain point, pancreas can no longer produce more amounts of insulin. In that situation, any increase in the blood glucose levels can not be handled, and glucose levels in the blood become abnormally high which is accompanied by the complete development of type II diabetes. Even in this circumstance, the body still reacts as there is no glucose in the system because the insulin produced is ineffective. This time, another blood glucose regulatory hormone, glucagon, steps in. Glucagon activates the hepatic glucose production, decreases glucose transport into muscle cells and increases breakdown of fats (lipolysis) in the tissues in order to meet the energy needs. Due to lipolysis, the levels of free fatty acids in plasma become elevated, which are then converted into ketone bodies by the liver. Urination is also increased due to high blood glucose levels. The excess glucose is eliminated from the body with some ions, such as Na^+ and K^+ and water through urination. Ketone bodies however, remain in the blood and decrease the pH of blood, which in turn leads to coma and death if not treated properly.

Development of type II diabetes is thought to arise from a combination of genetic and environmental factors. Genetically, there is a strong tendency to inherit type II diabetes from family members. As environmental factors, older age, obesity, sedentary life style and certain ethnicities can also play role in the development of type II diabetes.

Since the onset and the progression of type II diabetes is not as rapid as in the case of type I diabetes, it can be diagnosed and prevented much more easily via making alterations in the diet and in the lifestyle.

1.2.3. Gestational Diabetes Mellitus (GDM)

Gestational diabetes mellitus is defined as glucose intolerance in pregnant women who are in the second half of the pregnancy period. It is characterized by inadequate insulin secretion and responsiveness. Development of GDM is thought to be caused by the alterations in the hormone levels, or caused by insulin shortage due to pregnancy (Buchanan and Xiang, 2005). Even the degree of insulin resistance varies, a remarkable defect in the pancreatic beta cells have been consistently shown in GDM patients (Ryan *et al.*, 1995; Kautzky-Willer *et al.*, 1997; Buchanan 2001). Potential causes of inadequate beta cell function can be caused by autoimmunity, chromosomal mutations or a background of chronic insulin resistance (Buchanan and Xiang, 2005).

The GDM patients have never had diabetes before they only have high blood glucose levels during pregnancy. It is fully treatable, but requires attentive medical care and nutrition counseling. In some cases, these strategies may be insufficient to keep the blood glucose levels of pregnant women in normal ranges, at this stage, insulin therapy should be applied to the patients. If treated properly, the symptoms would probably disappear after delivery otherwise the women may develop type II diabetes later in their lives.

1.2.4. Complications of Diabetes Mellitus

Diabetic complications can be classified as acute (short-term) and chronic (long-term) complications. In the progress of diabetes, blood glucose levels become abnormally high, and have to be lowered to the normal ranges via diabetic medications. In this situation, there is a risk for blood glucose levels to drop below the normal values, due to the medication. On the other hand, if diabetes is not treated, then the glucose levels in the blood will continue to rise, fat breakdown will speed up, leading to accumulation of ketone bodies in the blood, which eventually ends up with ketoacidosis. All these effects mentioned above, emerging in a short period of disease progression, constitute the acute complications of diabetes.

Besides the short term effects, in the later stages of the disease, the complications become more serious and life-threatening. These complications are mainly resulted from the alterations in the cellular macromolecular structure, composition and function. In the literature, many studies present showing the diabetes-induced macromolecular alterations in different tissues, including bone (Boyar *et al.*, 2003), liver (Severcan *et al.*, 2000), heart (Toran *et al.*, 2006; Toyran *et al.*, 2007), muscle (Bozkurt *et al.*, 2007; Bozkurt *et al.*, 2010; Severcan *et al.*, 2010), platelets (Liu *et al.*, 2002), eye (Lin *et al.*, 1997) and in different membrane structures, including liver microsomal membrane (Severcan *et al.*, 2005a), heart crude membrane (Severcan *et al.*, 2003) and kidney brush border membrane (Severcan *et al.*, 2010).

The macromolecular alterations emerged due to diabetes, lead to destructions in all carbohydrate, fat and protein metabolisms. This situation eventually ends up with long-term complications affect nearly all of the organs and systems in the body, such as nervous system (neurological complications), eyes (blindness), heart (vascular complications of coronary artery disease), kidneys (renal failure) and testes (sexual dysfunction) (Hassan *et al.*, 1993; Harati 1996; Rolo and Palmeira 2006; Cohen *et al.* 2007).

According to the studies in the literature, diabetes has found to decrease the rat brain antioxidant capacity (Tahirovic *et al.* 2007), to provoke extensive neurochemical and structural changes (Chu *et al.* 1986; Wahba and Soliman 1988; Brands *et al.* 2003).

In addition to the neurological effects, diabetes also leads to breakdown of the blood-retinal barrier (BRB) in the structure of eye which takes place in the early stages of vascular dysfunction in the diabetic eye (Cunha-Vaz *et al.*, 1975; Enea *et al.*, 1989). BRB breakdown contributes to vasogenic macular edema in diabetic patients which is highly correlated with visual impairment in people with diabetic retinopathy (Klein *et al.*, 1995).

For the case of cardiovascular impairments, long-standing diabetes has documented to cause structural and functional cardiac impairment and to lead to ischemic heart disease, cardiomyopathy and congestive heart failure. It has mentioned in a previous study that, the structural changes in patients with diabetes are associated with impaired ventricular performance (Grossman and Messerli, 1996).

In the renal system, diabetes may cause nephropathy, a result of direct vascular abnormalities. In diabetic nephropathy, glomerular basement membrane becomes thickened, glomerular endothelium loose function and extracellular matrix components deposit into the mesangial area (Wolf *et al.*, 2005).

Long term effects of diabetes on testes of the reproductive system have shown as diminished reproductive organ weight, testicular sperm content, epididymal sperm content and sperm motility. Moreover, a decrease in the level of serum testosterone levels has also been determined. Thus, it is obvious that, sex behavior and reproductive tract functions are markedly affected by diabetes (Hassan, 1993).

1.2.5. Oxidative Stress in Diabetes Mellitus

Many diabetic complications are thought to be resulted from enhanced oxidative stress. Reactive oxygen species (ROS) are found at physiological concentrations in a healthy individual, which have a dramatic role in the maintenance of homeostasis. However in diabetics, production of free radicals is increased due to hyperglycemia and the antioxidant potential is reduced (Johansen *et al.*, 2005). Accumulation of ROS for a long period of time ends up with toxic effects of free radicals and oxidative stress.

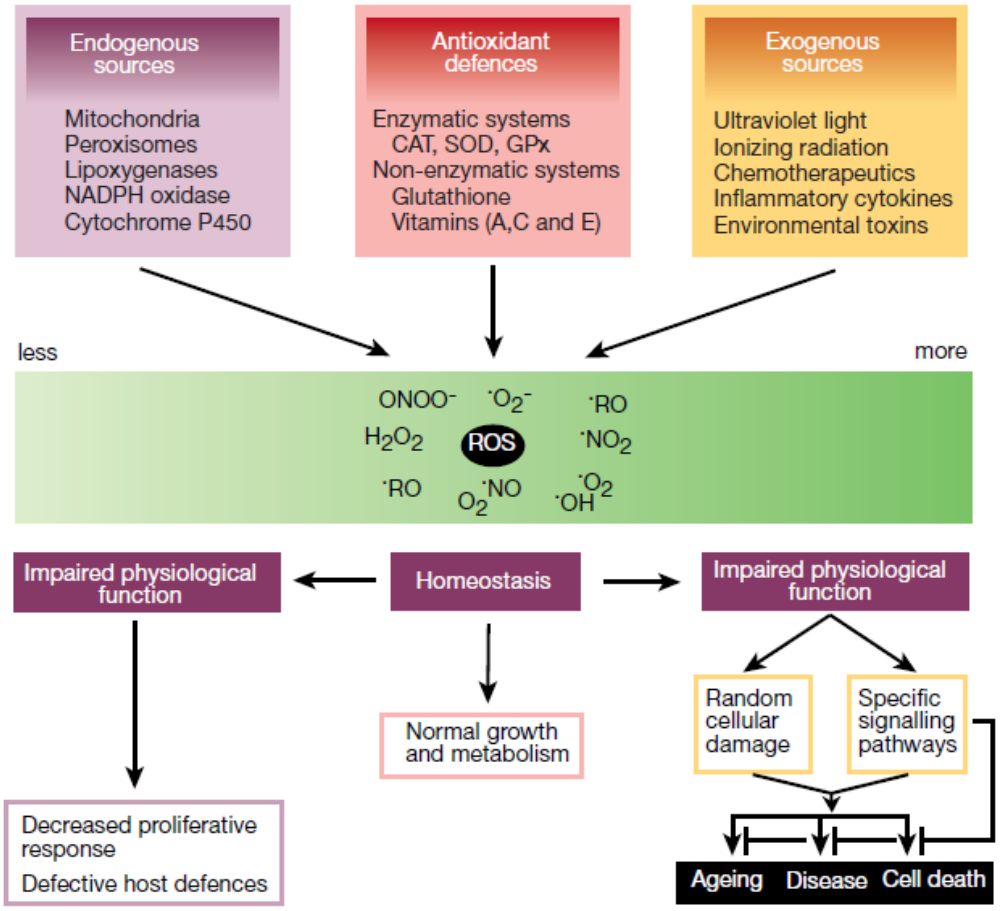

Figure 6. The sources and cellular responses to reactive oxygen species (ROS). (Kangralkar *et al.*, 2010).

In both type I and type II diabetes, it has been shown that enhancement of oxidative stress is accompanied by the increase in lipid peroxidation and protein oxidation levels (Velazquez *et al.*, 1991; Collier *et al.*, 1992; MacRury *et al.*, 1993; Neri *et al.*, 1994; Laaksonen *et al.*, 1996; Santini *et al.*, 1997; Laaksonen and Sen, 2000; Cederberg *et al.*, 2001; Severcan *et al.*; 2005a).

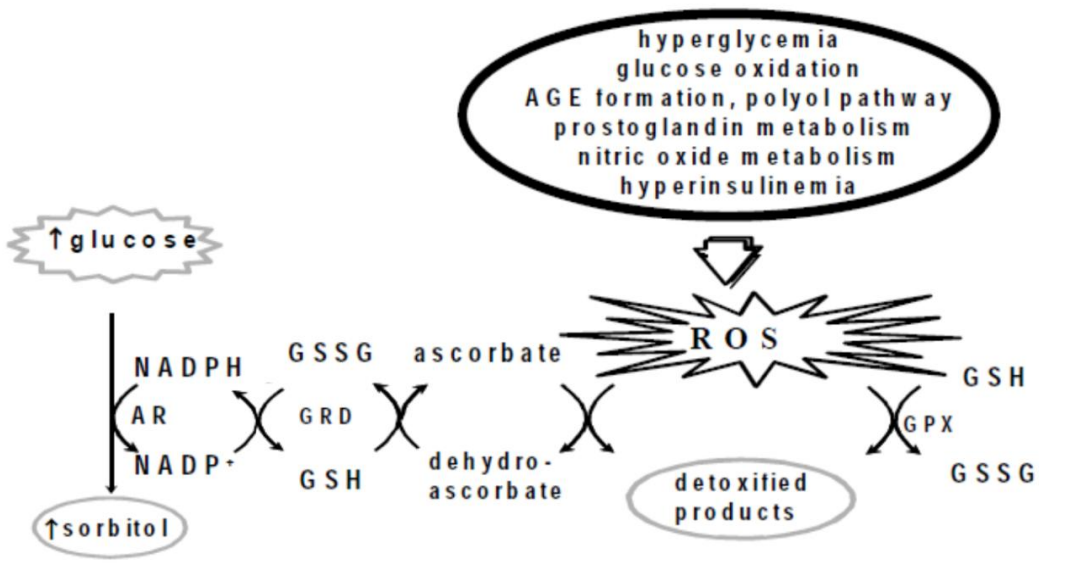

Figure 7. Mechanisms for increased oxidative stress in diabetes mellitus. ROS; reactive oxygen species, GSH; reduced glutathione, GSSG; oxidized glutathione, GRD; glutathione reductase, GPX; glutathione peroxidase, AR; aldose reductase. (Atalay and Laaksonen, 2002).

Oxidative stress is generated as a result of an imbalance between the systems that produce and scavenge the radicals. Even the exact mechanisms behind the enhancement of oxidative stress in diabetics has not been completely clarified yet, it is thought that combination of different, but interrelated mechanisms are responsible for this situation. These mechanisms include increment in the free radical formation,

such as superoxide radicals (Ceriello *et al.*, 1991; Wolff *et al.*, 1991; Baynes 1991; Dandona *et al.*, 1996) and antioxidant enzyme inactivation, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) inactivation, all of which have a role in the elimination of superoxide, hydrogen peroxide and hydroxyl radicals (Blakytny and Harding, 1992; Kawamura *et al.*, 1992, Yilmaz *et al.*, 2002; Sadi *et al.*, 2008; Kangralkar *et al.*, 2010). In addition, glycoxidation (Hunt *et al.*, 1990; Wolff *et al.*, 1991), formation of advanced glycation products (AGE) (Lyons, 1993; Schleicher *et al.*, 1997; Maritim *et al.*, 2003; Johansen *et al.*, 2005), activation of the polyol pathway (Grunewald *et al.*, 1993; Kashiwagi *et al.*, 1994; De Mattia *et al.*, 1994; Cameron *et al.*, 1996; Kashiwagi *et al.*, 1996), formation of lipid peroxides, decreased levels of ascorbic acid (Sinclair *et al.*, 1991), and perturbations in nitric oxide and prostoglandin metabolisms (Tesfamariam, 1994; Maejima *et al.*, 2001) constitute the other possible mechanisms which can end up with elevated oxidative stress (Figure 8).

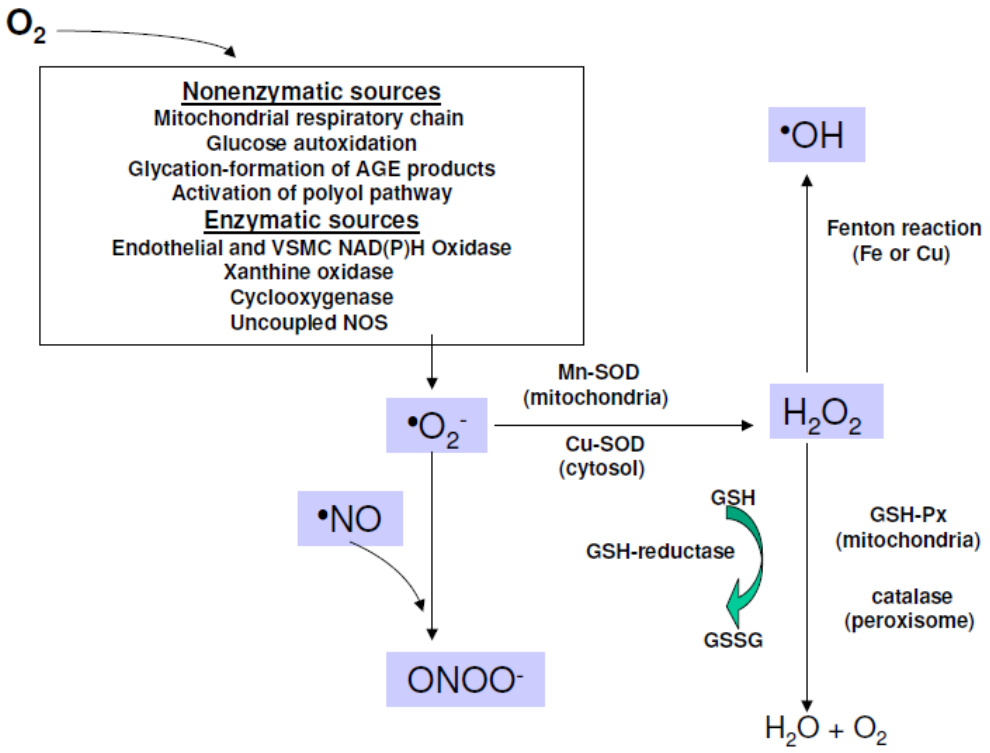

Figure 8. Generation of reactive species in diabetes, where superoxide dismutase is donated as SOD, reduced form of glutathione is donated as GSH, oxidized form of glutathione is donated as GSSG, glutathione peroxidase is donated as GSH-Px, and glutathione reductase is donated as GSH- reductase. (Johansen *et al.*, 2005).

1.2.6. Experimental Models of Diabetes Mellitus

Using experimental animal models in diagnosis and investigation of the pathogenesis of various diseases provide many advantages. In animal experiments, the number of the subjects in each experimental group should be chosen to allow statistically meaningful analysis. Besides, carrying out animal experiments give us chance to perform controlled experiments, in which the effects of only one parameter can be taken into consideration.

In the investigation of the pathogenesis and the molecular basis of diabetes mellitus, different kinds of experimental animal models have been commonly used. By this

way, the effect of each environmental factor can be examined separately, such as diet, drugs, toxins, viruses, physical activity and etc. Even these animal studies give valuable information about the disease, none of the animal models used, absolutely corresponds to the diabetes developed in human (Irer and Gulinnaz, 2004).

There are different methodologies used in order to construct experimental animal models for diabetes, which include spontaneous diabetes, surgical diabetes, viral diabetes and chemically induced diabetes.

Spontaneous diabetes is developed in animals which are genetically susceptible. BB Wistar rat is an example for type I diabetes model and, Goto-Kakizaki rat is an example for type II diabetes model which originates from selective breeding of glucose-intolerant non-diabetic Wistar rats over generations (Chen and Wang, 2005).

Surgical diabetes is generated by a process called pancreatectomy which includes the removal of the most pancreatic tissue. More than 80% of the pancreatic tissue is removed in order to obtain moderate hyperglycemia while, even more than 90% of pancreatic tissue has to be taken out in order to develop significant levels of hypoinsulinemia (Masiello, 2006; Etuk 2010).

Viruses are one of the most important environmental factors that lead to diabetes. More than 14 different viruses have been reported to be associated with the development of diabetes by inducing pancreatic beta cell autoimmunity. These viruses include Kilham rat virus (KRV), encephalomyocarditis, rubella, mumps and enteroviruses (Yoon *et al.*, 1977; Yoon *et al.*, 1980; Roivainen, 2006).

Besides the methodologies described above, some chemical agents, such as alloxan (ALL) and streptozotocin (STZ), are also used to induce diabetes in animals. These diabetogenic drugs can be administered intravenously, intraperitoneally or subcutaneously. The dose and the route administration is determined depending on the species of the animals used (Etuk, 2010). These agents selectively destroy the

beta cells of Langerhans islets in pancreas, thus the treatments end up with insulin deficiency and hyperglycemia (Jawerbaum and White, 2010). Among those diabetogens, streptozotocin is more selectively destroy pancreatic beta cells with respect to alloxan, so it is more commonly used in animal studies (Junod *et al.*, 1967). STZ prevents DNA synthesis in cells, and thus it prevents the cellular reproduction in mammalians, which in turn leads to destruction of pancreatic beta cells through necrosis (Sharad *et al.*, 2010).

1.2.6.1. Streptozotocin Induced Diabetes Mellitus

Streptozotocin [STZ; 2-deoxy-2(3-methyl-3-nitrosoureido)-Dglucopyranose] is an antibiotic isolated from *Streptomyces achromogenes* which also have oncolytic, oncogenic, and diabetogenic properties (Rossini *et al.*, 1977).

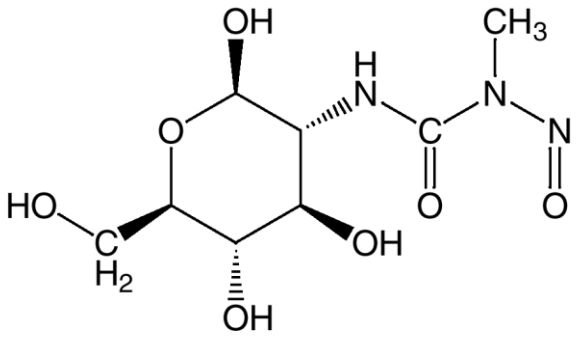

Figure 9. Chemical structure of Streptozotocin (STZ), (2-deoxy-2(3-methyl-3-nitrosoureido)-Dglucopyranose)**.**

As can be seen from the chemical structure, a glucose group is attached to the nitrosourea derivative, which provides the internalization of the STZ through the glucose transporters (Sharad *et al.*, 2010). Once the compound is taken into the cell,

the nitrosourea moiety is released, then it forms cross-linking with the vital structures and enforce the cell to die. Since pancreas is an excessively active organ in terms of glucose uptake, the poisonous effect of STZ would be more dramatic in this organ (Sharad *et al.*, 2010). Streptozotocin not only prevents DNA synthesis more selectively in mammalian pancreatic cells by leading to alkylation of DNA, but it also elevates the oxidative stress by enhancing the generation of hydrogen peroxide and hydroxyl radicals. Due to these properties, the molecular mechanism of the effect of STZ in the cell ends up with cell death through necrosis (Figure 10). Since the action of STZ results with destruction of pancreatic beta cells rather than development of insulin resistance, the model developed is considered as type I diabetic model (Jawerbaum and White, 2010).

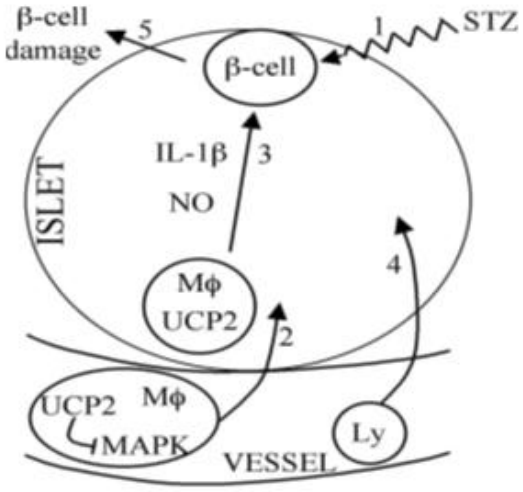

Figure 10. 1. STZ administration, 2. Recruitment of monocytes/ macrophages, 3. Deleterious environment, 4. Recruitment of lymphocytes, 5. Development of diabetes. (Reproduced from Yalin *et al.*, 2007).

STZ is administered to the animals as a single injection in order to induce diabetes. It has a serum half life as 15 min, meaning that STZ is cleared from bloodstream in 15

minutes (Rossini *et al.*, 1977). 1-2 days after the injection, hyperglycemia and 3 days after the injection, phagocytosis of necrotic cells can be observed in the diabetes-induced animals. The effect of STZ is dose-dependent. Administration of low doses (35 mg/kg) of STZ causes mild diabetes leading to acute ketoacidosis, moderate doses (55-65 mg/kg) results in high blood glucose levels and weight losses (Tomlinson *et al.*, 1992), while administration of high doses (100 mg/kg) ends up with death within 2 to 3 days.

1.3. Effects of Diabetes Mellitus on Testicular tissue

Diabetes mellitus leads to several disturbances in reproductive system. Severity of the disturbances is directly proportional with the duration and the degree of hyperglycemic status (Rodriguez-Rigau, 1980). It is thought that the complications of diabetes on testicular tissue can either be resulted from the decreased action of the hypothalamic pituitary-testicular axis, or be resulted from the direct influence of hyperglycemic condition on the testicles (Foglia *et al.*, 1969).

Testicular function is primarily controlled by the pituitary hormones. Considering the effect of diabetic state on hypothalamic pituitary-testicular axis, hypothalamus mediated control is altered which leads to decreased secretion of gonadotropin hormones from the pituitary, due to hyperglycemia or insulinopenia (Soulairac and Desclaux, 1948; Moguilevsky *et al.*, 1966; Foglia et al, 1969; Paz et al, 1978). Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), and growth hormone, which are secreted by pituitary, have found to be diminished in the blood serum of diabetic patients (Hutson et al, 1983; Benitez and Perez Diaz, 1985). Among these hormones, FSH is responsible for the regulation of spermatogenesis, and LH is responsible for the control of Leydig cell function (Ward et al, 1991). Even it is known that the changes in glucose and insulin metabolisms are correlated with the production of these two hormones, and thus associated with the function of the hypothalamic pituitary-testicular axis the exact molecular mechanism has remained unclear.

