Chapter 1: Review of literature
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1.1. Diabetes mellitus

Diabetes mellitus is a metabolic disease characterized by hyperglycemia resulting from the defects in insulin secretion to its action. The disorder initially affects carbohydrates, and later the metabolism of proteins and lipids. Most of the chronic complications of diabetes result from the existing hyperglycemic status due to the deranged metabolism of carbohydrates, which can cause macrovascular as well as microvascular diseases. The term diabetes was coined by Aretaeus of Cappadocia. It is derived from the Greek word “diabaínein” that literally means "passing through," or "siphon", a reference to one of diabetes' major symptoms - excessive urine production. In 1675, Thomas Willis added the word “mellitus” to the disease, a word from Latin meaning "honey", a reference to the sweet taste of the urine. This sweet taste had been noticed in urine by the ancient Greeks, Chinese, Egyptians, and Indians. In 1776, Matthew Dobson confirmed that the sweet taste was because of an excess of a kind of sugar in the urine and blood of people with diabetes (Dobson, 1776).

Diabetes is one of the major public health challenges of the 21st century. According to the WHO, the occurring epidemic of diabetes is strongly related to lifestyle and economic changes (King and Rewers, 1991). WHO estimates that by 2025 as many as 200–300 million people worldwide will have developed type 2 diabetes (King et al., 1998). This translates into an increase of nearly 6 million patients every year, according to statistics from the Centre for Disease Control (CDC). Diabetes is the sixth leading cause of death due to disease in the U.S., and the third leading cause among some ethnic populations. South East Asian countries have the highest burden of diabetes (King and Rewers, 1991; King et al., 1998), and the projections of the International Diabetes Federation on the prevalence of diabetes mellitus and impaired glucose tolerance (IGT) for the year 2005 is, respectively, 7.5 and 13.5% (King and Rewers, 1991; King et al., 1998).

1.2. Classification of diabetes mellitus

The WHO recognizes three main forms of diabetes mellitus: type 1, type 2, and gestational diabetes (occurring during pregnancy), which have similar signs, symptoms
and consequences but different causes and population distributions. Ultimately all forms are due to the β cells of the pancreas being unable to produce sufficient insulin to prevent hyperglycemia (Rother, 2007).

With type I diabetes, it has been demonstrated that destruction of the pancreatic beta cells (which are found in the islets of Langerhans) causes the disease by destroying the cells that produce insulin. Type I diabetes has been mainly associated with some type of destructive immune response: a large fraction of type I diabetics have antibodies against their own beta cells. Several other factors also implicate autoimmune disease in the development of type I diabetes, including lymphocyte infiltration of islets in patients with recent disease onset, correlation of certain human leukocyte antigens and incidence of diabetes, increased K-cell rosette formation and antibody- or complement-mediated cytotoxicity, and increased numbers of activated T lymphocytes. There are indications for other factors besides the immune response in the cause of type I diabetes. Viral infection and environmental agents have also been implicated. One possibility to tie the three agents together is that injury due to viruses or toxins kill some islet cells, resulting in the expression of antigens that cause an immune response to the remaining islet cells.

Type 1 may ultimately lead to diabetes mellitus in which “insulin is required for survival” to prevent the development of ketoacidosis, coma and death. An individual with a Type 1 process may be metabolically normal before the disease is clinically manifest but the process of β cell destruction can be detected. Type I is usually characterized by the presence of anti-GAD (glutamic acid decarboxylase), islet cell or insulin antibodies which identify the autoimmune processes that lead to β cell destruction in 85-90% of individuals. There are some forms of Type 1 diabetes which have no known aetiology. Some of these patients have permanent insulinopenia and are prone to ketoacidosis but have no evidence of autoimmunity (McLarty et al., 1990); this form of diabetes, known as idiopathic, is more common among individuals of African and Asian origin.

Type 2 is characterized by tissue-wide insulin resistance, but impairment of β cell function is necessary for its development. In the early stage, the predominant abnormality is reduced insulin sensitivity, characterized by elevated levels of insulin in the blood. At
this stage, hyperglycemia can be reversed by a variety of measures and medications that improve insulin sensitivity or reduce glucose production by the liver, but as the disease progresses the impairment of insulin secretion worsens and therapeutic replacement of insulin often becomes necessary (Lillioja et al., 1993; DeFronzo et al., 1997). Ketoacidosis is infrequent in this type of diabetes. Obesity is found in approximately 55% of patients diagnosed with type 2 diabetes.

Gestational diabetes involves a combination of inadequate insulin secretion and responsiveness, resembling Type 2 diabetes in several respects. It develops during pregnancy and may improve or disappear after delivery. Even though it may be transient, gestational diabetes may damage the health of the fetus or mother and about 20%–50% of women with gestational diabetes develop Type 2 diabetes later in life (DeFronzo et al., 1997).

Types 1 and 2 are incurable chronic conditions, but have been treatable since insulin became medically available in 1921, and today are usually managed with a combination of dietary treatment, tablets (in Type 2) and frequently insulin supplementation. Gestational diabetes typically resolves with delivery (Harris, 1993).

1.3. Complications of diabetes mellitus

The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision and weight loss. In its most severe forms, ketoacidosis or a non-ketotic hyperosmolar state may develop and lead to stupor, coma and in absence of effective treatment, death. Often symptoms are not severe or may be absent and consequently hyperglycemia sufficient to cause pathological and functional changes may be present for a long time before the diagnosis is made. If the diabetes is poorly controlled it can lead to diabetic complications.

The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels. The long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may
lead to renal failure and/or neuropathy with risk of foot ulcers, amputation, charcot joints and features of autonomic dysfunction including sexual dysfunction.

It damages the vessels and the basement membrane causing impaired delivery of nutrients and hormones to the tissues, resulting in tissue damage. The most sites affected are the retina, renal glomerulus and the nerve sheath. Vascular complications of diabetes occur in both micro and macro vascular vessels. Microvascular complications include retinopathy, nephropathy and neuropathy. Macro vascular complications comprise peripheral vascular disease and cardiovascular complications such as ischemic heart disease and hypertension. These are all chronic illnesses, which take 10-20 years to manifest. The severity of complications is also modified by genetic factors. Many of the diabetic patients do not develop complications even when their glycemic control is not optimal (Rosenstock and Raskin, 1988).

One of the most important factors in the pathogenesis of diabetic complications is the metabolic milieu of the diabetic patients, the main causative factor being hyperglycemia (Alessandro et al., 1999). The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin.

1.4. Conventional therapies for Diabetes

Diet and exercise are the first step of therapy for type 2 diabetes; if these do not keep blood sugar at goal levels, then antihyperglycemic agents are added. Drug therapy for type 2 diabetes aims to control blood sugar levels both in the basal (fasting) state and postprandially; rational combinations of agents with different mechanisms of action can be used (Fig. 1.1) Four major classes of antihyperglycemic agents can be used, either as monotherapy or, more appropriately, in combination with one another:

* Insulin secretagogues
* Insulin sensitizers
* Insulin
* \( \alpha \)-glucosidase inhibitors
Fig. 1.1: Pharmacological treatment of hyperglycemia according to site of action.
(GLP-1 glucagon like peptide 1. DPP4 - dipeptidyl peptidase 4. FFAs - free fatty acids).
Insulin secretagogues correct hyperglycemia by stimulating insulin secretion but only if the patient still has enough functioning β-cells. They close ATP sensitive potassium channels in the β-cells of the pancreas, increasing insulin production; slow-acting and rapid-acting agents are available. The major side effects of insulin secretagogues (and insulin replacement) are hypoglycemia and weight gain.

1.4.1. Sulfonylureas

They are insulin secretagogues, enhancing insulin secretion by binding to a unique receptor on pancreatic β-cells and having their greatest effect on fasting hyperglycemia (Uwaifo and Ratner, 2007; Nathan et al., 2009). In this group we can include the second-generation agents gliclazide, glyburide, and glimepiride, as well as the first-generation agents acetohexamide, chlorpropamide, tolazamide, and tolbutamide. When used as monotherapy, sulfonylureas generally result in HbA1C improvements of a magnitude similar to metformin (1.5%). Sulfonylureas are commonly associated with hypoglycemia and weight gain (∼2 kg). Based on long experience, efficacy, and low cost, sulfonylureas are recommended by the ADA-EASD as options for second step pharmacotherapy in patients whose HbA1C remains elevated on metformin (Nathan et al., 2009).

However, sulfonylureas are also hindered by limited durability of effect, with 3- and 9-year failure rates similar to those described for metformin (Turner et al., 2005), which is again consistent with progressive β-cell failure. It has been previously described that gliclazide improves vascular activity decreasing oxidative stress and inflammation.

1.4.2. Meglitinides

The recently introduced class of meglitinides consists of nateglinide, which binds to the same site of sulphonylurea receptor 1 as do the sulfonylurea derivatives, and repaglinide, which binds to a nearby site of the receptor, both leading to insulin release. They stimulate rapid, short-lived, insulin secretion. They lower postprandial glucose levels, although fasting hyperglycemia is also improved. Meglitinides are more specific than sulfonylureas and are associated with lower risk of hypoglycemia but clinical experience remains limited. These agents cannot further stimulate insulin release in patients on maximal doses of sulfonylurea derivatives. These drugs can be used in patients
with decreased renal function (Hasslacher, 2003) and for individuals with varying daily meal patterns. Experimental evidence suggests a better preservation of β-cell function compared to sulfonylureas (Blickle, 2006) and improved vascular effects that remain to be clinically proven (Ceriello, 2000).

1.4.3. Metformin

It is an insulin sensitizer well accepted as a first-line agent for treatment of type 2 diabetes (Nathan et al., 2009). It is a biguanide derivative that exerts an antihyperglycemic effect with minimal risk of hypoglycemia. Metformin lowers blood glucose concentration and improves insulin sensitivity by reducing hepatic gluconeogenesis and enhancing insulin-stimulated peripheral glucose uptake (Uwaifo and Ratner, 2007; Nathan et al., 2009). In addition, metformin reduces insulin resistance in muscle tissue and the liver, decreasing postprandial hyperglycemia and inhibits adipose tissue lipolysis thereby reducing circulating levels of FFAs (Kirpichnikov et al., 2002; Uwaifo and Ratner, 2007). Metformin may also suppress inflammation independently of action on glucose, insulin and FFAs (Dandona et al., 2003). Metformin also improves the lipid profile and lowers blood pressure and plasminogen activator inhibitor-1 levels in both patients and animals with impaired glucose tolerance and type 2 diabetes (Kirpichnikov et al., 2002; Verma et al., 2002).

Caution must be exercised when using metformin in a number of other patient populations, including those with impaired hepatic function or requiring drug therapy for heart failure, and metformin should be temporarily discontinued prior to surgical procedures or intravascular radiocontrast studies. In addition, metformin has been associated with rare cases of fatal lactic acidosis and is contraindicated in patients with renal dysfunction. Because of its efficacy, infrequency of weight gain or hypoglycemia, and low cost, metformin has been recommended by the ADA-EASD as first-line pharmacotherapy for type 2 diabetes (Nathan et al., 2009). However, the durability of metformin’s effectiveness as monotherapy is limited consistent with the progressive loss of β-cell function seen in type 2 diabetes.
1.4.4. α-glucosidase inhibitors

The α-glucosidase inhibitors, acarbose, miglitol and voglibose, slow digestion of oligosaccharides, thereby providing an alternative to reduce postprandial glucose levels (Scheen, 1998). The α-glucosidase inhibitors do not cause weight gain, can reduce postprandial hyperinsulinemia, and have been shown to lower plasma triglyceride levels in some studies (Krentz and Bailey, 2005). They must be dosed multiple times per day and are associated with frequent gastrointestinal side effects (Nathan et al., 2009).

They generally have less potent glucose-lowering effects than other oral anti-diabetics (Nathan et al., 2009). In the STOP-NIDDM (Study to Prevent Non-Insulin Dependent Diabetes Mellitus) trial, acarbose reduced the incidence of new cases of type 2 diabetes in high-risk subjects with impaired glucose tolerance (Chiasson et al., 2002). Acarbose therapy is also associated with a reduction in incidence of cardiovascular events (van de Laar et al., 2005) although some controversy is present.

