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STUDIES ON THE EFFECT OF ARTIFICIALLY CULTIVATED GANODERMA LUCIDUM ON STREPTOZOTOCIN INDUCED DIABETIC RATS

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3. REVIEW OF LITERATURE

3.1 GANODERMA LUCIDUM AND ARTIFICIAL CULTIVATION

The virtues of *Ganoderma lucidum* extracts, handed down from generation to generation, as a symbol of happy augury, good fortune, good health, longevity, and even immortality. It began with the Yuan Dynasty (1280–1368 A.D.). According to two famous Chinese plant medical books, *Shen Nong Ben Cao Jing* (25–220 A.D., Eastern Han Dynasty) and *Ben Cao Gang Mil* by Li Shi-Zhen (1590 A.D., Ming Dynasty), six Ling Zhi species= varieties were known in China at that time. Worldwide, more than 250 *Ganoderma* species have been described (Moncalvo and Ryvarden, 1997; Wasser and Weis, 1997). However, in therapeutic practices and literature citations, *Ganoderma* usually refers to the species of *Ganoderma lucidum*. Besides being treasured for its medicinal value in China for more than 1000 yr, the lack of availability of *Ganoderma lucidum* was also largely responsible for it being so highly cherished and expensive. During ancient times in China, any person who picked the mushroom from the natural environment and presented it to a high-ranking official was usually well rewarded. Even in the early 1950s, it was presented to Chinese leaders in Mainland China and Taiwan, following the occasional discovery in the wild. In the past, *Ganoderma lucidum* grew in small quantities only in the wild; therefore, it was very expensive.

The *Ganoderma lucidum* is extremely rare and difficult to find in the wild. Because the husks of the spore are very hard, the spores can’t germinate as readily as the spores of other mushrooms. To germinate, the right combination of oxygen and moisture conditions is needed. Fortunately, we are now able to recreate favorable growth conditions. It can be cultured on logs that are buried in shady, moist areas. *Ganoderma lucidum* can also be inoculated onto hardwood stumps. Under commercial cultivation conditions, *Ganoderma lucidum* is normally grown on artificial sawdust logs. The mushroom that was once the provenance of the emperors of China can now be purchased in health food stores (George, 2007). Recent increase in human consumption of *G. lucidum* is due to the fact that the mushroom is low in calories and rich in vegetable proteins, chitin, vitamins, and minerals (Kang et al., 2002; Wasser and Weis, 1999a).
Ganoderma lucidum (Fr.) Karst. is a basidiomycetes belonging to the genus Ganoderma of Polyporaceae, and it has been highly prized from ancient times as an ornamental mushroom and as a medicinal fungus. However, it only grows naturally and very rarely on aged trees in steep mountains. Recently, a method for artificially cultivating Ganoderma lucidum (Fr.) Karst. has been developed while utilizing the methods of artificially cultivating edible fungi. The artificial cultivation of the fungus is, however, extremely difficult due to unfavorable rooting since the physiological conditions of the fungus itself are different from those of the general edible fungi, for example, Lentinus edodes (Berk.) Singer, Pleurotus ostreatus (Fr.) Quel.

3.1.1 Growth requirements

3.1.1.1 Growth medium

Mycelial growth of Ganoderma lucidum was carried out on potato Dextrose Agar medium by tissue culture method. The fungus mycelium grew well at 25-27°C (Tiwari et al., 2004). It has been noted that under uniform environmental conditions, there is a high degree of radial symmetry in fungal colonies, with all portions of the mycelia front extending at the same rate (Edelstein and Segal, 1983) with no acceleration of growth over time (Brancato and Golding, 1953). Hence, it can be assured that the growth rate of fungi is constant (Trinci, 1971).

The culture medium is extremely important as it provides the nutrients for growth of the mycelium. Sone et al., (1985) who examined the growth of Ganoderma lucidum in a suite of different carbohydrates, including galactose, glucose, lactose, mannose, sucrose and xylose found that lactose produced the highest dry weight of mycelium after seven days, and glucose and glucitol produced the least. In addition, they observed that the mass of mycelium produced in the lactose medium was approximately 1.5 times greater than that in the glucose medium. The presence of nitrogen in the culture medium is another important factor and it has been observed that no mycelia growth occurs in its absence (Lin et al., 1973).

3.1.1.2 Temperature

Temperature is one of the most important environmental factors in mycelia growth requiring careful control (Brancato and Golding, 1953). Ganoderma lucidum has an optimal temperature of 28-30°C (Tiwari et al., 2004). Both polysaccharide
production and mycelia growth rate are favoured at temperature between 30 and 35°C, being drastically reduced outside this range (Yang et al., 1998a; Yang et al., 1998b).

3.1.1.3 pH

Fang and Zhong obtained similar pH profiles after the fourth day even though the initial value varied from 3.5 to 7.0 during the first four days the pH decreased to 3.2 and then remained constant for one week. After that, around days 10 to 14, when the glucose was almost exhausted, the pH increased rapidly to 7.0. When glucose was fed on day 8 in a quantity sufficient to increase its concentrations by 5 to 10g/l, the pH remained the same until day 14. The authors suggested that the relatively high glucose consumption may result in production of organic acids, which keep the pH low, although they did not do any analyses to support this (Fang et al., 2002).

3.1.1.4 Solid state fermentation

Solid state fermentation (SSF) is defined as the fermentation involving solids in absence (or near absence) of free flowing water; however, substrate must possess enough moisture to support growth and metabolism of microorganism (Pandey, 2003). In recent years, some bioprocesses using SSF have been developed for the production of bulk chemicals and value-added products such as ethanol, single-cell protein (SCP), mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, etc. (Soccol and Vandenberghe, 2003).

In Japan, *Ganoderma lucidum* are grown using the following methods: 1) Wood log cultivation, the most popular method. 2) Pot or bottle cultivation. 3) Tank cultivation. Over the past 30 years, a considerable number of studies have been made on the best method to harvest high-quality *Ganoderma lucidum*. These observations have shown that the best method to harvest high-quality *Ganoderma lucidum* is the wood log cultivation. Other particular conditions are necessary to get high-quality *Ganoderma lucidum* which may be consider under the following heads: (1) Selection of the highest strain (2) Using the right method of cultivation (3) To harvest them at the right time(4) To treat them properly after the harvest. (Yukio Naoi, Artificial
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cultivation of REISHI: To harvest high-quality *Ganoderma lucidum*, http://www.ganoderma.com

The above method for cultivating *Ganoderma lucidum* (Fr.) Karst. by planting a seed fungus (spore) into a material wood as in the case of *Lentinus edodes* (Berk.) Singer (Japanese Patent Application Laying-Open No. 88628/80) is disadvantageous from the viewpoint of productivity, since it takes much labor in inoculation and control of culture and long times for cultivating, maturing and growing of fungi. In addition, the conventional methods for cultivating *Ganoderma lucidum* (Fr.) Karst. have given only fungi having short stipe and much curvature and ramification, and the resulting fungi are, therefore, entirely different from the naturally growing fungi having long stipe (Japanese Patent Publication No. 38092/80 and Japanese Patent Laying-Open No. 105445/75).

Seed culture can be done by sawdust of a broad-leaved tree and rice bran are blended at a volume ratio of 4:1, and a suitable amount of water is added to the mixture to adjust the moisture content of the mixture to 60 to 70% by weight. After pushing the mixture into sterilized glass bottles and plugging the opening of the bottle with cotton wool with 3 holes for inoculation, the bottles are subjected to sterilization for the preparation of the solid medium in the bottle. Then, the aseptically proliferated mycelia on the agar culture medium is inoculated to the solid medium together with the agar piece. By cultivating the inoculated culture medium for about 20 days at about 25° C, the seed culture comprising mycelia of *Ganoderma lucidum* (Fr.) Karst. proliferated throughout the solid medium is obtained.

The seed culture (e.g. prepared above) is inoculated into the artificial solid medium and cultivated at a relative humidity of 40 to 80%, preferably 50 to 70% and at a temperature of 15° to 35° C., preferably 20° to 25° C. Usually, within about 20 to 30 days, the mycelia spreads throughout the culture medium, and a fungal tissue bed having mature rhizomorph on its upper surface is obtained. As the base material of the artificial solid medium of the invention, sawdust, rice bran, chaff, soya bean cake, bran, and the like may be used singly or as their mixture. In addition, a mixture of at least one of the base material and a covering material to be explained later may be used.

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The artificial solid medium is prepared by pressing a mixture of at least one of the base material and water together in a vessel such as glass bottle and plastic bottle and sterilizing the pressed material. The mixing ratio of the base material and water is usually 1:1.6 to 1:2 by weight. In the cases of preparing the solid medium, a carbon source such as glucose, maltose and the like, a nitrogen source such as yeast extract, peptone and the like, a pH-adjusting agent such as calcium carbonate, vitamins, inorganic salts as well as growth-promoting agents may be added there into. A mixture of 2 to 6 parts by weight of sawdust and 1 part by weight of rice bran is a preferable base material containing various nutrient components in suitable amounts and ratios. As the sawdust, those derived from a broad-leaved tree such as beech, oak, walnut and the like are preferably used, however, those derived from a coniferous tree such as pine, cryptomeria, Japanese hemlock and the like may be used.

The fungal tissue bed is cultivated at a temperature of 15° to 40° C., preferably 25° to 35° C. and at a relative humidity of at least 90%, preferably at least 95% under an illumination intensity of at most 500 lx, preferably 100 to 300 lx. Under such conditions, the primordium of fruit body begins to form within about 10 to 15 days and continues to grow.

Covering of the fungal tissue bed improves the retention of moisture in the bed to prevent the drying of the bed and also improves the heat-insulation resulting in promoting the active proliferation of the mycelia. Accordingly, covering of the bed is carried out to cover the bed well. In addition, it is not necessary to cover the upper surface of the bed, that is, the surface where the primordium grows, however, in order to promote the primordium growth, the covering is preferably carried out to the extent that it covers the rhizomorph of the bed. As the covering material, natural soil such as sand, loamy soil and the like, a soil-improving agent such as vermiculite, perlite and the like or rice straw, buckwheat chaff, or the like may be used. Kanuma-soil and Akadama-soil are the particularly preferable covering material from the viewpoints of moisture-retention and ventilation. The covering material is not necessarily sterilized particularly, in cases where it is clean. This step in cultivation depends on the desired length of the stipe of fruit body, and it takes ordinarily about 30 to 45 days.
Then the cultivation is carried out by altering the conditions of cultivation a little, i.e., at the same temperature of 15º to 40º C., preferably 25º to 35º C., however, at a relatively humidity of at least 40 and less than 90%, preferably, 60 to 80% under an illumination of at least 500 lx, ordinarily, 600 to 5000 lx. A fungal pileus is formed within about 10 to 30 days.

In addition, it is preferable to keep the moisture content of the fungal tissue bed during the cultivation steps 65 to 75% by weight. The method for cultivation of the invention may be executed indoors, under a controllable artificial illumination, however, it may be carried out in a vinyl house and in a glass house if a suitable control of illumination can be applied. The culture is preferably illuminated by natural light or incandescent electrical lamp (Wada et al., 1984).

Mushroom mycelia or spawns have normally been produced in solid cultures using substrates such as grain, sawdust or wood. The additions of agro-residues have gained wide attention in recent years, not only for the reutilization of the waste material, but also for the improvement of the production yield (Fan-Chiang et al., 2003).

Studies on mushroom dehydration are still scarce in the literature, (Gothandapani, et al., 1997; Yaparet et al., 1990) Most commercial production of *Lentinula (Lentinus) edodes* (Berk.) Pegler (shiitake) is done on synthetic logs that contain hardwood sawdust, straw or corncobs as the basal ingredients and starch-based supplements (10–60% dry weight) such as wheat bran, rice bran, millet, rye, and maize. Sufficient water is added to adjust the moisture content of the mix to about 60% (Royse et al., 1990; Royse and Sanchez, 2007).

For commercial production, the mix is weighed and filled into plastic bags in a uniform amount (usually about 2.7 kg). The bags are made of heat-resistant polypropylene and contain a special filter patch of laminated microporous plastic. The microporous patch provides a microbial barrier to contaminants and allows gas exchange with the outside environment during substrate colonization (Royse and Sanchez-Vazquez, 2001).
3.1.1.5 Biological efficiency

Closed (lamellae not exposed) mushrooms were harvested, counted and weighed daily. At the end of each break, yield and BE were determined as the ratio of fresh mushrooms (g) harvested per dry substrate weight (g) and expressed as a percentage. Yield was expressed in kg/m2. Moisture contents were used to calculate biological efficiency as described under "harvesting and determination of biological efficiency (BE)" Daniel and William, 2009. Edenes, et al., (2009) have prepared the substrate containing the straw and effluent in accordance with a 23 full factorial design to evaluate the effect of the medium composition (effluent concentration, pH and Pleurotus species) on the biological efficiency (fresh mushroom yield).