Besides the decreased functioning of Leydig cells, the total amount of these cells are also reduced due to the effect of diabetes on testicular tissue, which results in diminished production and secretion of the major male hormone, testosterone. Limited levels of testosterone lead to delayed sexual maturation and sexual dysfunction, which are the most frequent complications relevant with the reproductive systems of diabetic male patients. Moreover, sexual impotence, retrograde ejaculation (Klebanow and MacLeod, 1960; Greene *et al.*, 1963; Ellenberg and Weber, 1966; Kolodny *et al.*, 1974), premature ejaculation (Kolodny *et al.*, 1974), decreased libido (Klebanow and MacLeod, 1960), hyperplasia of the interstitial tissue (Oksanen 1963; Schoffling *et al.*, 1963; Fairburn, 1981), and degenerative alterations in the germinative epithelium, seminiferous channels and basal membrane thickness constitute the other complications of diabetes on reproductive system of males. All of these defects may eventually end up with infertility.

1.4. Vitamin C (Ascorbic acid)

Vitamin C (L-Ascorbic acid or L-Ascorbate) is a water-soluble, essential nutrient which is required for normal growth and development. Since Vitamin C is soluble in water, it can not be stored in the body, the excess amounts are readily eliminated through urine. Thus, it should be continuously provided by diet. Ascorbic acid is derived from glucose via the uronic acid pathway. Gulonolactone has to be converted into Ascorbic acid by the help of the enzyme, L-gulonolactone oxidase (Figure 11). Since this enzyme is not present in primates, they have to obtain the essential doses of Ascorbic acid from their diet. The good sources are majorly the fresh fruits and vegetables, particularly the citrus fruits.

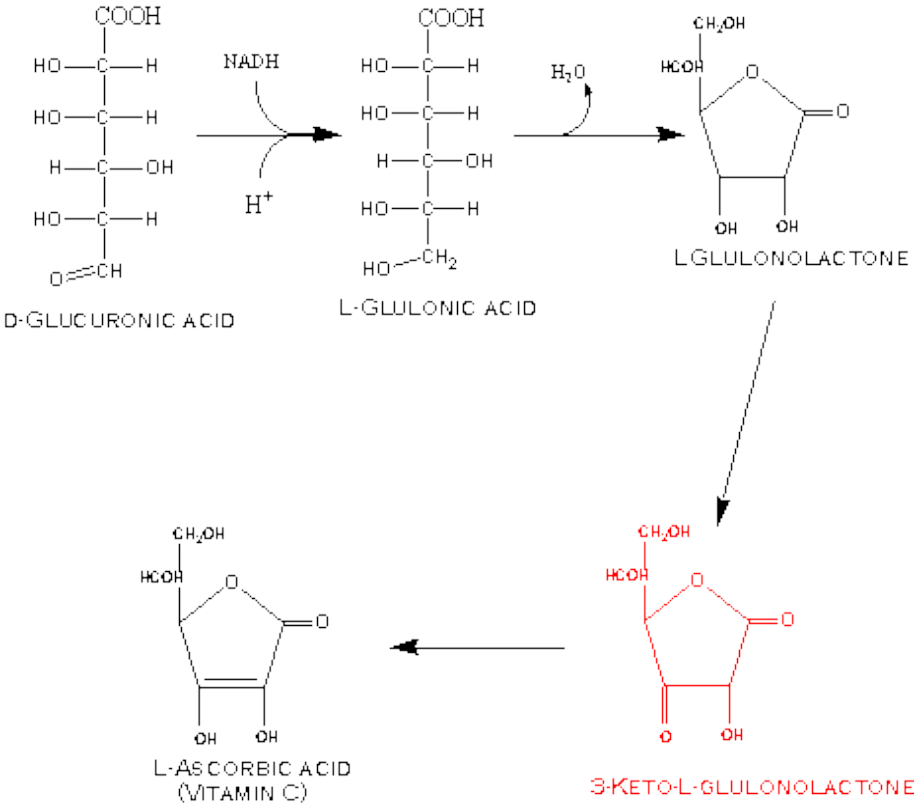

Figure 11. Synthesis of ascorbic acid.

Vitamin C is thought to be effective in the treatment of common cold, treatment of age-related macular degeneration (when used with other medicines), in lowering the high blood pressure, slowing the worsening of osteoarthritis, decreasing protein levels in the urine of type II diabetic patients, preventing atherosclerosis, reducing complications after a broken wrist, and also effective in treatment of lung diseases such as asthma and bronchitis (Anderson *et al.*, 1973; Seddon *et al.*, 1994; Carr and Frei, 1999; Upritchard *et al.*, 2000; Chen *et al.*, 2001a; Romieu and Trenga, 2001).

Vitamin C has many substantial functions in the body considering the molecular aspect. It is used as a cofactor or as a coenzyme, required for the normal functioning of the cellular structures. In the metabolism, it can also act as a reducing agent and as

an antioxidant (Niki, 1991; Retsky *et al.*, 1993; Maxwell *et al.*, 1997; Padayatty *et al.*, 2003).

The role of ascorbate as a reductant in the enzymatic reactions is to be a scavenger of free radicals including superoxide, hydrogen peroxide, hypochlorite, the hydroxyl radical, peroxyl radicals (Nishikimi, 1975; Bodannes and Chan, 1979; Cabelli and Bielski, 1983; Bendich, 1986; Halliwell, 1987; Kwon and Foote, 1988; Dwenger, 1992). Therefore, Vitamin C can be used to protect the tissues from harmful oxidative products and can also be used to keep certain enzymes in their required reduced forms (Padh, 1990).

As an antioxidant, ascorbic acid becomes oxidized to semidehydroascorbate, and to dehydroascorbate successively. Semidehydroascorbate is reconverted to ascorbate in the cytosol by cytochrome b_5 reductase and thioredoxin reductase in which NADH and NADPH required, respectively. Dehydroascorbate, which is the fully oxidized form of Vitamin C, is reduced spontaneously by glutathione, as well as it is reduced enzymatically in reactions using glutathione or NADPH (Padayatty *et al.*, 2003). This antioxidant property is so important not only because it is associated with lowered DNA damage but also because it is associated with diminished lipid peroxidation levels in the cells (Sies and Stahl, 1995). As an example, Vitamin C has been shown to protect the human sperm against the endogenous oxidative DNA damage (Fraga *et al.*, 1991). In addition, it has been shown to protect the membranes against peroxidation by enhancing the activity of tocopherol (Vitamin E), the major lipid-soluble, chain-breaking antioxidant (Niki *et al.*, 1985; Lambelet *et al.*, 1985; Wayner *et al.*, 1987).

1.5. Effects of Vitamin C on oxidative stress and diabetes

The common characteristic in both type I and type II diabetic patients, is the elevated blood glucose levels. As mentioned in the previous sections, hyperglycemic condition in accordance with the elevated levels of free fatty acids, leads to dramatic

increase in the production of ROS species and subsequent oxidative stress. Hyperglycemia gives rise to an increase in the input of metabolic substrate into mitochondria and overwhelms the electron transport system (ETS), which results in overproduction of ROS (Yu *et al.*, 2005).

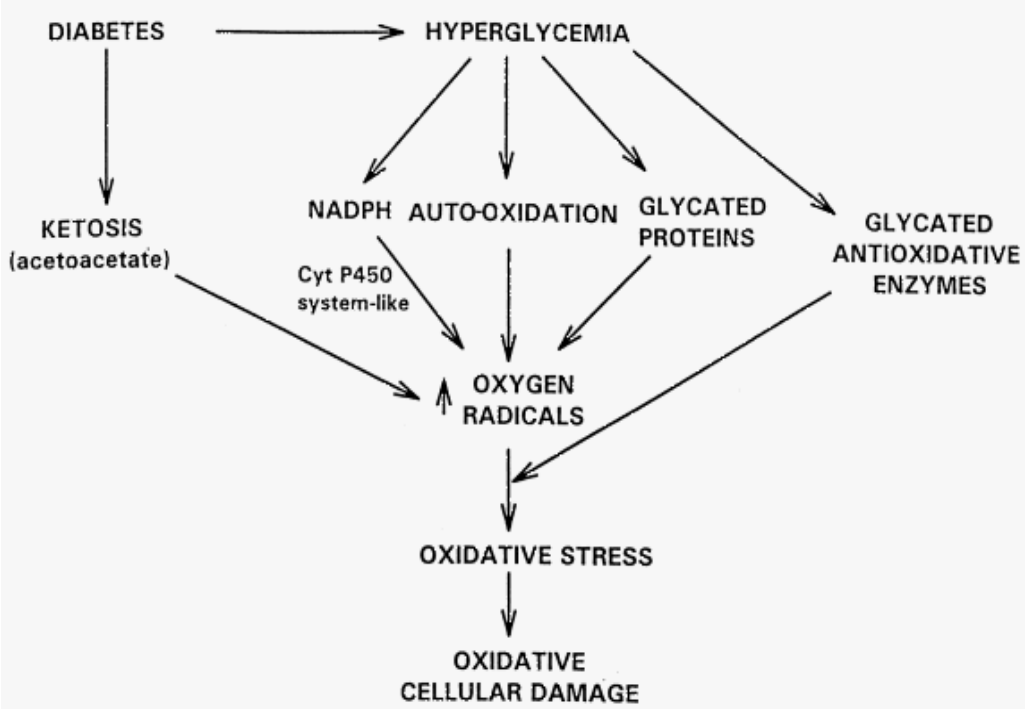

Figure 12. Mechanisms of oxidative cellular damage in diabetes**.**

Elevated production of free radicals causes not only the destruction of the pancreatic beta cells in the islets of Langerhans, but also the destruction on DNA due to oxidation in diabetic conditions (Evans *et al.*, 2003; Green *et al.*, 2004). Besides the destructions in DNA, ROS has capability to alter all other major classes of biomolecules (e.g., lipids, proteins) with concomitant changes in their structure and function (McCall and Frei, 1998).

In normal conditions, in healthy subjects, free radicals are regulated by a combination of enzymatic and non-enzymatic antioxidant defense mechanisms, including catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH). However, in the diabetic patients, the antioxidant potential is lowered (Yılmaz *et al.*, 2002; Sadi *et al.*, 2008). Thus, the antioxidant potential should also be enhanced in addition to the medical treatments to cope with the damage resulted from oxidative stress. Various antioxidant molecules, including Vitamin C, Vitamin E (tocopherol), urate, thiols and bilirubin are powerful reductants that can scavenge the free radicals (Maxwell *et al.*, 1997).

Among those antioxidant molecules, Vitamin C donates electrons sequentially in non-enzymatic reactions. When one electron is lost, semidehydroascorbic acid or ascorbyl radical is generated. Compared to other free radicals this compound is relatively stable with a half-life of 10^{-5} seconds and fairly unreactive. This property of Vitamin C explains why ascorbate is a more preferred antioxidant with respect to the other candidates (Padayatty *et al.*, 2003). In the literature, it has been also shown that Ascorbic acid is far more effective in inhibiting lipid peroxidation which is initiated by a peroxyl radical initiator than other antioxidant molecules, such as protein thiols, urate, bilirubin and tocopherol. Thus, by efficiently scavenging peroxyl radicals before they can initiate lipid peroxidation, Ascorbic acid can effectively protect biomembranes and the tissues against peroxidative damage (Sies and Stahl, 1995).

1.6. Basics of Spectroscopy

Electromagnetic radiation has two components, namely electrical field (E) and magnetic field (B). These interacting electrical and magnetic fields are oscillating at right angles to one another and also to the direction of propagation of the wave. This transverse wave is shown in Figure 13, where the direction of the electric field is donated as E, and the direction of the magnetic field is donated as B (Stuart, 1997).

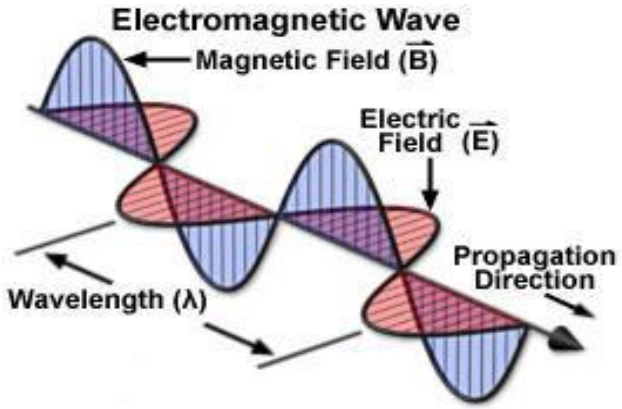

Figure 13. An electromagnetic wave.

Spectroscopy is defined as the study of interaction of electromagnetic radiation with matter. After interacting with an electromagnetic radiation, a matter will either absorb, emit, or scatter light particles. In the case of the energy of light is absorbed, the molecule is said to be excited to a higher energy level. An excited molecule can possess a quantum of energy described by the laws of quantum mechanics.

The typical energy-level diagram describing these energy levels is shown in Figure 14. The lowest energy level is defined as ground state, and all the higher energy levels are defined as excited state.

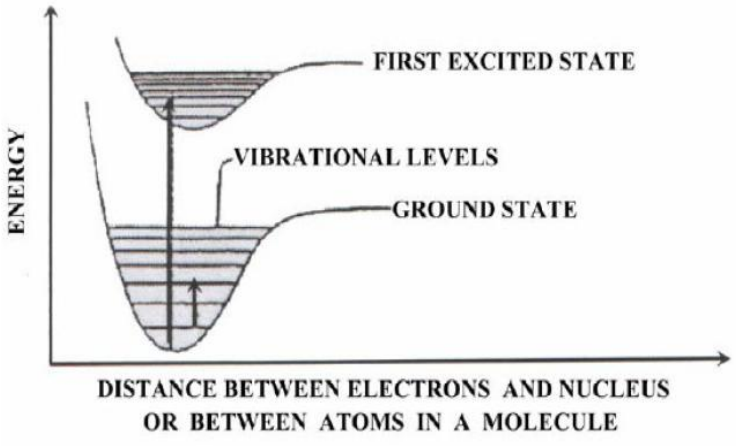

Figure 14. Typical energy-level diagram showing the ground state and the first excited state. Vibrational levels are shown as thin horizontal lines. A possible electronic transition between the ground state and the fourth vibrational level of the first excited state is indicated by the long arrow. A vibrational transition within the ground state is indicated by the short arrow**.** (Freifelder, 1982).

For most purposes, it is convenient to treat a molecule as if it possesses several distinct reservoirs of energy and the total energy of a molecule is given by the following equation (Campbell and Dwek, 1984).

E total = E transition + E electronic + E rotation + E vibration + E electron spin orientation + E nuclear spin orientation

The type of excitation depends on the wavelength of the light, which is in turn associated with the level of energy. As an example, when the light applied is from the infrared portion of the electromagnetic spectrum, the transition occurs from one vibrational energy level to another (Freifelder, 1982).

The energy (E) of the wave can be formulized by the Bohr equation as:

$$\Delta E = h.\ \upsilon$$

υ is the frequency of the applied radiation and *h* is the Planck's constant ($h=6.6x10^{-34}$ joule second).

$$\lambda = c.T = c/\ \upsilon$$

where c is the speed of light in vacuum ($3.0x108\ ms^{-1}$), T is the period and λ is the wavelength of light. These two equations given above can be used to identify the spectral unit called wavenumber, denoted by $\bar{\upsilon}$.

$$\bar{\upsilon} = \text{wavenumber} = (1/\ \lambda)\ [\text{has a unit of } cm^{-1}]$$

$$E = h.\ \upsilon = h.c/\ \lambda = h.c.\ \bar{\upsilon}$$

The plot of energy absorbed, emitted or scattered as a function of λ, υ or $\bar{\upsilon}$ is called a spectrum.

1.6.1. Infrared (IR) Spectroscopy

Infrared spectroscopy is a spectroscopic technique, in which the energy of most vibrational transitions results from the absorption of light in the infrared region of the

electromagnetic spectrum. The infrared portion of the electromagnetic spectrum can be divided into three major regions as near, middle and far infrared regions (Smith, 1999).

Region	**Wavenumber range (cm^{-1})**
Near	14000-4000
Middle	4000-400
Far	400-4

The infrared radiation applied to a matter is absorbed and makes the chemical bonds in the corresponding matter to vibrate. Thus, it can be said that, the infrared spectroscopy is based on the transitions between vibrational energy levels of the atoms of a molecule. There are various kinds of vibrations are present, which can be classified majorly in two groups, stretching and bending vibrations. The stretching and bending modes of vibrations are caused by the changes in bond length and bond angle respectively, due to the characteristic motions of various functional groups (e.g. methyl, carbonyl, amide etc.). Any alterations in these modes of vibrations correspond to the alterations in the function, conformation, and structure of the functional groups present in the system.

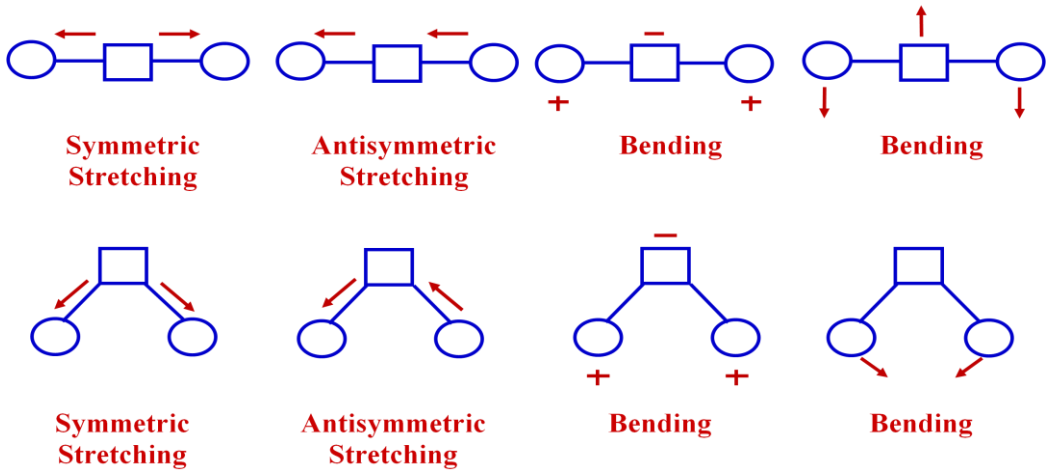

Figure 15. Types of normal vibrations in a linear and non-linear triatomic molecule**.**

1.6.2. Fourier Transform Infrared (FTIR) Spectroscopy

In FTIR spectroscopy, a beam of light which contains various frequencies is used instead of a monochromatic beam of light. The incoming light split into two paths using a half-silvered mirror. Then, both of the beams have been transmitted and reflected once as they are divided at the beamsplitter, reflected at either the movable or fixed mirror and eventually recombined at the beam splitter. Then the beam is transmitted to the sample and to the detector. Finally, a spectrum obtained as a function of the position of the moving mirror. Generally, two spectra are obtained, one of which is with the sample placed in the path of the light, and the other is without the sample. Then, ratios of those of two spectra are calculated point by point, and the resultant spectrum, which is frequency-dependent transmission of the sample, is obtained. For this purpose, most commonly Michelson Interferometer is used.

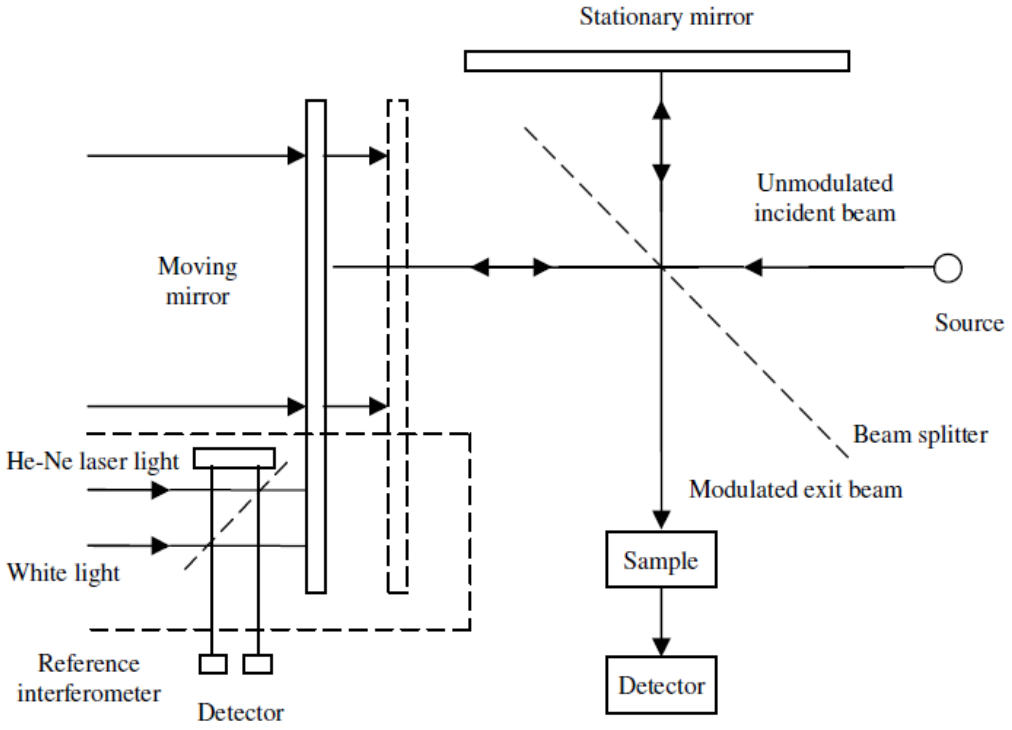

Figure 16. Basic component of an FTIR spectroscopy**.**

By this way, an interferogram is obtained, corresponding to a signal produced as a function of the change of path length between the two light beams (Stuart, 2004). The most important property of an interferogram is that, every single data point of the signal contains information all over the infrared region.

"The two domains of distance and frequency are interconvertible by the mathematical method of Fourier transformation" (Stuart, 2004). Therefore, by using the mathematical model called Fourier transformation, all the individual cosine waves that contribute to the interferogram are eventually denconvoluted by computer, and a plot of absorbance against wavenumber (cm^{-1}) is gathered, which constitutes the final representation of an infrared spectrum (Stuart, 1996).

The resulting spectrum represents the molecular fingerprint of the corresponding sample. Since there would be no two unique molecular structures have a possibility to produce the exactly same infrared spectrum, assignments for the functional groups can be done reliably prior to the spectral analysis.

1.6.3. Attenuated Total Reflectance (ATR) – FTIR Spectroscopy

Various kinds of samples, either solid or liquid can be studied by FTIR spectroscopy. By using ATR unit attached to the FTIR instrument, the sample preparation procedures can be reduced or even eliminated, and the spectral reproducibility can be enhanced. Attenuated total reflection attachment is based on the alterations in the total internal reflection of light, as the beam comes into contact with the sample (Figure 17).