1.4.5. Insulin

Several different insulin analogs are available for type-1 and advanced type-2 diabetic patients. Insulin administration is the most effective means of restoring glycemic control; because there is no maximum dose, any HbA1C level can be reduced to the target range if insulin is dosed adequately (Nathan et al., 2009). However, insulin has a number of limitations. It is typically administered by subcutaneous injection, often requiring multiple injections per day. Insulin carries a tangible risk of hypoglycemia, and regular self-monitoring of blood glucose is usually required (Nathan et al., 2009). It is typically associated with weight gain that significantly increases in patients on intensive insulin therapy (Henry et al., 1993) with adverse cardiovascular consequences (Xiang et al., 2006). The ADA-EASD guidelines recommend addition of insulin as a second-step option for patients who are not adequately controlled on metformin monotherapy or as a third-step option for patients who still do not reach the HbA1C target goal on oral combination therapy (Nathan et al., 2009). Insulin is also the treatment of choice for patients with severely uncontrolled or symptomatic type 2 diabetes (Nathan et al., 2009).
1.4.6. Novel antidiabetic agents

The currently available therapies used for type 2 diabetes do not significantly improve β-cell function. In addition, the current approach does not address defects in hormonal secretion thought to play key roles in the pathophysiology of type 2 diabetes. New emerging therapies for type 2 diabetes have become available in some countries in recent years. As a result of their recent availability, long-term studies are lacking and full safety profiles of such compounds are largely unknown, even though they are being used in large numbers of patients. These drugs offer a range of different mechanisms of action that complement established therapies. Several of the novel drugs are based on the incretin hormone, GLP-1. GLP-1 controls glucose levels through various mechanisms including glucose-mediated insulin secretion, suppression of inappropriate glucagon release, slowing gastric emptying and increasing satiety (Ducker, 2005; Barnett, 2007). The natural hormone is rapidly degraded by the enzyme dipeptidyl peptidase 4 (DPP-4), and the two approaches to new agents are inhibitors of DPP-4 activity and development of GLP-1 analogues resistant to degradation. An important property of these agents is their neutral or beneficial effect on bodyweight (Krentz, 2008).

1.5. Glucose metabolism

Glucose is the most important carbohydrate; most dietary carbohydrate is absorbed into the bloodstream as glucose and other sugars are converted into glucose in the liver. Glucose is the major fuel for tissues such as the brain and neural tissue and the sole fuel for red blood cells. Glucose is under the regulation of a homeostatic control system which aims to keep the fasting plasma concentration within narrow limits. Type-1 diabetes are prone to fluctuations out of range: hyperglycemia and hypoglycemia following treatment.

The liver plays a central and crucial role in the regulation of carbohydrate metabolism. It performs most of the reactions involved in the synthesis and utilisation of glucose. Its normal functioning is essential for the maintenance of blood glucose levels and of a continued supply to organs that require a glucose energy source. The liver uses glucose as a fuel and also has the ability to store it as glycogen and synthesize it from non-carbohydrate precursors by gluconeogenesis.
1.6. Glucose homeostasis

Glucose homeostasis is controlled primarily by the anabolic hormone insulin and also by several insulin-like growth factors. Several catabolic hormones, glucagon, catecholamines, cortisol and growth hormone oppose the action of insulin; they are known as anti-insulin or counter-regulatory hormones (Ashcroft and Ashcroft, 1992; Atkinson and Maclaren, 1995; Baynes and Dominiczak, 2004).

Glucose absorbed from the intestinal tract is transported via the portal vein to the liver. The biochemical pathways of the liver are adapted to release glucose in response to reductions in the plasma glucose concentration and modulation by various hormones. Pathways include glycogenolysis and gluconeogenesis (Ashcroft and Ashcroft, 1992; Baynes and Dominiczak, 2004).

Insulin is secreted in response to the increase in plasma glucose following a meal. The glucose concentration in the vicinity of the β cell is sensed by the β cell glucose transporter GLUT-2. Glucose is carried into the cell by GLUT-2, where it is phosphorylated into glucose 6-phosphate by glucokinase which also is a part of the glucose-sensing mechanism. Increased availability of glucose 6-phosphate increases the rate of glucose utilization and ATP production in the β cell. This changes the flux of ions across the cell membrane, depolarizes the cell and increases the concentration of cytoplasmic free calcium. The final result is insulin exocytosis.

Insulin decreases the plasma glucose concentration by promoting the uptake of glucose into tissues, intracellular glucose metabolism and glycogen synthesis. Many cells in the body, including fat, liver and muscle cells have specific cell membrane insulin receptors and insulin facilitates the uptake and utilization of glucose by these cells. Role of insulin and glucagon in glucose homeostasis is shown in Fig. 1.2.

Insulin consists of two peptide chains linked by two disulfide bonds. The α chain contains 21 amino acids and the β chain 30 amino acids. The molecular weight of insulin monomer is 5500 Dalton. The precursor of insulin within the β cells of the islet of Langerhans is the single chain preproinsulin. During insulin synthesis a 24-amino-acid signal sequence is first cleaved from preproinsulin by a peptidase, yielding proinsulin.
Fig. 1.2: Role of insulin and glucagon in glucose homeostasis
Further, maturation results in the conversion of proinsulin into insulin and release a smaller peptide called C peptide. A small amount of proinsulin enters the circulation. It has a half-life 3-4 times longer than that of insulin because it is not metabolized by the liver. However, proinsulin has <10% of the biological activity of insulin. Insulin is metabolized by insulinase in the liver, kidney and placenta. About 50% of insulin secreted by the pancreas is removed by first-pass extraction in the liver. Insulin promotes glycogen synthesis (glycogenesis) in the liver and inhibits its breakdown (glycogenolysis). It promotes protein, cholesterol and triglyceride synthesis and stimulates formation of very-low-density lipoprotein. It also inhibits hepatic gluconeogenesis, stimulates glycolysis and inhibits ketogenesis.

Glucagon is synthesized by the α cells of the pancreatic islet of Langerhans. Its secretion is stimulated by low and inhibited by high concentrations of glucose and fatty acids in the plasma. Glucagon stimulates glycogen breakdown and gluconeogenesis and inhibits glycogen synthesis and glucose oxidation. Its metabolic actions on target tissues are thus the opposite of those of insulin. The fine balance between insulin and glucagon action is a key factor in the control of fuel metabolism. The glucose level acts as a signal that initiates the islet hormonal response (Rang et al., 2003; Baynes and Dominiczak, 2004).

1.7. Glycolytic enzymes

The enzymes in the glycolytic pathway are of importance for the directional control of metabolic flow, since their prevalence over the opposing gluconeogenic enzymes will favour the glycolytic pathway.

Hexokinase, one of the key glycolytic enzymes, has a low Km for glucose and is also active with fructose. It is inhibited in an allosteric manner by the end product glucose-6-phosphate (White and Wilson, 1987). Colowick (1973) observed four distinct isoenzymic pattern of hexokinase on starch gel electrophoresis. Type I hexokinase predominates in kidney tissue and type IV in liver (Niymeyer et al., 1967).

Phosphoglucoisomerase, is a dimeric glycolytic enzyme in the kidney tissue. It competes with glucose-6-phosphatase, phosphoglucomutase and glucose-6-phosphate
dehydrogenase for the available glucose-6-phosphate. The enzyme alterations might be expected to influence the proportion of glucose-6-phosphate metabolised via the glycolytic route (Baumann et al., 1988).

Aldolase is responsible for splitting fructose-1,6-bisphosphate into two trioses namely dihydroxy aceton ephosphate and glyceraldehyde 3-phosphate.

Glucokinase and type II hexokinase are both insulin-dependent and sensitive enzymes and are almost completely inhibited or inactivated in diabetic rat liver in the absence of insulin (Gupta et al., 1999). Decreased enzymatic activity of glucokinase, hexokinase and phosphofructokinase has also been reported in diabetic animals, resulting in depletion of liver and muscle glycogen (Grover et al., 2000).

1.8. Gluconeogenic enzymes

The enzymes usually referred to as ‘key gluconeogenic enzymes’ include,

i. Glucose-6-phosphatase which splits glucose-6-phosphate to free glucose and inorganic phosphorus and is opposed by glucokinase.

ii. Fructose-1,6-diphosphatase, which dephosphorylates fructose-1,6-bisphosphate to fructose-6-phosphate and is opposed by phosphofructokinase.

Glucose-6-phosphatase is a microsomal enzyme, a regulatory calcium binding protein and three transport proteins (T1, T2 and T3) which transport glucose-6-phosphate, phosphate and glucose respectively, across the endoplasmic reticulum membrane (Burchell, 1990). Glucose-6-phosphatase plays an important role in the regulation of blood glucose levels. Any deficiency, drug or disease conditions that lower the activity of glucose-6-phosphatase will impair the regulation of blood glucose levels (Nordlie, 1985).

Fructose 1,6-bisphosphatase, another key enzyme that regulates gluconeogenesis is present in elevated concentrations in the organs capable of active gluconeogenesis, such as the liver and kidney and is located in the soluble fraction of cells.

The activities of the two gluconeogenic enzymes, glucose-6-phosphatase and fructose-1,6-bisphosphatase are increased during diabetes in the liver had been reported earlier by Shibib et al. (1993).
1.9. Diabetes and dyslipidemia

In general, individuals with diabetes who have insulin resistance also have higher circulating levels of triglycerides and lower high-density lipoprotein (HDL)-cholesterol concentrations and commonly manifest the metabolic syndrome (Grundy et al., 2005). The metabolic syndrome is a predictor of future risk of cardiovascular disease (Wilson et al., 2005). Excess of fatty acids in serum produced by diabetes promotes conversion of excess fatty acids into phospholipids and cholesterol in liver. These two substances along with excess triglycerides formed at the same time in liver may be discharged into blood in the form of lipoproteins (Boppana et al., 1997). The abnormal high concentration of serum lipids in diabetes is mainly due to the increase in the mobilization of free fatty acids from the peripheral depots, since insulin inhibits the hormone sensitive lipase. Due to insulin deficiency there is increased lipolysis and ketogenesis. On the other hand, glucagon, catecholamines and other hormones enhance lipolysis. The marked hyperlipidemia that characterizes the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on the fat depots (Al-shamaony et al., 1994).

Specifically, it is widely recognized that higher levels of small dense low-density lipoprotein particles are a major contributor of increased risk for cardiovascular disease in insulin-resistant patients (Kathiresan et al., 2006). The association of different lipid fractions (especially the total cholesterol to HDL-cholesterol ratio) with incident heart failure is not the same as that for incident atherosclerotic cardiovascular events. Evidence supports that higher triglyceride levels (Pillutla et al., 2005) or higher non-HDL-cholesterol levels (Velagaleti et al., 2009) increase the risk of developing heart failure, perhaps because of the presence of insulin resistance that predisposes to metabolic changes characterized by impaired myocardial fatty acid oxidation and greater uncoupling of mitochondrial proteins in the heart (Murray et al., 2004). Thus, in an insulin-resistant patient, myocardial glucose uptake is decreased. Indeed, patients with nonischemic cardiomyopathy may still exhibit relatively preserved myocardial glucose uptake in the presence of insulin sensitivity (Da´vila-Roma´n et al., 2002). Another possible lipid fraction that could be associated in raising the risk for heart failure in those with diabetes is higher levels of lipoprotein (a). Underexpression of peroxisome proliferator-activated
receptor α (PPAR-α) increases glucose metabolism and prevents cardiac dilatation, whereas its overexpression leads to severe cardiomyopathy. The multiple mechanistic steps implicating PPAR-α in the development of heart failure and the use of fibrates that act as agonists to PPARα in heart failure have been reviewed recently (Sarma et al., 2010).

1.10. Normal glucose tolerance

In normal glucose tolerant (NGT) subjects, the plasma glucose concentration during the postabsorptive state (e.g., after an overnight fasting) is maintained below 100 mg/dl. The majority (~70–75%) of the total body glucose uptake (~2 mg/kg min) takes place in insulin-insensitive tissues, mainly the brain, erythrocytes, and splanchnic (gut plus liver) tissues, whereas the remaining 20–25% occurs in insulin-sensitive tissues, primarily muscle (DeFronzo et al., 1997). The rate of glucose uptake by all tissues in the body during the postabsorptive state is precisely matched by the rate of endogenous glucose production, primarily by the liver (DeFronzo et al., 1997) and to a smaller extent by the kidney (Gerich et al., 2001).

After glucose ingestion, the increase in plasma glucose concentration stimulates insulin secretion. The combination of hyperglycemia plus hyperinsulinemia combines to suppress hepatic glucose production and to stimulate glucose uptake by splanchnic and peripheral tissues to dispose of the ingested glucose and restore normoglycemia (Katz et al., 1983; DeFronzo and Ferrannini, 1987; Mari et al., 1994). The rise in plasma glucose concentration after glucose ingestion is influenced both by the ability of hyperinsulinemia and hyperglycemia to suppress endogenous glucose production and to augment the disposal of the ingested glucose by peripheral tissues and the liver. Thus, the maintenance of normal glucose tolerance is dependent on adequate secretion of insulin from the β cells in response to nutrient stimuli and on the normal action of insulin in liver and peripheral tissues.

1.11. Mechanism of action of streptozotocin

Streptozotocin is a naturally occurring chemical that is particularly toxic to the insulin-producing β cells of the pancreas in mammals. It is used in medicine for treating
certain cancers of the islets of Langerhans and used in medical research to produce an animal model for Type 1 diabetes.