Fomina and Lysenkova (1989) have found that oak sawdust + rice husks (4:1) and oak sawdust + chaff (10:1) were the most suitable substrates, and strain cultivated on oak sawdust + rice husks gave 50–60 g fruiting bodies per 100 g of substrate. A yield of 540 g mushroom kg⁻¹ dry substrate was obtained from a mixture of oak sawdust with wheat bran at a ratio of 12.5% (Morales and Martines, 1990).

BE was significantly affected by the interaction between genotype, spawn run time, and substrate formulation (Royse and Bahler, 1986). The biodegradation efficiency of different substrates varies for L. edodes mushroom varieties. BE of substrates ranged from 46% to 100% in many studies (Royse, 1985; Przybylowicz and Donoghue, 1988; Kalberer, 1989; Triratana and Tantikanjana, 1989; Levanon et al., 1993a,b; Ilbay, 1994; Sobal et al., 1997; Leifa et al., 1999; Royse and Sanchez-Vazquez, 2003). BE values of shiitake strain IE-40 cultivated on pineapple crown bracts, sugarcane leaves and sugarcane bagasse were 37.5%, 82.7% and 130.2%, respectively (Salmones et al., 1999). Pire et al. (2001) informed that the BE was between 2.0% and 60.4%, using sterilized and supplemented wood shavings with incubation periods of 30–90 days. The BE values obtained by Morais et al. (2000) (18.9–59.5%) and Gaitan-Hernandez and Mata (2004) (24.8–55.6%) were lower than reported in this study (Elif and Aysun, 2007). Moisture content of pistachio kernels was determined by oven method (Kamangar & Farsam, 1977). About 3–5 g sample were ground and dried in a hot air oven at 103 ± 2°C for about 3 h until the differences between two weighing was slight (±0.05 g). Amount of moisture
evaporated was calculated and moisture content (MC) was expressed as % w.b. (wet basis). The method described above was adopted for all MC determinations using three replicates to get an average value of MC for any sample (Seyed and Masoud, 2007).

### 3.1.2 Composition of *Ganoderma lucidum*

*Ganoderma lucidum* contains mainly protein, fat, carbohydrate, and fiber. Artificially cultivated variety has similar contents of nutritional components compared with wild types, and the extraction significantly increases the amounts of crude protein and carbohydrates and deleted crude fiber. Mizuno, 1995 reported the composition of *Ganoderma lucidum* extract (% of dry weight), which consisted of folin-positive material (68.9%), glucose (11.1%), protein (7.3%), and metals (10.2%) (K, Mg, and Ca are the major components) (Chen, 1999; Stamets, 2000; Hobbs, 1995). However, there are qualitative and quantitative differences in the chemical composition of *Ganoderma lucidum* products depending on the strain, origin extracting process, and cultivation conditions (Wasser and Weis, 1993; Stamets, 2000; Hobbs, 1995, McKenna et al., 2002 and Mizuno, 1995). Major Bioactive Constituent over 300 reports have been published concerning the chemical constituents of *Ganoderma lucidum* and related species. The fruiting body, mycelia, and spores of *Ganoderma lucidum* contain approximately 400 different bioactive compounds, which mainly include triterpenoids, polysaccharides, nucleotides, sterols, steroids, fatty acids, proteins=peptides, and trace elements (McKenna et al., 2002; Gao et al., 2002; Mizuno, 1995, Kim and Kim, 1999 and Smith et al., 2002).

### 3.1.3 Hypoglycemic activity

*Ganoderma lucidum* polysaccharides have been shown to have hypoglycemic potentials in normal and glucose loaded mice and rats by increasing the plasma insulin levels, but did not affect the insulin binding to isolated adiposities (Hikino et al., 1985, 1989). Also, Hikino and Mizuno (1989) in a similar study reported the hypoglycemic activity of fifteen heteroglycans fractions of *Ganoderma lucidum* administered intraperitoneally at a dose of 100 mg/kg in rats. The hypoglycaemic activity of *Ganoderma lucidum* is thus due to an increase of the plasma insulin level.

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and an acceleration of glucose metabolism occurring not only in the peripheral tissues but also in the liver.

Glycans (ganoderans B and D) have shown significant hypoglycemic activity in mice (Wasser, 2005). Beta-glucans (ganodelan A and B) help release in insulin by facilitating the influx of calcium in the pancreas beta cells, lowering elevated blood sugar— a potential therapy for diabetes (George, 2007). Accordingly, after taking this herb, not only can insulin deficiency be supplemented, it can also make the pancreas regain its original function. In addition, it does not have any side effects like injections do. *Ganoderma lucidum* serves as a substitute to insulin to inhibit release of fatty acids. It thus improves symptoms in high blood glucose and high urine glucose patients. Blood glucose will be reduced from 173 to 116, cholesterol from 233 to 179, beat-protein from 580 to 465.

*Ganoderma lucidum* polysaccharides (Gl-PS) have hypoglycemic activity by increasing plasma insulin and by affecting the hepatic key enzymes in the carbohydrate metabolism in the normal and alloxan-induced diabetic mice (Hikino *et al.*, 1985, 1989). It has been generally accepted that alloxan-induced hyperglycemia is mainly due to its ability to induce oxygen free radicals, which damage the pancreas (Heikkila *et al.*, 1976) Since Gl-PS has the ability to scavenge the free radicals it will show hypoglycemic activity.

Animal studies have demonstrated that the polysaccharide fractions of *Ganoderma lucidum* have potential hypoglycemic and hypolipidemic activities. Treatment with Ganopoly in Diabetic patients significantly decreased the mean HbA1c from 8.4% at baseline to 7.6% at 12 weeks. The study by Goa *et al.*, (2002) demonstrated that Ganopoly is efficacious and safe in lowering blood glucose concentrations.

A 2-mo open label comparative clinical study of a reishi powder extract (1 g t.i.d.) for eight diabetic patients (four with NIDD and four with IDDM) found hypoglycemic effects comparable to those found in controls who were administered insulin or oral hypoglycemic agents (Wasser and Weis, 1997, Hobbs, 1995, McKenna *et al.*, 2002 and Goa *et al.*, 2002). A polysaccharide fraction of *Ganoderma frondosa* (SX fraction, p.o) showed hypoglycemic action in five patients with type 2
diabetes (Konno et al., 2002) Ganoderan A and B, glucans from *Ganoderma lucidum* fruiting bodies exhibited hypoglycemic effects in several test systems and ameliorated the symptoms of diabetes (Hikino et al., 1985)

*Ganoderma lucidum* serves as a substitute to insulin to inhibit release of fatty acids. It thus improves symptoms in high blood glucose and high urine glucose patients. Blood glucose will be reduced from 173 to 116, cholesterol from 233 to 179, beat-protein from 580 to 465. Water-solubility polysaccharides in *Ganoderma lucidum* suppress insulin-independent diabetes. Beta-glucans (ganodelan A and B) help release in insulin by facilitating the influx of calcium in the pancreas beta cells, lowering elevated blood sugar- a potential therapy for diabetes (George, 2007).

### 3.1.4 Hepatoprotective activity

Hepatoprotective mechanisms of ganosporeric acid A from the spores of *Ganoderma lucidum* were observed by Chen and Yu (1999). *Ganoderma lucidum* has been widely used for the treatment of chronic hepatopathy of various etiologies. Data from *in vitro* and animal studies indicate that *Ganoderma lucidum* extracts (mainly polysaccharides or triterpenoids) exhibit protective activities against liver injury induced by toxic chemicals (e.g., CCl4) and Bacillus Calmette-Guerin (BCG) plus lipopolysaccharide (LPS). Reishi also showed antihepatitis B-virus (HBV) activity in a duckling study. Recently, a randomized placebo-controlled clinical study (Zhou et al., 2002; Goa et al., 2003) showed that treatment with *Ganoderma lucidum* polysaccharides for 12 weeks reduced hepatitis B e antigen (HBeAg) and HBV DNA. The mechanisms of the hepatoprotective effects of *Ganoderma lucidum* have been largely undefined. However, accumulating evidence suggests several possible mechanisms. These include antioxidant and radicalscavenging activity, modulation of hepatic Phase I and II enzymes, inhibition of b-glucuronidase, antifibrotic and antiviral activity, modulation of NO production, maintenance of hepatocellular calcium homeostasis, and immunomodulating effects.

The mushroom could represent a promising approach for the management of various chronic hepatopathies. Further studies are needed to explore the kinetics and mechanisms of action of its constituents with hepatoprotective activities. The polysaccharide fractions and triterpenes isolated from *Ganoderma lucidum* have
shown protective effects on the liver in animal and human studies. Ninety patients with chronic hepatitis B, hepatitis B viral (HBV) DNA positivity, and aminotransferase elevation were included in this multicenter prospective randomized Phase I=II study. Subjects were randomized to be given Ganopoly (n = 60) or a placebo (n = 30) for 12 weeks, then followed up for 13 weeks.

**Fig. 3.1** Possible mechanisms for the hepatoprotective effects of *Ganoderma lucidum*. These include antioxidative and radical-scavenging effects, downregulation of activating enzymes, and upregulation of detoxifying enzymes, antiviral activities, inhibition of bglucuronidase, enhanced hepatic nucleic acid and protein synthesis, inhibition of hepatic collagen synthesis, immunomodulating effects, and modulation of nitric oxide (NO) production. GSH \( \equiv \) glutathione; GSSG \( \equiv \) oxidized glutathione; INOS \( \equiv \) inducible NO synthase; SOD \( \equiv \) superoxide dismutase; CYP \( \equiv \) cytochrome P450; UGT \( \equiv \) uridine diphosphate glucuronosyltransferases (Gao et al., 2003).

Ganoderic acids R and S and ganosporeric acid A from *Ganoderma lucidum* show *in vitro* antihepatotoxic activity in the galactosamine-induced cytotoxic test with primary cultured rat hepatocytes (Hirotani et al., 1986, Chen and Yu, 1993). *In vitro* fractions of a total triterpenoids extract of *Ganoderma lucidum* (75% ethanol) can
protect mice against hepatic necrosis induced by chloroform and D-galactosamine. The hepatoprotective effects were perhaps related to the ability to promote the activity of scavenging enzymes for hepatic free radicals in mice and thus it raise the ability of antioxidation in mice (Wang et al., 2002).

Ganopoly, the polysaccharide-containing preparation of *Ganoderma lucidum*, was proven in a double-blind, randomized and multicentered study in patients with chronic hepatitis B had cleared hepatitis B surface antigen from serum (Gao et al., 2002).

Liver Protection & Detoxification *Ganoderma lucidum* is able to protect the liver from damaged by various physiological and biological factors. Moreover, effect can be obtained either pre-or post-damage. *Ganoderma lucidum* speeds up metabolism of both medicine and toxic substances in liver, leading to curing of toxicated hepatitis. *Ganoderma lucidum* is also suitable for treating chronic hepatitis, effectively eliminating the related symptoms as dizzy, fatigue, and so on. *Ganoderma lucidum* can be used to treat chronic toxicosis, the various kinds of chronic hepatitis, and other hepatic diseases.

Ganoderic acid of *Ganoderma lucidum* had potent beta-glucuronidase-inhibitory activity and hepatoprotective effect against CCl4-induced liver injury, indicating that beta-glucuronidase seems to be closely related to liver injury (Kim et al., 1999). Total liver triglyceride and total liver cholesterol levels were also significantly lower in the Reishi-fed group (Kabir et al., 1988, Soo, 1996).

### 3.1.5 Cardiovascular and circulatory functions

Effect on Cardiovascular Clinical studies and experiments with animals confirm that *Ganoderma lucidum* can effectively dilate coronary artery, increase coronary vessel blood flow, and improve circulation in cardiac muscle capillaries, thus increase the supply of oxygen and energy to cardiac muscle. Therefore *Ganoderma lucidum* helps to protect the heart from shortage of blood supply, and it is ideal for both curing and preventing heart diseases like nausea. *Ganoderma lucidum* can obviously reduce the level of blood cholesterol, liporotein and triglycerides in hypertensive patients. It prevents the formation of arterial atheromatous patches. If patches are already formed, *Ganoderma lucidum* will reduce cholesterol in arterial...
wall and soften the blood vessel to avoid further damage. It also partially improves blood circulation, and inhibits platelet aggregation. All these effects contribute to preventing various kinds of stroke. It was prescribed routinely to those with a "knotted and tight chest" symptoms consistent with both stress and/or coronary artery disease-related angina. Researchers in China found that Reishi improved the blood flow and lowered oxygen consumption in the heart muscle (Chang and But, 1986).

3.1.6 Hypocholesterolaemic activity

During his work on Ganoderma's hypotensive activity, Kabir et al., (1988) investigated its effect on the lipid levels of the spontaneously hypertensive rats. After being fed the powdered fruit body for four weeks the plasma total cholesterol levels of these rats was significantly lower than the controls. Other investigators such as Shiao et al., (1987, 1988) have isolated specific triterpenes that have hypocholesterolaemic effects.

Hajjaj et al., (2005) have reported the isolation and identification of the 26-oxygenosterols ganoderol A, ganoderol B, ganoderal A and ganoderic acid Y and determined that the point of inhibition of cholesterol synthesis is between lanosterol and lathosterol. The 26-oxygenosterol could lead to novel therapeutic agents that lower blood cholesterol.