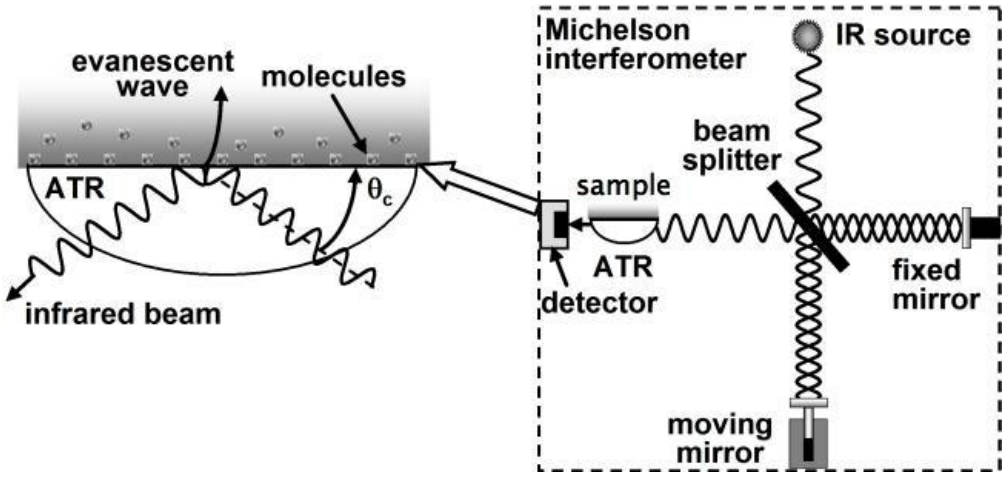

Figure 17. A multiple reflection ATR system**.**

An infrared beam directed to the ATR crystal with a high refractive index at a certain angle. If the angle of incidence at the interface among the crystal and sample is greater than the critical angle, which is defined as the function of the refractive indices of the two surfaces, then it undergoes to total internal reflectance. The infrared beam can get a signal when it is a few microns closely contact with the sample. Therefore, it is very important to have a close contact between the crystal and the sample, which can be achieved by applying a certain pressure to the sample.

When the sample absorbs the energy in certain regions of infrared spectrum, the remaining radiation will be attenuated. This attenuated radiation is detected by the spectrometer, and the infrared spectrum belongs to the sample will eventually be generated.

There are a number of crystal materials available for ATR. The crystal materials including Germanium (Ge), Silica (Si), Diamond, and zinc-selenide (ZnSe) which are chosen according to the characteristics of the samples analyzed (Table 1).

Table 1. Physical properties of commonly used ATR crystal materials.

Materials	Spectral range (cm^{-1})	Refractive index	Depth of penetration µm.
Diamond	4,5000-2,500 1,667-33	2.4	1.66
Ge	5,500	4	0.65
KRS-5	20,000-400	2.37	1.73
Si	8,300-1,500 360-70	2.37	1.73
ZnSe	20,000-650	2.4	1.66

Among these typical materials for ATR crystals diamond and zinc selenide are ideal for use in the mid-IR region while silicon is ideal for use in the far-IR region of the electromagnetic spectrum. Diamond and ZnSe crystals are relatively low cost ATR crystal materials, resistant to many chemicals, including many commercially available acids and alkalis. The crystal is also water resistant, which makes it easy to clean and makes it ideal to analyze wet or aqueous samples.

1.6.4. The Advantages of FTIR Spectroscopy

There are many advantages of FTIR spectroscopy are listed below.

- FTIR spectroscopy is a non-destructive, rapid and sensitive technique for structural and functional studies (Haris and Severcan, 1999; Melin *et al.*, 2000; Cakmak *et al.*, 2003).
- Instruments are relatively inexpensive when compared to other types of spectroscopic instruments.
- Regardless of the type and the phase of the sample used, small amounts would be sufficient for precise analysis (Mendelsohn and Mantsch, 1986).
- All kinds of samples, either solid, liquid or gases can be analyzed through FTIR spectroscopy.
- The samples used can be viscous liquids, in solutions, suspensions, inhomogeneous solids or powders (Colthup *et al.*, 1975).
- No long and sophisticated sample preparation procedures are needed.
- Any alterations in the functional groups of the macromolecules found in the system can be detected by FTIR spectroscopy simultaneously from the same spectrum (Kneipp *et al.*, 2000; Bozkurt *et al.*, 2007; Garip *et al.*, 2007).

- FTIR spectroscopy provides a precise measurement in which no external calibration is required (Rigas *et al.*, 1990; Manoharan *et al.*, 1993; Ci *et al.*, 1999).
- It has greater optical throughput.
- Rapid data acquisition is possible.
- The most dramatic advantage is its capability to increase the signal-to-noise ratio.
- With the utility of Fourier transformation, many scans can easily and rapidly be performed for a single sample in order to improve the signal-to-noise ratio. Therefore, signal-to-noise ratio can be enhanced by the averaging of numbers of scans per sample (Beaten *et al.*, 1998).
- Data processing can be performed simply with the aid of the computer software.
- The software also permits to store the data obtained permanently. In addition, by the software, it becomes possible to make some manipulations on the data and to make quantitative calculations (Rigas *et al.*, 1990; Manoharan *et al.*, 1993; Yano *et al.*, 1996; Ci *et al.*, 1999).

1.6.5. Applications of FTIR Spectroscopy in Biological Samples

Any alterations in the functional groups of cellular and/or membraneous macromolecules, such as proteins, lipids, nucleic acids and carbohydrates can be detected by FTIR spectroscopy (Fabian *et al.*, 1995; Moore *et al.*, 1995; Kneipp *et al.*, 2000; Toyran *et al.*, 2004; Toyran *et al.*, 2005; Akkas *et al.*, 2007). To get valuable information, shifts in band frequencies, alterations in the band areas and changes in the bandwidth values are determined in the spectra analyzed.

Different kinds of biological samples can be sensitively analyzed by FTIR spectroscopy. Among those samples, proteins in solution (Haris and Severcan, 1999; Severcan and Haris, 2003), isolated biological membranes (Severcan *et al.*, 2003; Severcan *et al.*, 2005a), and tissues (Severcan *et al.*, 2000; Boyar *et al.*, 2003; Bozkurt *et al.*, 2007; Bozkurt *et al.*, 2010; Ozek *et al.*, 2010a; Severcan *et al.*, 2010) are the ones that are most commonly studied.

In recent years, FTIR spectroscopy has become widely used in characterization of pathological tissues resulting from either a disease state, or from a chemical treatment. The disease states studied, include cancer (Ozek *et al.*, 2010b), obesity and diabetes (Lin *et al.*, 1997; Severcan *et al.*, 2000; Severcan *et al.*, 2003; Bozkurt *et al.*, 2007; Bozkuert *et al.*, 2010; Severcan *et al.*, 2010), on the other hand, the chemical treatments studied, includes the application of radiation (Cakmak *et al.*, 2011), and alcohol (Elibol-Can *et al.*, 2011). Besides, there have been also some studies performed to investigate the aquatic toxicological states (Cakmak *et al.*, 2003; Cakmak *et al.*, 2006), to determine the radiation-induced changes in foods (Dogan *et al.*, 2007), and to make characterization of different bacterial strains (Garip *et al.*, 2007).

1.6.6. FTIR Microspectroscopy

FTIR microspectroscopy, combination of an infrared spectroscopy and a light microscopy, is used as a comprehensive and sensitive technique for gathering rapid visual and chemical information for many sample types. By this way, any molecular changes in the samples can be detected together with the alterations in the morphological status. The microscope system automatically scans the sample and constructs a point-by-point map. This can be accomplished by taking the spectrum of the sample at one point and then moving the sample to the next point, and take the spectrum for that point, and continue like that until all the area of interest is scanned. At the end of scanning, the chemical map of the sample is obtained. With the aid of this method, larger areas can be analyzed in lesser time in contrast with other

traditional techniques This property allows FTIR microspectroscopy to collect much more statistical data.

One of the most important property of FTIR microspectroscopy is to get information about the morphology and about the macromolecular components with a greater spatial resolution and microscopic scale (Levin ang Bhargawa, 2005).

The most commonly applied areas of FTIR microspectroscopy includes the identification of dyes and pigments, identification of fibers, characterization of coatings, polymeric materials, and pharmaceuticals, analysis of forensic materials, and characterization and diagnosis of the diseased cells and tissues at very early stages (Gillard *et al.*, 1994; Katon 1996; Wetzel and LeVine, 1999; Scott *et al.*, 2001; Erukhimovitch *et al.*, 2005; Toyran *et al.*, 2006).

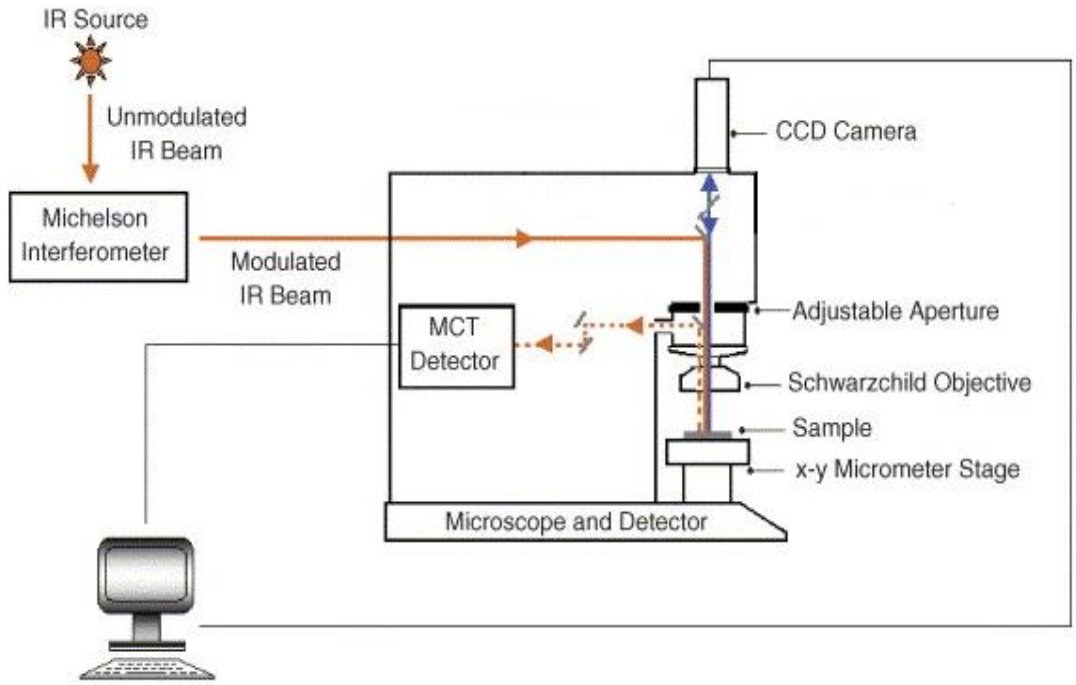

Figure 18. A simplified diagram of the Fourier transform infrared (FTIR) microscope system.

1.6.7. The Advantages of FTIR Microspectroscopy

- By FTIR microspectroscopy, high quality spectra can be obtained together with visual images of the sample of interest.
- Very small amount of sample is sufficient to make analysis.
- It takes relatively lesser time to gather the visual image, thus it is accepted as a fast imaging tool (Kretlow *et al.*, 2008).
- No sophisticated sample preparation procedure is required.
- Since FTIR imaging relies on the characteristic absorbance of the molecular vibrations in the sample any preprocessing, dying or labeling methods are not required for visualization, which make FTIR microspectroscopy a non-destructive technique (Kazarian and Chan, 2006; Kretlow *et al.*, 2008).
- The chemical maps of the sample are accomplished with a high spatial resolution (Jackson *et al.*, 1998).

1.6.8. Applications of FTIR Microspectroscopy in Biological Samples

Having a capability of rapid imaging and obtaining chemical maps with high spatial resolution, FTIR microspectroscopy has become a powerful tool in both diagnosis of different types of diseases and monitoring the progression of the diseases in various biological samples (Levin and Bhargava., 2005). In other words, it has recently become possible to characterize the pathologies in various cell and tissue types with the aid of FTIR imaging.

In the literature, there is a growing evidence found about the power of FTIR microspectroscopy in the identification of different tissue states, such as osteonal bone tissue (Paschalis *et al.*, 1996), lymphoid tumors in lymphs (Ramesh *et al.*, 2002), lung cancerous and noncancerous tissues (Yano *et al.*, 2000), cervical precancerous tissue (Chang *et al.*, 2003), brain tumors and tumor cells (Krafft *et al.*,

2007), Alzheimer's diseased brain tissue (Choo *et al.*, 1996), atherosclerotic human artery (Manoharan *et al.*, 1993), cirrhotic liver tissue (Diem *et al.*, 2000), diabetic heart tissue (Toyran *et al.*, 2006; Toyran *et al.*, 2007), and irradiated brain tissue (Cakmak, 2010). Besides, there have been successful studies performed about the characterization of different diseases, like colon cancer (Chen *et al.*, 2001b), breast cancer (Dukor *et al.*, 1998), pancreatic cancer (Chen *et al.*, 2006), acute human infections (Mordehai *et al.*, 2004) and inflammatory bowel disease (Argov *et al.*, 2004).

1.7. Aim of the Study

Diabetes mellitus is a chronic disease which causes about 5% of all deaths globally each year. Insufficient production, secretion or ineffective usage of insulin in the body leads to alterations in carbohydrate, fat and protein metabolisms, with both biochemical and anatomical/structural consequences. Therefore, diabetes is regarded as a multisystem disease affecting all vital organs and systems in the body.

In the literature, many studies are found about the complications of diabetes on various tissues. There have been also some studies performed in order to investigate the effects of diabetes on male reproductive system. However, the effect of diabetes on the structure and function of the macromolecular content of testicular tissue is still unknown. Therefore, in the current study, it was aimed to investigate the alterations in the composition, structure and function of the macromolecular content of rat testes by FTIR spectroscopic and microspectroscopic techniques together with the use of chemometric tools.

FTIR spectroscopy is a non-destructive technique, by which even small alterations in the functional groups of the macromolecules found in the system can be detected. This tool is also useful for the analysis of protein secondary structural components. Moreover, with the aid of FTIR microspectroscopy, high quality spectra can be obtained together with visual images of the samples. By this way, it would also

become possible to obtain the images of the alterations in the system. Therefore, these two techniques are very powerful in diagnosis and characterization of disease states and also in determination of the therapeutic effects of different drugs.

It has been known that, the oxidative stress, caused by the elevated free radical levels, leads to the development of diabetic complications. The main reasons which result in enhanced formation of free radicals are hyperglycemic condition and reduced antioxidant potential. Thus, the administration of antioxidant molecules is thought to be effective to lower the oxidative stress. Therefore, antioxidant treatment may have a recovery effect against the diabetic complications. For this reason, in the current study, besides the investigation of the physiopathology of diabetes on rat testes, it was also aimed to examine the recovery effect of Vitamin C, administered as an antioxidant, in diabetic subjects at macromolecular level.

CHAPTER II

MATERIALS & METHODS

2.1. Reagents

Streptozotocin (STZ), Ascorbic acid (Vitamin C), Insulin and Pentobarbital were purchased from Sigma Chemical Company, Saint Louis, in Missouri, USA. All chemicals were supplied at the highest grade of purity.

2.2. Animals and Feeding Procedures

With the approval of the experimental procedures by the Ethics Committee of Firat University, 12–14 weeks old male Wistar rats weight in the range of 250–300 gr were obtained (Firat University, in Elazig, Turkey). The adult male rats were housed in stainless steel, wire-bottomed cages in a conventional room with 12:12 dark: light cycle, at 22 ± 1^{o}C environmental temperature, with a 40–50 % of relative humidity. All groups of animals were fed with a standard diet with water *ad libitum.*

2.3. Construction of Experimental Groups

At the first stage, the animals were divided randomly, into 2 groups as control (n = 7) and diabetic (n = 35). After development of diabetes, at the day 7, diabetic rats were divided into 5 subgroups as diabetic (n = 7), diabetic + insulin administered (n = 7), diabetic + low dose Vitamin C treated (n = 7), diabetic + medium dose Vitamin C treated (n = 7) and diabetic + high dose Vitamin C treated (n = 7) groups.

2.3.1. Control Group

Control rats were injected with a single dose of citrate buffer (0.05 mol/L), intraperitoneally (i.p.). After one week of citrate buffer administration, physiological saline was injected i.p. daily, for 5 weeks. Blood glucose levels of the rats were measured from the tail vein blood by using a glucometer (One Touch Horizon Blood Glucose Monitoring System/Glucometer, USA) every week, for 6 weeks, till the end of the animal experiments. The rats were ultimately anaesthetized by pentobarbital (30 mg/kg). After decapitation of the rats, the testicles were isolated and stored at -80^{o}C for experimental analysis.

2.3.2. Diabetic Group

Streptozotocin (STZ) was used for the induction of type I diabetes in rats. Animals assigned to the diabetic groups (n = 7) were administered single dose of 55 mg/kg STZ in 0.05 mol/L citrate buffer, pH 4.5, i.p.. 6 hours after STZ administration, the diabetes induced animals were given 5% dextrose in order to prevent death due to a possible hypoglycemic shock. Blood glucose levels of diabetes induced animals were measured from the tail vein blood, by using a glucometer (One Touch Horizon Blood Glucose Monitoring System/Glucometer, USA), 3 days after the administration of STZ. Rats that have blood glucose levels above 300 mg/dl were then assigned as diabetic group. After one week of STZ administration, physiological saline was injected i.p. daily, for 5 weeks. At the end of 6 weeks, the diabetic rats were anaesthetized by pentobarbital (30 mg/kg). After decapitation of the rats, the testicles were isolated and stored at -80^{o}C for experimental analysis.

2.3.3. Insulin Administered Group

15 days after induction of diabetes by STZ, insulin was administered to n= 7 number of animals from the diabetes induced group. After one week of STZ administration, physiological saline was injected i.p. daily, for 5 weeks.

Administration of any chemical itself may lead to some alterations in the animal body due to the nature of the compound. Therefore, it is obligatory to show that, the alterations in the animals are resulted from the physiological effect of the chemical used. For this reason, insulin was administered to a group of diabetic animals, to prove that the alterations in the rats are caused by development of diabetes due to administration of STZ. 15 days after STZ administration, insulin is given to diabetic animals, 8-10 Insulin Unit (IU) per day i.p. for 4 weeks. At the end of 4 week period, the insulin administered diabetic rats were anaesthetized by pentobarbital (30 mg/kg). After decapitation of the rats, the testicles were isolated and stored at $-80^{o}C$ for experimental analysis.

2.3.4. Vitamin C Treated Groups

7 days after STZ injection, a group of diabetes induced rats were started to be treated with Ascorbic acid (Vitamin C) for 5 weeks. Three different doses of Ascorbic acid were applied to diabetics assigned as low (n= 7), medium (n= 7), and high dose (n= 7) Vitamin C treated animals.

2.3.4.1. Low Dose Vitamin C Treatment

7 days after STZ administration, the diabetic rats treated with low dose Vitamin C (n= 7) received 15 mg/kg/day Ascorbic acid i.p. for 5 weeks.

2.3.4.2. Medium Dose Vitamin C Treatment

7 days after STZ administration, the diabetic rats treated with medium dose Vitamin C (n= 7) received 50 mg/kg/day Ascorbic acid i.p. for 5 weeks.

2.3.4.3. High Dose Vitamin C Treatment

7 days after STZ administration, the diabetic rats treated with high dose Vitamin C (n= 7) received 100 mg/kg/day Ascorbic acid i.p. for 5 weeks.

As mentioned in previous chapters, during 5 weeks of Ascorbic acid treatment, the rats assigned as control, diabetic and insulin administered groups were injected physiological saline i.p. daily. At the end of 5 week treatment, different doses of Ascorbic acid administered diabetic rats were anaesthetized by pentobarbital (30 mg/kg). After decapitation of the rats, the testicles were isolated and stored at -80^{o}C for experimental analysis.

2.4. FTIR Spectroscopic Measurements

2.4.1. Sample Preparation for ATR-FTIR Spectroscopic Studies

Tissue samples were studied via ATR-FTIR spectroscopy, by directly placing the sample on the Diamond/ZnSe (Di/ZnSe) crystal plate of the Universal ATR unit of FTIR spectrometer. To ensure that the samples touch the crystal uniformly, a certain degree of pressure should be applied onto each sample. The good practice is that the applied pressure should allow for the strongest spectral bands to have an intensity extending beyond 70% T.

2.4.2. ATR- FTIR spectroscopy and spectral analysis

A one-bounce ATR mode in Perkin-Elmer Spectrum 100 FTIR spectrometer (Perkin-Elmer Inc., Boston, MA, USA) was used to carry out spectral collections. The air was scanned and subtracted from the sample spectra as a background. The collection of spectral data was performed in the middle infrared region, 4000-650 cm^{-1} frequency range, at room temperature. Each interferogram was collected with 50 scans at 4 cm^{-1} resolution. Each tissue sample was scanned as three different samples under the same conditions all of which gave nearly identical spectra. Thus, the average spectra of these triplicates for each sample were used for further analysis. Once the spectral data collection was completed, all the data manipulations were done by using SpectrumOne software (Perkin-Elmer).

Prior to the analysis of the spectra obtained, frequently preprocessing steps are applied to the data sets in order to make the spectra comparable. Depending on the analysis technique, the number of variables can either be reduced to prevent overfitting, or variables can be produced that incorporate domain knowledge, by inputting the data set into a feature extraction algorithm. The choice of method for each of these steps is highly dependent on the biological samples, instrumentation technique, and purpose of analysis. By this way, accounting and correction for noise, sloping baseline effects, differences in sample thickness/concentration and selection of the regions of interest become possible (Kelly *et al.*, 2011). However these preprocessing steps are applied only if it is necessary. If one of these steps is decided to be used it should be applied to each spectrum at identical conditions.

Among the pre-processing techniques, smoothing which is a simple separation of noise from signal, ideally without distortion and without de-resolution of the spectra used in corresponding experiments prior to spectral analysis. The widely used algorithm for smoothing in spectroscopy is the Savitzky-Golay method (Savitzky and Golay, 1964). So, the spectra obtained were first smoothed with nine-point Savitsky–Golay smooth function to remove the noise.

After smoothing process, three main parameters analyzed in infrared spectra, band wavenumber of the spectral bands, band area values and bandwidth values, all of which provide information about the macromolecules in the system. Firstly, the wavenumber value corresponding to the center of weight was assigned as the band positions. Determination of the band positions and the wavenumber shifts in these band positions provide structural information about the biomolecules. Secondly, band areas were calculated using the spectrum software. The ratios of the band areas were also examined. By analyzing the alterations in various ratios calculated, information about the relative concentrations of the respective biomolecules in the system can be obtained. In addition to these two parameters bandwidth values, calculated as the width at 0.75x height of the signal in terms of cm^{-1}, were also analyzed. Bandwidth analysis can give different information for different regions of the spectrum. For the lipid (C-H) region, alterations in the bandwidth values give information about the changes in the membrane fluidity, while for the protein region, the alterations in this parameter give information about changes in the protein conformation.

Classification and demonstration of the spectral data often requires a standardization step, so that information extracted from biologically equivalent signals can be quantified for comparison between classes. In this case, normalization allows for an effective comparison across a heterogeneous set of samples (Randolph, 2006). With normalization, information about the intensity of the spectrum is completely eliminated. Since this pretreatment is too tough for general data, it can be said that the use of normalization is limited to qualitative analysis (Kramer, 1998). Besides, baseline correction can also be preferably performed before the analysis of infrared spectra. It is frequently used to remove a sloping or curving baseline, which is essentially a wavelength-dependent intercept that can be unique for each sample spectrum (Franke, 2006). While all quantitative analysis were performed on non-normalized but pre-processed average spectra, for visual demonstration of the variations, the baseline corrected and normalized data were taken into consider.

2.4.3. Cluster Analysis

Cluster analysis was performed to make an efficient discrimination between the experimental groups, by using OPUS 5.5 Software (Bruker Optic, GmbH). Prior to cluster analysis, second derivatives of the spectra were calculated and subsequently vector normalized with a nine smoothing point Savitzky–Golay algorithm over the frequency range of 4000-1000 cm^{-1}. Spectral distances were calculated between pairs of spectra as Pearson's correlation coefficients (Helm *et al.*, 1991). Among different methods, Ward's algorithm together with Euclidian distances was used to construct a two dimensional plot, called dendograms, which gives one of the best predictions (Lasch *et al.*, 2004). Even small differences between groups can be visualized easily by this method.