Streptozotocin was originally identified in the late 1950’s as an antibiotic (Vavra et al., 1959). The drug was discovered in a strain of the soil microbe Streptomyces achromogenes. In the mid-1960s streptozotocin was found to be selectively toxic to the β cells of the pancreatic islets, the cells that normally regulate blood glucose levels by producing the hormone insulin. This suggested the drug's use as an animal model for diabetes (Mansford and Opie, 1968) and as a medical treatment for cancers of the β cells (Murray et al., 1968).

Streptozotocin is a glucosamine-nitrosourea compound. It is a 2-deoxy-D-glucose derivative of the carcinogen N-methyl-N-nitrosourea (Herr et al., 1967). As with other alkylating agents in the nitrosourea class, it is toxic to cells by causing damage to the DNA, though other mechanisms may also contribute. When streptozotocin was administered to rats by i.v. injection, DNA was methylated with the formation of 7-methylguanine, O⁶-methylguanine, 3-methyladenine, and 7-methyladenine in liver, kidney, intestine and pancreas.

Streptozotocin is considered cell cycle phase non-specific, although it particularly inhibits progression out of the G2 phase of cell division. The mechanism of action of streptozotocin actively appears to occur as a result of formation of methylcarbonium ions, which alkylate or bind with many intracellular molecular structures including nucleic acids. Its cytotoxic action is probably due to cross linking of DNA strands, resulting in inhibition of DNA synthesis. Streptozotocin has little effect on RNA or protein synthesis.

Streptozotocin is similar enough to glucose to be transported into the cell by the glucose transport protein GLUT2, but is not recognized by the other glucose transporters. This explains its relative toxicity to β cells, since these cells have relatively high levels of GLUT2 (Wang and Gleichmann, 1998 and Schnedl et al., 1994). Its diabetogenic action has been ascribed to the enhancement of intracellular methylation reactions (Wilson et al., 1984) and production of NO (Corbett et al., 1991; Kwon et al., 1994) and free radicals (Gandy et al., 1982). Mechanism of action of streptozotocin is shown in Fig. 1.3.
Fig. 1.3: Mechanism of action of streptozotocin

- STZ
  - Mitochondria
    - Aconitase
      - ATP
    - XOD
      - $O_2^-$
      - $H_2O_2$
      - 'OH'
      - ONOO
  - DNA Alkylation
    - DNA damage
      - poly (ADP-ribosylation)
        - NAD$^+$
        - ATP
          - Impaired insulin section and β cell death
STZ is thought to damage both nuclear and mitochondrial DNA as well as protein (Okamoto, 1982, Pettepher et al., 1991). In the process of DNA repair the nuclear enzyme poly (ADP-ribose) polymerase (PARP) depletes the cell of its substrate NAD. It is thought the depletion of the cellular NAD to non physiological levels is what ultimately results in β cell death.

It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. However, the synergistic action of both NO and reactive oxygen species may also contribute to DNA fragmentation and other deleterious changes caused by STZ. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate. Therefore intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.

1.12. Alloxan

Alloxan (2,4,5,6-tetraoxohexahydropyrimidine) is an unstable and reactive chemical substance that exists in several tautomeric forms. Alloxan is readily reduced to dialuric acid, which is the toxic form of the compound (Rerup, 1970). The drug is now widely used for the induction of type I diabetes in a variety of animals (Rerup, 1970; Dulin et al., 1983). It should be emphasized at this point that there is no evidence linking this drug to diabetes in humans. The alloxan-dialuric acid couple caused oxygen consumption and produced hydrogen peroxide when a reducing agent was present. The production of $\text{H}_2\text{O}_2$ was attributed to the quinine structure of alloxan (Dulin et al., 1983). The autooxidation of dialuric acid yielded, $\text{H}_2\text{O}_2$, $\text{O}_2$, $\text{O}_2^-$ and $\text{OH}$. Hydroxyl radical production detected by ethylene formation was inhibited by SOD, CAT, and the hydroxyl radical scavengers benzoate and ethanol, suggesting the Haber-Weiss reaction as the source of hydroxyl radicals (Dulin et al., 1983).

1.13. Free radicals

A free radical is a molecule with one or more unpaired electrons in its outermost orbital. The reactivity of free radicals results from their desire to attain an electron of opposing spin direction (Assmann et al., 2000). The ability of radicals and other reactive species to induce cellular damage has been demonstrated in a variety of experimental systems. Numerous studies have shown that free radicals have the potential to produce
most of the tissue changes associated with the expression of a variety of toxicities and disease processes (Kehrer, 2000). Therefore, free radicals have been implicated as contributors to a wide range of disorders (Valko et al., 2007). There are many types of radicals, but in biological systems most significant are those derived from oxygen. Oxygen has two unpaired electrons in separate orbitals in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation.

1.13.1. Superoxide anion radical (\( \cdot \text{O}_2^- \))

The acceptance of a single electron by O2 generates cellular \( \cdot \text{O}_2^- \). The mitochondrial respiratory chain is the major source of \( \cdot \text{O}_2^- \) (Nohl and Hegner, 1978). The superoxide anion radical is abundant and can reach an intracellular concentration of about \( 10^{-11} \) M. \( \cdot \text{O}_2^- \) is not highly reactive with biological molecules however once formed it quickly undergoes dismutation to generate hydrogen peroxide, which is highly reactive. This reaction is markedly accelerated by a family of enzymes, the superoxide dismutases (SODs). \( \cdot \text{O}_2^- \) can react with H+ to form \( \text{HO}_2^- \) (hydroperoxy radical) which is much more reactive than \( \cdot \text{O}_2^- \). Other enzymes can generate superoxide anions. A notable example is NADPH oxidase, primarily located in phagocytes, neutrophils and monocytes, this enzyme produces large amounts of \( \cdot \text{O}_2^- \) and other reactive oxidants that are used for fighting invading microorganisms.

1.13.2. Hydroxyl radical (\( \mathbf{\cdot \text{OH}} \))

The hydroxyl radical is an extremely reactive oxidant (Halliwell, 1999). It is also a short-lived molecule with an estimated half-life of nanoseconds at 37°C, during which it can travel only a few Ångstroms. Despite its short life span, \( \cdot \text{OH} \) is capable of inducing considerable damage to nuclear and mitochondrial DNA. This radical alone can cause over a 100 types DNA modifications. In addition, \( \cdot \text{OH} \) can lead to lipid peroxidation and oxidation of carbohydrates and proteins. The brain is particularly susceptible to lipid peroxidation since it is rich in lipids and contains high levels of iron. The hydroxyl radical is a major product of IR due to radiation-induced dissociation of water molecules.
1.13.3. Peroxyl radical (L·)

Lipid peroxidation is a process in which lipids undergo oxidation and peroxyl radicals are formed. The hydroxyl radical is not the only radical that can initiate lipid peroxidation: ¹O₂ and the peroxynitrite anion can also do so (Hogg and Kalyanaraman, 1999). Lipid peroxidation, and decomposition of fatty acids can lead to the formation of toxic products, such as lipid hydroperoxides (LOO·) and peroxyl radicals. LOO· in turn can attack adjacent polyunsaturated fatty acids (PUFA) and reinitiate the process. Thus, this complex, self-propagating process, can lead to oxidation of most or all of the cellular lipids, making it highly destructive.

1.13.4. Hydrogen peroxide (H₂O₂)

This is one of the most stable ROS and acts as a messenger in cellular signaling pathways. In addition to SOD, there are several other cellular systems that generate H₂O₂, including monoamine oxidase (MAO), diamine and polyamine oxidase, and glycolate oxidase. H₂O₂ is quite stable and under normal conditions is not toxic up to a cellular concentration of about 10⁻⁸ M (Imlay et al., 1988) However, H₂O₂ is highly diffusible through cell membranes and organelles and it is this capacity to travel long distances from its site of generation that makes it hazardous. In the presence of transition metals such as Fe²⁺ or Cu⁺, H₂O₂ it can be converted to the highly reactive hydroxyl radical, either by Fenton or Harber–Weiss reactions (Imlay et al., 1988; Halliwell, 1999). H₂O₂ is detoxified by a set of enzymes that includes the selenium-dependent glutathione peroxidase (GPx) and catalase.

1.13.5. Nitric oxide (NO) and generation of the peroxinitrite anion (ONOO⁻)

NO is synthesized from l-arginine by any of the three NO synthase isoforms. NO is quite stable and benign for a free radical, with a lifetime of several seconds. Under normal conditions NO has many physiological functions as a neuronal messenger and modulator of smooth muscle contraction. However, when its intracellular level is increased it can induce a cascade of events that can eventually lead to cell death. NO can interact with ·O₂⁻ to generate the peroxynitrite anion (ONOO⁻) (Radi et al., 1991b). This molecule accounts for much of the NO toxicity. The reactivity of ONOO⁻ is roughly the same as
that of ·OH and NO2·. Its toxicity is derived from its ability to directly nitrate and hydroxylate the aromatic rings of amino acid residues and to react with sulfahydryls (Radi et al., 1991b), lipids (Radi et al., 1991a), and DNA. Peroxynitrite anion can also effect cellular energy status by inactivating key mitochondrial enzymes and it may trigger calcium release from the mitochondria. The peroxynitrite anion and perhaps a few other reactive nitrogen species (RNS) with the exception of NO, can nitrate tyrosine residues, potentially leading to protein dysfunction. This strong ubiquitous activity can have devastating effects on cellular physiology and viability.

1.14. Damages produced by the free radicals

Free radicals such as ROS and RNS are attractive species to blame as mediators of toxicities. They have been extensively studied in recent years, but with few exceptions their roles in the etiologies of specific disorders remain largely undefined. At low or moderate concentrations, ROS and RNS are necessary for the maturation process of cellular structures and can act as weapons for the host defense system.

The reactive nature of these species makes all cellular macromolecules potential targets, and a vast array of changes has been identified that could provide mechanistic explanations for the observed injury, cause protein oxidation, DNA damage, and lipid peroxidation and thereby leads to different diseases and abnormalities in body. Free radicals induced DNA damage can be described both chemically and structurally and shows a characteristic pattern of modifications. The forms of DNA damage produced by free radicals include modification of all bases, production of base-free sites, deletions, frame shifts, strand breaks, DNA–protein cross-links, and chromosomal rearrangements. The endogenous reactions that are likely to contribute to ongoing DNA damage are oxidation, methylation, depurination, and deamination (Ames, 1989). Double strand break is the most serious type of DNA damage caused by free radicals because neither strand is able to provide physical integrity or information content.

Oxidization of proteins occurs through multiple reactions, including side chain alterations and backbone cleavage, and disrupts protein structure, causing denaturation, aggregation, and susceptibility to degradation (Dean et al., 1997). Oxidized proteins are functionally inactive and the consequent unfolding in some cases make them susceptible to
proteinases. Alterations of protein structure and function induced by ROS and RNS may contribute to carcinogenesis. ROS produced from various sources can directly damage DNA, activate transcription factors, kinases, or genes, inactivate the same factors, or modulate signal transduction pathways. ROS and RNS react with proteins to modify amino acid residues by oxidation, nitrosation, nitration, and halogenation. Tyrosine residues in protein react with various RNS to form 3-nitrotyrosine (NTYR) (Blanchard et al., 2001). Myeloperoxidase (MPO) and Eosinophil peroxidase (EPO) can also nitrate tyrosine to form NTYR using \( \text{H}_2\text{O}_2 \) and nitrite (NO\(^2^-\)) as substrates (Eiserich et al., 1998).

1.15. Free radicals and diabetic complications

Diabetes is associated with formation of the ROS by different pathogenic ways. Increased glucose flux into endothelial cells promoted by GLUT1 transporters in consequence of high plasma glucose causes acceleration of intracellular glucose metabolic pathways. Advanced generation of ROS in the respiratory chain of mitochondria has been repeatedly demonstrated. In addition, it may further accelerate glycation, sorbitol, protein kinase C or hexosamine pathways which are activated by high glucose itself (Giardino et al., 1996). Relationship between oxidative stress and complications of diabetes mellitus is shown in Fig. 1.4. It is apparent that different pathogenic mechanisms are closely linked to the oxidative stress which has been recognized as the main cause for development of the endothelial dysfunction.

Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Wolff and Dean, 1987; Jiang et al., 1990). Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Halliwell and Gutteridge, 1990; Hogg et al., 1993). Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Kawamura et al., 1994; Tsai et al., 1994). Another important source of free radicals in
Fig. 1.4: Relationship between oxidative stress and complications of diabetes mellitus
diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then advanced glycation end products (AGEs) (; Mullarkey et al., 1990; Hori et al., 1996). These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions (McCarthy et al., 2001), promote free radical formation and quench and block antiproliferative effects of nitric oxide (Wautier ET AL., 1994; Vlassara, 1997). By increasing intracellular oxidative stress, AGEs activate the transcription factor NF-κB, thus promoting up-regulation of various NF-κβ controlled target genes (Mohamed et al., 1999). NF-κβ enhances production of nitric oxide, which is believed to be a mediator of islet beta cell damage.