Hypercholesterolaemia is another recognised risk factor for atherosclerosis. If Ganoderma has both hypocholesterolaemic and hypotensive activities then it should also significantly reduce the incidence of atherosclerosis. Ganoderma's oxygenated sterols inhibit the synthesis of LDL (or bad) cholesterol better than statin drugs (George, 2007).

3.1.7 Renal protective activity and others

Kidney disease is one of the most difficult diseases to cure. They include acute or chronic nephritis, diabetic renal syndrome, nephrosis and rheumatic fever. These may due in part to high cholesterol count and high blood sugar level. The kidneys are overloaded and blood can not reach renal capillaries. Nephrosis results, with edema, urine retention, fatigue and possibly serious uremia.

Prof. Fumio Tsurudani of Nagoya University Medical Institute, Japan did an experiment with patients suffering from renal disease to check the effectiveness of...
Ganoderma lucidum. It was found that it could not only lower proteinuria and cholesterolemia, but could maintain renal proper function. Based on the experimental results, renal disease can be cured significantly by taking Ganoderma lucidum.

Anti nephrotoxicity the methanolic extract of Ganoderma lucidum could help prevent nephrotoxicity manifested consequent to cisplatin chemotherapy. The effect is mainly due to the capacity of the extract to restore renal antioxidant defence system. (Thulasi et al., 2006)

3.1.8 Antioxidant and Antiaging

Oxidative stress is the main cause in several of neuronal degeneration processes as well as in the physiological decline associated with aging (Harman, 1981). Decreased functional efficiency in antioxidant defence system has been suggested to be one of the preliminary factor that contribute the aging process (Reiter et al., 1996). Ming-Fu Wang et al., (2004) have reported that Ganoderma has significantly increased the enzymes such as superoxide dismutase (SOD) and Glutathione peroxidase (GSH-Px) which involved in antioxidant defence in senescence accelerated mice (SAMP8) suggesting that the Ganoderma may improve learning and memory ability and promote the activities of antioxidation.

The polysaccharides and polypeptides found in Ganoderma lucidum can effectively delay aging by the following mechanisms.

1. To enhance and regulate immune function. Such enhancement and regulation can effectively delay aging in adult and aged people. For the youngster, the immune system will be optimized to ensure healthy growth.

2. To regulate metabolism and enhance synthesis of nucleic acids and proteins. Research works have shown that Ganoderma lucidum enhances synthesis of nucleic acids and proteins in blood plasma, liver, and bone marrow, hence effectively prevent aging. It is observed that the use of Ganoderma lucidum to prevent aging benefits not only the aged, but also the young, since growth and development process will ultimately lead to aging.

3. Effect on Free Radicals. One cause of aging is the reduction of self-originated antioxidant or antioxidant-like material (such as SOD) in the body. These antioxidants are essential for encountering damage to the body by free...
radicals. *Ganoderma lucidum* polysaccharides possess the properties that are very similar to SOD which can remove the free radicals and prevent its damage to the body by stopping over-oxidation of lipid. Such action protects the cells and delays their aging.

4. *Ganoderma lucidum* polysaccharides prominently enhance DNA synthesis in cell nucleus, and increase the number of cell divisions, which results in delayed aging.

Recent years have witnessed a renewed interest in plants as pharmaceuticals because they synthesize a variety of secondary metabolites with antioxidant potential which can play a major role in protection against molecular damage induced by reactive oxygen species (ROS) (Cao *et al.*, 1997; Vaya *et al.*, 1997). Hundreds of plants are used traditionally for the management of Diabetes mellitus. To date, however, only a few of these medicinal plants have received scientific scrutiny, despite the fact that the World Health Organization has recommended that medical and scientific examinations of such plants should be undertaken (WHO, 1980).

Chinese medical texts traditionally call for using 1.5 to 9 grams of dry mushroom per day which approximates to 150 to 900 mg of concentrated Reishi extract. For serious problems like cancer, Dr. Morishige adjusted the dosage from 2 to 10 grams of Reishi extract per day (Carlson, 1996; Kenneth, 1992)

### 3.2 DIABETES MELLITUS

Diabetes mellitus (DM) is a major health problem all over the world. Globally, the number of people that has been diagnosed with diabetes has exploded in the past two decades. In 2000, 151 million people in the world were diabetic. With the current rate of increase (6% per annum), it has been projected that 221 million people will be diabetic in 2010 and 324 million by 2025 (Zimmet *et al.*, 2001).

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by hyperglycemia resulting from defective insulin secretion, resistance to insulin action or both (Gavin *et al.*, 1997). Type 1 diabetes is the consequence of an autoimmune-mediated destruction of pancreatic β-cells, leading to insulin deficiency, while type 2 diabetes is characterized by insulin resistance and relative, rather than absolute, insulin deficiency (Malaisse, 1983). Growing evidence indicates that

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oxidative stress is increased in diabetes, due to the overproduction of reactive oxygen species (ROS), and decreased efficiency of antioxidant defences (Giron et al., 1999; Wiernsperger, 2003). Oxidation of lipids, proteins and other macromolecules (such as DNA) occurs during the development of diabetes (Ohkawa et al., 1979). Mitochondrial DNA mutations have also been reported in diabetic tissues, suggesting oxidative stress-related mitochondrial damage (Lee et al., 1997).

In diabetes, oxidative stress seems mainly to be due to an increased production of free radicals and/or a sharp reduction of antioxidant defences (Cross et al., 1987; Oberley, 1988; Hunt et al., 1992; Young et al., 1992; Thompson and Lee, 1993; Giugliano et al., 1996; Low et al., 1997). It is well known that superoxide anion is the primary radicals formed by the reduction of molecular oxygen that may lead to secondary radicals or reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radicals (Grisham and McCord, 1986; Katusic, 1996).

Jang et al., (2000) found that increased oxidative stress has been suggested to be involved in the pathogenesis and progression of diabetic tissue damage. On the other hand, there is evidence that diabetes induces changes in the activities of antioxidant enzymes in various tissues (Oberley, 1988).

Diabetes mellitus is characterized by increased generation of glycoxidation products associated with the advanced oxidative stress (mullarkey et al., 1990). The presence of higher glucose or glycated protein concentration enhances lipid peroxidation (Kawamura et al., 1994) and reversely, lipid peroxides may increase the extent of advanced glycation end-products (Hicks et al., 1989).

Oxidative stress in diabetes was thought to be a result of free radicals generated during autoxidation of glucose (Miyata et al., 1999). Increased levels of ROS in type-2 DM was implicated to contribute to a hypercoagulable state (Collier et al., 1992) and most recently, evidence was provided for the accumulation of oxidation products prior to the development of diabetes (Matteucci et al., 2000). The causes of enhanced free radical production are hyperglycaemia (Hammes et al., 1994) and hyperinsulinemia (Paolisso and Giugliano, 1996).

Oxidative stress is an imbalance between oxidants and antioxidants leads to many biochemical changes and is an important causative factor in several human
chronic diseases, such as atherosclerosis and cardiovascular disease, mutagenesis and cancer, several neurodegenerative disorders and the aging process (Frei, 1999). Diabetic mellitus is one such disease and it is estimated that the number of diabetic patients will continue to increase in the future (Furusho et al., 2002). It has been postulated that the etiology of the complications of diabetes involves oxidative stress perhaps as a result of hyperglycemia (Hunt et al., 1990). The elevated levels of blood glucose in diabetes produce oxygen-free radicals (OFR), which cause membrane damage due to peroxidation of membrane lipids and protein glycation (Sato et al., 1979). Evidence that oxidative stress is present in diabetes originates from the frequent observation that both ROS and antioxidants are increased. The latter is logically rather seen in early stages of diabetes and should be interpreted as a tentative compensation of cells against increasing oxidative stress (Turk et al., 2002; Zobali et al., 2002). According to tissue and cell type, the nature of antioxidant elevation may vary, indicating specificities which, again, be important for therapeutic interventions.

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko et al., 2001; Maritim et al., 2003). ROS include free radicals such as superoxide (\(\cdot O_2^-\)), hydroxyl (\(\cdot OH\)), peroxyl (\(\cdot RO_2\)), hydroperoxyl (\(HRO_2^-\)) as well as nonradical species such as hydrogen peroxide (H2O2) and hydrochlorous acid (HOCI) (Evans et al., 2002). RNS include free radicals like nitric oxide (NO) and nitrogen dioxide (\(\cdot NO_2\)) as well as non-radicals such as peroxynitrite (ONOO\(^-\)), nitrous oxide (HNO2) and alkyl peroxynitrates (RONOO) (Evans et al., 2002). Oxygen derived free radicals have been implicated in the pathophysiology of various disease states, including diabetes mellitus (Giugliano et al., 1996).

Under normal conditions, \(O_2^-\) is quickly eliminated by antioxidant defence mechanisms. \(\cdot O_2^-\) is dismutated to H2O2 by manganese superoxide dismutase (Mn-SOD) in the mitochondria and by copper (Cu)-SOD in the cytosol (Evans et al., 2003). H2O2 is converted to H2O and O2 by glutathione peroxidise (GSH-Px) or catalase in the mitochondria and lysosomes, respectively.
H\textsubscript{2}O\textsubscript{2} can also be converted to the highly reactive 'OH radical in the presence of transition elements like iron and copper.

While ROS are generated under physiological conditions and are involved to some extent as signalling molecules and defence mechanisms as seen in phagocytosis, neutrophil function, and shear-stress induced vasorelaxation, excess generation in oxidative stress has pathological consequences including damage to proteins, lipids and DNA. These detrimental effects are briefly summarized in this section. ROS can stimulate oxidation of low-density lipoprotein(LDL) and ox-LDL, which is not recognized by the LDL receptor, can be taken up by scavenger receptors in macrophages leading to foam cell formation and atherosclerotic plaques (Boullier et al., 2001).

\textbf{O}_2^- can activate several damaging pathways in diabetes including accelerated formation of advanced glycation end products (AGE), polyol pathway, hexosamine pathway and PKC, all of which have been proven to be involved in micro and macrovascular complications. In endothelial cells, H\textsubscript{2}O\textsubscript{2} mediated apoptosis and pathological angiogenesis (Taniyama and Griendling, 2003). Furthermore, O_2^- immediately reacts with NO generating cytotoxic ONOO- and this reaction itself has several consequences. First, ONOO- alters function of biomolecules by protein nitration as well causing lipid peroxidation. For example, potassium channels, which regulate the vasorelaxation response, are inhibited by nitration (Liu and Gutterman, 2002; Liu et al., 2002).

**3.2.1 Mechanisms of oxygen free radicals production by hyperglycaemia**

Hyperglycaemia is a widely known cause of enhanced plasma free radical concentration (Hammes et al., 1994). Free radical production caused by hyperglycaemia may occur via at least four different routes:

i) Increased glycolysis (Vaag et al., 1992);

ii) Intercellular activation of sorbitol (Polyol) pathway (Williamson et al., 1993);

iii) Autooxidation of glucose (Wolff et al., 1991) and

iv) Non-enzymatic protein glycation (Ceriello et al., 1992)
i) Increased glycolysis (Vaag et al., 1992)

Hyperglycaemia seems to enhance non-oxidative metabolism (glucose conversion to lactate) through increasing glucose-6-phosphate (Vaag et al., 1992). Increased glucose metabolism to lactate is associated with an increase in NADH/NAD⁺ ratio (Williamson et al., 1993). Under this condition of markedly accelerated glycolysis, oxidation of glyceraldehydes-3-phosphate (GAP) to 1,3-biphosphoglycerate (1,3- DPG) by glyceraldehydes 3-phosphate dehydrogenase appears to become the rate limiting step in glycolysis (Kobayashi and Neely, 1979), this reaction is coupled to reduction of NAD⁺ to NADH. In the cytosol, NADH is oxidized to NAD⁺ by lactate dehydrogenase (LDH), coupled by reduction of pyruvate to lactate. Thus, the increase in the ratio of NADH/NAD⁺ will reflect increased lactate/ pyruvate ratio (Williamson et al., 1993). The mechanism by which an increased rate of glycolysis in turn increases free cytosolic NADH/NAD⁺ ratio (redox imbalance) appears to result from a disequilibrium between the rate of oxidation of GAP to 1,3-DPG and the rate of reduction of pyruvate (Kobayashi and Neely, 1979). This result indicates that, the increased glycolysis as a consequence of hyperglycaemia is closely related to an increase in NADH/NAD⁺ ratio due to impaired oxidation of NADH to NAD⁺.

ii) Intercellular activity of sorbitol (Polyol) pathway

The increased glucose flux via sorbitol pathway (a pathway of a minor significant under normal glycemic condition) which leads to the accumulation of both sorbitol and fructose is thought to be one of the main metabolic disturbances related to diabetic hyperglycemia (Ciuchi et al., 1996). In this pathway, glucose is reduced to sorbitol by aldose reductase (AR), coupled with oxidation NADH/NAD⁺. Sorbitol is then oxidized to fructose coupled with reduction of NAD⁺ to NADH by sorbitol dehydrogenase (SDH)( Cameron et al., 1997).