Via the hierarchical cluster algorithm, two spectra showing the highest similarity, such as the spectra with the smallest spectral distance, were merged into a cluster, and the distance between the cluster constructed and all the remaining spectra were calculated. After that, the two spectra with the smallest distance merged into another new cluster again and again. Then, the distance between the newly formed cluster and the remaining spectra were calculated once more. These two sequential events of forming a cluster with the spectra having the smallest distance and calculating the distance between the clusters constructed and the remaining spectra were repeated till only one eventual cluster was obtained.

Ward's Algorithm gives opportunity to find the most homogenous groups to be merged. Thus, the heterogeneity factor (H) would be the smallest in the two groups merged. In Wards Algorithm, the heterogeneity factor is calculated by the formula below:

$$H(\mathrm{i, r}) = \frac{\lfloor n(\mathrm{p}) + n(\mathrm{i}) \rfloor D(p,i) + \lfloor n(i) + n(q) \rfloor D(q,i) - n(i) D(q,i)}{n + n(i)}$$

When the clusters, p and q, are merged into a new cluster assigned as r, *n*(i) represents the number of spectra merged in the cluster i, where $D(p,i)$, corresponds to the spectral distance between p and i clusters, and $D(q,i)$ corresponds to the spectral distance between q and i clusters (Severcan *et al.*, 2010).

Sensitivity and the specificity values of the FTIR spectroscopy were then calculated by using the clusters obtained which gives an idea about the performance and the accuracy of the method that is used in the diagnosis of a disease (Fung *et al.*, 1997; Rhijn *et al.*, 2009).

Table 2. Definitions for sensitivity and specificity (Reproduced from Severcan *et al.*, 2010)

Cluster Analysis Results based on FTIR Data			
	Positive*	Negative*	
Diabetic	A	B	Sensitivity = A/(A+B)
Non-diabetic	C	D	Specificity = D/(C+D)

* Positive and negative values are deduced as follows:

A- The number of diabetic animals clustered in diabetic group (true positives)

B- The number of diabetic animals clustered in control group (false negatives)

C- The number of control animals clustered in diabetic group (false negatives)

D- The number of control animals clustered in control group (true negatives)

2.4.4. Protein secondary structure analysis

Alterations in the protein secondary structures can be analyzed by normalized second derivative (peak picking) method. In order to apply this method, first the second derivatives of the spectra were obtained by applying a Savitzky–Golay algorithm with nine smoothing points. Then the second derivative spectra were vector normalized at 1700-1600 cm^{-1} region, by OPUS NT data collection software package (Bruker Optics, Reinstetten, Germany) and thus the peak intensity values were calculated. While calculating the peak intensity values, the peak minima of the second derivatives were taken into consideration, since they correspond to the peak positions of the original absorption spectra (Toyran *et al.*, 2006). Each peak minima values in the second derivative corresponds to the secondary structural components of proteins, namely turns, alpha helices, beta sheets, aggregated beta sheets and random coils. Changes in the peak intensity values of these bands in the second derivative spectra of amide I give information about the relative concentrations of the secondary structural components of proteins. Thus, through this approach, a general idea can be provided about the variations in the secondary structures of proteins in different conditions.

2.4.5. Statistical Test

The results obtained were expressed as 'mean ± standard error of mean (SEM)'. Since the number of experimental groups were more than two, the significance of the differences between the groups were calculated with the aid of ANOVA, and then Tukey test, as a post test with the Minitab Statistical Software Release13.0 program. The diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. Due to the statistical analysis, p value less than or equal to 0.05 was accepted as statistically significant. The degree of significance was denoted as less than or equal to $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ showing increasing order of significance for the diabetic group, and as

$p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ showing increasing order of significance for the Vitamin C treated groups.

2.5. FTIR Microspectroscopic Measurements

2.5.1. Sample Preparation for FT-IR Microspectroscopic Studies

Two successive tissue sections of 8 μm were obtained from testes of all experimental groups with a small amount of optimum cutting tool by using a cryotome and the sections were readily thaw-mounted on IR-transparent BaF_2 windows. One set of these sections were used for FTIR microspectroscopic measurements in order to determine the pathological regions in the tissue, and the subsequent set of tissue sections were used for Hematoxylin & Eosin (H&E) staining in order to observe the tissue regions defined and their pathological status under the light microscope (Leica).

2.5.2. FT–IR microspectroscopy and spectral analysis

FTIR microspectroscopy (Perkin-Elmer Inc., Boston, MA, USA) was used to gather the maps from the tissue sections. A computer-controlled *x*/*y* stage found in the microscope allows obtaining spectral maps of the samples in a rectangular area. The IR spectra were collected in 4000-750 cm^{-1} frequency range, with 16 cm^{-1} resolution and one scan per pixel where the pixel size is 6.25 μm, by using a mercury–cadmium–telluride detector. Dry air was purged to the FTIR microspectroscopy in order to reduce the contributions resulting from interfering water vapor and CO_2.

FTIR microspectroscopy provides spatially-resolved IR spectral information by scanning the sample point-by-point across the surface of a stationary detector, collecting an IR spectrum, namely IR mapping, at each point without access to multichannel detectors (Harthcock and Atkin 1988). Tens of thousands of individual

spectra can be collected rapidly and in parallel, each referring to a distinct and identifiable spatial location on the sample. Besides the information about chemical composition and molecular structure, which can also be obtained from conventional IR spectra, the integration of spatial and spectral information in vibrational imaging data sets opens new possibilities in data analysis (Kidder *et al.*, 2001). The data analyzed the interpretation of data in FTIR microspectroscopic studies is similar to that of FTIR spectroscopy mentioned above. However, in FTIR microspectroscopy the most valuable information is gathered by the calculation of different ratios about the macromolecular content of the system.

Perkin Elmer Spotlight software was used to analyze the data obtained from FTIR microspectroscopy. The comparison between the control diabetic and different doses of Vitamin C treated groups were performed by calculating the ratios of the integrated areas of the infrared bands belongs to different functional groups. By using the ratios in the spectral analysis, the effect of the sample thickness would be eliminated. The areas of the baseline corrected spectra were used while obtaining the images of the functional groups. The integrated spectral regions and the corresponding baseline points for the infrared bands used in this study are presented in Table 3.

Table 3. The spectral regions and baseline points used for particular infrared bands.

Infrared Band	**Integrated Spectral Range (cm^{-1})**	**Baseline Points (cm^{-1})**
Olefinic=CH band	3025-3005	3100-2740
CH_2 asym stretch. band	2936-2916	3100-2740
C-H stretch. region	2970-2847	3100-2740

2.5.3. Statistical Test

The ratio values of the functional groups of interest were obtained for every pixel in every FTIR Microspectroscopic image. A mean and standard error of mean (SEM) value were calculated for each animal used in the experiment. Then, the individual animal values were averaged for each experimental group (control, diabetic, diabetic + low dose Vitamin C treated, diabetic + medium dose Vitamin C treated and diabetic + high dose Vitamin C treated). Since the number of experimental groups were more than two, the significance of the differences between the groups were calculated with the aid of ANOVA, and then Tukey test, as a post test with the Minitab Statistical Software Release13.0 program. Due to the statistical analysis, *p* value less than or equal to 0.05 was accepted as statistically significant. The degree of significance was denoted as less than or equal to $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ showing increasing order of significance for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ showing increasing order of significance for the Vitamin C treated groups.

CHAPTER III

RESULTS

This part of the study is addressed to investigate the effects of diabetes on rat testes and the recovery role of administration of Vitamin C against the diabetic complications with the aid of ATR-FTIR spectroscopy and FTIR microspectroscopy.

3.1. General FTIR Spectrum and Band Assignment of Testes

In this current study, 6 (six) experimental groups of Wistar rats were used, assigned as control (n=7), diabetic (n=7), diabetic + insulin treated (n=7), diabetic + low dose Vitamin C treated (n=7), diabetic + medium dose Vitamin C treated (n=7) and diabetic + high dose Vitamin C treated (n=7) animals, to reveal the effect of diabetes on rat testes and furthermore, to investigate the recovery role of Vitamin C, as an antioxidant, against the diabetes-induced complications.

In ATR-FTIR spectroscopy, the infrared absorption bands belong to different functional groups present in the system. Since the vibrations due to absorption of light at certain wavelengths are unique to specific vibrational modes of particular functional groups, it becomes possible to assign the positions of these molecular absorption bands. Moreover, any alterations in different modes of vibrations correspond to the alterations in the conformation, structure and therefore, in the functions of different functional groups found in the system. Through this approach, changes in the macromolecular content and composition, such as, alterations in the unsaturation and/or saturation levels of lipids, in the chain

lengths of lipids, and changes in the lipid and protein contents in the corresponding system, could be revealed among the experimental groups. Additionally, alterations in the secondary structural components of proteins were also investigated.

Figure 19, 20, and 21 demonstrate the representative infrared spectrum of control testes in the 4000-3025 cm^{-1}, 3025-2820 cm^{-1} and 1800-950 cm^{-1} spectral regions, respectively. The main bands of the infrared spectra labeled in these figures, assigned with regard to this study and other studies cited in the literature. The detailed spectral band assignments were presented in Table 4.

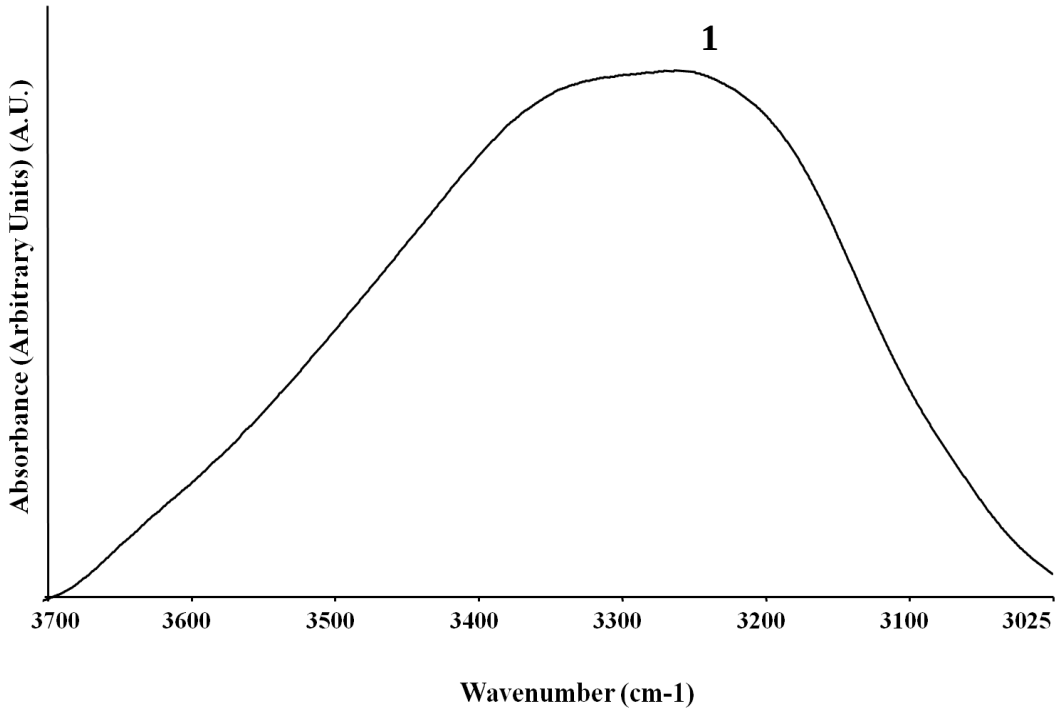

Figure 19. The representative ATR-FTIR spectra of control rat testes in the 4000-3025 cm^{-1} region**.**

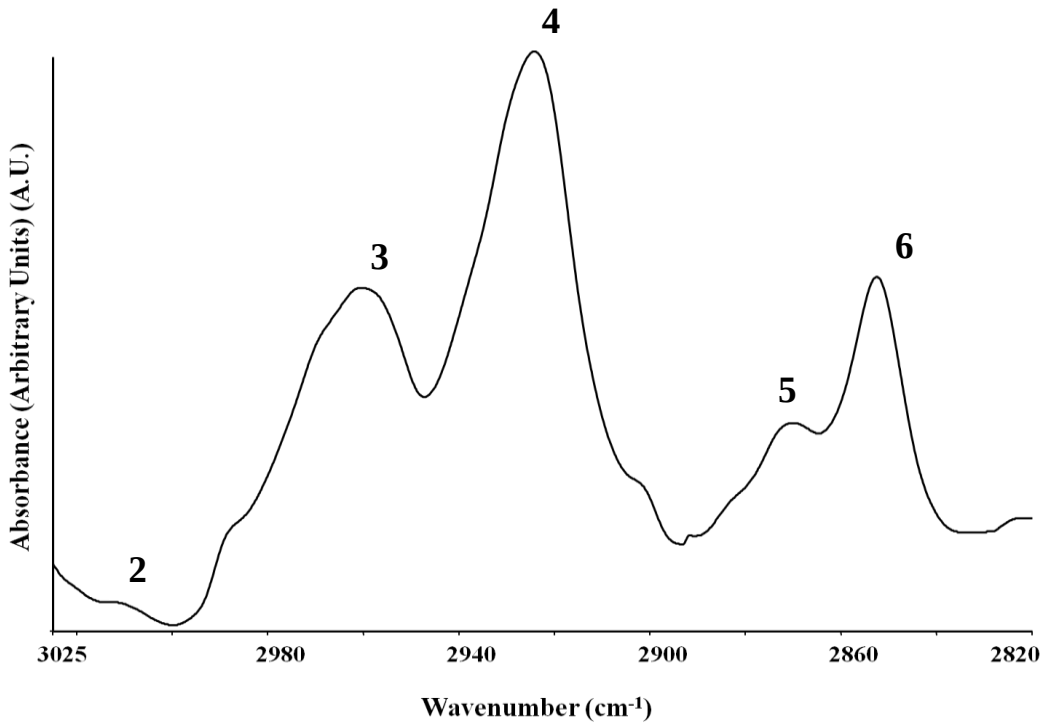

Figure 20. The representative ATR-FTIR spectra of control rat testes in the 3025-2820 cm^{-1} region**.**

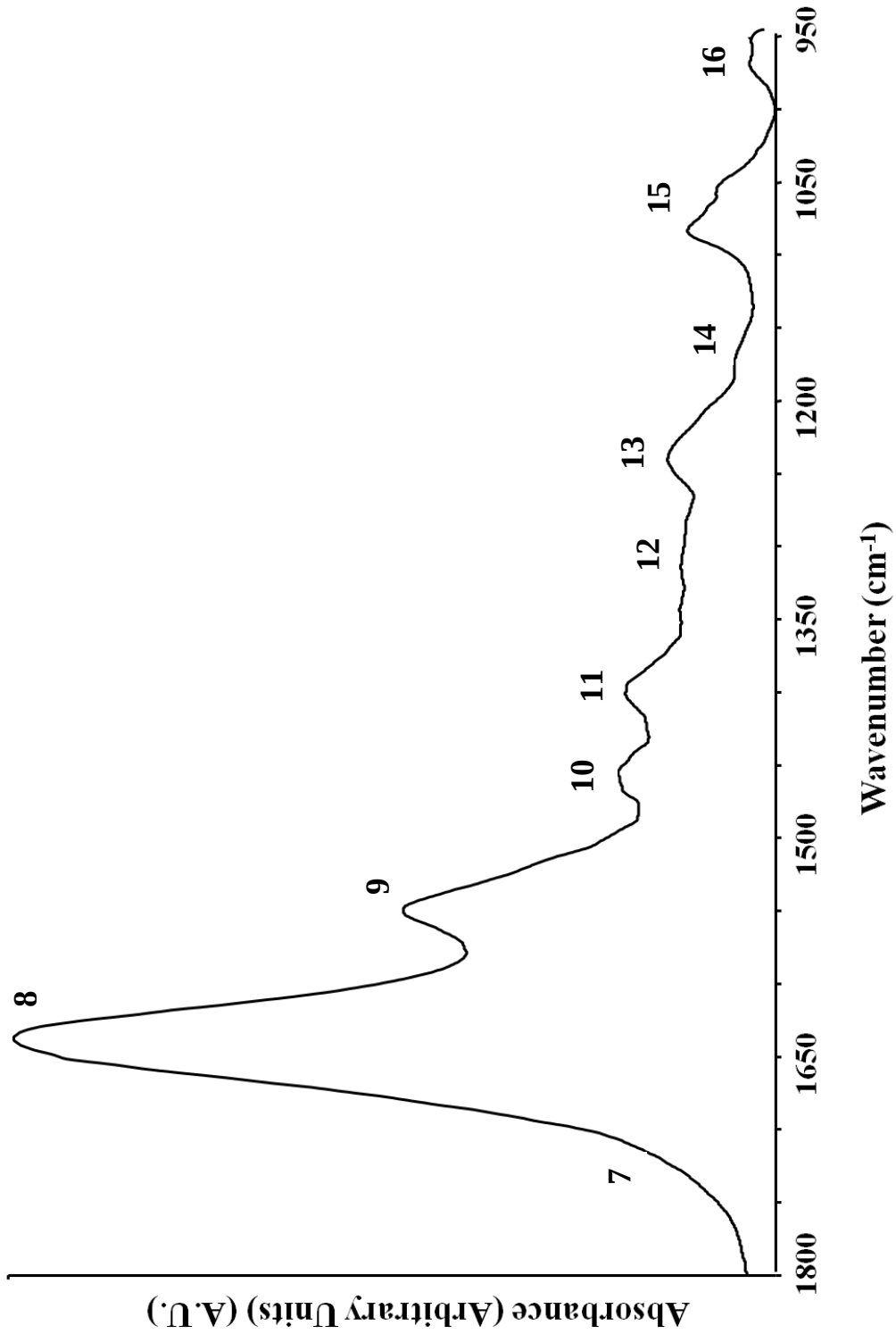

Figure 21. The representative ATR-FTIR spectra of control rat testes in the 1800-950 cm^{-1} region**.**

Table 4. ATR- FTIR band assignment of testes tissue.

Band No	Wavenumber (cm^{-1})	Band Assignment
1	3305	**Mainly N-H stretching** (Amide A) of proteins with contribution from O-H stretching of polysaccharides (Cakmak *et al.*, 2003)
2	3011	**Olefinic HC=CH**: unsaturated lipids (Liu *et al.*, 2002; Severcan *et al.* 2005a)
3	2958	**CH_3 anti-symmetric stretching**: equal contribution from lipids, protein side chains (Szalontai 2009, Severcan et al, 2010)
4	2923	**CH_2 anti-symmetric stretching**: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids (Cakmak *et al.* 2003)
5	2872	**CH_3 symmetric stretching**: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids (Ozek *et al.*, 2010a)
6	2852	**CH_2 symmetric stretching**: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids (Cakmak *et al.*, 2006)
7	1742	**Carbonyl C=O stretching**: cholesterol esters (Mendelsohn *et al.*, 2003)
8	1638	**Amide I**: protein C=O stretching (Haris and Severcan 1999; Krafft *et al.*, 2004)
9	1548	**Amide II**: protein N-H bend, C-N stretch (Kneipp *et al.*, 2000; Krafft *et al.*, 2004)
10	1458	**CH_2 bending**: lipids (Bozkurt *et al.*, 2010)
11	1393	**COO- symmetric stretching**: fatty acids (Cakmak *et al.*, 2006)
12	1311	**Amide III**: Peptide side chain (Steiner *et al.*, 2003)
13	1237	**PO_2^- anti-symmetric stretching**: mainly nucleic acids with the little contribution from phospholipids (Kneipp *et al.*, 2000; Akkas *et al.*, 2007)
14	1173	**CO–O–C anti-symmetric stretching**: glycogen and nucleic acids (Rigas *et al.*, 1990; Cakmak *et al.*, 2006)
15	1084	**PO_2^- symmetric stretching**: nucleic acids and phospholipids C-O stretch: glycogen, polysaccharides, glycolipids (Kneipp *et al.*, 2000; Akkas *et al.*, 2007)
16	970	**C-N^+-C stretching**: RNA (Cakmak *et al.*, 2003)

3.2. Effects of Diabetes on Rat Testes

ATR-FTIR spectral data were collected in the range of 4000-650 cm^{-1}. In order to be more clear, in the visualization of the alterations between the control and diabetic spectra, the analysis were performed in two spectral ranges. The first range was 3025-2820 cm^{-1} which corresponds to mainly lipid region and the second, range was 1800-950 cm^{-1}, which corresponds to fingerprint region. The figures showing the spectral alterations between the experimental groups in representative spectra, since all the control spectra are overlapped with each other and all the diabetic spectra, which are different than control spectra, are also overlapped.

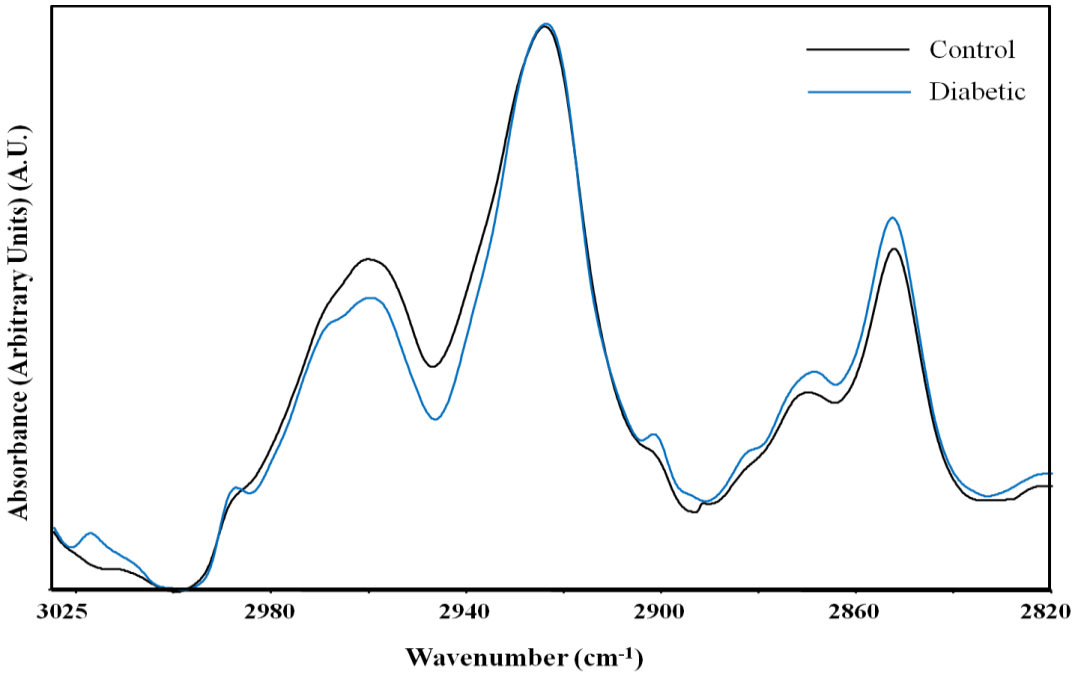

Figure 22. The representative infrared spectrum of the control and diabetic rat testes in the 3025-2820 cm^{-1} region. The spectra were normalized with respect to the CH_2 anti-symmetric stretching band**.**

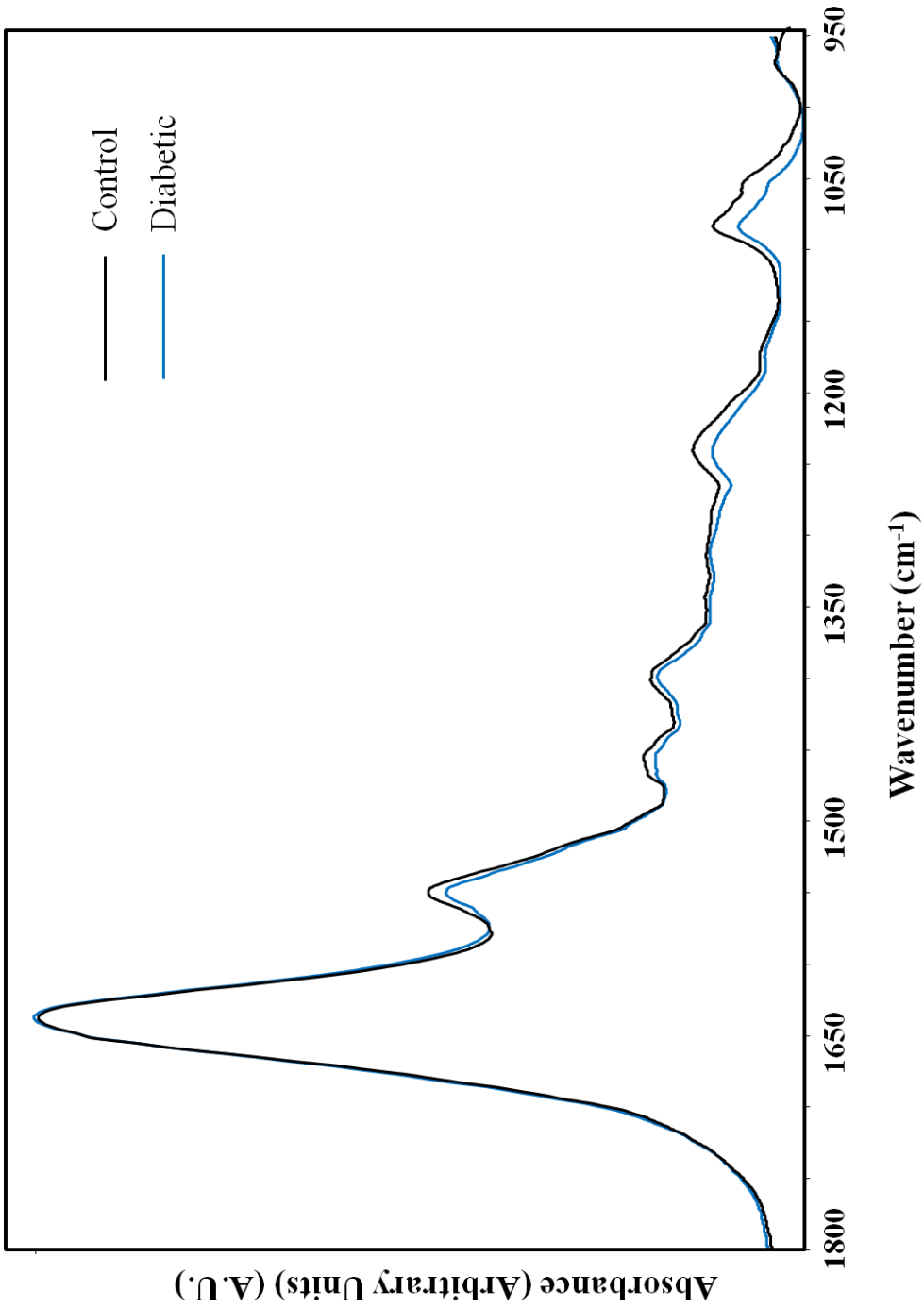

Figure 23. The representative infrared spectrum of the control and diabetic rat testes in the 1800-950 cm^{-1} region. The spectra were normalized with respect to the amide I band**.**

Regarding these spectral differences, control and diabetic rat testes samples were differentiated using cluster analysis, with a hundred percent accuracy. The result is demonstrated in Figure 24.