Considerable evidence also implicates activation of the sorbitol pathway by glucose as a component in the pathogenesis of diabetic complications, for example, in lens cataract formation or peripheral neuropathy (Greene et al., 1992; Obrosova et al., 1997). Efforts to understand cataract formation have provoked various hypotheses. In the aldose reductase osmotic hypothesis, accumulation of polyols initiates lenticular osmotic changes. In addition, oxidative stress is linked to decreased glutathione levels and depletion of NADPH levels (Cheng et al., 1986; Gonzalez et al., 1986). Alternatively, increased sorbitol dehydrogenase activity is associated with altered NAD+ levels (Williamson et al., 1993), which results in protein modification by nonenzymatic glycosylation of lens proteins (Yano et al., 1989; Ramalho et al., 1996).

Mechanisms linking the changes in diabetic neuropathy and induced sorbitol pathway are not well delineated. One possible mechanism, metabolic imbalances in the neural tissues, has been implicated in impaired neurotrophism (Mizisin et al., 1997; Delcroix et al., 1998), neurotransmission changes (Ralevic et al., 1995; Stevens et al., 2000) cell injury (Kalichman et al., 1998; Mizisin et al., 1998), and axonopathy (Chokroverty et al., 1988; Fernyhough et al., 1999).

1.16. Antioxidant defenses in body

Anti-oxidants are substances capable to mop up free radicals and prevent them from causing cell damage. Halliwell, a leading scientist in the field of antioxidant research, formulated the definition of an antioxidant as: “any substance that, when present at low
concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” (Halliwell et al., 1995). Antioxidants cause protective effect by neutralizing free radicals, which are toxic byproducts of natural cell metabolism.

Antioxidant defense mechanisms involve both enzymatic and nonenzymatic strategies. Common antioxidants include the vitamins A, C, and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Other antioxidants include α-lipoic acid, mixed carotenoids, coenzyme Q10, several bioflavonoids, antioxidant minerals (copper, zinc, manganese, and selenium), and the cofactors (folic acid, vitamins B1, B2, B6, B12). They work in synergy with each other and against different types of free radicals. Vitamin E suppresses the propagation of lipid peroxidation; vitamin C, with vitamin E, inhibits hydroperoxide formation; metal complexing agents, such as penicillamine, bind transition metals involved in some reactions in lipid peroxidation and inhibit Fenton and Haber-Weiss-type reactions; vitamins A and E scavenge free radicals (Halliwell and Gutteridge, 1990; Chow, 1991; Laight et al., 2000).

Discrepancies in observed biomarkers for oxidative stress was seen, especially in the activities of SOD, catalase, and glutathione peroxidase in experimentally diabetic animals. Decreased levels of glutathione and elevated concentrations of thiobarbituric acid reactants are consistently observed in diabetes. In addition, changes in nitric oxide and glycated proteins are also seen in diabetes.

1.17. Biomarkers of oxidative stress: in vivo diabetes studies

1.17.1. Lipid Peroxidation

Hydroperoxides have toxic effects on cells both directly and through degradation to highly toxic hydroxyl radicals. They may also react with transition metals like iron or copper to form stable aldehydes such as malondialdehydes that will damage cell membranes. Peroxyl radicals can remove hydrogen from lipids, producing hydroperoxides that further propagate the free-radical pathway (Halliwell and Gutteridge, 1990).

Induction of diabetes in rats with streptozotocin (STZ) or alloxan uniformly results in an increase in thiobarbituric acid reactive substances (TBARS), an indirect evidence of
intensified free-radical production. Preventing the formation of hydroxyl radicals would be an efficient means to reduce hydroxyl induced damage, and several compounds have been tested as antioxidants in diabetic animals with varying success. For example, the increase in TBARS associated with diabetes is prevented by treatment with nicotinamide (Melo et al., 2000), boldine (Jang et al., 2000), melatonin (Montilla et al., 1998), aspirin (Caballero et al., 2000), L-arginine or sodium nitroprusside (Mohan and Das, 1998), α-lipoic acid (Obrosova et al., 2000), aminoguanidine, captopril, enalapril, or nitecapone (Lal et al., 2000), if this treatment is given before or immediately after the diabetogen.

Even after diabetes is established, the buildup of TBARS may be reversed by treatment with combined vitamins C, E, and β-carotene (Mekinova et al., 1995), melatonin (Maritim et al., 1999), gemfibrozil (Ozansoy et al., 2001), probucol (Kaul et al., 1996), and vitamin E (Kim et al., 2000). Dietary supplementation with α-lipoic acid, evening primrose oil or sunflower oil lowers plasma lipids and hemostatic risk factors (Ford et al., 2001). These normalization effects are seen in kidney (Jang et al., 2000), liver (Melo et al., 2000), heart (Ozansoy et al., 2001), brain (Pierrefiche et al., 1993), intestine (Maritim et al., 1999), lung (Cinar et al., 2001), pancreas, plasma (Mohan and Das, 1998), red blood cells, lens (Obrosova and Stevens, 1999), and retina (Obrosova et al., 2000). In addition, increased lipid peroxidation in genetically diabetic C57BL/Ksdb+/db+ mice, as measured by conjugated dienes at wound sites, returns to normal levels after raxofelas treatment (Altavilla et al., 2001).

In contrast, both basal and iron-stimulated TBARS levels are significantly elevated in livers of rats treated with vanadyl sulfate compared to untreated STZ-induced diabetic rats, highlighting the importance of using multiple indicators of peroxidative change. Similarly, quercetin (Sanders et al., 2001) and the sorbitol dehydrogenase inhibitor SDI-157 (Obrosova et al., 1999) exacerbate the increased TBARS concentrations in livers (Sanders et al., 2001) and nerves (Obrosova et al., 1999) of untreated diabetic rats. On the other hand treatment with coenzyme Q10 (Rauscher et al., 2001), piperine (Rauscher et al., 2000a), isoeugenol (Rauscher et al., 2001a), or experimental antioxidants PNU-104067F or PNU-74389G (Rauscher et al., 2000), results in no change in lipid peroxidation in liver, kidney, heart, and brain of diabetic rats.
1.17.2. Superoxide dismutases (SOD)

O$_2^{•−}$ is one of the most frequently generated ROS in the cell. The first line of defense against O$_2^{•−}$ is SODs. Isoforms of SOD are variously located within the cell. CuZn-SOD is found in both the cytoplasm and the nucleus. Mn-SOD is confined to the mitochondria, but can be released into extracellular space. SOD converts superoxide anion radicals produced in the body to hydrogen peroxide, thereby reducing the likelihood of superoxide anion interacting with nitric oxide to form reactive peroxynitrite.

The effect of diabetes on the activity of SOD is erratic, with no discernable pattern based on gender or species of animal, or duration of diabetes, or tissue studied. Alterations of SOD activity in diabetic animals are normalized by probucol (Kaul et al., 1996), captopril, α-lipoic acid (Obrosova et al., 2000), melatonin, boldine (Jang et al., 2000), nitecapone (Lal et al., 2000), and stobadine (Stefek et al., 2000), all of which were administered prior to or concomitant with the diabetogen. When treatment is initiated in animals with well established diabetes, coenzyme Q10 (Rauscher et al., 2001) and piperine (Rauscher et al., 2000a) normalize renal activity, but no reversal of diabetic effects is seen with melatonin (Maritim et al., 1999), aminoguanidine or desferrioxamine, or gemfibrozil (Ozansoy et al., 2001). Treatment with vitamin C, vitamin E, and β-carotene for 8 weeks elevates hepatic SOD activity in diabetic rats, which is normal without treatment (Mekinova et al., 1995).

1.17.3. Catalase (CAT)

This antioxidant enzyme is a homotetrameric protein (mol. wt. 240 kDa) that catalyzes the conversion of hydrogen peroxide to water and oxygen.

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

CAT is present in most aerobic cells in animal tissues and is especially concentrated in the liver and erythrocytes. The brain, heart, and skeletal muscle contain only low amounts. Mammalian CAT is a heme protein, which reduces H$_2$O$_2$ to water by utilizing electrons from either H$_2$O$_2$ (catalase reaction) or from other small molecules such as methanol or ethanol (peroxidase reaction). CAT is found in peroxisomes and cytoplasm and is especially localized in the alveolar type II pneumocytes and macrophages.
Catalase activity is found to be elevated in heart (Rauscher et al., 2001), aorta (Ozansoy et al., 2001), brain (Aragno et al., 1999), kidney (Maritim et al., 1999), liver (Maritim et al., 1999) and red blood cell of diabetic rats. These alterations of catalase activity due to diabetes are normalized by treatment with captopril, aminoguanidine, melatonin (in liver) (Maritim et al., 1999), acetylsalicylic acid (Caballero et al., 2000), DHEA (Aragno et al., 1999), probucol (Kaul et al., 1996), α-lipoic acid (Kocak et al., 2000), and stobadine (Stefek et al., 2000), all of which were administered before or at the same time as the diabetogen. Effects of diabetes on cardiac catalase activity are exacerbated by treatment with quercetin (Sanders et al., 2001) or coenzyme Q10 (Rauscher et al., 2001).

1.17.4. Glutathione peroxidase and glutathione reductase

Glutathione peroxidase and reductase are two enzymes that are found in the cytoplasm, mitochondria, and nucleus. Glutathione peroxidase metabolizes hydrogen peroxide to water by using reduced glutathione as a hydrogen donor (Santini et al., 1997). Glutathione disulfide is recycled back to glutathione by glutathione reductase, using the cofactor NADPH generated by glucose 6-phosphate dehydrogenase. Glutathione peroxidase activity is seen to be elevated in liver (Sanders et al., 2001, kidney (Rauscher et al., 2001a), aorta (Kocak et al., 2000), pancreas (Jang et al., 2000), blood (Mohan and Das, 1998), and red blood cells, whereas decreased activity was seen in heart (Kaul et al., 1996) and retina (Obrosova et al., 2000) of diabetic animals.

Diabetes-induced alterations in glutathione peroxidase activity are reversed by treatment with probucol (Kaul et al., 1996), DHEA (Aragno et al., 1999), combined vitamins C, E, and β-carotene (Mekinova et al., 1995), quercetin (in liver and brain, though not in kidney or heart) (Sanders et al., 2001), coenzyme Q10 and isoeugenol (only in liver) (Rauscher et al., 2001), aminoguanidine, and α-lipoic acid (Kocak et al., 2000). Aminoguanidin treatment attenuates erythrocyte glutathione peroxidase activity, exceeding control values after both 6 and 12 weeks of induced diabetes. Activity of glutathione reductase, which regenerates cellular glutathione, is reduced in retina (Obrosova et al., 2000) and plasma but increased in heart (Sanders et al., 2001) of diabetic
animals. None of these effects is reversed by treatment with antioxidants, including α-lipoic acid, quercetin, piperine, isoeugenol, coenzyme Q10, Larginine, or nitroprusside.

1.17.5. Vitamins

Vitamins A, C, and E are diet-derived and detoxify free radicals directly. They also interact in recycling processes to generate reduced forms of the vitamins. α-Tocopherol is reconstituted when ascorbic acid recycles the tocopherol radical; dihydroascorbic acid, which is generated, is recycled by glutathione. These vitamins also foster toxicity by producing prooxidants under some conditions. Vitamin E, a component of the total peroxyl radical-trapping antioxidant system (Weber et al., 1997), reacts directly with peroxyl and superoxide radicals and singlet oxygen and protects membranes from lipid peroxidation. The deficiency of vitamin E is concurrent with increased peroxides and aldehydes in many tissues. There have been conflicting reports about vitamin E levels in diabetic animals and human subjects. Plasma and/or tissue levels of vitamin E are reported to be unaltered (Martinoli et al., 1993) increased (Asayama et al., 1994), or decreased (Cinar et al., 2001) by diabetes. Discrepancies among studies in terms of preventive or deleterious effects of vitamin E on diabetes induced vascular aberrations may arise from the variety of examined blood vessels or the administered dose of vitamin E.

1.18. Enzymes as biochemical markers in toxicity

Liver is the largest organ in the body performing multiple metabolic functions to maintain and promote health. Several enzymes present in the liver cell are responsible for the metabolic activities of the liver. Some of the enzymes by detoxification process offer protection against hepatotoxic agents.

The serum levels of enzymes - aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin are the most sensitive tests employed in the diagnosis of hepatic diseases. Elevated levels of serum enzymes like acid phosphatase are indicative of cellular damage and loss of functional integrity of the liver cell membrane. The following enzymes are employed to estimate the functions of the liver.
1.18.1. Serum transaminase

It catalyses the transfer of amino group from an amino acids to keto acids.