The studies suggested several hypotheses for tissue injury caused by increased sorbitol pathway activity:

1. The decreased availability of NADPH (required for maintenance of
reduced glutathione) which is oxidized to NADP⁺ through reduction of glucose to sorbitol by aldose reductase (Tilton et al., 1995). Furthermore, the competition between aldose reductase and glutathione reductase for NADPH cofactor depletes reduced glutathione (Ciuchi et al., 1996). Attention has been focused on this GSH depletion, because it can play a role in increased oxygen free radicals production, which is thought to cause oxidative tissue damage in diabetes.

2. Increased NADH/NAD⁺ ratio, which is related to accelerated oxidation of sorbitol to fructose by NAD⁺ dependent sorbitol dehydrogenase (Tesfamariam and Cohen, 1992). Williamson et al., (1993) and Ceriello et al., (1996) have reported that NADH produced in the cytosol by oxidation of sorbitol to fructose can remain there temporarily but for a long run it has to be transported into the mitochondria to be oxidized by respiratory chain causing generation of superoxide radical and other oxygen reactive species derived from it. Thus, an increase in the cytosolic NADH may be accompanied by increased load of mitochondria NADH, which in turn leads to increased oxygen radicals generation.

iii) Glucose auto-oxidation

Glucose can be auto-oxidized in a cell-free system under physiological conditions via enediol tautomer formation which generates hydrogen peroxide; reactive intermediate such as hydroxyl and superoxide radicals and ketoaldehydes (Brownlee et al., 1988 and Packer, 1993). Transition metals such as iron are believed to be of crucial importance in the cascade of these reactions, as they catalyze auto-oxidation of glucose auto-oxidation can actually occur and could be responsible for increased oxygen radicals in diabetes (Monnier, 1990; Baynes, 1991 and Santini et al., 1997).

iv) Non-enzymatic protein glycation (glycosylation)

Non-enzymatic glycation is a spontaneous chemical reaction between glucose and the amino groups of proteins in which reversible shift bases and more stable Amadori products are formed (Vlassara et al., 1994). Advanced glycation end
products (AGEs) are then produced by auto-oxidation of Amadori product (The Diabetes Control and Complication Trials Research Group, 1993). AGEs elicit their cellular effects by binding to specific cellular receptors (Esposito et al., 1989; Yang et al., 1991; Schmidt et al., 1994; Vlassara et al., 1996), one of which the RAGEs (receptor for AGEs), has been identified on endothelial cells (Neeper et al., 1992; Abel et al., 1995; Rithaler et al., 1995), monocytes/macrophages, mesangial cells neurons and smooth muscle cells. Interaction of AGEs with endothelial surface RAGEs generates intracellular oxidative atress and therapy modulates cellular functions, even in the presence of intact antioxidant mechanisms (Yan et al., 1994; Schmidt et al., 1995; Wautier et al., 1996). This process is probably enhanced and amplified when antioxidant defence mechanisms are reduced (Bierhaus et al., 1997).

3.2.2 Mechanisms of oxygen free radicals production by hyper-insulinemia

Decline in physical fitness, increase in body fatness and upper body fat distribution are frequently associated with hyperinsulinemia and insulin resistance (De-Fronzo et al., 1991). Several lines of evidence seem to indicate the relationship between hyperinsulinemia and free radical production. Kriegger-Brauer and Kather (1992) reported that in intact human fat cells, exposure to insulin leads to a time and dose-dependent accumulation of hydrogen peroxidise in the suspension medium. This effect, which has been related to the presence of a membrane-bound NADPH oxidase was found to persist after cell disruption and not required ATP indicating that the receptor kinase step was by passed. In addition, increased insulin concentration in rats following intraperitoneal injection of dextrose has been found to be associated with increased free radical production (Habib et al., 1994).

Treverse et al., (1998) and Betteridge (2000) reported that the imbalance of generation and scavenging of free radicals play an important role in determining tissue damage associated with diabetes. Lipid peroxidation is the primary cellular damage resulting from free radical reactions. Also, significant changes in cellular lipid structures are generally occurring in diabetic states (Armstrong and Al-Awadi, 1991; Toborek et al., 1992). In these states, the structure changes are oxidative in
nature due to peroxidation of the lipids that defined as peroxidative deterioration of unsaturated fatty acids of cellular membrane phospholipids, via intermediate radical reactions (Rungby et al., 1992; Cameron et al., 1994), with a result of producing hydroperoxides. The net effect of these combined reactions is the generation of highly toxic peroxy radicals (ROO-) which generate new lipid hydroperoxides because of their close proximity in biomembranes to other lipids (Betteridge, 2000; Sakamoto, 1985; Kajanachumpol et al., 1997).

3.2.3 Mechanisms of oxygen free radicals production by hypo-insulinemia

Hypoinsulinemia increase the activity of fatty acyl-CoA oxidase that indicates 3-oxidation of fatty acids resulting in increased production of H$_2$O$_2$ (Horie et al., 1981). Kakkar et al., (1995) and Tatsuki et al., (1997) recorded significant increase of erythrocytic and pancreatic catalase (CAT) activity in streptozotocin diabetic rats and ascribed this increase to the accentuated oxidative stress in diabetes. However, Matkovics et al., (1998) demonstrated a significant decrease of CAT activity in erythrocyte hemolysate of streptozotocin diabetic rats.

3.2.4 Changes in antioxidant enzyme activities due to diabetes

The studies examined the tissue levels of the enzymatic antioxidant defences in diabetes with varying results. Piper et al., (1995) have demonstrated that, in experimental diabetes the activity of catalase was increased in vascular tissues with absence of any significant changes in the activity of the major antioxidant enzymes (superoxide dismutase and glutathione peroxidise). In addition, Wohaieb and Godin (1987) have showed increased activities of catalase and superoxide dismutase (SOD) in the pancreas of diabetic rats, while the liver showed a generalized decrease in the activities of catalase, SOD and glutathione peroxidise (GSH-Px). In the study, the increase in the activities of both CAT and SOD occurred in the tissue with the lowest antioxidant enzymatic activities (pancreas) before onset of diabetes, suggesting a compensatory response to an increase in endogenous oxidant radicals in pancreas by diabetes.

A decrease in the concentration of reduced glutathione (GSH) has been observed in erythrocytes from diabetic subjects, as a result of decrease in activities
of the enzymes involved in GSH synthesis (such as γ-glutamylcysteine synthetase) or in the transport rate of oxidized glutathione (GSSG) from erythrocytes (Murakami et al., 1989) and enhanced sorbitol pathway (Ciuchi et al., 1996).

In addition, a decrease in the activity of glutathione reductase (GSSG-R) which acts to reduce GSSG to GSH has also been reported (Tagami et al., 1992). Kazuhiro et al., (1989) and Matkovics et al., (1998) elucidated that GSSG-R activity decreased in erythrocyte hemolysates of streptozotocin diabetic rats and attributed this decrease to the enzyme glycation by the uncontrolled hyperglycaemia (Jain and Me Vie, 1994).

Also, Jos et al., (1990) and Dominguez et al., (1998) have reported a significant decrease of erythrocyte GSH-Px activity in diabetic children and adolescents compared with control subjects. They attributed this decrease to a decline in blood glutathione content in those diabetics, since GSH is a substrate and cofactor for this enzyme. Therefore, low GSH content indicates low GSH-Px activity, which may produce increased oxidative stress propensity. Moreover, enzyme inactivation either through glycation process (Arai et al., 1987) or under conditions of increased oxidative stress might also contribute to low GSH-Px activity (Lyons, 1991).

3.2.5 Lipid peroxidation in diabetes

Both radical and non-radical oxidants can induce lipid peroxidation particularly of those lipoproteins that contain unsaturated fatty acids. A product of the reaction between a superoxide anion and nitric oxide, known as peroxynitrite, is a particularly powerful oxidant of low-density lipoproteins (LDLs) (Violi et al., 1999). The evidence for oxidative damage in diabetes has been reported by Sato et al., (1979). They reported that the average level of lipid peroxides in plasma is higher in diabetic patients than in normal people and the diabetic patients with angiopathy had higher lipid peroxide levels than other diabetic patients.

Further suggestion is that the high levels of lipid peroxide in plasma may cause an increase in lipid peroxide levels in the intima of the blood vessel, which may then initiate atherosclerosis. Recent studies have found increased and similar in
vitro oxidizability of LDL fractions of plasma from diabetic patients and identified autoantibodies against oxidatively modified LDL in type-1 diabetic patients against suggesting that LDL oxidation occurs in vivo in diabetes (Jain et al., 1998).

Lipid peroxidation has been implicated in the pathogenesis of many degenerative disorders (Armstrong et al., 1982) including naturally occurring (Nishigaki et al., 1981) and chemically induced diabetic mellitus (Rerup, 1970; Higuchi, 1982). Consequently, mechanisms in the formation of lipid hydroperoxides and biologically active metabolites, together with their effect on cellular structure and function are becoming of increasing importance to the study of diabetogenesis (Crabbe, 1987).

Lipid hydroperoxides (LHP) produced from a variety of long-chain polyunsaturated fatty acid precursors via intermediate radical reactions, involve oxygen and metal cations (iron and copper). The net result of these combined reactions is the generation of highly reactive and cytotoxic lipid radicals, which generate new LHP because of their close proximity in biomembranes to other lipids. Extracellularly, lipid hydroperoxides are transported in the systemic circulation by low- and high- density lipoproteins (Nishigaki et al., 1981). When released locally, LHP produce structural damage (Berdanier, 1988) Peroxidative regulation occurs through intervention by lipid and water soluble antioxidants, as well as by specific antioxidant enzymes, i.e., dioxide (1-) dismutase, peroxidise and catalase. The formation of LHP and their metabolites are important in clinical medicine because they alter membrane structure and function, especially in the retinal portion of eye which is very sensitive to oxidative stress. For example, a steady decline is observed in the electroretinogram not only in the streptozotocin (STZ) model (Pautler and Ennis, 1980) but also when synthetic LHP is injected into the vitreous of experimental animals (Armstrong et al., 1982). These changes are irreversible. Moreover, support for the concept of increased oxidative stress (increased generation of free radicals) in diabetes is derived principally from in vitro experiments (Wolff, 1993; Giugliano et al., 1996).

The primary causal factor is hyperglycaemia and this operates via several
mechanisms, although the individual contribution of each mechanism to hyperoxidative stress remains undefined, as does the response relationship between hyperglycaemia and overall oxidative stress in diabetes. Glycoxidation of glucose generates reactive oxygen species, such as superoxide, hydrogen peroxide and hydroxyl radical (Giugliano et al., 1996). These accelerate the formation of advanced glycosylation end-products (AGEs) which in turn generate more free radicals (Giugliano et al., 1996; Vlassara et al., 1994). Increased cellular uptake of glucose stimulates protein kinase C activity (Lee et al., 1989) which, amongst other effects, activates peroxidise enzymes and the cyclo-oxygenase (COX) pathway (Lee et al., 1989; Feener and King, 1997), with resultant over production of oxidative molecules. By elevating endothelial cell calcium, hyperglycaemia also stimulate the synthesis of NO (Cohen, 1993; Poston et al., 1995), but in the presence of superoxide, nitric oxide (NO) is converted into the highly potent oxidant molecule peroxynitrite (ONOO⁻) (Beckman et al., 1990).

Antioxidant defences may also be impaired in diabetes, thereby contributing to net oxidative stress. Decreased tissue concentrations of antioxidants, such as vitamin E, SOD and CAT, have also been demonstrated in vitro (Wohaieb and Godin, 1987). Although there are extreme difficulties in measuring free radicals in vivo, some support for the notion of increased oxidative stress in diabetes and its association with poor metabolic control and coronary heart disease has been derived from observations in patients with diabetes mellitus (Nourooz-Zadeh et al., 1997; Griffin et al., 1997). Increased oxidative stress may provide a plausibly pathobiological basis for the direct association between hyperglycaemia and increased cardiovascular persuasive risk in diabetes mellitus (Barrett-Connor, 1997; Lehto et al., 1997).

In spite of recent persuasive evidence (Griendling and Alexander, 1997), definitive clinical proof for the role of oxidative stress in the pathogenesis of atherosclerosis in both diabetic and non diabetic subjects remains outstanding. In addition, there were inverse relationships between insulin action and oxidative stress or hypofibrinolysis. Insulin resistance and increased oxidative stress have been observed in obese Type 2 diabetic patients (Skrha et al., 1996). The relationship
between insulin action and oxidative stress was therefore suggested (Paolisso and Giugliano, 1996).

Fig. 3.2 In boxes is shown mechanisms that are directly related to hyperglycaemia. In circles are some mechanisms that result from the reaction of free radicals (e.g., superoxide \( \cdot O_2^- \)) with lipoproteins (e.g., small, dense low-density lipoprotein, sd LDL) and nitric oxide (NO), ox LDL, oxidized LDL-ONOO, peroxynitrite.