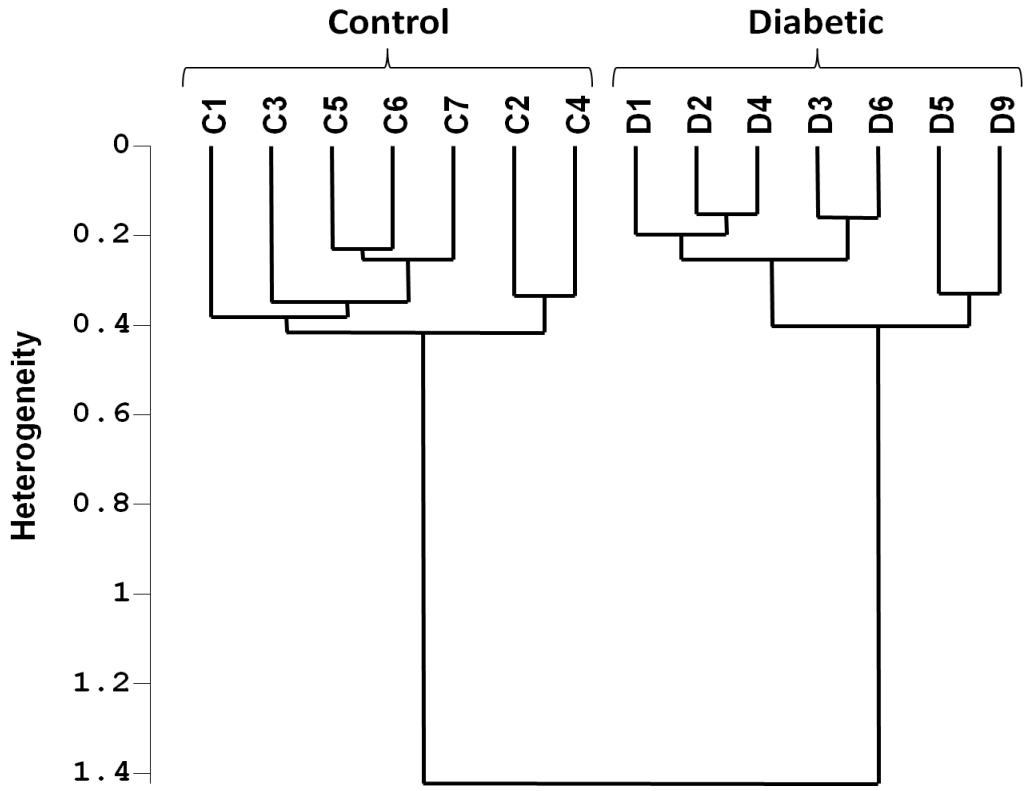

Figure 24. Hierarchial clustering of control (C) and diabetic (D) rat testes. Clustering was performed using Ward's algorithm, and second derivative vector normalized spectra in the spectral range of 4000-1000 cm^{-1}.

It has been widely known that, administration of any chemical itself may lead to some alterations in the biological systems due to the nature of the chemical compound. Therefore, in this kind of studies, it is obligatory to show that, the alterations in the animals are resulted particularly from the physiological effect of the chemical used.

In this current study, a diabetes inducing agent, namely Streptozotocin (STZ) was used to develop type I diabetes in rats. However, it should be clarified that, the alterations observed in the testicular tissues of STZ administered rats, were particularly caused by the development of type I diabetes due to STZ administration, instead of causing by the chemical nature of STZ, itself. For this reason, in this study, insulin was administered to a group of diabetic animals, in order to prove that the alterations in the STZ administered rats are literally caused by the development of diabetes due to STZ administration. By performing cluster analysis, it was shown that, the insulin treated diabetic animals are healed and they become undistinguishable from their control counterparts (Figure 25). This evidence assures that, the alterations in the rat testes are indeed resulted from the induction of diabetes by STZ.

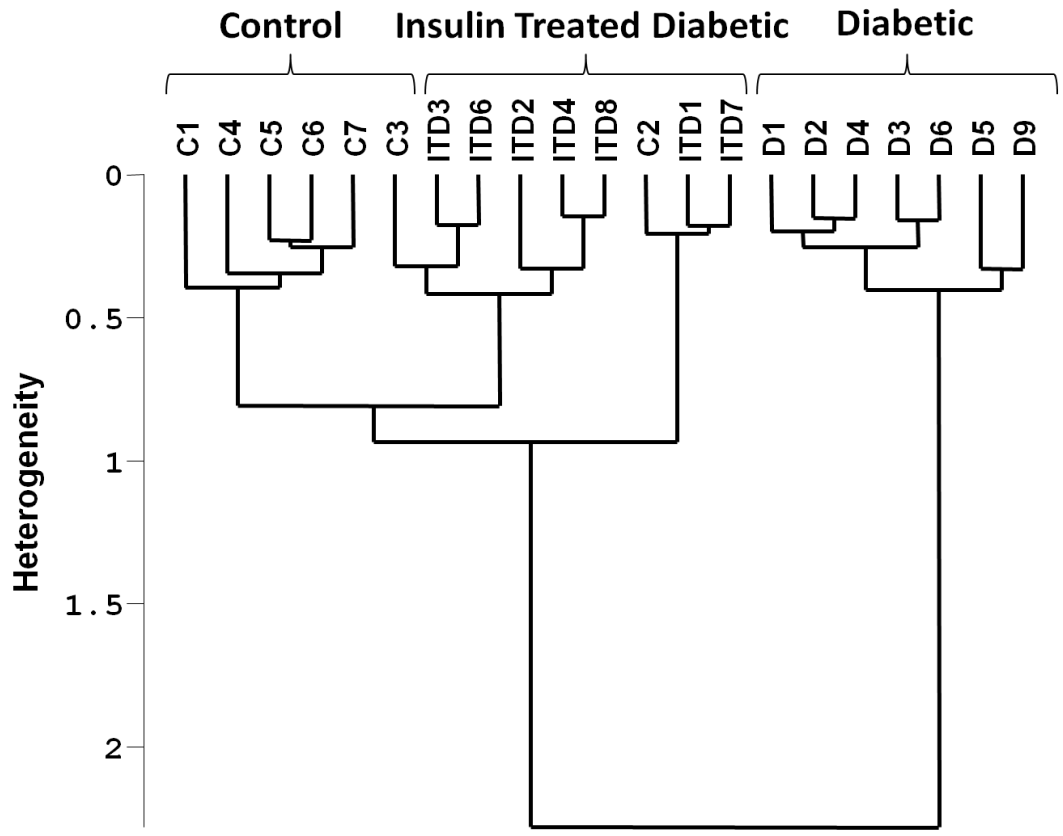

Figure 25. Hierarchial clustering of control (C), diabetic (D) and insulin treated diabetic (ITD) rat testes. Clustering was performed using Ward's algorithm, and second derivative vector normalized spectra in the spectral range of 4000-1000 cm^{-1}.

3.3. Effects of different doses of Vitamin C on Diabetic Rat Testes

ATR-FTIR spectral data were collected in the range of 4000-650 cm^{-1}. In order to be more clear in the visualization of the alterations between the control, diabetic, low dose Vitamin C treated, medium dose Vitamin C treated and high dose Vitamin C treated spectra, the analysis were performed in the same two spectral ranges, mentioned above. The first range, corresponding to mainly lipid region, was 3025-2820 cm^{-1}, and the second range, corresponding to fingerprint region, was 1800-950 cm^{-1}.

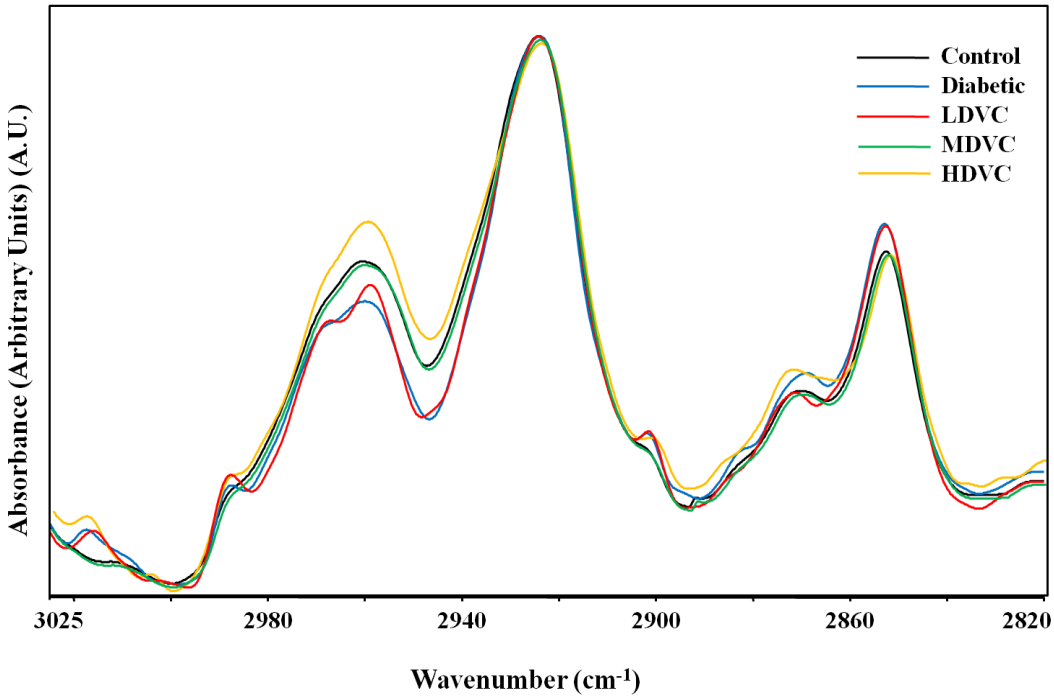

Figure 26. The representative infrared spectrum of the control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes in the 3025-2820 cm^{-1} region. The spectra were normalized with respect to the CH_2 anti-symmetric stretching band**.**

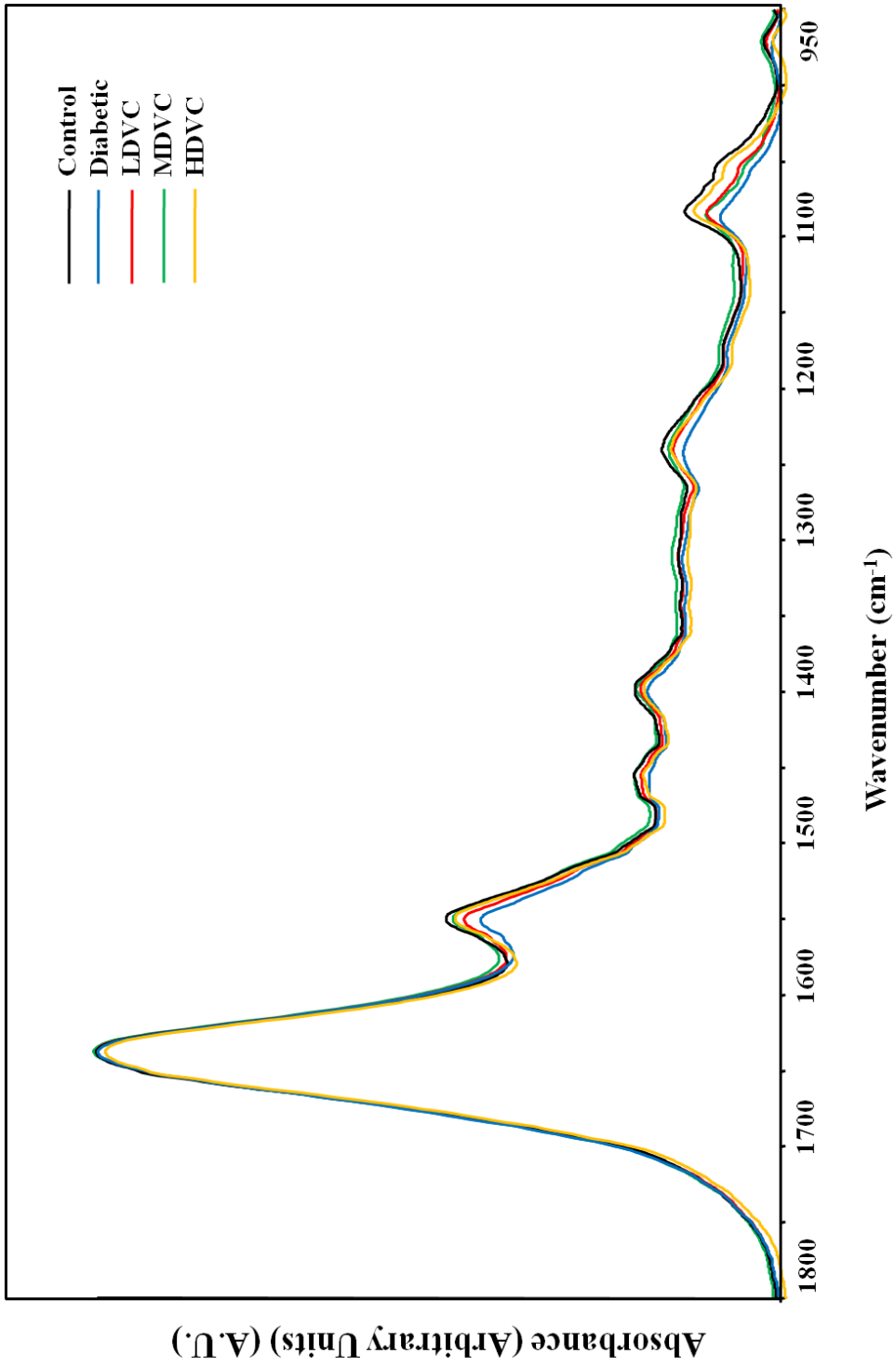

Figure 27. The representative infrared spectrum of the control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes in the 1800-950 cm^{-1} region. The spectra were normalized with respect to the amide I band**.**

Regarding these spectral differences, the control (C), diabetic (D), and different doses (low dose (LDVC), medium dose (MDVC) and high dose (HDVC)) of Vitamin C treated diabetic rat testes were differentiated using cluster analysis, with high accuracy. The result is demonstrated in Figure 28.

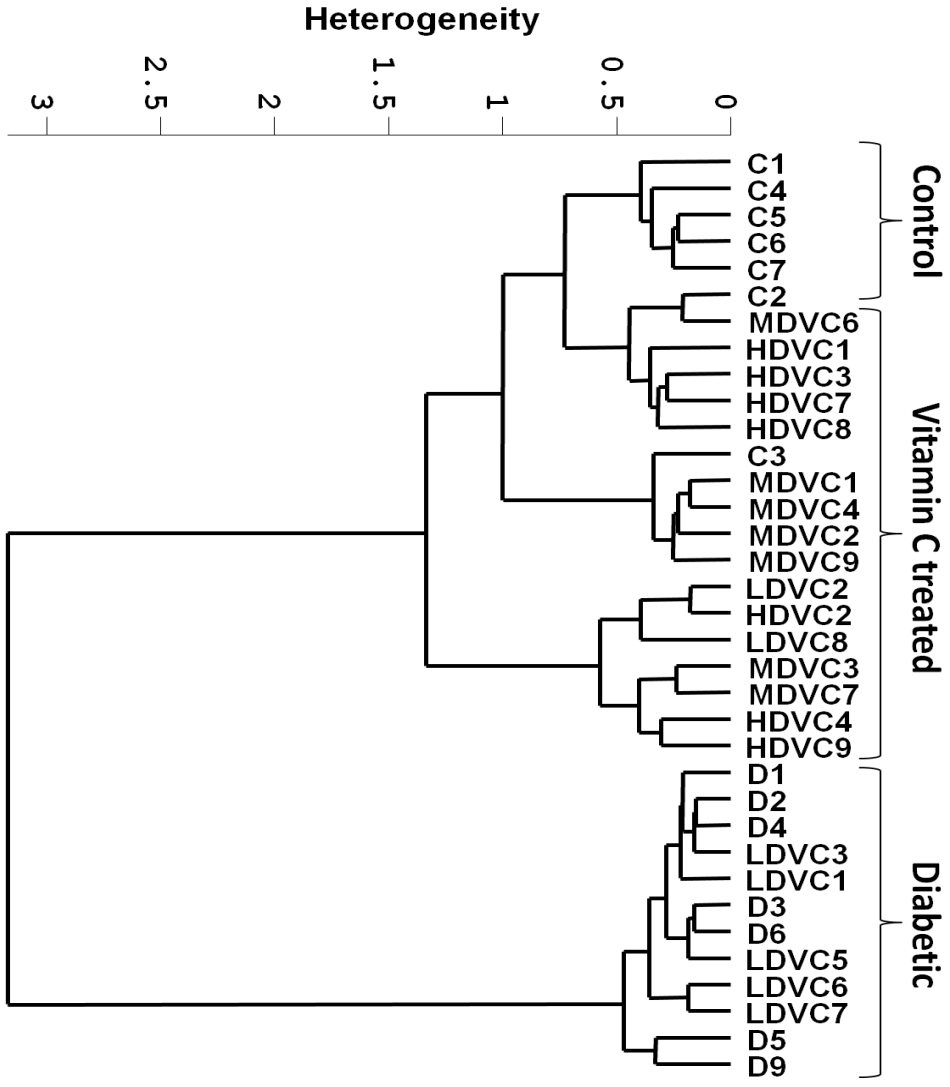

Figure 28. Hierarchial clustering of control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Clustering was performed using Ward's algorithm, and second derivative vector normalized spectra in the spectral range of 4000-1000 cm^{-1}.

3.4. Secondary Structure Analysis of Main Protein Band (Amide I band)

Protein secondary structural analysis was performed by vector normalized second derivative method, using the amide I band, 1700-1600 cm^{-1}, to gather information about the alterations in the components of protein secondary structures.

For this purpose, the second derivative of amide I band was obtained, and then the second derivative spectra were vector normalized at 1700-1600 cm^{-1} region. After that, the peak intensity values of the sub-bands in the second derivative spectra of amide I absorption were calculated. From the intensity values, the secondary structural components of proteins can be analyzed relatively among the experimental groups.

Figure 29 shows representative spectra for average absorbance and the second derivative spectra of control rat testes in the 1700-1600 cm^{-1} region. In the second derivative of amide I band, the bands around 1693 cm^{-1} arises from antiparallel β-sheet structures and the bands located in the 1685-1670 cm^{-1} spectral range arise from turns. Besides, 1659 cm^{-1} and 1651 cm^{-1} are both resulted from α-helix structure, the band around 1638 cm^{-1} is resulted from β-sheet structure and finally, the band at around 1629 cm^{-1} arises from aggregated β-sheet structure. In addition to these secondary structural components, random coils can also be observed at around 1644 cm^{-1} (Pelton, 2000; Palaniappan and Vijanasundaram, 2008; Garip *et. al.*, 2010).