Aspartate aminotransferase (Serum glutamate oxaloacetate transaminase)

\[
L\text{-Aspartate} + \alpha\text{-Oxoglutarate} \xrightarrow{\text{ALP}} \text{Oxaloacetate} + L\text{-Glutamate}
\]

Alanine aminotransferase (Serum glutamate pyruvate transaminase)

\[
L\text{-Alanine} + \alpha\text{-Oxoglutarate} \xrightarrow{\text{ALP}} \text{Pyruvate} + L\text{-Glutamate}
\]

Increases in both transaminases are found in hepatic diseases. Highest values of enzyme activity are seen in acute viral hepatitis. ALT is more abundant in liver cells than in any other organs in the body and is primarily used as a specific marker for hepatic damage (Chattopadhyay et al., 1992).

1.18.2. Alkaline phosphatase (ALP)

Alkaline phosphatases play an important role in carbohydrate metabolism and oxidative phosphorylation. Phosphatases are enzymes which catalyses the splitting of phosphoric acid from certain monophosphoric esters.

\[
R\text{-PO}_4 + \text{HOH} \xrightarrow{\text{ALP}} R\text{-OH} + \text{HPO}_4^{2-}
\]

It is elevated in both infective hepatitis and obstructive jaundice. It is involved in lipid transport in intestine. ALP is present in all tissues especially in cell membrane and serves as membrane marker. It is an important marker in hepatobiliary disease (Chatterjee and Shinde, 1994).

1.19. TCA cycle

This cycle is of central importance in all living cells that use oxygen as part of cellular respiration. In eukaryotic cells, the citric acid cycle occurs in the matrix of the mitochondrion (Fig. 1.5). The major pathways of fuel oxidation generate acetyl CoA, which is the substrate for the TCA cycle. In the first step of the TCA cycle, the acetyl portion of acetyl CoA combines with the 4-carbon intermediate oxaloacetate to form
Fig. 1.5: TCA cycle
citrate (6 carbons), which is rearranged to form isocitrate. In the next two oxidative
decarboxylation reactions, electrons are transferred to NAD to form NADH, and 2
molecules of electron-depleted CO$_2$ are released. Subsequently, a high-energy phosphate
bond in GTP is generated from substrate level phosphorylation. In the remaining portion of
the TCA cycle, succinate is oxidized to oxaloacetate with the generation of one FAD (2H)
and one NADH. The net reaction of the TCA cycle, which is the sum of the equations for
individual steps, shows that the two carbons of the acetyl group have been oxidized to two
molecules of CO$_2$, with conservation of energy as three molecules of NADH, one of FAD
(2H), and one of GTP.

1.20. Dehydrogenases of TCA cycle and their importance
1.20.1. Isocitrate dehydrogenase (ICDH)

Isocitrate dehydrogenase (ICDH) is an enzyme that participates in the citric acid
cycle. It catalyzes the third step of the cycle: the oxidative decarboxylation of isocitrate,
producing alpha-ketoglutarate (α-ketoglutarate) and CO$_2$ while converting NAD$^+$ to
NADH. This is a two-step process, which involves oxidation of isocitrate (a secondary
alcohol) to oxalosuccinate (a ketone), followed by the decarboxylation of the carboxyl
group beta to the ketone, forming alpha-ketoglutarate. Mammalian tissues contain two
isoforms of this enzyme. One exists entirely in mitochondria, and the other exists in
mitochondria and also in cytosol. Both the forms catalyze the same reaction (Corpas et al.,
1999). ICDH provides the first connection between the TCA cycle and the electron
transport pathway and OXPHOS, via its production of NADH.

Isocitrate + NADP+ Mg$^{2+}$ (metal ion) → alpha-ketoglutarate + NADPH + H$^+$ + CO$_2$

1.20.2. Alpha-Ketoglutarate dehydrogenase (α-KGDH)

α-ketoglutarate dehydrogenase complex is an enzyme catalyzes the oxidative
decarboxylation of an alpha-keto acid, releasing the citric acid cycle's second CO$_2$ and
NADH.

α-ketoglutarate + NAD$^+$ + CoA → Succinyl CoA + CO$_2$ + NADH
There are a number of features that make this enzyme distinct from other enzymes important in the bioenergetic processes. First of all, it is highly regulated and is the primary site of control of the metabolic flux through the Krebs cycle (Hansford, 1980). The mammalian enzyme is inhibited by its end products, succinyl-CoA and NADH (Smith et al., 1974). α-KGDH could be a crucial target of ROS in cells and, being an important regulatory site in the mitochondrial metabolism, could play a key role in the bioenergetic deficit evolving in oxidative stress. On the other hand, it has been revealed recently that the enzyme itself is able to generate ROS (Starkov et al., 2004; Tretter and Adam 2004), therefore could contribute to the induction of oxidative stress. α-KGDH appears to be more sensitive to disturbed homeostatic factors than other enzymes (Mastrogiacomo et al., 1993).

1.20.3. Succinate dehydrogenase (SDH)

SDH catalyzes stereospecific dehydrogenation of succinate to fumarate. The enzyme is strongly inhibited by malonate, a structural analog of succinate and an example of competitive inhibitor. Succinate dehydrogenase (SDH) is bound to the inner mitochondrial membrane. It catalyses the direct transfer of H2 from the substrate to a flavoprotein without the participation of NAD+. Succinate enzyme contains FAD+ and iron sulphur (Fe:S) protein. Its activity is promoted by succinate, phosphate and ATP.

\[
\text{Succinate} + \text{FAD}^+ \rightarrow \text{Fumarate} + \text{FADH}_2
\]

1.20.4. Malate dehydrogenase (MDH)

MDH catalyzes the conversion of malate into oxaloacetate (using NAD+) and vice versa (this is a reversible reaction).

\[
\text{L-Malate} + \text{NAD}^+ \leftrightarrow \text{oxaloacetate} + \text{NADH} + \text{H}^+
\]

Several isozymes of MDH exist, depending on where they are localized in the cell and their specific dependence on NAD+ or NADP+ (only in chloroplasts). There are two main isoforms in eukaryotic cells (Minarik et al., 2002). One is found in the mitochondrial
matrix participating as a key enzyme in the citric acid cycle that catalyzes the oxidation of malate. The other is found in the cytoplasm, assisting the malate-aspartate shuttle with exchanging reducing equivalents so that malate can pass through the mitochondrial membrane to be transformed into oxaloacetate for further cellular processes (Musrati et al., 1998). The activity of SDH and MDH in tissues was decreased in diabetic animals (Lemieux et al., 1984).

1.21. The electron transport chain and oxidative phosphorylation

The Electron Transport Chain (ETC) and the Oxidative phosphorylation (OXPHOS) system is made up of five complexes (complex I-V) and consists of approximately 90 subunits, of which only 13 are encoded by the mtDNA. Complexes I-IV comprises the ETC. Oxidation of carbohydrates in the TCA cycle and lipids via β-oxidation generate the electron carriers NADH and FADH\(_2\), which donate electrons to the ETC. In the ETC, the transport of electrons is coupled to the generation of a proton gradient across the inner mitochondrial membrane, which is further used by the fifth enzyme complex to synthesize ATP from ADP+P\(^-\) (Saraste, 1999).

1.21.1. Complex I (NADH: ubiquinone oxidoreductase)

This is the largest of the enzyme complexes of the OXPHOS system consisting of 45 subunits in bovine heart, of which seven are encoded in mitochondria. The enzyme contains multiple prosthetic groups, one flavinmononucleotide (FMN) and eight iron-sulphur clusters. Another common name for this enzyme complex is NADH dehydrogenase. NADH in the inner membrane space is oxidised to NAD\(^+\) transferring two electrons to the FMN moiety of complex I. The electrons are then further transferred via a series of iron-sulphur clusters to the matrix side of the inner membrane to reduce ubiquinone to ubiquinol. This transfer is coupled to the translocation of four protons across the inner membrane into the intermembrane space.

1.21.2. Complex II (Succinate ubiquinone oxidoreductase)

This is the only exclusively nuclear encoded complex, oxidizes succinate to fumarate in the TCA cycle, and donates electrons to the ETC. Complex II consists of a
catalytic subunit, SDH, and two membrane subunits, anchoring the complex into the inner mitochondrial membrane (Capaldi et al., 1977). Electrons from succinate are donated to the covalently bounded FAD of SDH, reducing it to FADH$_2$. The electrons are then further transported via a number of iron/sulphur clusters to ubiquinone, reducing it to ubiquinol. Complex II feeds electrons to the ETC without translocating protons across the membrane (Lancaster and Kroger, 2000). This enzyme has a mass of approximately 100 to 140 kD and is composed of four subunits: two Fe-S proteins of masses 70 kD and 27 kD, and two other peptides of masses 15 kD and 13 kD. Also known as flavoprotein 2 (FP2), it contains an FAD covalently bound to a histidine residue, and three Fe-S centers: a 4Fe-4S cluster, a 3Fe-4S cluster, and a 2Fe-2S cluster. When succinate is converted to fumarate in the TCA cycle, concomitant reduction of bound FAD to FADH$_2$ occurs in succinate dehydrogenase. This FADH$_2$ transfers its electrons immediately to Fe-S centers, which pass them on to ubiquinone (UQ). Electron flow from succinate to UQ.

1.21.3. Complex III (Ubiquinol-cytochrome c oxidoreductase)

Complex III catalyzes the transfer of electrons from ubiquinol, to cytochrome c. It consists of a homodimer, with each monomer composed of eleven subunits, of which one is encoded by the mitochondrial genome (Yu et al., 1998). Ubiquinol is a two-electron carrier, whereas cytochrome c is a single electron carrier. Ubiquinol donates its two electrons consecutively to complex III, releasing two protons at the inner membrane space. One electron is transferred to cytochrome c via the Rieske iron-sulphur protein, while the second electron is transferred back to the matrix side, to cytochrome b of complex III. Cytochrome b is able to accept two electrons, which in turn, are donated to ubiquinone at the matrix side, generating ubiquinol. The reduction of ubiquinone to ubiquinol via cytochrome b also requires the removal of two protons from the matrix side, thus adding to the proton gradient of the respiratory chain. Due to the recycling of ubiquinone this process is also termed the Q-cycle (Darrouzet et al., 2001).

1.21.4. Complex IV (Cytochrome c oxidase; Ferrocytochrome c- oxidoreductase)

Complex IV is a water-soluble protein that donates electrons on the cytoplasmic side of the inner mitochondrial membrane to complex IV, the final step in the respiratory chain. Complex IV is composed of 13 subunits of which three are encoded by the mtDNA.
It catalyzes the transfer of electrons from the reduced cytochrome c pool to molecular oxygen, reducing it to water. In this step, four electrons have to be donated from complex IV to two molecules of oxygen, without generating any ROS. This is achieved by complex IV storing the four electrons on haem and copper atoms, before releasing them only in the presence of two molecules of oxygen and four protons at the matrix side of the mitochondrial inner membrane. Additionally, four protons are translocated across the inner mitochondrial membrane during this reaction (Schultz and Chan, 2001).

1.21.5. Complex V (ATP synthase; F1F0-ATPase)

The overall outcome of the action of the ETC is the removal of protons from the matrix side, transferring them to the inner membrane space side; thus rendering the matrix side negatively charged, while storing protons on the cytoplasmic side of the inner mitochondrial membrane. This electrochemical gradient is finally utilised by the fifth component of the OXPHOS system, the Complex V, which drives the generation of ATP from ADP and P· (Saraste, 1999). Complex V is composed of a membrane-bound subcomplex (F0), a large extra-membranous complex (F1) that resides in the matrix space, and a stalk connecting the two complexes. Protons from the intermembrane space are allowed to enter complex V through the F0 complex leading to subunit rotation within the enzyme complex. The energy from this rotation is then used for ATP synthesis, which takes place in the F1 complex (Schultz and Chan, 2001).

1.22. ROS production via the mitochondrial respiratory chain a causal link between high glucose and the main pathways responsible for hyperglycemic damage

Mitochondria are the principal source of ROS in cells as the result from imperfectly coupled electron transport. Oxidative stress is widely accepted as playing a key mediatory role in the development and progression of diabetes and its complications due to increased production of free radicals and impaired antioxidant defenses (Ceriello, 2003).

Pyruvate derived from glycolysis is transported into the mitochondria, where it is oxidized by the tricarboxylic acid (TCA) cycle to generate NADH. Electrons deriving from oxidation of substrates are funneled through the redox carriers of the respiratory chain (complexes I, III, and IV) to the final electron acceptor, molecular oxygen. Through four-electron reduction, oxygen is converted to water. However, during normal metabolism,
reactive incompletely reduced forms of oxygen, such as superoxide (Fig. 1.6), are produced. Normally, only 0.1% of total oxygen consumption leaks from the respiratory chain to generate ROS.