A decrease of oxidative stress could therefore improve insulin action in subjects with insulin resistance. Drugs acting like scavengers of oxygen radicals are promising tool in the treatment of patients with increased oxidative stress. On the other hand, lipid peroxide levels in plasma of diabetic patients have been found to be significantly higher than in healthy individuals (Kaji et al., 1985). Furthermore, Sato et al., (1979) reported an increase in thiobarbituric acid reaction in these...
patients especially in poorly controlled diabetic and diabetic with angiopathy. This elevation has been considered as a cause of organ or tissue degeneration. Significantly higher values of thiobarbituric acid reactive substances (TBARS), which provide an indirect measurement of lipid peroxidation and decreased erythrocyte antioxidant enzyme activities, have been observed in serum of adult diabetic patients (Arai et al., 1987; Sharma et al., 2000), heart, and pancreas and blood of streptozotocin diabetic rats (Kakkar et al., 1995).

On other instance, TBARS is considered as an indicator of free radical production. An increase in TBARS level in liver may therefore be due to increased oxidative stress that might remote DNA and protein alterations (Sato et al., 1979) including changes in the enzyme activities implicated in lipid metabolism and free radicals scavenging process (Douillet et al., 1998). Also, increased oxidative stress in diabetes mellitus may be a reason for such decrease in erythrocyte count. Hyperglycaemia can burden the cells with extra free radicals (Fujiwara et al., 1989).

This, coupled with reduced GSH content secondary to its increased utilization in diabetic erythrocyte (Jain and Me Vie, 1994) can cause peroxidative breakdown of phospholipids fatty acids in the erythrocytes membrane. This is supposed by the fact that erythrocytes of diabetic patients are more susceptible.

### 3.2.6 Mechanisms of hyperglycaemia-induced oxidative stress

In physiologic concentrations, endogenous ROS (Wolff and Dean, 1987) help to maintain homeostasis. However, when ROS accumulate in excess for prolonged periods of time, they cause chronic oxidative stress and adverse effects. This is particularly relevant and dangerous for the islet, which is among those tissues that have the lowest levels of intrinsic antioxidant defences.

Multiple biochemical pathways and mechanisms of action have been implicated in the deleterious effects of chronic hyperglycaemia and oxidative stress on the function of vascular, retinal and renal tissues. Considerable less work has been performed using islet tissue. At least six pathways are emphasized in the literature as being major contributors of ROS.
1) Glyceraldehyde Autoxidation

Glyceraldehyde 3-phosphate is a phosphorylation product formed from glucose during anaerobic glycolysis. The partner product, dihydroxyacetone phosphate, also contributes to intracellular glyceraldehyde concentrations via enzymatic conversion by triose-phosphate isomerase. Thereafter, glyceraldehyde 3-phosphate is oxidized by glyceraldehyde-phosphate dehydrogenase (GAPDH). Continuance of glycolysis yields pyruvate, which enters the mitochondria where it is oxidized to acetyl-CoA, and the processes of the tricarboxylic acid cycle and oxidative phosphorylation begin.

One alternative to this classic pathway of glucose metabolism is the less familiar route of glyceraldehyde autoxidation. The potential relevance of this pathway to diabetes mellitus was pointed out by Wolff and Dean (1987), who emphasized that autoxidation of α-ketoaldehydes. In the presence of redox active metals, H2O2 can form the highly toxic radical. This pathway, therefore, forms two potentially toxic substances, α-ketoaldehydes, which contribute to glycosylation-related protein chromophore development, and the hydroxyl radical, a reactive oxygen species that can cause mutagenic alterations in DNA.

Although glyceraldehyde is characteristically thought of as an insulin secretagogue, when present in excess it may also inhibit insulin secretion (Sakai et al., 2003). Long term exposure to high glucose concentrations decreases GAPDH activity in islets (Sakai et al., 2003), which favours excess glyceraldehydes accumulation. Exposure of endothelial cells to 30mM glucose caused GAPDH inhibition through the mechanism of ROS-activated poly(ADP-ribosylation of GAPDH by poly-(ADP-ribose) polymerase. This intum was associated with intracellular advanced glycation end product (AGE) formation and activation of protein kinase (PKC), the hexosamine pathway, and NF-α B (Sakai et al., 2003).

2) PKC Activation

Dihydroxyacetone can undergo reduction to glycerol 3-phosphate and acylation and thereby increase de nova synthesis of diacylglycerol, which activates protein kinase C, of which has at least 11 isoforms. Activation of PKC has many
biochemical consequences that relate to microvascular disease in diabetes. PKC activation associated with increases in TGF-α1, vascular endothelial growth factor, endothelin-1, NAD(P)H oxidase, NF-αB, and ROS (Inoguchi et al., 1992; Ishii et al., 1996).

Fig. 3.3 Six biochemical pathways along which glucose metabolism can form ROS

Under physiologic conditions, glucose primarily undergoes glycolysis and oxidative phosphorylation (Inoguchi et al., 2000). Under pathological conditions of hyperglycemia, excessive glucose levels can swamp the glycolytic process and inhibit glyceraldehyde catabolism, which cause glucose, fructose-1,6-bisphosphate, and glyceraldehydes-3-phosphate to be shunted to other pathways:1, enolization and...

3) Methyglyoxal, Glycation and Sorbitol

Three reactive intracellular dicarbonyls (glyoxal, methylglyoxal, and 3-deoxyglucosone) form AGEs by reacting with amino groups on intracellular and extracellular proteins (De-Fronzo and Ferrannini, 1991). AGEs play important roles in the pathogenesis of secondary complications of diabetes, especially with regard to microvascular disease in the retina, nerves and kidney and likely islets. When GAPDH-mediated catabolism of glyceraldehydes-3-Phosphate is impaired, such as in the presence of high glucose concentrations, accumulation of glyceraldehydes-3-Phosphate and dihydroxyacetone favors formation of methylglyoxal.

Additionally, increased flux along the polyol pathway as a result of hyperglycaemia results in aldose reductase-mediated NADPH-dependent reduction of glucosw to form sorbitol. Oxidation of sorbitol by NAD+ increases the cytosolic NADH:NAD+ ratio, which tends to inhibit GAPDH activity. This can lead to increased levels of triose phosphates, methylglyoxal and diacylglycerol. This chain of events is also associated with consumption of NAD+ by activated poly(ADP ribose) polymerase, which can be activated by hyperglycaemia via increased production of ROS and DNA strand breaks (Wiernsperger, 2000).

Above and beyond the damage that reactive dicarbonyls can cause through enhancement of glycation and the formation of AGEs, the Maillard reaction between carbohydrates and proteins also generates ROS (Wiernsperger, 2000). Thus, hypoglycaemia simultaneously enhances both glycative and oxidative stress, which together synergistically contribute to the development of diabetic complications.

4) Hexosamine Pathway

In states of excess intracellular glucose, fructose 6-phosphate via glutamine: fructose-6-phosphate aminotransferase can form glucosamine 6-phosphate and then UDP-N-acetylglucosamine, which supports proteoglycan synthesis and the formation of (O)-linked glycoproteins. This pathway has been shown to be related...
to increases in transcription of TGF-α TGF-β1 and PAI-1 and has been implicated in insulin resistance (Brownlee, 1994). Glucosamine infusions in rodents and in humans have been associated with interference with glucose sensing by the beta cell and with insulin sensitivity (Brownlee, 1994).

5) Oxidative Phosphorylation

High glucose concentration increase the mitochondrial proton gradient as a result of overproduction of electron donors by the tricarboxylic acid cycle, which in turn increase production of mitochondrial superoxide (Krieger-Brauer and Kather, 1992). In these experiments, inhibition by Mn-SOD or UCP-1 of hyperglycaemia-induced overproduction of mitochondrial superoxide prevented the increases in polyol pathway flux, intracellular AGE formation, PKC activation, and hexosamine pathway activity in endothelial cells (Garber, 1980).

High glucose concentrations were shown to increase mitochondrial superoxide production, proton leak, lower ATP levels and impaired glucose-induced insulin secretion in islets from wild type but not from UCP-2-knock-out animals (Krieger-Brauer and Kather, 1992), suggesting that superoxide-mediated activation of UCP-2 could play a role in type II diabetes. It has also been reported that a 2 mM glyceraldehyde concentration in 24-h islet incubations increased ROS levels and inhibited insulin secretion, effects that were abrogated by n-acetylcysteine (Habib et al., 1994). Thus, when the glycolytic pathway is swamped by glucose, it seems likely that both mitochondrial and non-mitochondrial pathways contribute ROS to the glucotoxic process that impairs beta cell function (Mahadev et al., 2001).

3.2.7 Other factors generating oxidative stress

i) Hormones

Most type 2 diabetes patients are hyperinsulinemic for a long period. Insulin can stimulate oxidative stress by various mechanisms: the hormone induces production of H$_2$O$_2$ when activating its receptors and, although hydrogen peroxide is not a strong oxidant itself, it can indirectly activate oxidative reaction. Insulin also stimulates the sympathetic nervous system, which leads to activation of neurotransmitters and their enzymatic systems, several of which induce oxidative
stress. For example diabetic vessel walls contain high levels of NAD(P)H oxidase, which may be activated by prenylation of p21rac (Kashiwagi et al., 1999; McCarty et al., 2002). Leptin is another hormone reportedly stimulating oxidative stress (Yamagishi et al., 2001).

ii) Lipids

Increased fasting and postprandial plasma levels of triglycerides, free fatty acids and cholesterol are common in type 2 diabetes. They are known to generate ROS (Ohara et al., 1993; Inoguchi et al., 2000). In the vessel wall, import of-or local formation of oxLDL is a cardinal mechanism involving oxidative stress in the atherosclerotic process.

iii) Varies

Angiotensin II generates oxidative stress in blood vessels by stimulating NADH oxidase and is claimed to mediate the effect of hyperinsulinemia. A major source of oxidative stress in vascular pathophysiology is the alternance of ischemia/reperfusion, since the hypoxic period characterizing ischemia is followed by a brutal oxidative burst suffering from complications such as arthritis or diabetic foot experience numerous daily repetitive episodes of ischemia/reperfusion. These phenomena is even more frequent in patients suffering snoring and/or sleep apnoea and exciting data were recently reported linking degradation of glycemic control with oxidative stress generated along these night episodes, which are particularly frequent in patients presenting with obesity (Boethel, 2002; prabhakar, 2002). Nitric oxide (NO) is both a scavenger and a prooxidant when it is attacked by radical such as becoming transformed into peroxynitrite (Hogg and Kalyanaraman, 1991). It may represent an important contributor to oxidative stress because NO levels are frequently elevated in early stages of diabetes.

3.2.8 Antioxidant strategies to protect the β-cell from hyperglycaemia

The theme of this miniseries is that continuation of high glucose levels after onset of either type 1 or type II diabetes has secondary adverse effects on the beta cell itself, not that glucose toxicity is the initial cause of either disease. In the case of type 1 diabetes it has been published that increased glucose levels are associated
with increased beta cell generation of cytokines (Maedler et al., 2002), which are prooxidants. In a model of type 2 diabetes, high glucose concentrations increased intracellular peroxide levels in islets (Randle et al., 1994).

3.2.8.1 Beneficial effects of antioxidant enzyme over expression in islets

Injections of superoxide dismutase have been reported to act prophylactically against alloxan-induced diabetes (Gopaul et al., 2001). Transgenic animals over expressing superoxide dismutase have enhanced beta cell tolerance to oxidative stress-induce diabetes (Kubisch et al., 1994). Over expression of antioxidant enzymes in β-cell lines provide protection against prooxidants, and combinatorial rather than single over expression of antioxidant enzymes is more efficacious. Transgenic over expression of catalase provided protection for the beta cell against streptozotocin and hydrogen peroxide.

Adenovirus over expression of catalase and superoxide dismutase has been shown to protect human islets (Niskanen et al., 1995) and a beta cell line (Ohara et al., 1993) against oxidative stress. In our laboratory adenoviral over expression of glutathione peroxidase (Paget et al., 1998) and γ-GCL (Ohara et al., 1993) have been shown to protect islets against the adverse consequences of prooxidants on insulin gene expression, insulin content, and insulin secretion.

3.2.8.2 Protection by antioxidant drugs against beta cell oxidative stress

Several antioxidant drugs have been evaluated as protectors against beta cell oxidative stress. N-Acetylcysteine protects against oxidative stress and diabetes in rats and mice (Postic et al., 1999; Kaneto et al., 1999). In both instances, this drug provided preserved insulin content and insulin gene expression as well as PDX-1 binding to the insulin promoter. The oral hypoglycaemic agents’ metformin and troglitazone have antioxidant properties and prevent hyperglycaemia in the rat (Srivastava et al., 1993). Vitamin E has beneficial effects on glycemic control in rats (Ihara et al., 2000). Glicazide, a commonly used in the treatment of type 2 diabetes, has been shown to protect pancreatic beta cells from damage by hydrogen peroxide (Kimoto et al., 2003). These findings suggest that adjunct therapy with antioxidants may represent a useful ancillary pharmacologic approach to the
management of type 2 diabetes.

Antioxidants may act negatively when overdosed. However cells have learned to live with and to some extent to integrate ROS into their biochemistry. Actually ROS are involved in the mediation of cells signals, where they may play positive roles in normal physiology: phagocytosis, insulin signalling or shear-stress induced vasodilation are such examples (Mahadev et al., 2001). It could thus be that at least excessive concentrations of antioxidants interfere with physiological processes and alleviate the beneficial effects of these substances.