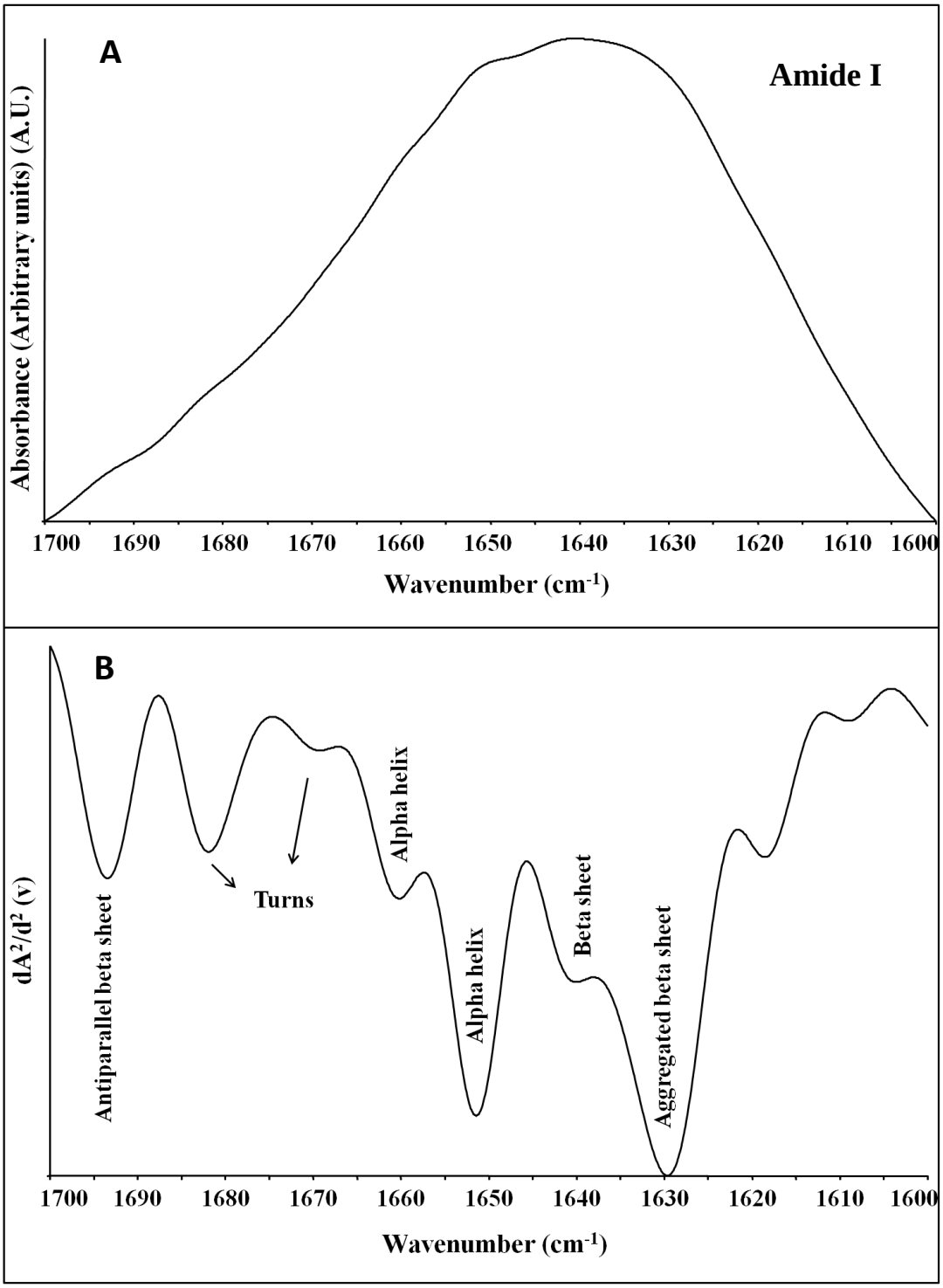

Figure 29. The average absorbance (A), and second derivative (B) ATR- FTIR spectra of control rat testes in the 1700-1600 cm^{-1} region**.**

Table 5. The numerical comparisons of the intensities of the main protein secondary structures for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. The values are the mean ± Standard Error of Mean (SEM) for each sample. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

	PROTEIN SECONDARY STRUCTURES				
	Control (n=7)	**Diabetes (n=7)**	**LDVC (n=7)**	**MDVC (n=7)**	**HDVC (n=7)**
α - helix	-0.158±0.007	-0.173±0.002**	-0.172±0.004	-0.156±0.004$^{\#\#\#}$	-0.160±0.004$^{\#\#}$
Random coil	-0.102±0.002	-0.105±0.001*	-0.101±0.005	-0.096±0.004$^{\#}$	-0.086±0.004$^{\#\#}$
β - sheet	-0.147±0.006	-0.121±0.005**	-0.123±0.002	-0.146±0.009$^{\#\#}$	-0.151±0.012$^{\#\#}$
Aggregated β - sheet	-0.206±0.003	-0.213±0.001*	-0.206±0.004$^{\#}$	-0.208±0.002$^{\#}$	-0.212±0.004

The numerical comparisons of the alterations in the protein secondary structures in all experimental groups of rat testes were shown in the Table 5 given above, and in the following bar graphs. As seen from Figure 30, and from Table 5, the peak intensity of random coil structure increased slightly and the intensities of aggregated β-sheet structures increased dramatically in the diabetic group compared to the control group. As also can be seen from these figures, Vitamin C treated diabetic groups have peak intensities for secondary structural components of proteins become much closer to control group. These results demonstrate the recovery effect of Vitamin C on the protein secondary structures against the complications of diabetes.

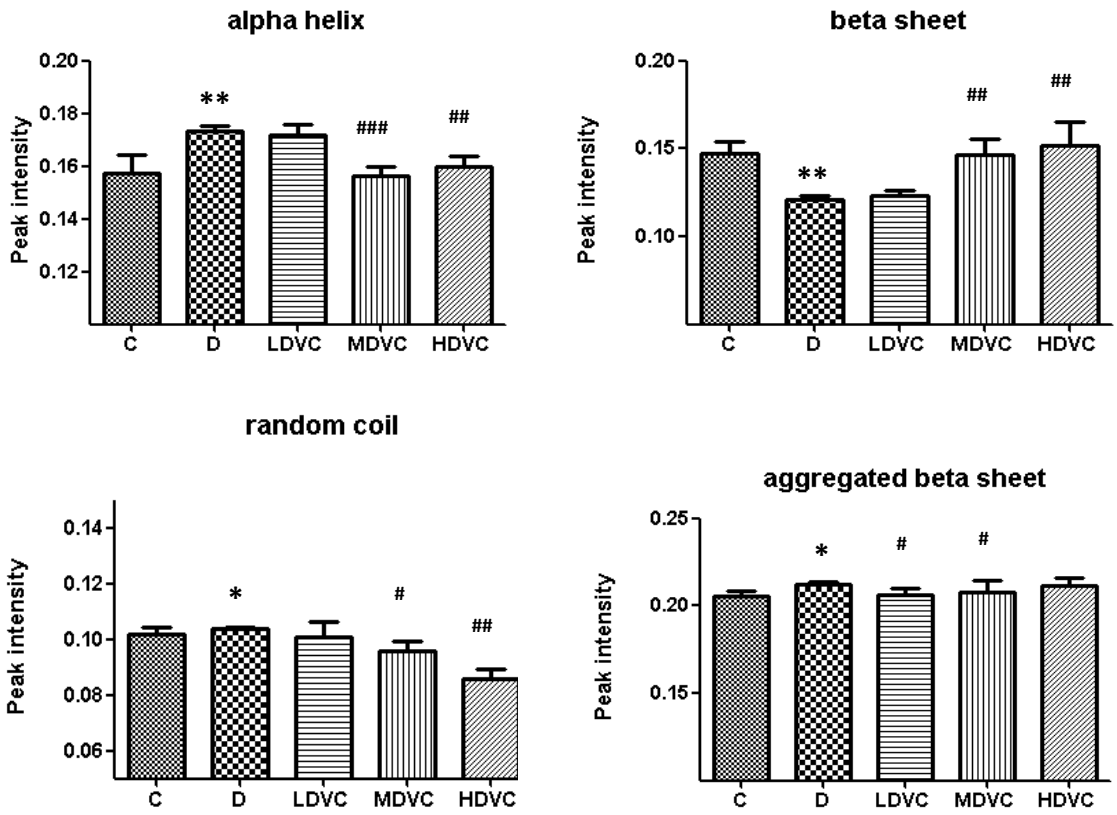

Figure 30. The comparisons of the intensities of the main protein secondary structures (α-helix, β- sheet, aggregated β- sheet and random coil) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes**.** Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

3.5. Numerical Comparisons of the Bands of Control, Diabetic, Low Dose Vitamin C Treated Diabetic, Medium Dose Vitamin C Treated Diabetic, and High Dose Vitamin C Treated Diabetic Groups

3.5.1. The effects of diabetes and different doses of Vitamin C treatment on the structure of rat testes

Determining the position of a band and specifying the shifts in the band positions enables us to identify the bands and to obtain structural information about the biomolecules in the corresponding biological system, respectively. In order to take the possible spectral differences, that could be observed between the experimental groups, into account, the mean and the standard error of mean (SEM) values of the wavenumber values of the bands, and direction of the shifts were calculated. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. All experimental groups' means of rat testes were compared with the aid of ANOVA, and then Tukey test, as a post test, and the significance values were calculated. The results of significant changes in the wavenumber values of main bands were given in Table 6.

Table 6. Numerical summary of the detailed differences in the band wavenumbers control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic spectra. The values are the mean ± Standard Error of Mean (SEM) for each sample. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

	BAND WAVENUMBER (cm^{-1})				
Band No	**Control (n=7)**	**Diabetes (n=7)**	**LDVC (n=7)**	**MDVC (n=7)**	**HDVC (n=7)**
2	3011.40±0.91	3014.67±0.65*	3010.87±0.27$^{\#}$	3012.78±0.22$^{\#}$	3014.53±0.22
9	1548.80±0.29	1548.42±0.07	1548.69±0.16	1549.41±0.09$^{\#}$	1549.42±0.11$^{\#}$
12	1311.30±0.16	1309.66±0.16**	1311.46±0.06$^{\#\#}$	1311.31±0.12$^{\#\#}$	1311.56±0.36$^{\#}$

3.5.2. The effects of diabetes and different doses of Vitamin C treatment on the composition of rat testes

By analyzing the alterations in the band area values, information about the concentrations of the corresponding biomolecules in the system can be obtained. In order to take the possible spectral differences, that could be observed between the experimental groups, into account, the mean and the standard error of mean (SEM) values of the band area values and direction of the alterations in the areas were calculated. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. All experimental groups' means of rat testes were compared with the aid of ANOVA, and then Tukey test, as a post test, and the significance values were calculated. The results of significant changes in the area values of main bands assigned were given in Table 7.

Table 7. Numerical summary of the detailed differences in the band areas of control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic spectra. The values are the mean ± Standard Error of Mean (SEM) for each sample. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

	BAND AREA				
Band No	**Control (n=7)**	**Diabetes (n=7)**	**LDVC (n=7)**	**MDVC (n=7)**	**HDVC (n=7)**
2	1.69±0.01	$2.88\pm0.03^{***}$	$1.50\pm0.02^{\#\#\#}$	$2.20\pm0.01^{\#\#}$	$1.78\pm0.07^{\#\#\#}$
4	1.34±0.03	$1.29\pm0.01^{*}$	$1.35\pm0.04^{\#}$	$1.35\pm0.04^{\#}$	$1.50\pm0.06^{\#\#}$
6	0.40±0.01	$0.36\pm0.01^{*}$	$0.39\pm0.01^{\#}$	$0.40\pm0.01^{\#}$	$0.41\pm0.02^{\#}$
9	5.96±0.15	$5.76\pm0.04^{*}$	$6.73\pm0.40^{\#}$	$6.27\pm0.29^{\#}$	$7.09\pm0.39^{\#}$
11	2.58±0.03	$2.52\pm0.01^{*}$	$2.60\pm0.06^{\#}$	$2.61\pm0.05^{\#}$	$2.62\pm0.07^{\#}$
15	1.38±0.04	$1.29\pm0.03^{*}$	$1.43\pm0.07^{\#}$	$1.38\pm0.02^{\#}$	$1.89\pm0.21^{\#\#}$

3.5.3. The effects of diabetes and different doses of Vitamin C treatment on different band area ratios of various functional groups in rat testes

The ratios of the band areas were also examined in order to get information about the relative concentrations of the respective biomolecules in the system. The mean and the standard error of mean (SEM) values of the band ratios of some specific bands, and direction of the alterations in the ratios were calculated. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. All experimental groups' means of rat testes were compared with the aid of ANOVA, and then Tukey test, as a post test, and the significance values were calculated. The assignments of the band ratio values

calculated, and the results of changes in the band ratio values were given in the following Table 8 and 9.

Table 8. FTIR band ratios and their assignments

Olefinic/Total Lipid*	Unsaturation level of the system
Olefinic/Total CH_2	Unsaturation level of the system
CH_2 sym + CH_2 asym / Total Lipid*	Saturation level of the system
CH_2 asym / Total Lipid*	Chain length of lipids

* Total lipid = CH_2 asym + CH_2 sym + CH_2 bending + Ester + Olefinic

Table 9. Numerical summary of the detailed differences in different ratios of functional groups of control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic spectra. The values are the mean ± Standard Error of Mean (SEM) for each sample. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

	BAND RATIOS				
Ratio of Peak Areas	**Control (n=7)**	**Diabetes (n=7)**	**LDVC (n=7)**	**MDVC (n=7)**	**HDVC (n=7)**
Olefinic/Total CH_2	0.991± 0.021	1.738± 0.016***	0.865± 0.028$^{\#\#\#}$	1.280± 0.038$^{\#\#\#}$	0.935± 0.057$^{\#\#\#}$
Olefinic / Total Lipid	0.316± 0.004	0.443± 0.001***	0.278± 0.008$^{\#\#\#}$	0.366± 0.005$^{\#\#\#}$	0.301± 0.014$^{\#\#\#}$
CH_2 sym + CH_2 asym / Total Lipid	0.320± 0.005	0.255± 0.002***	0.322± 0.003$^{\#\#\#}$	0.287± 0.006$^{\#\#}$	0.324± 0.005$^{\#\#\#}$
CH_2 asym / Total Lipid	0.248± 0.004	0.198± 0.001***	0.250± 0.002$^{\#\#\#}$	0.221± 0.006$^{\#}$	0.252± 0.004$^{\#\#\#}$

3.6. Detailed Spectral Analysis

The 3025-2820 cm^{-1} region is mainly composed of the bands that originate from the lipids in the biological system of interest. In this spectral region, a weak band assigned as olefinic band is located at around 3011 cm^{-1}. The olefinic band is originated from C-H stretching vibrations of the olefinic=C-H groups in unsaturated lipids, therefore this band can be used to determine the unsaturation level of the system (Liu *et al.*, 2002; Severcan *et al.*, 2005a). Moreover, olefinic band can also be used to obtain information about the concentration of double bonds in the lipid structure due to unsaturated fatty acyl chains (e.g. linoleic acid, arachidonic acid, etc.), and/or due to lipid peroxidation (Severcan *et al.*, 2005a, Liu *et al.*, 2002; Severcan *et al.*, 2003). The wavenumber of the olefinic band located at around 3011 cm^{-1} shifted to a higher value from 3011.40±0.91 cm^{-1} to 3014.67±065 cm^{-1} ($p<0.05^{*}$) in diabetic rat testes. After the Vitamin C treatment, a recovery in low and medium doses of Vitamin C administration was observed, as the wavenumber values shifted back to 3010.87±0.27 ($p<0.05^{\#}$) and 3012.78±0.22 ($p<0.05^{\#}$), respectively. However in the high dose Vitamin C administration, there was only very slight recovery which is insignificant. Considering the band area values, the olefinic band area increased significantly in diabetic group ($p< 0.001^{***}$). As can be seen from Figure 31 after the administration of different doses of Vitamin C, a significant recovery was observed for all doses of treatment with respect to the diabetic state.

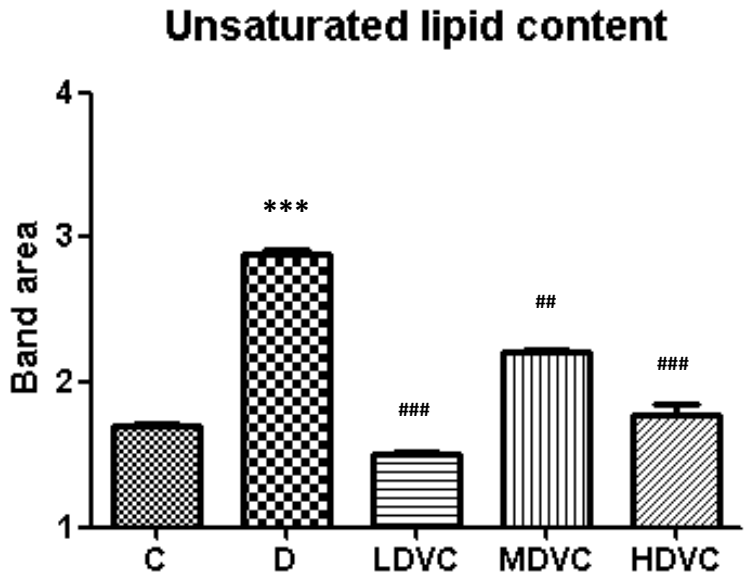

Figure 31. Comparison of unsaturated lipid contents (=CH olefinic band area) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

The bands located in the 3000-2820 cm^{-1} region of the spectrum, are assigned to C-H stretching modes. Among these bands, CH_3 anti-symmetric stretching vibration, around 2958 cm^{-1} arises from equal contribution of lipids and protein side chains (Szalontai 2009, Severcan *et al.*, 2010). CH_2 anti-symmetric stretching vibration, located at around 2923 cm^{-1}, results mainly from lipids, with the little contribution from proteins, carbohydrates, nucleic acids (Cakmak *et al.* 2003). On the other hand, CH_3 symmetric stretching mode, around 2872 cm^{-1}, is mainly due to proteins, with the little contribution from lipids, carbohydrates, nucleic acids (Ozek *et al.*, 2010). Finally, CH_2 symmetric stretching vibration at around 2852 cm^{-1} mainly results from lipids, with the little contribution from proteins, carbohydrates, nucleic acids (Cakmak *et al.*, 2006). Among these vibrational modes, especially CH_2 stretching vibrations, can be used as a marker for the detection of changes in lipid order,

content, and/or composition (Severcan *et al.*, 2005b). CH_3 symmetric and anti-symmetric stretching vibrations are not used to infer about the lipid status of a system, because these vibrations are due to mainly protein side chains and to equal contributions from proteins and lipids, respectively, rather than resulting purely from lipids. Instead, CH_3 symmetric and antisymmetric stretching vibrations are useful for getting information about proteins of the corresponding system. Besides these structural and concentrational information, functional information about lipids can also be obtained from this region of the spectra. For this purpose, alterations in the bandwidth values of CH_2 anti-symmetric and symmetric bands are taken into consideration, and information about lipid dynamics in the system can be obtained (Korkmaz and Severcan 2005; Severcan *et al.*, 2005b; Severcan *et al.*, 2000).

No significant change was observed in the wavenumber shifts and in the bandwidths of the stretching bands in the 3000-2820 cm^{-1} among all experimental groups. Considering the alterations in the band area values, the area under the CH_3 anti-symmetric band increased insignificantly, whereas the CH_2 anti-symmetric band area decreased significantly ($p<0.05^{*}$) in the diabetic animals with respect to their controls. Additionally, administration of low and medium doses of Vitamin C were seen to have significant recovery role against the diabetic alterations ($p<0.05^{\#}$) (Figure 32).

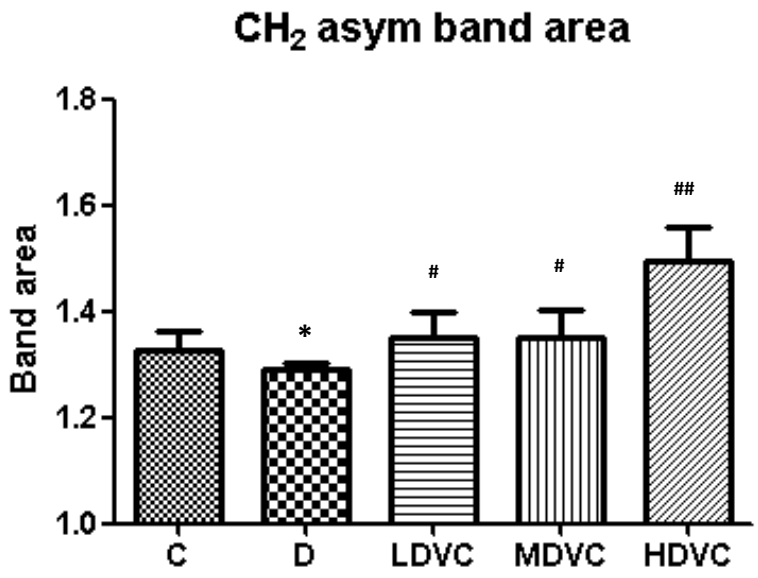

Figure 32. Comparison of CH_2 anti-symmetric stretching band area for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

On the other hand, the peak area of the CH_3 symmetric stretching band decreased slightly, while the CH_2 symmetric band area decreased significantly in diabetic group of animals ($p < 0.05^{*}$). It can be also seen from Figure 33, Vitamin C treatment has a recovery role when compared to diabetes-induced animals. All low, medium and high doses of Vitamin C administration indicated significant recovery role against the effects of diabetes ($p<0.05^{\#}$).

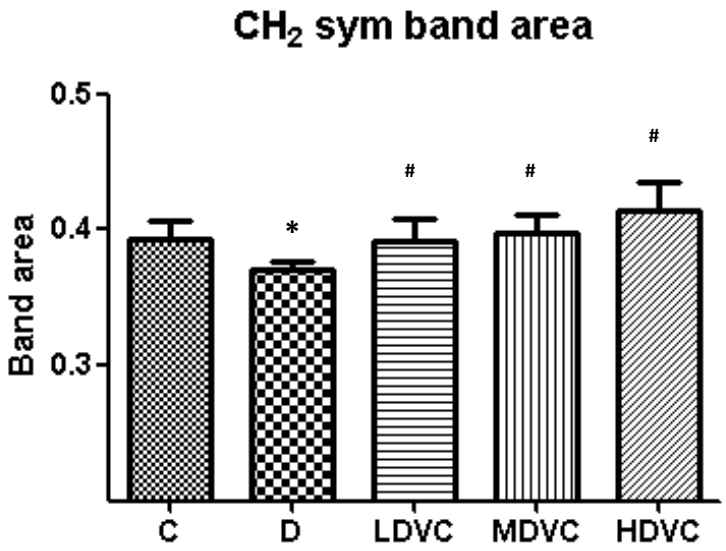

Figure 33. Comparison of CH_2 symmetric stretching band area for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

1800-950 cm^{-1} region of the spectra corresponds to the fingerprint region. The first band in this spectral range is the cholesterol ester band located at around 1742 cm^{-1} which is resulted from C=O stretching vibrations of ester carbonyl groups in lipids (Mendelsohn *et al.*, 2003). This band was very small in all experimental groups, and there were no significant shifts observed either in the wavenumber, or in the band area values of this band.

This fingerprint region also includes two main protein bands located in between 1700-1480 cm^{-1} spectral region, namely amide I, which arises from mainly C=O stretching (80%) and C-N stretching vibrations of the protein backbone, and amide II, which is originated from N-H bending (60%) and C-N stretching (40%) vibrations of the proteins, respectively (Haris and Severcan, 1999; Melin *et al.*, 2000; Takahashi *et al.*, 1991; Cakmak *et al.*, 2006; Bozkurt *et al.*, 2010). The changes in

protein conformation and protein secondary structures can be determined by analyzing the amide I and amide II protein bands. The secondary structural components of the amide I band, antiparallel β-sheet, turns, α-helices, β-sheet, and aggregated β-sheet, were observed at approximately 1693 cm^{-1}, 1685-1670 cm^{-1}, 1651-1659 cm^{-1}, 1638 cm^{-1}, and 1629 cm^{-1}, respectively. Besides, the random coil structures can also be detected at a wavenumber of approximately 1644 cm^{-1}. Therefore, any significant shifts in the wavenumber values of amide band indicate either an alteration in the secondary structures or alteration in the conformation of proteins. In addition, since the amide bands are originated from the superimposition of certain sub-bands resulting from different types of protein secondary structures, alterations in bandwidth values of protein bands can also be analyzed in order to provide information about changes in protein conformation (Dousseau and Pezolet, 1990). There were no significant shift observed either in the wavenumber, or in the bandwidth values of amide I and amide II bands. Considering the alterations in the band area values, the band areas of amide I and amide II bands decreased in diabetic groups. The decrease was seems to be slight in amide I, but the decrease was more prominent in amide II ($p<0.05^{*}$). Through the analysis of band area of amide II band, administration of different doses of Vitamin C was also observed to reverse the effects of diabetes on the protein content in a significant manner ($p<0.05^{\#}$) (Figure 34).