The primary factor governing mitochondrial ROS generation is the redox state of the respiratory chain (Lambert and Brand, 2004a). Electron transfer through the mitochondrial respiratory chain generates a proton (voltage) gradient. Under normal conditions, much of the energy of this voltage gradient is used to generate ATP as the collapse of the proton gradient through ATP synthase drives the ATP synthetic machinery. The amplitude of the electrochemical proton gradient, which is known as respiratory control, regulates the overall rate of electron transport in the respiratory chain. When the electrochemical potential difference generated by the proton gradient is high (such as in high glucose states), the life of superoxide-generating electron transport intermediates, such as ubisemiquinone, is prolonged. This occurs because the activity of the respiratory chain complexes as proton pumps is inherently governed by the transmembrane proton gradient ($\Delta p$H) and the membrane potential ($\Delta \Psi$mt). When sufficiently high, $\Delta p$H and $\Delta \Psi$mt inhibit the proton pumps. It is evident that each of the ROS-generating sites has a different redox potential, and thus each will respond differently to changes in $\Delta p$H and $\Delta \Psi$mt, resulting in a complex regulation of ROS generation by these membrane gradients (Lambert and Brand, 2004b). There appears to be a threshold value above which even a small increase in $\Delta \Psi$mt gives rise to a large stimulation of superoxide production by mitochondria.

How is ROS production via the mitochondrial respiratory chain a causal link between high glucose and the main pathways responsible for hyperglycemic damage? The prevailing hypothesis is that hyperglycemia-induced increase in electron transfer donors (NADH and FADH$_2$) increases electron flux through the mitochondrial electron transport chain. Consequently, there is an increase of the ATP/ADP ratio and hyperpolarization of the mitochondrial membrane potential. This high electrochemical potential difference generated by the proton gradient leads to partial inhibition of the electron transport in complex III, resulting in an accumulation of electrons to coenzyme Q. In turn, this drives partial reduction of O$_2$ to generate the free radical anion superoxide. It is this accelerated reduction of coenzyme Q and generation of ROS that is believed to be the fundamental
Fig. 1.6: Electron transport chain ROS generation by mitochondria and dissipation of the proton gradient by UCPs. Scavenging by antioxidant defenses is insufficient to prevent oxidative stress in hyperglycemia. CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, ATP synthase; TCA, tricarboxylic acid cycle; UCP, uncoupling protein.
source for mitochondrial dysfunction that plays a critical role in diabetes-related metabolic disorders and tissue histopathology.

1.23. Cardiovascular diseases

Cardiovascular diseases are among the most common causes of death in the Western world and transition countries. The aetiological risk markers that have been shown to be specially modified by the diet are related to lipid and lipoprotein metabolism, haemostatic function, oxidative damage, homocysteine metabolism and blood pressure changes (Mensink et al., 2003). LDL and HDL cholesterol, triacylglycerol, homocysteine and blood pressure are well-validated and generally accepted biomarkers, being the rest of them just recommended. However, currently only LDL and blood pressure are considered diet-related biomarkers (Mensink et al., 2003).

The management of dyslipidemia has advanced over the past 50 years. In the 1970s, the low-density lipoprotein–cholesterol (LDL–C) receptor was identified, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase was characterized, and an HMG-CoA reductase inhibitor was developed. Since the 1990s, LDL–C has been recommended as the primary treatment target because the substantial benefits of statins have been demonstrated in many clinical trials of patients with atherosclerotic cardiovascular disease (Cheung and lam, 2010). Statins clearly have beneficial effects on atherosclerosis mediated by decreased LDL–C and improving endothelial function, in part, through anti-inflammatory actions (Koh, 2000). In addition to high LDL–C, including low concentrations of high-density lipoprotein– cholesterol (HDL–C) and high concentrations of triglycerides (TG). Even with the aggressive reduction of LDL–C, the risk of cardiovascular events in patients with CHD remains substantial (Chapman, 2005). This may result, in part, from their low levels of HDL–C and elevated levels of TG (Ballantyne et al., 2001). Many studies have also found that non-HDL–C (total cholesterol—HDL–C) perform better than LDL–C in cardiovascular risk prediction (Ramjee et al., 2011).

Epidemiological evidence has established a strong link between low levels of HDL–C and cardiovascular events (Gordon et al., 1977). An increase in HDL–C of 1 mg/dL is associated with a 3% reduction in CHD outcomes (Gordon et al., 1977).
Therefore, increasing HDL–C may potentially be a promising treatment strategy for preventing cardiovascular disease, particularly in subjects with low HDL–C levels.

1.24. Dietary fatty acids and cholesterolemia

Correlations between fatty acid intake and cholesterolemia were described in the second half of the twentieth century, when the Seven Countries Study by Keys and colleagues demonstrated how total plasma cholesterol levels increases together with the proportion of fat in the diet. Several subsequent studies that examined the effects of individual fatty acids on both total plasma cholesterol levels and the different lipoprotein subclasses, namely LDL and HDL, improved our understanding of this correlation (Clarke et al., 1997). Several epidemiological and interventional studies examined the effects of dietary fatty acids on lipoprotein metabolism and cholesterol transport. Overall, the available data suggest that, when isocalorically substituted for nutrients with neutral effects on cholesterolemia (such as carbohydrates), saturated and trans fatty acids tend to increase total and plasma LDL cholesterol levels. Conversely, polyunsaturated, cis fatty acids (namely, those of the n-6 series such as linoleic acid) induce the opposite effects. Monounsaturates, such as oleic acid, also reduce total and plasma LDL cholesterol levels, although to a lesser extent than do n-6 polyunsaturates. The effects of fatty acids on plasma HDL cholesterol levels are (Lichtenstein, 2006):

a) an increase after saturates intake;

b) an increase (to a lesser degree) after monounsaturate consumption;

c) no change after polyunsaturates;

d) a decrease after trans ingestion.

In terms of fat content, it is important to base food choices on up-to-date composition tables, since changes in the fatty acid composition of several food items (e.g., meat) as a consequence of new diets for animals and new food-processing techniques are sometimes relevant. Most international guidelines suggest that to improve cholesterol levels saturated fat intake should be lower than 10% of the total caloric intake. Reasonably, the optimal intake of saturated fatty acids could be 7 - 10% of total calories.
1.25. LDL–cholesterol and the risk of cardiovascular events

The total cholesterol level has been shown to correlate with CHD risk over a large range of values. The reason for this is that total cholesterol strongly reflects LDL cholesterol levels, which constitute approximately 65% of total cholesterol. The plasma LDL cholesterol is currently the primary target for lipid modification in primary prevention. At present, the armamentarium for lowering LDL cholesterol mainly includes dietary intervention and the use of drugs. The dietary intervention involves limitation of fat calories, saturated fats, and cholesterol and limited increase in polyunsaturated fats and, perhaps, in monounsaturated fats. Drug intervention primarily includes hydroxymethylglutarylcoenzyme A (HMG-CoA) reductase inhibitors, with resins and niacin as supplementary agents.

Considerable study has been focused on the relationship of LDL and other lipoproteins to the endothelium, thrombogenesis, and vascular dysfunction and on the effects of intervention to lower LDL cholesterol levels or possible changes in the constitution of the LDL molecule (Selwyn et al., 1997). It is known that native LDL has little effect on cells within the arterial wall. On the other hand, oxidized or acetylated LDL binds avidly with macrophages and other sites, and produces toxic effects that affect the endothelium, platelet adhesion, platelet-dependent thrombin generation, monocyte recruitment, free radical production, and other proliferative processes that facilitate atherogenesis (Deckert et al., 1997). Small, dense LDL particle appear particularly susceptible to oxidation and appear to induce endothelial vasomotor dysfunction (Anderson et al., 1996).

In other studies, the association between LDL and platelet metabolism has been studied. In vitro studies have shown that the contact of LDL with washed platelets results in a relative acidification of the platelets, although this effect is maximum at relatively low LDL concentrations (25-50 mg/dL). Acidification appears to increase platelet responsiveness to activating agents and thus increase thrombogenesis and adverse effects on the endothelium. The evidence that relatively low concentrations of LDL are needed to produce a maximum effect suggests that there may be inter individual variations in short-term and long-term effects of LDL on platelet pH, which may play a role in susceptibility to atherogenesis (Tracy and Tracy, 1997).
The interrelations of LDL, HDL, and very low density lipoprotein (VLDL) particles are important in the determination of the relative importance of LDL and VLDL in the increasing risk of atherosclerosis and in the determination of the importance of HDL in the decreasing risk of atherosclerosis. VLDL is produced in the liver and primarily contains triglyceride. As VLDL passes through the circulation, it is converted by biochemical interactions with HDL particles to an intermediate density lipoprotein. Some of these VLDL remnant particles are taken up by the liver, and some of the intermediate density lipoprotein particles, through an increase in cholesterol, a decrease in triglycerides, and a removal of apolipoproteins other than apolipoprotein B (apoB), are converted into LDL. The major apolipoprotein class of VLDL and LDL, apoB, is associated with increased risk for CHD. The major apolipoprotein of HDL, apoA1, is associated with decreased risk for CHD.

1.26. Triglycerides and atherogenesis

Elevated TG levels are thought to increase CHD risk through the atherogenic effects of TG-rich remnant lipoproteins, which are partially degraded, TG-rich lipoprotein remnants of hepatic and intestinal origin that have lost TG through the action of lipoprotein lipase and have picked up cholesterol ester through the action of cholesterol ester transfer protein (Brewer et al., 1999). Specifically, chylomicrons are produced in the gut from dietary fat and are not thought to be atherogenic until their TG core is removed by lipoprotein lipase. It is the resulting chylomicron remnants that are atherogenic, perhaps because they are sufficiently small to infiltrate arterial walls. Similarly, very low-density lipoprotein (VLDL) particles are produced in the liver from hepatic TG and become VLDL remnants after cleaving of the TG core. Assays for remnant cholesterol have been developed and levels of these lipoproteins are a better predictor of CHD risk than TG levels. In individuals with diabetes, the elevations in remnant lipoprotein cholesterol levels are much greater than those in TG levels, which may partially account for the high CHD risk associated with diabetes (Schaefer et al., 2002).

Elevated TG levels, together with low levels of HDL-C and an increased prevalence of small LDL particles, constitute a lipid triad termed atherogenic dyslipidemia that is associated with premature CHD (Austin et al., 1990). An elevated TG level (>150 mg/dL) is also one of the determinants of risk for the metabolic syndrome, a cluster of
metabolic abnormalities related to insulin resistance and elevated free fatty acid levels that is associated with an increased risk for development of type 2 diabetes (Lorenzo et al., 2003) and premature CHD.

1.27. Antiatherogenic and antidiabetic mechanisms of HDL–cholesterol

HDL plays an important role in modulating atherogenesis by mediating reverse cholesterol transport (Toth, 2008). This process is promoted by apolipoprotein A-I and involves the transport and uptake of free cholesterol from the peripheral tissues, such as the arterial wall, and its subsequent delivery to the liver for its reuse or excretion into the bile. Other beneficial properties of HDL–C are anti-inflammatory activity (Charvet et al., 2010) and antioxidative effects. HDL suppresses macrophage toll-like receptor 4 -mediated inflammatory responses, which are dependent on cholesterol efflux via ATP-binding cassette protein A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) (Charvet et al., 2010). HDL–C also has vasodilatory properties and ameliorates endothelial dysfunction (Yuhanna et al., 2001).

Insulin resistance is the core component of metabolic syndrome and is associated with low HDL–C. Both contribute to the development of overt diabetes and cardiovascular diseases (Navab et al., 2000; Cha and Kim, 2003). Low HDL–C is a frequent finding in overweight and obesity as well as insulin-resistant states. Several studies in mice with either knockout or overexpression of apoA-I have provided evidence that HDL may exert insulin-sensitizing effect by increasing adiponectin levels (Van et al., 2010). Intravenous reconstituted HDL reduced plasma glucose in T2D patients by increasing plasma insulin and activating AMP-activated protein kinase in skeletal muscle (Drew et al., 2009). Treatment with a cholesteryl ester transfer protein (CETP) inhibitor, torcetrapib, a potent HDL–Cincreasing drug, reduced the plasma glucose and insulin levels and improved insulin resistance in atorvastatin-treated T2D patients (Barter et al., 2011). These findings indicate that interventions that increase HDL–C levels are beneficial in glucose homeostasis, particularly in the insulin resistant milieu (Fryirs et al., 2010). HDL has been reported to inhibit pancreatic β-cell apoptosis by preventing activation of c-Jun N-terminal kinases pathway (Abderrahmani et al., 2007) and promote β-cell survival by inhibiting caspase-3 cleavage and activating Akt/protein kinase B (Rutti et al., 2009). Taken together, HDL has antidiabetic properties by both increasing insulin sensitivity and improving
pancreatic β-cell function. Antioxidative and anti-inflammatory mechanisms of HDLs also contribute to antidiabetic effect of HDL.