Since we know the aetiology of diabetes to a large extent, it is possible to find preventive measures. Interestingly many of our dietary ingredients can not only prevent the occurrence of certain diabetes but also control diabetes. Free radicals mediated oxidative damage is one of the common causes of pancreatic $\beta$-cell dysfunction or damage induced by toxins. Many of our dietary ingredients like polyphenols from tea and gooseberry, curcumin from turmeric etc., are powerful antioxidants and free radical scavengers.

3.2.8.3 Beta cell host mechanisms against chronic oxidative stress

Clinical reports of elevated levels of oxidative stress markers in patients with type 2 diabetes are numerous. Unfortunately, the islet is among the least well endowed tissues in terms of intrinsic antioxidant enzyme expression, including SOD-1, SOD-2, catalase, and glutathione peroxidase (Grankvist et al., 1981; Tiedge et al., 1997). Thus, type 2 diabetes mellitus is associated with elevated markers of chronic oxidative stress, pancreatic islets contain relatively low levels of antioxidant gene expression, and elevated glucose levels down-regulate the rate-limiting enzyme for glutathione synthesis.

3.2.8.4 Natural defence against oxidative stress and antioxidants

 Reactive species can be eliminated by a number of enzymatic and nonenzymatic antioxidant mechanisms. As discussed above, SOD immediately $^{\cdot}\text{O}_2^-$ converts to $\text{H}_2\text{O}_2$, which is then detoxified to water either by catalase in the lysosomes or by glutathione peroxidase in the mitochondria. Another important enzyme is glutathione reductase, which regenerates glutathione that is used as a
hydrogen donor by glutathione peroxidise during the elimination of $H_2O_2$. Maritim et al. (2003) have reviewed in detail that diabetes has multiple effects on the protein levels and activity of these enzymes, which further augment oxidative stress by causing a suppressed defence response. Nonenzymatic antioxidants include vitamins A, C and E, glutathione, $\alpha$-lipoic acid, carotenoids, trace elements like copper, Zinc and selenium, coenzyme Q10 (Co Q10) and cofactors like folic acid, uric acid, albumin and vitamin B$_1$, B$_2$, B$_6$ and B$_{12}$. Alternations in the antioxidant defence system in diabetes have been reviewed (vega-Lopez et al., 2004).

Glutathione (GSH) acts as a direct scavenger as well as a co-substrate for GSH peroxidise. It is a major intracellular redox tampon system. Vitamin E is a fat-soluble vitamin that prevents lipid peroxidation. It exists in 8 different forms, of which $\alpha$-tocopherol is the most active form in humans. Hydroxyl radical reacts with tocopherol forming a stabilized phenolic radical which is reduced back to the phenol by ascorbate and NAD(P)H dependent reductase enzymes (Hensley et al., 2000; Hensley et al., 2004).

Vitamin C (ascorbic acid) increases NO production in endothelial cells by stabilizing NOS cofactor BH4 (Heller et al., 2001). $\alpha$-Lipoic acid is a hydrophilic antioxidant and can therefore exert beneficial effects in both aqueous and lipid environments. $\alpha$-lipoic acid is reduced to another active compound dihydrolipoate. Dihydrolipoate is able to regenerate other antioxidants such as vitamin C, vitamin E and reduced glutathione through redox cycling (Heller et al., 2001). Thus, both experimental and clinical studies summarized in the next sections utilized these naturally occurring antioxidants, especially vitamins C, E and $\alpha$-lipoic acid, in order to delineate the role of oxidative stress in the development of vascular complications of diabetes.

3.2.9 Evidence from experimental models

A multitude of in vivo studies have been performed utilizing antioxidants in experimental diabetic models. The effect of antioxidants on oxidative stress are measured through certain observable biomarkers. These markers include the enzymatic activities of catalase, SOD, GSH-Px and GSH-reductase, as well as
thiobarbituric acid reactants (TBARS) levels, an indirect measurement of free-radical production that has been shown to be consistently elevated in diabetes. Normalization of the activity levels of any of these markers, and ultimately, the balance of free-radical production/removal, would be an effective method to reduce ROS-induced damage (Heller et al., 2001).

Countless publications exist showing existence of various indicators of oxidative stress in vitro and this has finally led to a lively ongoing debate about the pertinence and relevance of parameters such as TBARS, manlondialdehyde, isoprostanes or nitrotyrosines as typical examples (De Zwart et al., 1999). Oral intake of high glucose in animals increases TBARS and reduces the activity of hepatic enzymes susceptible to thiol group oxidation (Folmer et al., 2002). In humans, oxidative stress is also seen in postprandial periods in normal individuals but diabetic patients are unable to compensate for the increased ROS (Ceriello et al., 1998). This increase may be attributed to acute effects of high glucose and/or lipids.

The studies examined the tissue levels of the enzymatic antioxidant defence in diabetes with varying results. Piper et al., (1995) demonstrated that, in experimental diabetes the activity of catalase was increased in vascular tissues with absence of any significant changes in the activity of the other major antioxidant enzymes (superoxide dismutase and glutathione peroxidase). In addition, Wohaieb and Godin (1987) showed increased activities of catalase and superoxide dismutase (SOD) in the pancreas of diabetic rats, while the liver showed a generalized decrease in the activities of catalase, SOD and glutathione peroxidase (GSH-Px). In the study, the increase in the activities of both CAT and SOD occurred in the tissue with the lowest antioxidant enzymatic activities (Pancreas) before onset of diabetes, suggesting a compensatory response to an increase in endogenous oxidant radicals in pancreas by diabetes. A decrease in the concentration of reduced glutathione (GSH) has been observed in erythrocytes from diabetic subjects, as a result of decreases in activities of the enzymes involved in GSH synthesis (such as γ-glutamylcystein synthetase) or in the transport rate of oxidized glutathione (GSSG)
from erythrocytes (Murakami et al., 1989) and enhanced sorbitol pathway (Ciuchi et al., 1996). In addition, a decrease in the activity of glutathione reductase (GSSG-R) which acts to reduce GSSG to GSH has also been reported (Tagami et al., 1992) Kazuhiro et al., (1989) and Matkovics et al., (1998) elucidated that GSSG-R activity decreased in erythrocyte hemolysate of streptozotocin diabetic rats and attributed this decrease to the enzyme glycation by the uncontrolled hyperglycemia (Jain and Mc Vie, 1994).

Also, Jos et al., (1990) and Dominguez et al., (1993) reported a significant decrease of erythrocyte GSH-Px activity in diabetic children and adolescents compared with control subjects. They attribute this decrease to a decline in blood glutathione content in those diabetics, since GSH is a substrate and cofactor for this enzyme. Therefore, low GSH content indicates low GSH-Px activity, which may produce increased oxidative stress propensity. Moreover, enzyme inactivation either through glycation process (Arai et al., 1987) or under conditions of increased oxidative stress might also contribute to low GSH-Px activity (Lyons, 1991).

Increased oxidative stress is also found in the basal state in both types of diabetes, some studies suggesting that it is much more pronounced in type 2 than in type 1 diabetes (Seghrouchni et al., 2002). Type 2 diabetes exhibit increases in TBARS and reduction in catalas activity but, surprisingly correlation was found in a recent study between TABARS and level or duration of hyperglycaemia (Turk et al., 2002). Plasma glutathione (GSH) levels are decreased and oxidized purines increase, illustrating DNA damage (Dincer et al., 2002). In blood vessels, increased levels of superoxide have been recorded in both arterial and venous segments (Guzik et al., 2002).

Many studies report the presence of oxidative stress already in prediabetic stages; for example children have increased oxidative stress and SOD levels at the onset of type 1 diabetes (Dominguez et al., 1998). In glucose-intolerant (IGT) subjects, the increased level of inflammatory cytokines is linked to oxidative processes (Exposito et al., 2002). Increased levels of vitamin A but not vit E, increased TABARS, increased isoprostanestances as well as reduced antioxidants have
all been reported in IGT (Tavridou et al., 1997; Niskanen et al., 1995; Gopaul et al., 2001; Vijayalingam et al., 1996). Data on antioxidants are variable since, according to the origin of the insult and tissue/cell type, antioxidants may even be increased (Paget et al., 1998; Coasta et al., 2002). In view of data with insulin and some lipid fractions, it is likely that normoglycemic insulin-resistant subjects may already exhibit oxidative stress. Indeed obesity, characterized by hyperinsulinemia and dyslipidemia, is accompanied by elevated oxidative stress (Ciccone et al., 1999).

Mulberry-treated diabetic rats showed a significant decrease in fasting blood glucose concentrations indicating a good glycemic control. Increased lipid peroxidation and the activity of catalase (CAT) in erythrocytes observed in diabetic controls were significantly decreased by mulberry leaves (48% and 33%, respectively). Decreased GSH concentrations and the activity of glucose-6-phosphate dehydrogenase and antioxidant enzymes viz., glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and superoxide dismutase (SOD) observed in uncontrolled diabetes were improved (52%, 69%, 151%, 95%, 24% and 106%) by mulberry treatment very efficiently (Bondada and Varadacharyulu, 2003).

Glutathione peroxidase has a key role in enzymatic defense systems and acts on peroxides (H\textsubscript{2}O\textsubscript{2}, lipid or organic peroxides) to remove them. In the present investigation, it was observed that mulberry therapy could effectively normalize the activity of GPx and SOD but only partially restored that of GST (Bondada and Varadacharyulu, 2003).

Ramesh and Pugalendi (2006) have revealed that Diabetic rats had an elevation in the levels of lipid peroxidation markers (Thio barbituric acid reactive substances (TBARS) and lipid hydroperoxides (HP) and a reduction in nonenzymic antioxidants (vitamin C and reduced glutathione (GSH) except vitamin E in the plasma and liver, and enzymatic antioxidants (Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (GPx) in the liver. Umbelliferone treatment has decreased lipid peroxidation markers and enhances antioxidants status. The GSH contents of liver, kidney and heart tissues were augmented by the

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administration of *Vitis vinifera* aqueous extract at a dose of 500mg/kg (Nilufer Orhan *et al.*, 2006).

Hepatic superoxide dismutase, catalase and glutathione peroxidise activities of the STZ-induced diabetic rats were significantly decreased in comparison with the control rats. Those enzymes activities were also increased by administering genistein and ISP to the STZ-induced diabetic rats. The concentration of Thiobarbituric acid reactive substance in the STZ-induced diabetic rats was significantly elevated, while the genistein and ISP supplement decreased it to the control concentration.

Interestingly, even in a healthy population, variations in insulin sensitivity are related to lipid hydroperoxide levels and reduced catalase and Vit E levels (Facchini *et al.*, 2000). Again in the general population, various markers of glucose metabolism and of insulin resistance were associated with oxidative stress (Trevisan *et al.*, 2001). One should also remind that low vitamin E levels revealed to be better predictors of diabetes than age, BMI or smoking (Salonen *et al.*, 1995). Thus, oxidative stress may be a very early event in the long history of diabetes, similar to what is seen for functional microvacular defects (Wiemspenger *et al.*, 2000).

Some of the edible plants such as bottle gourd (*Cucurbita ficifolia*), kidney beans (*Phaseolus vulgaris*), spinach (*Spinacea oleracea*), curcumber (*Cucumis sativus*), cumin seeds (*Cuminum cyminum*), onion (*Allium cepa*), garlic (*Allium sativum*), cauliflower (*Brassica oleracea var botrytis*), bitter guard (*Momordica charantia*), plantain (*Musa sapientum*) are known to have antidiabetes properties (Romans-Ramos *et al.*; 1995; Augusti and Sheela, 1996; Grover *et al.*; 2002; Khanna *et al.*; 1974; Srivastava *et al.*; 1993; Lotliker and Rajarama, 1996).

Studies suggest that *Allium cepa* bulb extract stimulates the β - cells of pancreas. Similarly active principles of *Euphorbia prostrata* and *Ficus bengalensis* stimulate the secretion of insulin from β- cells of pancreas (Akhtar *et al.*, 1984; Augusti *et al.*, 1994). The extract of *Salvia lacendifolia* is reported to increase the number of islets of Langerhans (Jimenez *et al.*, 1995).
3.2.10 Hypolipidaemic Activity

Oxidative stress caused by free radicals damages the endothelial cells in the blood vessels, increases blood cholesterol levels, promotes lipid peroxidation (LPO) and plays a central role in pathogenesis of secondary complications of DM. Hypothetically, since oxidative stress plays a pathogenic role in diabetes, supplementation with antioxidants should attenuate oxidative stress and improve oxidative stress-mediated damage in diabetes.

Diabetes produces disturbances in lipid profiles and, especially, an increased susceptibility to lipid peroxidation (Lyons, 1991). *Fomes fomentarius* is a kind of mushroom of the genus Fomes, native to north of the temperate zone of the northern hemisphere. Crude extract of this mushroom has been used in traditional medicine because of its antioxidation, diuretic, alleviation of fever, antitumor, and anti-inflammatory properties (Yahagi et al., 1999).