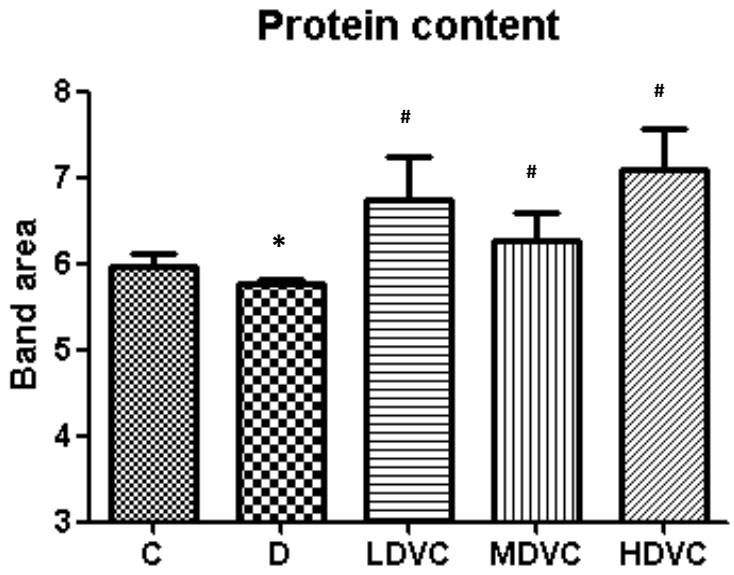

Figure 34. Comparison of protein contents (amide II band area) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

The other protein band in the fingerprint region, namely amide III band, located at around 1311 cm^{-1}, is resulted from the peptide side chains. More specifically, this band is originated from the C-N and the C-C stretching and the N-H bending vibrations (Steiner *et al.*, 2003). As can be seen from Table 6, the wavenumber of this band which is located at 1311 cm^{-1} shifted to lower values in diabetic rat testes significantly ($p<0.01^{**}$). Besides, the reversal effect of Vitamin C treatment was also observed. The reversal effect was more prominent in low and medium doses of Vitamin C administration ($p<0.01^{\#\#}$). In addition to the wavenumber analysis of this band, there was no significant change found in the area of the same band in diabetic rats.

Moreover, there are some lipid bands in this region located at around 1458 cm^{-1}, 1393 cm^{-1}, and 1173 cm^{-1} which are assigned to the CH_2 bending mode of protein and lipids (Manoharan *et al.*, 1993; Cakmak *et al.*, 2003), COO^- symmetric stretching vibrations in fatty acids (Cakmak *et al.*, 2003) and CO–O–C anti-symmetric stretching vibrations of phospholipids, triglycerides, cholesterol esters (Mendelsohn *et al.*, 2003), respectively. There were no significant alteration detected in the wavenumber and band area values from the analysis of CH_2 bending band, and no significant alteration detected in the wavenumber values from the analysis of COO^- symmetric stretching band. However, a slight decrease was found in the band area of COO^- symmetric stretching band, and this decrease was reversed after different doses of Vitamin C treatment.

The bands located in between 1300-1000 cm^{-1} spectral range, are assigned to anti-symmetric and symmetric phosphate stretching modes mainly due to the vibration of the P=O bond present in the phosphate moieties (PO_2^-) of phospholipids and nucleic acids (Kneipp *et al.*, 2000). The changes in the hydration state of the head group of phospholipids and the degree and position of phosphorylation of the nucleic acids in DNA and RNA can be monitored by analyzing these stretching modes in the 1300-1000 cm^{-1} region. In the wavenumber values of the bands, no dramatic alterations were observed. Considering the band area values, the band area of PO_2^- symmetric stretching band decreased dramatically ($p<0.05^*$). Moreover, Vitamin C treated diabetic samples were found to have recovery effect against diabetes-induced alterations in this band, and thus the band area values became closer to their control values. In this manner, low and medium doses of Vitamin C administration seemed to be much more effective ($p<0.05^\#$), when compared to high dose of Vitamin C treatment (Figure 35).

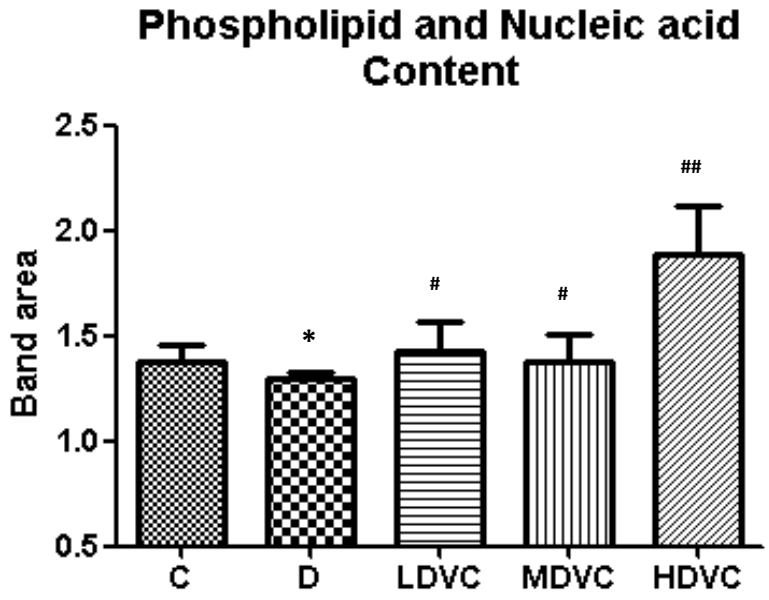

Figure 35. Comparison of phospholipid and aminoacid content in the system (PO_2^- symmetric stretching band area) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

Besides, no significant alterations were observed for the relatively weak band centered at 1173 cm^{-1}, which is due to anti-symmetric stretching mode of the CO-O-C groups present in glycogen and nucleic acids (Rigas *et al.*, 1990; Cakmak *et al.*, 2006). Additionally, no significant alterations were found in the other main band in the fingerprint region, which is the RNA band at around 970 cm^{-1} due to C-N-C stretching vibrations of nucleic acids (Cakmak *et al.*, 2003).

In FTIR spectroscopic analysis, band areas of the bands can also be calculated in order to get direct information about the macromolecular concentrations of the corresponding system. Moreover, calculation of different ratios of the band areas is also important in order to get information about the relative concentrations of the respective biomolecules in the system. Among those, olefinic/total lipid ratio,

olefinic/total CH_2 ratio and CH_2 sym + CH_2 asym/total lipid ratio are used to get information unsaturation and the saturation levels of the system, respectively. Besides, CH_2 asym/total lipid ratio gives information about chain length of lipids.

On the other hand, FTIR microspectroscopy, which enables to construct a point-by-point chemical map of the sample of interest by scanning the whole sample, also enables to gather images of macromolecular variations, in terms of distribution, concentration and content. Through this approach, any changes in the macromolecular components of the sample can be detected together with the alterations in the morphological status with a greater spatial resolution and microscopic scale. In other words, FTIR microspectroscopy is a beneficial tool in the differential visualization of the distribution of macromolecular components and determining the changes in the corresponding macromolecular components. Through the FTIR images obtained by FTIR microscope, information about the concentration of biomolecules in the tissues can be obtained. As can be seen from following figures, alterations in the macromolecular status of control, diabetic, and different doses of Vitamin C treated diabetic rat testes tissues can easily be discriminated by the help of FTIR imaging, all of which are supporting the findings of ATR- FTIR spectroscopic studies.

The average maps from FTIR microspectroscopic images were colored according to different macromolecular ratios, in all of which red color corresponds to the highest ratio and blue color corresponds to the lowest ratio as shown on the color bar in the corresponding figures (Figure 39 and 41).

The following maps belong to different macromolecular ratios obtained by FTIR microspectroscopy, support the numerical results which obtained from the ATR-FTIR spectroscopic data.

As seen from the figures below, (Figure 36, 37, and 38) showing the alteration in the unsaturation (olefinic/total lipid band area ratios and/ or olefinic/total CH_2 band area

ratios) and saturation levels (CH_2 asym+CH_2 sym/total lipid band area ratio) of experimental groups, degree of unsaturation increased dramatically in diabetic group of animals, with respect to their controls ($p<0.001^{***}$). Besides, all doses of Vitamin C administered to diabetic animals were shown to decrease the level of unsaturation, and to increase the saturation level dramatically ($p<0.001^{\#\#\#}$). These results imply the recovery effect of Vitamin C against the diabetes-induced alterations. Supporting these results, in the olefinic/CH region ratio image, which is an indicator of unsaturation level of the system, the increase in the unsaturation level of diabetic animals was also clearly observed (Figure 39).

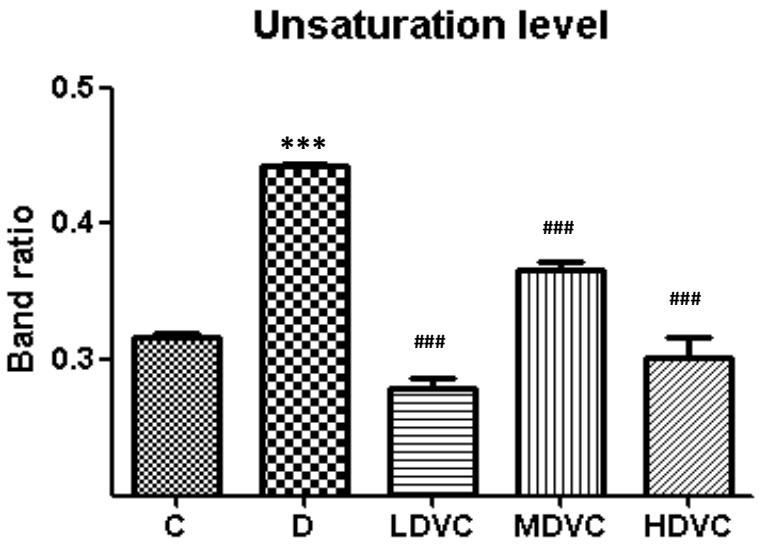

Figure 36. Comparison of unsaturation levels (olefinic/total lipid band area ratios) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

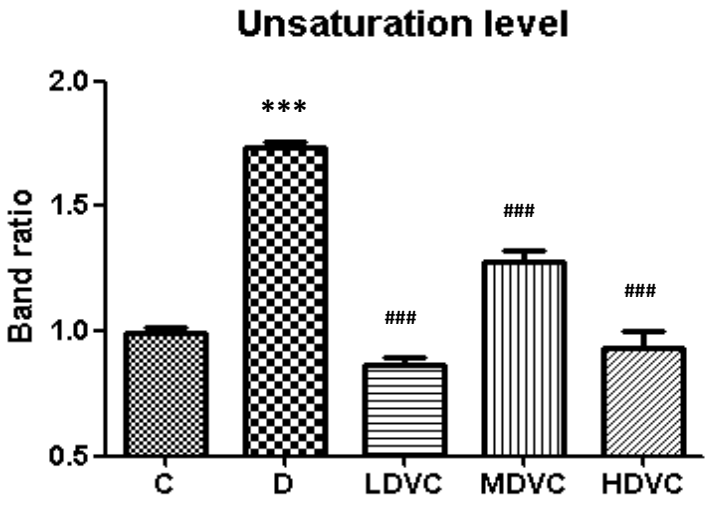

Figure 37. Comparison of unsaturation levelss (olefinic/total CH_2 band area ratios) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

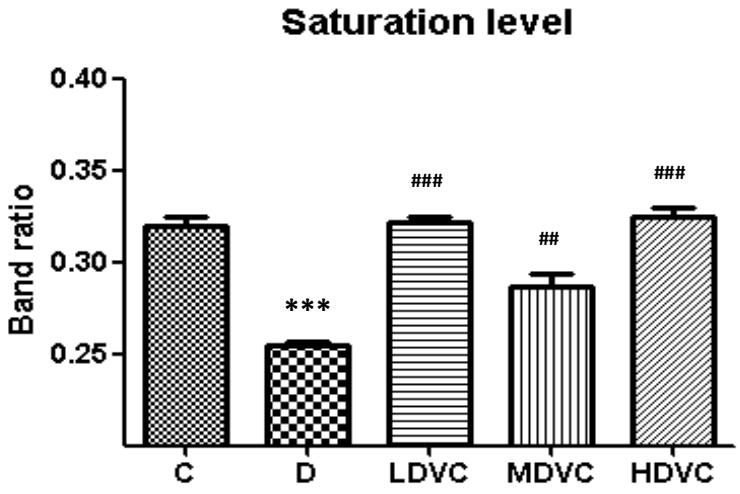

Figure 38. Comparison of saturation levels (CH_2 asym + CH_2 sym/total lipid band area ratios) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

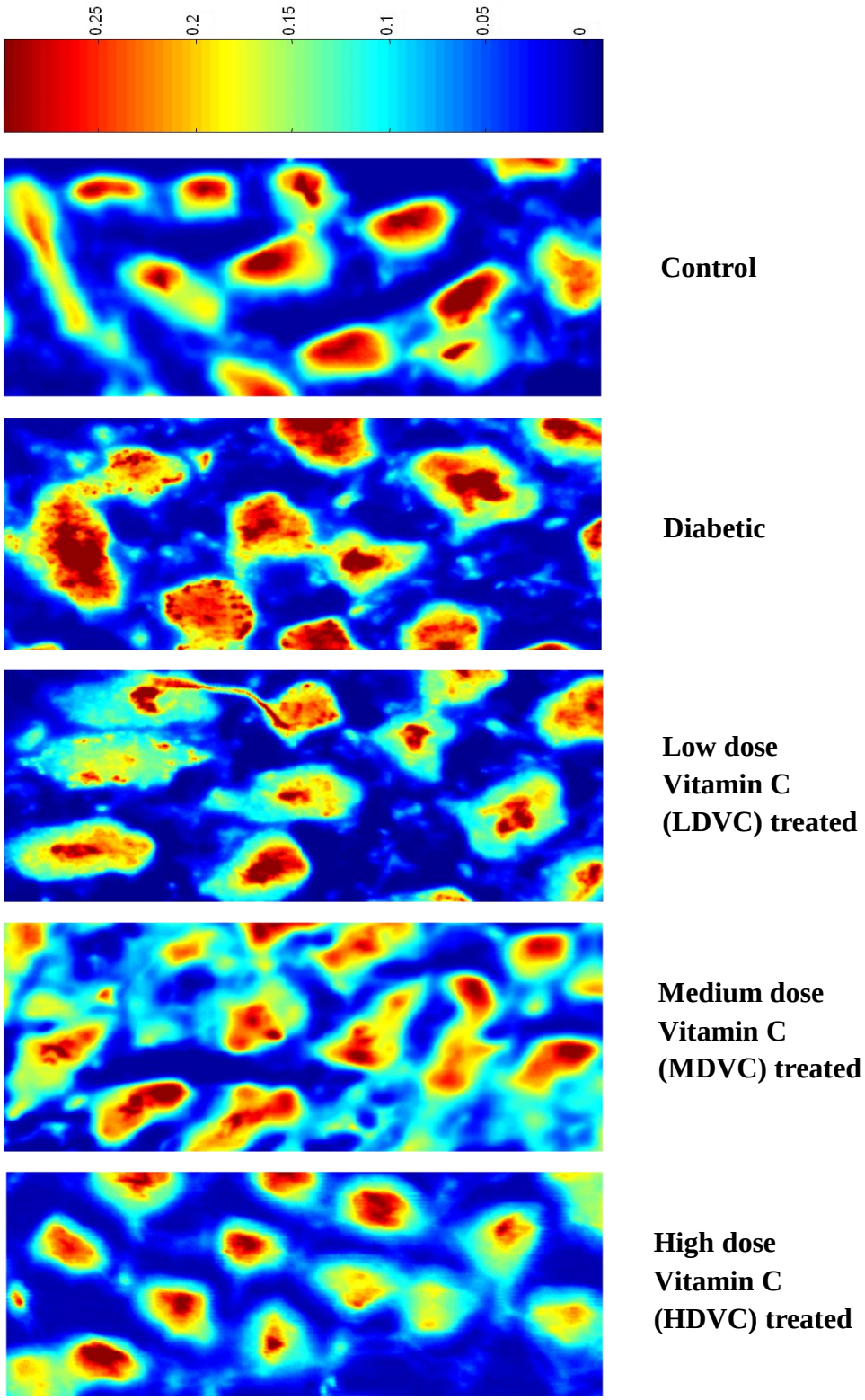

Figure 39. Olefinic/CH region ratio images of control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes for comparison of unsaturation levels**.**

As mentioned before, calculation of CH_2 asym/total lipid band area ratio gives information about the alterations in the chain lengths of lipids present in the system. It can be seen from Figure 40, in the diabetic group, chain lengths of lipids were found to decrease dramatically ($p<0.001^{***}$). As also can be seen from the same figure, administration of Vitamin C was shown to reverse the alterations that occur due to induction of diabetes. Low and high doses of Vitamin C treatment seemed to have high recovery effects against the diabetic complications ($p<0.001^{\#\#\#}$), while medium dose of Vitamin C treatment seemed to have a less significant recovery effect ($p<0.05^{\#}$). Moreover, it can be clearly seen that the numerical results obtained from the ATR-FTIR spectroscopic data is highly supported with the FTIR microspectroscopic images, which was also obtained by calculation of CH_2 asym/total lipid band area ratio of the experimental groups (Figure 41).

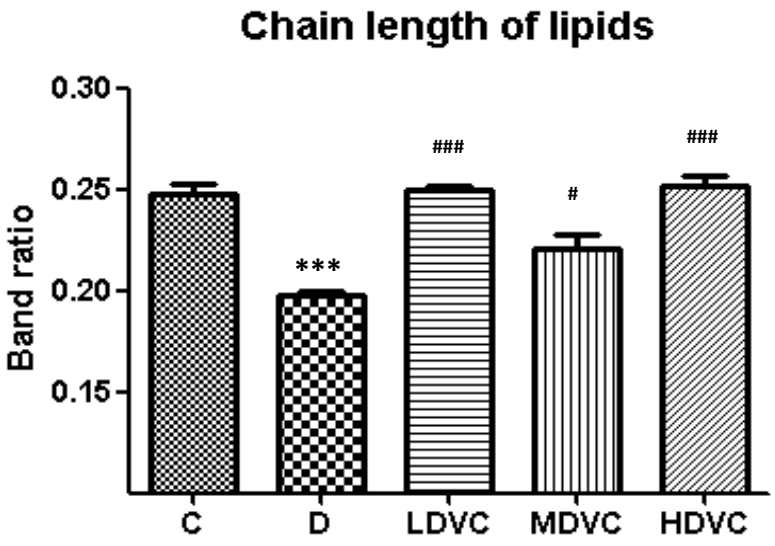

Figure 39. Comparison of chain lengths of lipids (CH_2 asym/total lipid band area ratios) for control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes. Diabetic group was compared with respect to the control group, while all other groups were compared with respect to the diabetic group. The degree of significance was denoted as $p<0.05^{*}$, $p<0.01^{**}$, $p<0.001^{***}$ for the diabetic group, and as $p<0.05^{\#}$, $p<0.01^{\#\#}$, $p<0.001^{\#\#\#}$ for the Vitamin C treated groups.

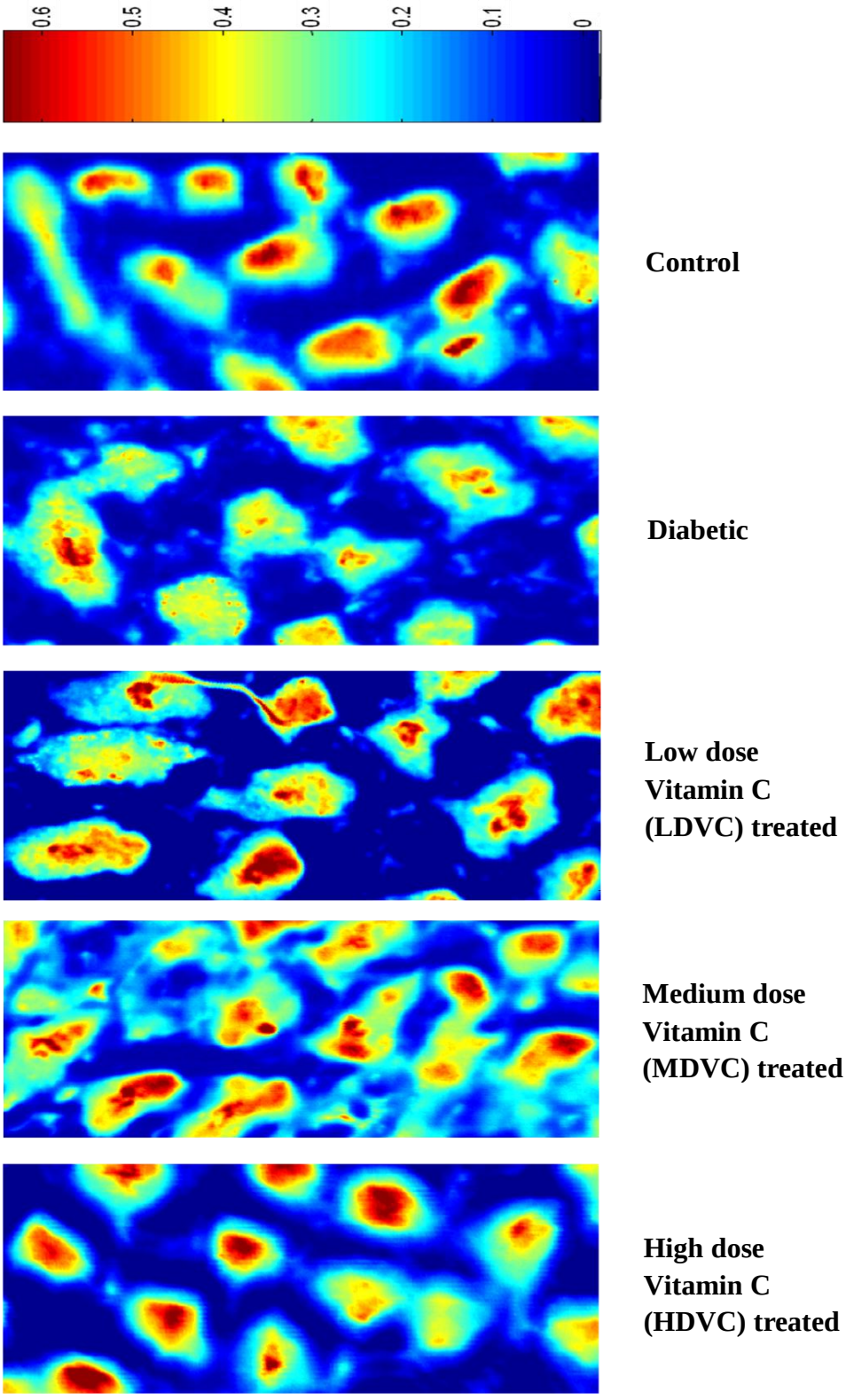

Figure 41. CH_2 asym/total lipid band area ratio images of control (C), diabetic (D), low dose Vitamin C (LDVC) treated diabetic, medium dose Vitamin C (MDVC) treated diabetic and high dose Vitamin C (HDVC) treated diabetic rat testes**.**

CHAPTER IV

DISCUSSION

Diabetes mellitus is a heterogeneous, metabolic disorder characterized by hyperglycemia. Type I diabetes, the insulin-dependent diabetes mellitus, is caused by the autoimmune destruction of insulin producing beta cells of the islets of Langerhans, in pancreas. Since the beta cells are destructed, either no insulin can be produced, or the level of insulin produced is extremely low (Akerbhlom *et al.*, 1998; Jun *et al.*, 2003). Even it has been shown that different pro-inflammatory cytokines have a significant role in the pathogenesis of type 1 diabetes, the underlying molecular mechanisms responsible for the cytotoxic effects of these cytokines still remains unclear (Rabinovitch, 1998). It has been thought that, the possible explanation for this cytotoxic effect is the generation of reactive oxygen species (ROS), due to oxidative stress in diabetic state. ROS generation, mainly caused by the elevated levels lipid peroxidation products, is believed to be the major cause of cytokine-mediated death of β-cells in the pancreatic islets of Langerhans (Rabinovitch *et al.*, 1996).