1.28. 3β-hydroxy3-methylglutharyl coenzyme A reductase (HMGCR)

3β-hydroxy-3-methylglutharyl coenzyme A reductase (HMGCR) is the key enzyme of the pathway, catalyzing a NADPH-dependent reduction of HMG-CoA to mevalonate, the first committed step in cholesterol biosynthesis. This step is the most regulated, involving at least three different regulatory mechanisms. The enzyme is inactivated by: i) phosphorylation via a cAMP-dependent protein kinase, which can be reversed by phosphatase, ii) shortening the half-life by higher cholesterol levels (normal half-life~3 h) and iii) at the transcriptional level via transcription factors of the SREBP (sterol regulatory element-binding protein) family. SREBP-2 regulates transcription of all cholesterogenic genes.

The primary target of statins is HMGCR, which catalyzes the rate limiting step in the biosynthesis of sterols, cholesterol and isoprenoids. The enzyme is a transmembrane glycoprotein anchored to the endoplasmic reticulum. The functional enzyme is a tetramer with the dimeric active sites at the interface of the two monomers (Istvan et al., 2000). The HMG moiety of the substrate, which is bound to a single HMGCR monomer, comes into proximity of the nicotinamide ring of a NADPH molecule, whose binding pocket is located in the neighbouring monomer. The substrate-binding pocket is characterized by a loop of residues 682–694. The cis-peptide (Cys688–Thr689) within this region that bends over the top of HMG is essential in the formation of the HMG-binding site, and is critical in positioning the residues participating directly in HMGCR mediated reduction (Istvan et al., 2000). The substrate-binding pocket also accommodates statin molecules upon competitive inhibition of the enzyme (Istvan, 2002).

1.29. Statins

Statins (eg, atorvastatin, simvastatin) inhibit hydroxymethylglutaryl coenzyme A-reductase, the rate limiting enzyme in cholesterol biosynthesis. Inhibition of cholesterol synthesis results in reduced hepatic cholesterol content, which may increase the expression of LDL-C receptors (Bilheimer et al., 1983). The upregulation of LDL-C receptors lowers concentrations of TG-rich lipoproteins because intermediate-density lipoprotein and VLDL
remnants are also removed from the circulation via the LDL receptor (Vega and Grundy, 1990). Statins have also been reported to lower the production of VLDL apo B-100, probably as a result of decreased availability of hepatic cholesterol, thereby further lowering TG levels (Watts et al., 2003).

1.30. Importance of fungi

Human relationships with mushrooms are ancient and fascinating. The Egyptians believed that they were a gift from the god Osiris, while the ancient Romans called them a "divine food" because they thought that mushrooms resulted from the lightning thrown to earth by Jupiter during storms. Higher fungi (mushrooms) have been used by mankind for millennia. Basidiomycetous fungi (mushrooms) can be defined as "macrofungi" with distinctive fruiting bodies that are large enough to be seen by the naked eye and to be picked by hand. Many mushrooms have long been valued as tasty, nutritious food by different societies worldwide. To the ancient Romans they were "the foods of the Gods" resulting from bolts of lightning thrown to the earth by Jupiter during thunder storms; the Egyptians considered them as "a gift from the God Osiris"; while the Chinese viewed them as "the elixir of life". It is estimated that there are approximately 1.5 million species of fungi in the world of which approximately 70,000 species are described (Hawksworth, 2001). About 14,000 of the known species belong to the macro fungi, of which about 5,000 species are edible and over 1,800 species are considered to have medicinal properties (Chang, 1995). More varieties of mushrooms have been isolated and identified, and the number of mushrooms being cultivated for food or medicinal purposes has been increasing rapidly (Chang, 1995).

1.31. Nutritional composition of mushrooms

In general, mushrooms are quite high in protein, with an important content of essential amino acid, but low in fat (Mattilda et al., 2001). Furthermore, these fungi supply a large amount of carbohydrates and fiber and a nutritionally significative content of vitamins (B1, B2, B12, C and D) and mineral elements (Ca, K, Mg, Na, P, Cu, Fe, Mn and Se) (Mattilda et al., 2001).

Crude protein is found in high levels in edible mushrooms and range can vary between 15.2 g/100 g dried weight (DW) in L. edodes to 80.93 g/100 g dried weight in A.
bisporus (Manzi et al., 1999). The levels of essential amino acids in mushrooms have been reported to vary widely among species (Manzi et al., 1999). According to FAO/WHO, they are considered rich in glutamic acid, aspartic acid and arginine, however, their proteins are deficient in methionine and cysteine. The limiting amino acids result to be leucine and lysine in L. edodes and P. ostreatus and P. eryngii (Manzi et al., 1999). Interestingly, two uncommon aminoacids:γ-amino butyric acid (GABA) and ornithine have been detected, which have shown important physiological activities (Manzi et al., 1999). Therefore, the dietary importance of mushrooms is expected to grow in the coming years due to the protein demand of the increasing world population and the interest of reducing the risks related to the consumption of animal foods sources.

Edible mushrooms provide low amounts of fat. In general, unsaturated fatty acids are predominant over saturated fatty acids. The range of reported concentrations of carbohydrates varies from 35 to 70% DW with some heterogeneity among species. Mushrooms are good source of vitamins based on the high levels of riboflavin (vitamin B2), niacin and folates and traces of vitamin C, vitamin B1, vitaminD, β-carotene (precursor of vitamin A), vitamin E and vitamin B12 (Mattilda et al., 2001). Mushrooms appear as the only non-animal-based food source containing vitamin D, and hence they are the only natural vitamin D source for vegetarians. Hence, vitamin D2 content is considerable in a number of wild mushroom species, but unfortunately, it almost absent in cultivated species (Mattilda et al., 2001). As compared with vegetables, mushrooms proved to provide a reasonable content of many mineral elements (6–10.5% DW) (Manzi et al., 1999). The main constituents in the ash are potassium and, depending on the mushroom, phosphorus or magnesium (Manzi et al., 1999), in addition to calcium, copper, iron and zinc.

A great variability can also be observed among mushrooms in the dietary fiber supply. In general, a remarkably high or appreciable level of total dietary fiber ranging from 81.7 to 96.3% sample dry matter was obtained from the Boletus group, Agrocybe aegerita, A. bisporus, Pleurotus eryngii and ostreatus, inwhich β-glucans are the major fiber polysaccharides together with chitin (Manzi et al., 2001). The β-glucans represent from 4 to 13% of the total dietary fiber with a variability of the dietary fiber fractions depending on mushrooms species. In this context, polysaccharide β-glucans from
mushrooms are considered as functional compounds because they appear to stimulate the immunomodulatory response, modulate humoral and cellular immunity, and thereby having a beneficial effect in fighting against infections besides those substances also exhibit hypocholesterolemic. β-glucans are therefore promising candidates as anticoagulant agents. Recently, they have been demonstrated to have anti-cytotoxic, antimutagenic and antitumorogenic properties, being promising candidates as pharmacological agents (Mantovani et al., 2008).

In addition to their nutritional components, some edible mushrooms are rich in bioactive compounds. Different bioactive compounds of edible mushrooms are responsible for their antioxidant (Lo and Cheung 2005), antitumor/anticancer (Wasser and Weis, 1999a), antimicrobial (Rao et al., 2009), immunomodulatory, antiatherogenic (Yamada et al., 2002) and hypoglycemic reported properties.

1.32. Anti diabetetic effect of mushrooms

A large number of animal studies, using both normal and diabetic animals, have demonstrated a hypoglycemic effect of mushrooms and mushroom components. This effect appears to be mediated via mushroom polysaccharides (possibly both alpha- and beta-glucans) via a direct interaction with insulin receptors on target tissues, although this mechanism remains to be confirmed.

A randomized, double-blinded, and placebo-controlled clinical trial showed that *A. blazei* Murill supplementation in combination with metformin and gliclazide improved insulin resistance in these subjects. An increase in adiponectin concentration after *A. blazei* Murill extract consumption for 12 weeks may be the mechanism that resulted in the reported effect. Clinical investigation in diabetic patients has also shown that Oyster mushroom consumption significantly reduced systolic and diastolic blood pressure, lowered plasma glucose, total cholesterol and triglycerides significantly, with no significant change in body weight, and no deleterious effects on liver or kidney function (Khatun et al., 2007). These results in humans mirror the decreases in plasma glucose, cholesterol and triglyceride concentrations following *A. bisporus* consumption observed in rats and the reduction in blood pressure in Zucker fatty rats following oral administration of Maitake mushroom fractions.
Aqueous extracts of various mushrooms have been shown to possess hypoglycemic activity against diabetes-inducing compounds in obese and diabetic animal models. An aqueous extract of *G. lucidum* (0.03 and 0.3 g/kg) lowered the serum glucose level in obese/diabetic (+db/+db) mice after one week of treatment through the suppression of hepatic PEPCK gene expression (Seto et al., 2009). Aqueous extracts of *Pleurotus pulmonarius* also have been shown to possess hypoglycemic activity. Beta-glucans and their enzymatically hydrolyzed oligosaccharides from *A. blazei* have anti-hyperglycemic, anti-hypertriglyceridemic, anti-hypercholesterolemic, and anti-arteriosclerotic activity indicating overall anti-diabetic activity in diabetic rats.

Extracellular polysaccharides (EPS) from *Laetiporus sulphureus* var. *miniatus* have been shown to both stimulate insulin secretion and insulin sensitivity possibly via regulation of lipid metabolism in diabetic mouse models. A polysaccharide isolated from *P. linteus* reportedly inhibited the development of autoimmune diabetes by regulating cytokine expression in non-obese diabetic mice (Kim et al., 2010). The hypoglycemic potential of EPS was also confirmed by histopathological examination that showed that EPS administration is able to restore impaired kidneys to almost normal architecture as well as pancreatic islets of Langerhans (Yamac et al., 2008) in streptozotocin induced rats.

### 1.33. Lipid and lipoprotein metabolic effects of mushroom

Traditionally, edible mushrooms have been prescribed in Oriental medicine due to their hypocholesterolemic effects (Sun et al., 2007). In general, the intake of edible mushroom reduces the cardiovascular risk (Yamada et al., 2002), due to the occurrence of specific substance and other bioactive compounds (Table 1.1). The mechanisms related to cholesterol metabolism involved in the hypocholesterolemic effect of edible mushrooms are depicted in Fig. 1.7. The fatty acid pattern of edible mushrooms seems to contribute to reduce serum cholesterol (Barros et al., 2007). When the fatty acid profile of some edible mushrooms was analyzed, considerable amounts of polyunsaturated fatty acid were found. The presence of trans isomers of unsaturated fatty acids is associated with the strongest effects on raising the serum total cholesterol to high-density lipoprotein ratio, increasing cardiovascular diseases risk (Mauger et al., 2003). The trans isomers of unsaturated fatty acids have not been detected in mushrooms (Barros et al., 2007).
Fig. 1.7: Effect of edible mushrooms on the cholesterol metabolism.

- HMG-CoA reductase
- Bile acid synthesis
- Enterohepatic circulation
- Fecal Sterol Excretion
- Potential hypcholesterolemic mechanism of edible mushroom
Table 1.1: Edible mushrooms with reported hypocholesterolemic properties.