The consumption of sapogenin extract from bitter yam has been demonstrated to beneficial by lowering blood glucose and lipid profile in STZ-induced diabetic rats (McAnuff et al., 2003).

Administration of the active fraction (100 mg/kg bw) of *Vernonia anthelmintica* seeds for 45 days resulted in significant reduction in plasma glucose, HbA1C, cholesterol, triglycerides, free fatty acids, phospholipids and HMG-CoA reductase in STZ diabetic rats. In addition to that, significant decrease in plasma insulin, protein, HDL and hepatic glycogen observed in STZ diabetic rats, was normalized after 45 days of treatment with the active fraction of *Vernonia anthelmintica* seeds.

The marked increase in serum triglycerides, total cholesterol, LDL-cholesterol and decreased HDL cholesterol observed in diabetic rats is in agreement with the findings of Nikkila and Kekki (1973). The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia (Mitra et al., 1995). In the present study, administration of the dihydroxy gymnemic triacetate to the STZ-induced diabetic rats significantly (p < 0.05) improved these parameters. The observed hypolipidaemic effect may be because of decreased
cholesterogenesis and fatty acid synthesis. Significant lowering of total cholesterol, triglycerides, LDL-cholesterol and raise in HDL-cholesterol is a very desirable biochemical state for prevention of atherosclerosis and ischaemic conditions (Luc and Fruchart, 1991).

The level of HDL-cholesterol, which increased after Gymnemic triacetate administration, might be due to the increase in the activity of lecithin cholesterol acyl transferase (LCAT), which may contribute to the regulation of blood lipids (Patil et al. 2004). Oral administration of Gymnemic triacetate for 45 days to diabetic rats lowered serum lipids, LDL-cholesterol level in diabetic rats.

Kasiappan et al., 2005 have showed that STZ induced diabetic rats, showed significant increase in the levels of cholesterol, phospholipids, triglycerides and free fatty acids which were considerably restored to near normal in Eugenia jambolana seed kernel or glibenclamide treated animals. The plasma lipoproteins (HDL, LDL, VLDL-cholesterol) and fatty acid composition were altered in STZ-induced diabetic rats and these levels were also reverted back to near normalcy by EJs-Kernel or glibenclamide treatment.

3.2.11 Biochemical parameters associated with Diabetes.

Gymnema sylvestre and Momordica charantia have been studied in some detail for their mechanism of action. Gymnema sylvestre leaf has been shown to stimulate to release and/or secretion of insulin from β— cells of pancreas (Shanmugasundaram et al., 1990; Siddiqui et al., 2000). The aqueous extract of this plant is also reported to inhabit the absorption of glucose from the intestine. The extract is also reported to increase the activities of enzymes responsible for utilization of glucose by insulin dependent pathways (Shanmugasundaram et al., 1999). The same drug can also give protection to β— cells of pancreas from toxic chemicals (Shanmugasundaram et al., 1990).

Viable β— cells of pancreas are necessary for Momordica charantia to exert its anti-diabetic effects. It acts on the β—cells stimulate them to release or secrete more insulin (Karunanayake et al.; 1990; Rokeya et al.; 1995). Further, this plant extract has antioxidant activity and it increase drug metabolizing is enzymes in rat.
liver (Rokeya et al.; 1995). The weight gain was directly associated with peripheral serum insulin levels and total exogenous insulin dose (Edelman Steven, 1998).

The increased insulin levels in diabetics will normalize the plasma and tissue protein levels by increasing protein synthesis or decreasing protein degradation or reducing protein glycosylation. A study with the treatment of active fraction of *Vernonia anthelmintica* seeds ethanol extract. The total protein levels in plasma, of diabetic rats which were lower than in normals, were increased after the treatment with the active fraction.

The liver is the primary site of endogenous glucose production (Roden and Bernroider, 2003) with a minor contribution from the kidney (Cersosimo et al., 1997; Meyer et al., 2004; Stumvoll et al., 1997) and produces glucose either from gluconeogenesis or via glycolysis. Elevated endogenous glucose production is a common abnormality associated with diabetes that, in concurrence with deprived pancreatic function and reduced glucose clearance, contributes to the hyperglycemia characteristic of the disease, diabetes (Wajngot et al., 2001). Insulin regulates the metabolism by modulating the uptake and utilization of glucose in target organs such as liver, kidney, skeletal muscle and adipose tissue by controlling the activities of numerous metabolic enzymes. Recently, Deng et al. have reported that resveratrol enhances muscular glucose uptake via both insulin dependent and non-insulin dependent pathways (Deng et al., 2008).

The daily oral treatment of resveratrol (5 mg/kg body weight) to diabetic rats for 30 days demonstrated a significant ($p < 0.05$) decline in blood glucose and glycosylated hemoglobin levels and a significant ($p < 0.05$) increase in plasma insulin level. The altered activities of the key enzymes of carbohydrate metabolism such as hexokinase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphatase, fructose-1,6-bisphosphatase, glucose-6-phosphate dehydrogenase, glycogen synthase and glycogen phosphorylase in liver and kidney tissues of diabetic rats were significantly ($p < 0.05$) reverted to near normal levels by the administration of resveratrol (Palsamy and Subramanian, 2009).

Bitter gourd is shown to ameliorate the total sugar and uronic acid contents
during diabetes and was correlated to increase in non-enzymatic glycation of tissue proteins and the resultant non-enzymatic glycation adducts, including serum proteins, hemoglobin, and increase in the content of sugar nucleotides during diabetes (Spiro, 1985; Bucala and Vlassara, 1995).

Serum enzymes including AST and ALT are used in the evaluation of hepatic disorders. An increase in these enzyme activities reflects active liver damage. Inflammatory hepatocellular disorders result in extremely elevated transaminase levels (Foreston et al., 1985; Hultcrantz et al., 1986). In accordance with these findings, streptozotocin treatments has a significant role in the alteration of liver functions since the activity of AST and ALT were significantly higher than those of normal value. On the other hand, treatment of the diabetic rats with the garlic extract caused reduction in the activity of these enzymes in plasma compared to the mean values of the diabetic group and this is in agreement with that of Sheweita et al. (2001).

The significant reduction in the activities of AST, ALT and ALP and the levels of urea, uric acid and creatinine on administration of active fraction of Vernonia anthelmintica seeds ethanol extract, indicated the protective effect of extract on liver and kidney of diabetic rats (Shaik et al., 2009). Treatment with bitter yam steroidal sapogenin extract resulted in a significant decrease in the activities of the kidney transaminases compared to the normal and diabetic control groups (Marie et al., 2006).

The reduction in liver enzyme activities (El-Demerdash et al., 2005) is mainly due to leakage of these enzymes into the blood stream as a result of alloxan toxicity which leads to the liver damage. A possible explanation for the differential effects of onion and garlic on the activities of AST, ALT, LDL, ALP and ACP in plasma and liver is that these treatments may inhibit the liver damage induced by alloxan.

Streptozotocin treatment has a significant role in the alteration of liver functions since the activity of AST, ALT, ALP and ACP was significantly higher than those of normal value. On the other hand, treatment of the diabetic rats with
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dihydroxy gymnemic triacetate caused reduction in the activity of these enzymes in plasma compared to the mean values of the diabetic group (Pitchai Daisya, 2009).

Inflammatory hepatocellular disorders result in extremely elevated transaminase levels (Foreston et al. 1985; Hultcrantz et al. 1986). In accordance with these findings, streptozotocin treatments has a significant role in the alteration of liver functions since the activity of AST, ALT, ACP and ALP were significantly higher than those of normal value. Administration of *Gymnemic triacetate* improved the liver function by decreasing the serum ALT, AST, ACP and ALP levels in diabetic rats.

Oral administration of novel compound dihydroxy gymnemic triacetate partially improved the body weight after 45 days of treatment. In uncontrolled or poorly controlled diabetes, there is an increased glycosylation of a number of proteins, including Hb (Alberti and Press, 1982). The level of HbA1c is monitored as a reliable index of glycemic control in diabetes (Gabbay, 1976) and useful in the management of diabetes mellitus. Significant fall in glycosylated hemoglobin indicated the efficiency of the compound in glycemic control.

The oral administration of resveratrol to diabetic rats regulated the activity of glycogen metabolizing enzymes by stimulating the remnant β-cells to secrete more insulin thereby normalized the altered glycogen content (Palsamy and Subramanian, 2009). Administration of *Gymnemic triacetate* increased the skeletal muscle and liver glycogen content. Improvement of plasma protein was observed after oral administration eremanthin to diabetic rats (Eliza et al., 2009).

Jeong-Sook (2006) had reported that a significant reduction in glucose-6-phosphate was observed in the groups treated with genistein and ISP in comparison with the diabetic control. The supplementation increased the plasma insulin level but decreased the HbA1c level of the streptozotocin-induced diabetic rats. It increased the glucokinase level of STZ-induced diabetic rats. Glycosylated hemoglobin can be used as an excellent marker of overall glycemic control. Since it is formed slowly and does not dissociate easily, it reflects the real blood glucose level (Bunn et al., 1981; Guoyan et al., 1992) Glycosylated hemoglobin had been

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found to increase in patients with diabetes mellitus (Baskaran et al., 1990). Oral administration of eremanthin decreased hyperglycemia and therefore the level of HbA\textsubscript{1c} decreased. Treatment with Gymnemic triacetate to STZ-induced diabetic rats for 45 days reduced the HbA\textsubscript{1c} level.

An increase in the glycoprotein components namely hexoses, fucose, sialic acid was observed in hyperlipidemic patients with/without diabetes. Increase in glycoprotein level could also be due to increased synthesis to repair the damaged membrane structure by peroxidation (Kaviarasan et al., 2005).

Sirichai et al., 2005 have reported that p-methoxycinnamic acid (p-MCA) have reduced the excessive activities of hepatic glucose-6-phosphatase, hepatic hexokinase, glucokinase and phosphofructokinase in diabetic rats and increased hepatic glycogen in these rats.

The glycogen content of the skeletal muscle and liver, which markedly decrease in diabetes (Prasannan and Subrahmanyam, 1965; Grover et al., 2000), increased significantly in the compound treated animals as compared to the diabetic control. This may be due to the activation of glycogen synthase system by the compound treatment. Administration of Gymnemic triacetate increased the skeletal muscle and liver glycogen content (Perfumi et al., 1991).

Treatment with ethanolic extract of seeds of Eugenia jambolana prevented the depletion in liver and skeletal muscle glycogen content in alloxan induced diabetic rabbits (Sharma et al., 2003). Similar to the above findings, an increase in skeletal muscle and liver glycogen content was found in STZ-diabetic rats by the oral administration of eremanthin. STZ induced a significant ($P < 0.001$) increase in renal glycogen content this was almost normalized by Caralluma sinaica ($P < 0.001$) (Mohammed Habibuddin, 2008). The increase in plasma bilirubin (hyperbilirubenimia) may be resulted from the decrease of liver uptake, conjugation or increase bilirubin production from hemolysis and this finding coincides with the decrease in total erythrocyte counts (data not showh). Also, the elevation in plasma bilirubin indicates liver damage as confirmed by the changes in the activities of plasma and liver enzymes.
3.3 STREPTOZOTOCIN (STZ) ADMINISTRATION

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is synthesized by Streptomyces achromogenes and is used to induced both IDDM and NIDDM, respectively. The range of the STZ dose is not as narrow as in the case of alloxan. The frequently used single intravenous dose in adult rats to induce IDDM is between 40 and 60 mg/kg b.w. (Ganda and Rossi 1976), but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single dose below 40mg/kg b.w. may be ineffective (Katsumata et al., 1992).

![Structure of Streptozotocin](image)

**Fig. 3.4 Structure of Streptozotocin**

STZ may also be given in multiple low doses. Such treatment is used predominantly in the mouse and the induction of IDDM is mediated by the activation of immune mechanisms. NIDDM can easily be induced in rats by intravenous or intraperitoneal treatment with 100 mg/kg STZ on the day of birth. This method of NIDDM induction was described for the first time by Portha et al., (1974). At 8-10 weeks of age and thereafter, rats neonatally treated with STZ manifest mild basal hyperglycaemia, an impaired response to the glucose tolerance test (Portha et al., 1979) and a loss of β-cell sensitivity to glucose (Giroix et al., 1983).
Fig.3.5 The mechanism of streptozotocin (STZ) induced toxic events in β-cells of rat pancreas. MIT-mitochondria; XOD-xanthine oxidase

Streptozotocin action in β-cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, the hyperglycaemia is observed with a concomitant drop in blood insulin. About six hours later, hypoglycaemia occurs with high levels of blood insulin. Finally, hyperglycaemia develops and blood insulin levels decreased (West et al., 1996). These changes in blood glucose and insulin concentrations reflect abnormalities in β-cell function. STZ impairs glucose oxidation (Bedoya et al., 1996) and decreased insulin biosynthesis and secretion (Bolaffi et al., 1987, Nukatsuka et al., 1990 b). It was observed that STZ at first abolished the β cell response to glucose. Temporary return of responsiveness then appears which is followed by its permanent loss and cells are damaged (West et al., 1996).