Elevated production of free radicals can both lead to destruction of the pancreatic beta cells, and lead to distortions on DNA due to oxidation in diabetic conditions. Moreover, elevated levels of ROS species have also capability to alter the biomolecules (e.g., lipids, proteins) in the system with concomitant changes in their structure and function. Since all the carbohydrate, lipid and protein metabolisms are affected by the increased levels of free radicals in diabetic state, the complications of diabetes are observed nearly in all of the organs and systems in the body, such as eyes, heart, kidneys, nervous system, and reproductive system. The complications of

diabetes on testicular tissue of reproductive system can either be shown up by the decreased functioning or decreased amount of Leydig cells. This situation results with reduced production and secretion of the major male hormone, testosterone, which in turn leads to delayed sexual maturation and sexual dysfunction (Hassan, 1993).

In healthy individuals, levels of free radicals are controlled through series of enzymatic and non-enzymatic antioxidant defense mechanisms, such as, catalase (CAT), superoxide dismutase (SOD) and reduced glutathione (GSH). While in the case of diabetes, the antioxidant potential lowers, and this decrease in the antioxidant potential leads to increased oxidative stress radicals (Maxwell *et al.*, 1997). In order to increase the antioxidant capacity, some supplementary antioxidants, such as Vitamin C, can be administered.

Vitamin C, as an antioxidant, functions as being a scavenger of free radicals including superoxide, hydrogen peroxide, hypochlorite, the hydroxyl radical and peroxyl radicals. Scavenging by the antioxidants, the tissues can be protected against the free radicals. The antioxidant property of Vitamin C is important both because it is associated with lowered DNA damage and because it results with diminished lipid peroxidation levels in the cells, which in turn ends up with a kind of recovery effect against the diabetic complications (Sies and Stahl, 1995).

In the literature, even some studies have performed to reveal the diabetic complications on male reproductive system, the effect of diabetes on the structure and function of the macromolecular content of testicular tissue still remains unclear. Therefore, in the current study, it was aimed to reveal the effects of STZ induced-type I diabetes and to investigate the recovery role of Vitamin C in the composition, structure and function of the macromolecular content of rat testes with the aid of ATR-FTIR spectroscopic and FTIR microspectroscopic techniques.

In infrared spectroscopic analysis, assignment of the band positions and the shifts in the positions of the bands enable to provide structural information about the biomolecules, on the other hand, determination of the alterations in the band area values gives information about the concentrations of the corresponding biomolecules in the system. Moreover, through the calculation of various band area ratios, information about the relative concentrations of the respective biomolecules in the system can be obtained. Additionally, bandwidth of the spectral bands can also be examined to gather information about the changes in the membrane fluidity for the lipid (C-H) region, and to gather information about the alterations in the protein conformation, for the protein region of the spectra.

Since type I diabetes and its complications are mainly associated with increased ROS production due to elevated levels of lipid peroxidation products, the spectral analysis of the lipid bands will be particularly emphasized in this chapter.

The 3025-2820 cm^{-1} region of the infrared spectra is mainly composed of the bands originated from the lipids. The first band in this lipid region is the olefinic band, which gives information about the unsaturation state of acyl chains in the system (Takahashi *et al.*, 1991; Severcan *et al.*, 2005a, Liu *et al.*, 2002; Severcan *et al.*, 2003). In the literature, it has reported that, the shift in the band position of olefinic band to lower values is an indicator of an increase in the orderness of the system (Mantsch, 1984; Lopez-Garcia *et al.*, 1993; Severcan *et al.*, 2003). In the current study, the shift in the band position of the olefinic band to higher degrees in diabetic samples with respect to the control samples indicates that diabetes has a disordering effect on the membrane structure of the testicular tissue. This disordering effect of diabetes is possibly due to the increased levels of lipid peroxidation, and so increased levels of lipid peroxidation end products. Especially after low and medium doses of Vitamin C treatment, the band position of olefinic band shift to lower degrees with respect to diabetic samples, and the wavenumber value of this band came closer to that of control samples. However, in the high dose Vitamin C administration, only very slight reversal effect was detected. These results reveal that, Vitamin C has a

recovery effect against the diabetic alterations in the membrane orderness/ disorderness degree.

Besides the structural analysis, as mentioned above, information about concentration of macromolecules can also be obtained through the calculation of the band area values. The band area of olefinic band was shown to increase significantly in diabetic group. This result also suggested that, the lipid peroxidation and thus the lipid peroxidation end products increased in diabetic state (Severcan *et al.*, 2005a, Liu *et al.*, 2002). For all doses of treatment, Vitamin C administration was shown to result in a significant reversal in the olefinic band area with respect to diabetic state. It can be concluded from this result that, Vitamin C has a significant recovery role against the diabetic complications associated with lipid peroxidation levels.

As mentioned in the previous chapters, type I diabetes is characterized by insufficient production of insulin by pancreatic beta cells. Since the signal coming from the presence of insulin is required in order for the cells to take up the glucose from blood, in the absence or in the scarce of insulin, the glucose will remain in the blood. So, in the progress of type I diabetes, blood glucose levels become abnormally high. Although the blood glucose levels are elevated, the body cells are not able to use these high amounts of glucose as a fuel. Thus, the cells become searching for another source to meet the energy needs. For this reason, lipids are begun to use as energy source. Through this way, the levels of lipids, namely ketone bodies for this particular case, increases in the blood, and the lipid constituents are taken up by the cells to meet the energy needs (Jain and McVie, 1999). This situation leads to abnormal lipid metabolism in the cells, by increasing the lipid, lipoprotein and lipid peroxides levels abnormally (Suryawanshi, *et al.*, 2006). It also leads to inhibition of the activity of antioxidant enzymes leading to accumulation of free radicals in the cells, and eventually ending up with elevated levels of lipid peroxidation and so lipid peroxidation end products in diabetic conditions (Davi *et al.*, 2005; Jain and McVie, 1999). Besides these defects in the lipid metabolism, oxidative stress and free radical formation may also increase due to exposure to prolonged periods of hyperglycemia,

by causing non-enzymatic glycation of proteins (binding of glucose to protein molecules) (Hanachi *et al.*, 2009; Mahboob *et al.*, 2005). Both high glucose levels and protein glycation enhance low density lipoproteins (LDL) oxidation by metal ions, and these reactions also yield advanced glycosylation end (AGE) products. LDLs isolated from diabetics contain higher levels of AGE products were shown to more easily be oxidized by copper than non-diabetic LDL (Davi *et al.*, 2003; Johansen *et al.*, 2005; Maritim *et al.*, 2003; Schleicher *et al.*, 1997; Lyons, 1993). In addition to the mechanisms explained above, mitochondria have also role in the elevation in the free radical and in the lipid peroxide products levels due to increased lipid peroxidation. Mitochondria contain relatively large amount of polyunsaturated fatty acid in their phospholipid structure. These phosholipids include unsaturated fatty acids containing 2, 4, 5 and 6 double bonds. Containing high amounts of double bonds (for example containing 3 or more double bonds) make the fatty acid to be likely to be more sensitive for attacking by free radicals which results in higher levels of lipid peroxidation (Green *et al.*, 2004). Due to all these possible reasons, and also due to simultaneous decrease in the antioxidant potential in diabetic conditions, there would be an imbalance between the production and scavenging of free radicals (Johansen *et al.*, 2005). These factors associated with the increase in lipid peroxidation levels, constitute the basis of the diabetic complications. In order to recover, or reduce the damages caused by diabetes, antioxidant defense system should be increased by means of capacity. At this point, usage of a supplementary antioxidant, such as Vitamin C or Vitamin E, becomes precious. With the administration of Vitamin C, free radicals such as oxygen related radicals (superoxide, hydroxyl radical, peroxyl radicals), are scavenged efficiently just before they can initiate lipid peroxidation (Padayatty *et al.*, 2003; Maxwell *et al.*, 1997; Retsky *et al.*, 1993; Niki, 1991). Through this way, Vitamin C can effectively recover the diabetes induced macromolecular alterations and be able to protect the cells and the biomembranes against peroxidative damage (Sies and Stahl, 1995; Padh, 1990).

In the literature, there is a controversy about the relationship between the olefinic band area and lipid peroxidation levels. Some studies suggested that, the area of olefinic band decreases in the diabetic state (Sills *et al.*, 1994), while others suggested an increase for the same case (Severcan *et al.*, 2005a, Liu *et al.*, 2002). This difference observed in the alterations of the olefinic band area due to lipid peroxidation may be resulted from the structural differences between the samples. The tissue samples, platelets or microsomal membranes surround with cytoplasmic material include lipid peroxidation end products in their environment. Therefore, the cytoplasmic lipid peroxidation end products are able to have a contribution towards the increased olefinic band intensity and area in diabetic samples when compared to the control samples. On the other hand, for instance erythrocyte membranes, having pure membrane phospholipids without cytoplasm, do not contain cytoplasmic lipid peroxidation end products. In this case, there would be no contribution of lipid peroxidation end product to the olefinic band area. Thus, no increment is observed in the band area or band intensity of olefinic band. This structural difference between the samples of interest could explain the reason why there were different findings achieved in different studies. In this current study, tissue samples were used which surrounds with cytoplasmic material containing lipid peroxidation end products. Therefore, the elevation in the amounts of lipid peroxidation end products leads to an increase in the olefinic band area in this case, indicating an increase in the unsaturated lipid content.

The bands located in the 3000-2820 cm^{-1} region of the spectrum, are assigned to C-H stretching modes. Considering the alterations in the band area values, CH_2 anti-symmetric and CH_2 symmetric band area values were found to decrease significantly in diabetic animals with respect to controls. CH_2 anti-symmetric and CH_2 symmetric bands which are originated mainly from lipids, gives information about lipid content of the system. The decrease in the area of these two bands suggests that, the lipid concentration decreased in diabetic state. While, by the administration of Vitamin C, the band areas of CH_2 anti-symmetric and CH_2 symmetric bands were found to increase, implying that Vitamin C has a recovery effect on lipid content of diabetic

rat testes, as approaching the band area values to those in control group. In the presence of insulin, in other words when the glucose levels is sufficient to meet the energy needs of the cells, the release of free fatty acids (FFA) are inhibited. These free fatty acids are converted to triglycerides and then, they are stored in the form of triglycerides. Since the insulin levels are insufficient in diabetic state, the free fatty acids can not be stored as triglycerides on the contrary they are released into the blood. Despite the high levels of blood glucose, the glucose can not be taken into the cells, instead the free fatty acids are taken and used as an energy source through lipolysis (Bergman and Ader, 2000; Eriksson *et al.*, 1999). This situation explains the reason underlying the decrease in the lipid content of diabetics. Vitamin C, as an antioxidant, helps to recover the complications of diabetes, and the cells can become their normal homeostatic state.

The alterations in the unsaturation and the saturation levels of the lipids were also shown by calculating the ratios of olefinic/total lipid band area ratios and olefinic/total CH_2 (CH_2 asym + CH_2 sym) band area ratios for indicating the unsaturation level and CH_2 asym + CH_2 sym/total lipid band area ratio for indicating the saturation level. The olefinic/total lipid band area ratio and olefinic/total CH_2 band area ratio were shown to increase dramatically in diabetic group, however the ratio of CH_2 asym + CH_2 sym/total lipid band area was shown to decrease significantly. These results also proved that, the degree of unsaturation increased dramatically in diabetic group of animals, with respect to their controls, while saturation level decreased. Besides, all doses of Vitamin C administered to diabetic animals were shown to decrease the level of unsaturation, and to increase the saturation level dramatically. Since antioxidant capacity decreases in diabetic conditions, administration of Vitamin C, as an antioxidant, ends up with recovery effect against diabetes-induced complications. These results also supported the findings mentioned above, implying that Vitamin C has a recovery effect against the diabetes-induced alterations in lipid un/saturation due to elevated levels lipid peroxidation.

Calculating another ratio, namely CH_2 asym/total lipid band area ratio makes it possible to gather information about the alterations in the acyl chain lengths of lipids present in the system. In diabetic state, acyl chain lengths of lipids were found to decrease dramatically. While the administration of Vitamin C was shown to reverse the alterations, that occur due to development of diabetes. The structural phospholipids, such as in the membrane structure and in the mitochondria, contain relatively large amount of polyunsaturated fatty acid in their phospholipid structure. These unsaturated lipid contents are susceptible to be attacked by free radicals in the case of elevated lipid peroxidation levels as mentioned above, and in turn it results with the breakdown of longer chains into smaller lipid acyl chains (Bozkurt *et al.*, 2010). This breakdown of the structural phopholipid chains explains the reason why the chain lengths of the lipids decreased in diabetic conditions.

The 1800-950 cm^{-1} spectral region corresponds to the fingerprint region. The first band in this spectral range is the cholesterol ester band located at around 1742cm^{-1} which is due to the C=O stretching vibrations of ester carbonyl groups in lipids (Mendelsohn *et al.*, 2003). This band was very small in all experimental groups, which disabled us to carry out trustable detailed spectral analysis. Therefore, no assumption will be made considering this cholesterol ester band.

There are also two main protein bands located in this fingerprint region, in the 1700-1480 cm^{-1} spectral region specifically. Among these two protein bands, amide I, arises from mainly C=O stretching (%80) and C-N stretching vibrations of the protein backbone, and amide II, is originated from N-H bending (%60) and C-N stretching (%40) vibrations of the proteins (Haris and Severcan, 1999; Melin *et al.*, 2000; Takahashi *et al.*, 1991; Wong *et al.*, 1991; Cakmak *et al.*, 2006). Besides, a third smaller protein band is also present in the fingerprint region, which is named as amide III. Amide III band is located at around 1311 cm^{-1}, and is mainly originated from the C-N and C-C stretching and N-H bending vibrations of proteins (Bozkurt *et al.*, 2007; Steiner *et al.*, 2003). Alterations in the frequency values of these protein bands indicate an alteration in the protein conformation or in the protein structure.

The frequency of amide III band was observed to shift to lower values significantly in diabetic state, suggesting that there is an alteration in the protein conformation or in the protein structure. Besides, the reversal effect of Vitamin C treatment was also observed, which was more prominent in low and medium doses of Vitamin C administration.

Diabetes-induced changes in protein secondary structures and the recovery role of Vitamin C on these diabetes-induced alterations in proteins were also determined. For this purpose, protein secondary structural analysis was performed by vector normalized second derivative method, using the amide I band, between 1700-1600cm^{-1} spectral region. Supporting to the wavenumber analysis of amide III band, some alterations were observed in the secondary structural components of proteins in the system. According to the secondary structural analysis, the peak intensity of random coil structure was found to increase slightly and the intensity of aggregated β-sheet structure was found to increase dramatically in diabetic group with respect to their controls, while the β-sheet content was found to decrease significantly. With the administration of Vitamin C to the diabetic animals, the peak intensities for secondary structural components of proteins become much closer to the values observed in control group. These results demonstrate the recovery effect of Vitamin C on the protein secondary structure against the complications of diabetes. It can be concluded from these findings that, diabetes leads to distortions in protein conformation and in the secondary structures of proteins. The reason why β-sheet content was found to decrease in diabetic state is possibly the conversion of the β-sheet structure to mainly aggregated β-sheet structures. In addition, Vitamin C treatment increased the β-sheet content again, indicating that the mainly the aggregated β-sheet structure again turn back to β-sheet.

As mentioned, alterations in the band area values of different experimental groups, give us information about the changes in the macromolecular contents. For the protein bands, the calculation of the band areas is important in order to obtain information about the protein content of the corresponding system. The band areas of

amide I and amide II were found to decrease in diabetic group. However, the decrease is more prominent in amide II. After Vitamin C treatment, the protein content seemed to be recovered, and the band area values become much closer to the values observed in control group. As mentioned above, one of the reasons for diabetic complications is the elevated oxidative stress and free radical formation due to glycation of proteins in the cells due to hyperglycemic conditions (Hanachi *et al.*, 2009; Mahboob *et al.*, 2005). Proteins modified consequently contain glycation and oxidation adduct residues, and undergo cellular proteolysis with release of corresponding free adducts (Wang *et al.*, 2006; Ahmed *et al.*, 2005). Therefore, in addition to the lipolysis, diabetic conditions also induce protein degradation, which explains the reduction in the protein content of diabetes induced group of animals. The increased amounts of urinary excretion of protein glycation and oxidation products in diabetic patients also suggests that there is an increased damage in proteins through glycation and oxidation processes in diabetic conditions, with subsequent cellular proteolysis and elimination of free adducts (Ahmed *et al.*, 2005). With the administration of Vitamin C, the antioxidant capacity is able to be increased, and through this approach, the diabetic alterations can be reversed. Since the cells call back more or less to their healthy state with the elevation in the antioxidant capacity, the macromolecular metabolisms are rearranged. By this way, with the administration of Vitamin C, proteolysis level decreased, just as in the case of decrease that we have observed in lipolysis levels.

Among the lipid bands present in the fingerprint region, only COO^- symmetric stretching band located at around $1393cm^{-1}$, was found to decrease significantly in terms of band area, in diabetic conditions. This decrease was able to be reversed after administration of different doses of Vitamin C. COO^- symmetric stretching band is originated from the COO^- symmetric stretching vibrations in fatty acids and amino acid side chains (Jackson *et al.*, 1998; Cakmak *et al.*, 2003). The decrease observed in the band area of COO^- symmetric stretching band demonstrates that, there is a decrease in the fatty acids and amino acid content of the corresponding system, while Vitamin C can reverse the diabetes induced alterations, and recover the fatty acids

and amino acid content, also consistent with the findings of our study explained above.

The 1300-1000 cm^{-1} spectral range mainly includes anti-symmetric and symmetric phosphate stretching modes, mainly originated from the vibration of the P=O bond present in the phosphate moieties (PO_2^-) of phospholipids and nucleic acids (Kneipp *et al.*, 2000). The band positions for PO_2^- anti-symmetric stretching and PO_2^- symmetric stretching bands are 1237 cm^{-1} and 1084 cm^{-1}, respectively. These particular phosphate stretching vibrations make it possible to gather information about the hydration state of the head group of phospholipids in the membrane structures (Mendelsohn and Mantsch, 1986) and to gather information about the alterations in the quantity, conformational state or in the phosphorylation levels of the nucleic acids in DNA and RNA, in the cell structures (Dovbeshko et al, 2000; Kneipp et al, 2000). In the analysis of the shifts in the wavenumber values, no dramatic alterations were observed for these two bands. Thus, any comments can not be made either about the hydration levels of phospholipids in membrane structures and in the nucleic acids, or about the alteration in the conformation of RNA, DNA and disorderness/orderness of phospholipids. On the other hand, considering the band area values, the band area of PO_2^- symmetric stretching band was shown to decrease dramatically in diabetic group. Moreover, Vitamin C treated diabetic samples were shown to become closer to the values observed in the control group, revealing that administration of Vitamin C has a recovery effect against the diabetes-induced alterations. This decrease in the area of the PO_2^-symmetric stretching band indicates a decrease phospholipid content of the membrane structures and also a decrease in the nucleic acids content of the cells. These results are also compatible with the findings mentioned above, in which the reason of the decrease in the phospholipid and nucleic acid contents were clarified.

Lastly, no significant alterations were observed for the bands located at around 1173 cm^{-1}, and 970 cm^{-1} originated from CO-O-C groups present in glycogen and nucleic

acids (Rigas *et al.*, 1990; Cakmak *et al.*, 2006) and C-N-C stretching vibrations of nucleic acids (Cakmak *et al.*, 2003), respectively.

Although many studies are found in the literature, about the complications of diabetes on various tissues, including male reproductive system, the effects of diabetes on the macromolecular structure, composition and function of testicular tissue have not been demonstrated yet. In this manner, the current study fills the missing part of the literature about the diabetes induced changes in testicular tissue at macromolecular level.

By the help of the findings obtained from this study, new therapies for the treatment of diabetes can be taken into account, in order to recover the diabetes-induced complications. Moreover, it was revealed that, a different approach from the main clinical strategies, antioxidant supplementation, can also be suggested as a complementary medication strategy for diabetic patients. Because it is obvious that, antioxidant administration, Vitamin C for this particular case, can effectively have a recovery effect on the biomembrane structures and the cells or tissues against peroxidative damage due to the development of diabetes. By this way, life quality of the diabetic patients can be significantly increased.

Besides, early diagnosis has a great deal of importance for all kinds of diseases in order to have a chance to treat the patients much more effectively. Through FTIR spectroscopy and FTIR microspectroscopy, rapid visualization of the early macromolecular alterations due to the presence or the degree of progress of various diseases can be demonstrated sensitively. Therefore, by the improvement and the common usage of these rapid and sensitive biophysical techniques in clinic, it will be very helpful for the patients by having a chance to make early diagnosis for different diseases, such as diabetes or even cancer, in near future. As a conclusion, this current study provides a new insight on the etiological and therapeutic aspects of type I diabetes.

CHAPTER V

CONCLUSION

The results of the current study indicate that, STZ induced type I diabetes leads to dramatic alterations in the macromolecular structure and composition of rat testes. Moreover, through the administration of Vitamin C, partial recovery of diabetes-induced alterations was demonstrated.

The detailed spectral analysis of the ATR-FTIR spectroscopy and FTIR microspectroscopy revealed the following interpretations:

The saturation level of the lipids in the corresponding biological system altered due to diabetes. The unsaturated lipid content was shown to increase in diabetic state, due to increased levels of free radical formation through elevated lipid peroxidation levels. Since the antioxidant potential decreased in diabetic conditions, there would be an imbalance between the production and scavenging of free radicals and this situation constitutes the basis of the development of diabetic complications. Administration of Vitamin C provides scavenging of free radicals before they initiate lipid peroxidation. Through this way, Vitamin C can effectively recover the damages and protect the cells and the biomembranes against peroxidative damage.

The orderness of the system was also found to be altered due to diabetes related to the elevated lipid peroxidation levels, and thus increased amounts of lipid peroxidation end products.

Besides the alterations in the un/saturation levels of lipids, contents of lipids and proteins also altered in diabetic state. The lipid and protein contents both decreased

in diabetic conditions. However, the reduction in the lipid content was much more prominent when compared to the decrease in the protein content. These reductions in lipid and protein contents are caused by the elevated levels of lipolysis and proteolysis processes due to hyperglycemia resulted from insufficient levels of insulin. In this situation, the fats are begun to be used as the main energy source. Therefore, lipolysis is highly elevated in the diabetic conditions with respect to proteolysis. After treatment of diabetic animals with Vitamin C, the lipid and protein contents were shown to become much closer to the control group, indicating that Vitamin C, as an antioxidant, has a recovery effect on diabetes-induced alterations. Besides, structural phospholipid chains also started to be degraded, which ends up with a decrease in the chain lengths of the lipids in diabetic conditions.

In addition to the changes in the protein content, the protein conformation and protein secondary structures also altered in diabetes. Through the analysis of protein secondary structures, the aggregation and denaturation of proteins were observed in the case of diabetes. While the Vitamin C treatment showed a recovery effect on diabetes-induced alterations in protein structure, by decreasing the aggregated and denatured protein contents.

Moreover, fatty acid levels, phospholipid amounts in the membrane structure, amino acid content and also nucleic acids content were also shown to decrease in diabetic conditions as consistent with the previous findings mentioned above. However, Vitamin C can reverse the diabetes-induced alterations, and the fatty acid levels, phospholipid amounts in the membrane structure, amino acid content and also nucleic acids content were all shown to be recovered after the antioxidant treatment.

To conclude, structure and composition of the rat testes were revealed to be altered due to STZ-induced type I diabetes, and administration of Vitamin C was indicated to have a recovery effect against the diabetic complications.

This was the first study that demonstrates the effects of STZ-induced type I diabetes on rat testes, and was also first to suggest the recovery role of Vitamin C, as an antioxidant, on diabetic rat testes, using FTIR spectroscopic and microspectroscopic imaging tools.

This study has insights about the therapeutic capacity of Vitamin C on rat testes, which requires further studies in order to be certified. In future, Vitamin C treated control rat testes will also be studied in detailed, in order to see the effects of Vitamin C on healthy testicular tissue. Besides, combinations of different antioxidants will also be investigated to obtain a better recovery effect. Through this way, in near future, new complementary therapies for the treatment of diabetes will be able to taken into consideration.

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