<table>
<thead>
<tr>
<th>Edible mushroom</th>
<th>Hypocholesterolemic properties</th>
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</thead>
<tbody>
<tr>
<td><em>Agaricus bisporus</em></td>
<td>↓ LDL cholesterol ↑ Serum total cholesterol</td>
</tr>
<tr>
<td></td>
<td>↓ HDL cholesterol</td>
</tr>
<tr>
<td></td>
<td>↑ Hepatic LDL receptor mRNA</td>
</tr>
<tr>
<td></td>
<td>↓ Serum adipocytokine/↑ Fat depositon/↑ Triglycerides in liver</td>
</tr>
<tr>
<td><em>Auricularia auricula</em></td>
<td>↓ LDL cholesterol and serum total cholesterol</td>
</tr>
<tr>
<td><em>Lentinus edodes</em></td>
<td>↓ Cholesterol levels</td>
</tr>
<tr>
<td></td>
<td>↓ Phospholipids of plasma</td>
</tr>
<tr>
<td></td>
<td>Modification of hepatic phospholipids metabolism</td>
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<tr>
<td></td>
<td>Hyperhomocysteinemic effect</td>
</tr>
<tr>
<td><em>Pleurotus citrinopilettus</em></td>
<td>↓ Total lipids ↑ Total cholesterol</td>
</tr>
<tr>
<td></td>
<td>↓ Triglycerides in liver and plasma</td>
</tr>
<tr>
<td></td>
<td>↑ Bile acid excretion</td>
</tr>
<tr>
<td></td>
<td>Inhibition of HMG-CoA reductase</td>
</tr>
<tr>
<td><em>Pleurotus florida</em></td>
<td>↓ Total lipids ↑ Total cholesterol</td>
</tr>
<tr>
<td></td>
<td>↓ Triglycerides in liver and plasma</td>
</tr>
<tr>
<td></td>
<td>↑ Bile acid excretion</td>
</tr>
<tr>
<td></td>
<td>Inhibition of HMG-CoA reductase</td>
</tr>
<tr>
<td><em>Pleurotus ostreatus</em></td>
<td>↓ VLDL cholesterol ↑ LDL cholesterol</td>
</tr>
<tr>
<td></td>
<td>↓ Serum total cholesterol</td>
</tr>
<tr>
<td></td>
<td>↓ Plasma triglycerides</td>
</tr>
<tr>
<td></td>
<td>↓ Blood pressure</td>
</tr>
<tr>
<td></td>
<td>↓ Antioxidative glutation peroxidase activity</td>
</tr>
<tr>
<td></td>
<td>Inhibition of HMG-CoA reductase</td>
</tr>
<tr>
<td><em>Tremella fuciformis</em></td>
<td>↓ LDL cholesterol ↑ Serum total cholesterol</td>
</tr>
<tr>
<td></td>
<td>↓ Plasma triglycerides</td>
</tr>
<tr>
<td></td>
<td>↓ Hepatic total cholesterol</td>
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The dietary fiber intake may affect plasma lipid concentrations and lower the cardiovascular diseases risks (Grundy and Denke, 1990). The soluble dietary fiber has shown healthy effects on serum lipid levels, reducing total cholesterol and LDL-cholesterol amounts (Erkkila and Lichtenstein, 2006). The formation of viscous gels from soluble dietary fiber such as glucans might contribute to inhibit the cholesterol and triglycerol absorption (Ebihara and Schneeman, 1989). Their viscous properties are related to an increase on the fecal excretion of bile acids and short-chain fatty acids (propionate), which inhibits acetate incorporation (substrate for sterols and fatty acid synthesis) to serum lipids (Wolever et al., 1995). The results of some reports suggest that the hypocholesterolemic effects of some fruiting bodies of edible mushroom could be mainly attributed to the dietary fiber supply. Two mushrooms with a high dietary fiber content such as *Auricularia auricula* and *Tremella fuciformis* have proved to produce similar lowering in LDL cholesterol levels, and therefore in the serum total cholesterol. Neither of them affect serum HDL cholesterol, however the *A. bisporus* fiber can elevate the hepatic LDL receptor messenger RNA in male rats, reducing the HDL cholesterol concentration and serum total cholesterol by lowering VDL+ILD+LDL cholesterol concentrations (Fukushima et al., 2000). In addition to the reduction in serum cholesterol, *T. fuciformis* also lowered plasma triacylglycerol levels and hepatic total cholesterol. This finding might be the result of an inhibition of the synthesis of hepatic triglycerides by increasing the short chain fatty acids production (acetate, propionate and butyrate) during the dietary fiber fermentation by colonic microflora (Suzuki et al., 1983).

Mushrooms are also of interest because they contain large amounts of β-glucans polysaccharides, which exhibit hypocholesterolemic and anticoagulant functions (Zekovic et al., 2005). Other interesting fungal polysaccharide with similar characteristics to dietary fiber is chitosan (D-glucosamine polymer) or chitin (N-acetyl-D-glucosamine polymer). The high fat diet-induced obese mice supplied with a chitosan supplement (5%) from *A. bisporus* during ten weeks, showed lower lipid absorption and serum adipocytokine levels (Neyrinck et al., 2009). Consequently, those actions could contribute to reduce fat deposition in the liver (a decrease of triglyceride content by 39%) and muscle (a decrease of triglyceride content by 66%) besides to a lowering in fat mass. Nowadays, fungal
chitosan is being commercialized as a dietary supplement for obesity and cholesterol management.

Investigations in the last decade have revealed that fruiting bodies of *P. ostreatus* exhibit a hypocholesterolemic effect on rats with normocholesterolemia and hypercholesterolemia induced by intake of a high fat diet (Bobek et al., 1991) or alcohol intake and diabetes or hereditary cholesterol disorder. A pronounced cholesterol-lowering effect of oyster mushroom consumption (*P. ostreatus*) has been demonstrated on rats through a decrease in very-lowdensity lipoproteins (Bobek et al., 1998) and also by suppressing the activity of HMG-CoA reductase (Bobek et al., 1995) and an enhanced fractional catabolic rate of cholesterol (Bobek et al., 1994). Recently, it has been evidenced by Kantun et al. (Khatun et al., 2007) that *P. ostreatus* reduced total cholesterol, triglycerides plasma, glucose and blood pressure in diabetic subjects. The results of this trial suggest that eating mushrooms (*P. ostreatus*) provides health benefits by acting on the atherogenic lipid profile under hypercholesterolemic and normcholesterolemic conditions (Khatun et al., 2007).

Microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMG-CoA reductase) is the major rate-limiting enzyme in cholesterol biosynthesis. Inhibition of HMG-CoA reductase decreases intracellular cholesterol biosynthesis (Steinberg et al., 1989). Mevinolin is a known pharmacological HMG-CoA reductase inhibitor (Endo and Monacolin, 1979). A high quantity of this inhibitor has been found in *P. ostreatus* fruiting bodies, especially in the *pileus*. Furthermore, it has been found in sporocarps of *P. cornucopiae* and *P. eryngii* (Gunde-Cimerman and Cimerman, 1995). The biological activity of mevinolin produced by mycelia from three different species of fungus *Pleurotus* (*P. sapidus, P. saca and P. ostreatus*) had been previously assessed.

1.34. Anti-atherosclerosis effect of mushrooms

Atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries, constitutes the single most important contributor to the growing burden of cardiovascular diseases.

In this context, several mechanisms involved in the anti-atherosclerotic effect of some edible mushrooms have been reported (Fig. 1.8). Research in the last two decades
Fig. 1.8: Antiatherosclerotic effects and potential involved mechanisms of different edible mushrooms.

ANTIATHEROSCLEROTIC EFFECT OF EDIBLE MUSHROOMS

IMPROVEMENT OF VASCULAR REACTIVITY

ANTI-INFLAMMATORY EFFECTS
- Grifola frondosa
- Hypsizigus marmoreus
- Pleurotus eryngii
- Pleurotus florida
- Pleurotus ostreatus

ANTI-HYPERTENSIVE EFFECTS
- Ganoderma lucidum
- Grifola frondosa
- Lentinus edodes
- Pleurotus narbonensis

ANTI-OXIDATIVE EFFECTS
- Agrocybe aegerita
- Boletus edulis
- Flammulina velutipes
- Lactarius deterrimus
- Lentinus edodes
- Pleurotus citrinopileatus
- Pleurotus ostreatus
- Serocomus chrysebteron
- Suillus collinus
- Volvariella volvacea

ANTI-PLATELET AGGREGATING EFFECTS

INHIBITION OF LDL OXIDATION

INHIBITION OF ADENOSIN 5’ DIPHOSPHATE
- Lentinus edodes
- Pleurotus florida
- Pleurotus ostreatus
has revealed that inflammatory and oxidative processes are common features in several cardiovascular conditions, such as atherosclerosis. *Phellinus rimosus* showed higher anti-inflammatory and antioxidant properties.

**1.35. *Phellinus***

*Phellinus* is a large and widely distributed genus of the family Hymenochaetaceae (Donk). *Phellinus* species are mostly tropical mushrooms and 18 species are known from Kerala. Some of the species of *Phellinus* are extensively studied in China, Japan and Korea especially *Phellinus linteus*, which has been considered to be an important traditional Chinese medicine (Ying et al., 1987). *Phellinus* has been used to treat abdominal pain, stomach problems, lymphatic tumor and menses disorders. There are 220 known species of *Phellinus* mushroom in the world and they were found mainly in tropical areas of America, Africa and Asia (Kim et al., 2003a). Different species of *Phellinus* (*e.g.* *P. linteus*, *P. baumi*, *p. igniarius*, *P. tremulae*, *P. robustus*, *P. gilvus*, *P. weirii*, *P. pini*) are known to have different medicinal effects (Jung et al, 1992; Cho et al, 2002; Park et al, 2003; Lahiri et al; 2010). It is usually used in traditional oriental medicine and has been reported to have many pharmaceutical attributes, including anti-mutagenicity and anticancer and anti-diabetic and antioxidant properties (Kim et al, 1996; Han et al, 1999; Ji et al, 2000; Kim et al., 2010; Song et al, 2002). Several mushrooms belonging to the genera *Inonotus* and *Phellinus*, such as *Inonotus obliquua*, *Phellinus linteus*, *Phellinus ribis* and *Phellinus igniarius* often contain a bundle of yellow antioxidant pigments which are composed of hispidin derivatives and polyphenols.

![Hispidin](image)

**Hispidin (6-(3,4-dihydroxystyr)-4-hydroxy-2-pyrone)**
Phellinus linteus, which has been considered to be an important traditional Chinese medicine (Ying et al., 1987). Polysaccharides of Phellinus linteus and Phellinus baumi were reported to possess antidiabetic activity (Hwang et al., 2005; Kim et al., 2010). P. linteus demonstrated anticancer effect by activating cytotoxic cells and macrophages thus increasing potential immune response potential. The methanolic extract of basidiocarps of Phellinus linteus demonstrated antioxidative effect (Chung et al, 1998) and antimutagenic activities (Sohn and Nam, 2001). Phellinus ribis and Phellinus igniarius have been used for the treatment of gastrointestinal cancer, cardiovascular disease, tuberculosis, liver or heart disease, fester, bellyache, bloody gonorrhea, stomach ailments and diabetes (Nakamura et al, 2004), as a traditional medicine. Hispolon and hispolon derivatives were isolated from the fungus Phellinus igniarius (Mo et al., 2004). Hispolon, a yellow pigment was first found in Inonotus hispidus in 1996 (Ali et al., 1996b). Hispolon has been reported to exhibit apoptosis effect on human epidermoid KB cells (Chen et al., 2006b) and antivirus activities (Awadh et al., 2003). Hispolon also inhibit chemiluminescence response of human mononuclear cells and suppress mitogen induced proliferation of spleen lymphocytes of mice (Ali et al., 1996a).

![Hispolon structure](image)

**Hispolon** 6-(3,4-dihydroxyphenyl)-4-hydroxy-hexa-3,5-dien-2-one)

1.35.1. Phelliuns rimosus

Phellinus rimosus is a parasitic host specific poly pore mushroom often found growing on jack fruit tree trunks in Kerala (Fig. 1.9). It is a less extensively studied species of the genus Phellinus. Basidiocarps of this mushroom have been reported to be used by some tribes in Kerala for curing mumps (Ganesh, 1988). Earlier investigations showed that
Fig 1.9: *Phellinus rimosus* growing on tree trunk
ethyl acetate and methanol extract of *P. rimosus* possessed antioxidant, antitumor and hepato protective activities (Ajith and Janardhanan 2001, 2002 and 2003). Further chemoprotective as well as antimutagenic activities of *P. rimosus* has also reported (Ajith and Janardhanan 2006, 2011). Anti-inflammatory, anti-arthritic as well as radio protective activities of a polysaccharide protein complex (PPC-Pr) isolated from *P. rimosus* is also reported (Meera et al., 2009 a, b). Recent investigation demonstrated the radio protective activity of polysaccharide protein complex isolated from *P. rimosus* (Jini et al., 2011).

The significant antioxidant and anti-inflammatory activities of methanolic extract of *P. rimosus* was first reported by Ajith and Janardhanan, (2001). The ethyl acetate extract of *P. rimosus* exhibited significant hepatoprotective effect against CCl$_4$ induced acute hepato toxicity in rats. The extract also possessed significant free radical scavenging activity in a concentration dependent manner that could exert a beneficial effect against hepatotoxicity in experimental animals (Ajith and Janardhanan, 2002a). The ethyl acetate extract of *P. rimosus* was also found to be effective in amelioration of cisplatin induced nephrotoxicity in mice (Ajith and Janardhanan, 2002b). Ajith and Janardhanan (2003) have also reported the cytotoxic and anti-tumor activities of ethyl acetate, methanol and aqueous extracts of *P. rimosus*. This study showed that ethyl acetate and methanol extracts possessed in vitro cytotoxic activity against Dalton’s lymphoma ascites (DLA) and Ehrlich’s ascites carcinoma (EAC) cell lines. All three extracts were highly effective in inhibiting growth of solid tumor induced by DLA cell line in mice. The ethyl acetate extract was also effective in preventing the EAC induced ascites tumor development in mice.