Intracellular action of STZ results in changes of DNA in pancreatic β cells comprising its fragmentation (Yamamoto et al., 1981, Morgan et al., 1994). Recent experiments have proved that the main reason for the STZ-induced β-cell death is alkylation of DNA (Delaney et al., 1995, Elsner et al., 2000). The alkylation activity of STZ is related to its nitrosourea moiety, especially at the O-6 position of guanine.
After STZ injection to rats, different methylated purines were found in tissues of these animals (Bennett and Pegg, 1981).

Since STZ is a nitric oxide (NO) donor and NO was found to bring about the destruction of pancreatic islet cells, it was proposed that this molecule contributes to STZ-induced DNA damage (Kroncke et al., 1995, Morgan et al., 1994). The participation of NO in the cytotoxic effect of STZ was confirmed in several experiments (Turk et al., 1993, Kroncke et al., 1995). On the other hand, the lowering of NO concentration in pancreatic islet cells by inhibition of the inducible form of nitric oxide synthase partially counteracted DNA cleavage induced by STZ (Bedoya et al., 1996).

STZ was found to generate reactive species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells (Takasu et al., 1991; Bedoya et al., 1996). The formation of superoxide anions results from both STZ action on mitochondria and increased activity of xanthine oxidase. It was demonstrated that STZ inhibits the Krebs cycle (Turk et al., 1993) and substantially decreases oxygen consumption by mitochondria (Nukatsuka et al., 1990b). These effects strongly limit mitochondrial ATP production and cause depletion of this nucleotide in β cells (Nukatsuka et al., 1990b, Sofue et al., 1991).

Augmented ATP dephosphorylation increases the supply of substrate for xanthine oxidase (β cells possess high activity of this enzyme) and enhances the production of uric acid the final product of ATP degradation (Nukatsuka et al., 1990a). Then, xanthine oxidase catalyses reaction in which the superoxide anion is formed (Nukatsuka et al., 1988). As a result of superoxide anion generation hydrogen peroxide and hydroxyl radicals are formed (Nukatsuka et al., 1990a, Takasu et al., 1991). The inhibition of xanthine oxidase by allopurinol restricts the cytotoxic effect of STZ in vitro.

Pre-treatment of β cells with this inhibitor prevented the STZ-induced decrease of insulin secretion (Nakatsuka et al., 1990a). It can be stated that potent alkylating properties of STZ are the main reason of its toxicity. NO and reactive oxygen species can act separately or form the highly toxic peroxynitrate...
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(Figure 2.3). Therefore, intracellular antioxidants or NO scavengers substantially attenuate STZ toxicity.

3.3.1 Mechanism of STZ in diabetes

Streptozocin is a diabetogenic agent that is thought to damage pancreatic \( \beta \)-cells by causing DNA and chromosomal damage via alkylation and/or free radical generation. The DNA damage causes a critical reduction in NAD, which leads to dysregulation of cellular functions and eventually cell death. While this hypothesis is generally accepted, it remains to be determined if the STZ-induced alkylation and free radical generation work together or independently to cause DNA damage. Due to these different chemical properties of STZ, further work is required to better understand the precise mechanism by which STZ destroys pancreatic cells and causes diabetes mellitus.

![Fig. 3.6 Mechanism of STZ in diabetes](image)

The Okamoto model hypothesizes that STZ causes DNA damage, which leads to a critical reduction of NAD levels thus causing cell death. The current proposal is that once inside the cell, STZ causes free radical generation (1a), which

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can cause DNA damage (2) or STZ decomposes to carbonium ion (1b), Carbonium ion can cause DNA damage via alkylation(2), The damaged DNA is repaired by activated poly (ADP-ribose) synthetase (3), which uses the cells source of NAD (4). The decreased levels of NAD (5) lead to cellular dysfunction and eventually cell death (6).

3.4 HISTOPATHOLOGY

3.4.1 Liver

Histopathological examination of liver in control animals showed normal hepatic lobules. The central venule with radiating columns of liver cells of normal shape and size were seen. There were no signs of congestion, inflammation, cellular necrosis or cholestasis in control liver sections. Liver sections of STZ-diabetic control rats showed appreciable histological changes compared to controls. It was with multifocal areas of hepatocellular vacuolations and hypertrophy. Focal areas of chronic inflammation with eosinophil infiltration were also seen. Livers of STZ-diabetic rats treated with chromium picolinate showed similar pathological changes as those of STZ-diabetic control rats; however their intensity was much lower. Diabetes mellitus is associated with increased frequency of hepatic histopathologic lesions. The most common lesions seen are an increase in liver glycogen leading to vacuolization in cytoplasm and hepatocyte nuclei (Glick, 1987).

Taeho et al., 2006 showed that the untreated control rat showed a well hepatic lobule structure. The hepatocytes had a rich acidohilic cytoplasm and a round nucleus. The hepatic laminae were also kept to one layer. The L-ascorbic acid treated rats without diabetes also showed a similar result to the untreated control. However, the STZ-induced diabetic rat, on the contrary, displayed microvesicular steatosis with variable sizes in the cytoplasm of hepatocytes. Nuclei had an irregular form around the central vein. This histological change directly indicates the type 1 diabetes induced hepatotoxicity. On the contrary, the hepatic lesions were lowered by the administration of L-ascorbic acid to diabetic rats, which hepatocytes showed an aspect similar to normal control group being arranged generally evenly. Nuclei of hepatocytes also had a similar shape to the nuclei of the control group.
3.4.2 Kidney

Histological examination of the sections of kidneys from STZ-diabetic rats showed marked microscopic changes like multifocal areas of moderate cortical tubular vacuolations and interstitial mononuclear cell infiltration. Dilatation of cortical tubules especially at the corticomedullary junction was also observed. However, the classical signs of diabetic nephropathy such as nodular lesions of glomerulus were not evident. The incidence and intensity of tubular vacuolations and interstitial cellular infiltration in STZ-diabetic rats treated with chromium picolinate was much lower compared to the diabetic control kidneys. Histological examination of kidney sections of STZ-diabetic rats showed multiple areas of tubular vacuolations with tubular epithelial hypertrophy. Multifocal tubules also revealed degeneration of epithelium characterized by eosinophilic appearance with pyknosis of nuclei. However, these changes of epithelial hypertrophy and degeneration were significantly lower in the kidney sections of STZ-diabetic rats treated with chromium picolinate. Kidney sections of healthy rats treated with chromium picolinate showed no pathological changes and were comparable to those of control rats (Glick, 1987).

Yong-Gui et al., 2006 reported that rats in the Diabetes group had an increase in the glomerular tuft and mesangial area and glomerular volume when compared with the values in the control group. Mycophenolate mofetil treatment ameliorated the increase of the glomerular tuft and mesangial area and glomerular volume. Rats in Diabetic group had an increase in the tubulointestinal injury index when compared to the control group. Mycophenolate mofetil treatment was associated with a reduction in the tubulointestinal injury index as compared with DM group; but this did not reach statistical significance.

The characteristic renal changes occurring in early stages of diabetic nephropathy include an increase in glomerular filtration rate (GFR) due to hyperfiltration, renal and glomerular hypertrophy, and accumulation of extracellular matrix (ECM) proteins (Mason & Wahab, 2003). Glomerular hypertrophy precedes glomerular basement membrane (GBM) thickening, leading to glomerular hyperfiltration, microalbuminuria and the development of proteinuria. Advanced mesangial matrix expansion reduces the surface area of glomerular capillaries.

Studies on the Effect of Artificially Cultivated Ganoderma lucidum on Streptozotocin Induced Diabetic rats.
available for filtration, leading to declining glomerular function (Steffes et al. 1993). Progressive loss of renal function correlates with the development of tubulointerstitial fibrosis in addition to glomerulosclerosis (Gilbert & Cooper, 1999). Glomerular hypertrophy occurs in both Type 1 and Type 2 diabetic patients in both the early and the late stages of disease, although the mechanisms leading to increase in glomerular size may differ (Moriya et al. 2000; Ramage et al. 2002). Previous studies (Osterby & Gunderson, 1980; Hirose et al. 1982; Nyengaard & Rasch, 1993) provide convincing evidence of glomerular capillary growth and glomerular hypertrophy as an earliest response to hyperglycaemia in diabetic nephropathy. Evidence from both human and animal studies indicates that glomerular filtration surface area is increased in the early stages of diabetes, and quantitative morphological (stereological) studies have shown this to be the result of increases in length, surface area and number of capillaries per glomerulus (Osterby & Gunderson, 1980; Nyengaard & Rasch, 1993).

Results from several studies indicate that capillary growth contributes to glomerular growth prior to focal and segmental glomerulosclerosis (FSGS) (Yoshida et al. 1989; Doi et al. 1990; Fogo et al. 1990; McKay et al. 1990; Osterby, 1992). Nagata et al. (1992) found an increase in the length of capillaries in glomeruli following unilateral nephrectomy, and Nyengaard & Rasch (1993) demonstrated a significant increase in the surface area, length and number of capillaries in streptozotocin (STZ)-induced diabetic rats at 10 days.

Vascular endothelial cells are a major target of hyperglycaemia-induced damage, but the mechanisms underlying this damage are not completely understood. Hyperglycaemic injury may induce microvascular changes via glucose-induced activation of protein kinase C (PKC), increased formation of advanced glycation end-products (AGE) and increased glucose flux through the aldose reductase pathway (Koya et al. 2000; Nishikawa et al. 2000; Brownlee, 2001). The production of various growth factors and cytokines, including vascular endothelial growth factor (VEGF), may be an important mediator of the vascular changes observed in diabetic nephropathy. VEGF is a potent cytokine known to stimulate angiogenesis and markedly increase microvascular permeability. Up-regulation of VEGF and VEGF receptor-2 (VEGF-R-2) have been reported in diabetic nephropathy (Tsuchida et al.
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1999; Mason & Wahab, 2003). Tsuchida et al. (1999) demonstrated that long-term administration of an AGE inhibitor (OPB-9195) afforded renoprotection and decreased renal VEGF mRNA and glomerular VEGF immunoreactivity in a rat model of Type 2 diabetes. Recent studies have identified VEGF as a pathogenic link between hyperglycaemia and early renal dysfunction in diabetes (Vriese et al. 2001; Flyvberg et al., 2002). Cooper et al. (1999) administered a neutralizing VEGF antibody to db/db mice and observed attenuation of glomerular hypertrophy, basement membrane thickening and urinary albumin excretion. VEGF antibody administration also reduced the expansion in total mesangial volume of db/db mice without impact on body weight, blood glucose, insulin levels or food consumption (Flyvberg et al. 2002). Vriese et al. (2001) also used VEGF blockade via neutralizing antibody administration in STZ-induced diabetic rats and observed decreased hyperfiltration, albuminuria and glomerular hypertrophy in these animals. It is unclear if the increased length of the glomerular capillaries observed with progressive diabetic nephropathy in the present study is associated with angiogenesis or the lengthening of existing capillaries. Angiogenesis occurs as a result of migration and proliferation of endothelial cells, endothelial tube formation, and maturation of blood vessels. The angiogenic factor angiopoietin (Ang)-1, a ligand for Tie-2, is involved in the recruitment and attachment of pericytes during embryonic angiogenesis (Suri et al. 1996). By contrast, Ang-2 competitively inhibits binding of Ang-1 (Maisonpierre et al. 1997). Collectively, Ang-1, Ang-2 and Tie-2 play pivotal roles in the formation of the renal vasculature and glomeruli during kidney development (Woolf & Yuan, 2001). Recently, Yamamoto et al. (2004) demonstrated the therapeutic effects of tumstatin, an angiogenic inhibitor, in ameliorating early glomerular hypertrophy, hyperfiltration and urinary albumin excretion in STZ-induced diabetes in rats. Increased renal expression of VEGF, flk-1 and Ang-2 were inhibited by tumstatin treatment in these animals.

3.4.3 Pancrease

DM is a pathological condition, resulting in severe metabolic imbalances and non physiological changes in many tissues, where oxidative stress plays an important role in the etiology (Baynes and Thorpe, 1996). Oxidative stress markers in diabetic...
rates and increased ROS was observed in pancreatic islets. Diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycaemia, thereby depleting the activity of the antioxidative defence system and thus promote free radical generation (Baynes and Thorpe, 1996). Such models include alloxan or streptozotocin (STZ) induced diabetic rats and mice.

Studies with electron microscope showed that STZ causes picnotic and lobular nuclei, swelling in granular endoplasmic reticulum cisternae and dilations in intracellular areas between pancreatic β-cells (Bolkent et al., 2000). In another study, it was demonstrated that type 2 diabetes increases number of immature secretory granules, mitochondria and of hypertrophied cytoplasmic organelles such as golgi complex and endoplasmic reticulum (Hong et al., 2002). In STZ - induced diabetes, specific toxicity of STZ on pancreatic β-cells have been studied for many years in various experimental studied (Das et al., 1996; Aughsteen, 2000; Hong et al., 2002).

Razieh et al., 2005 when compared with the untreated diabetic rats, histopathological examination of the *Teucrium polium* plant extract treated diabetic rats revealed an increase in the number of pancreatic islets and the number of β-cells, along with a reduction in the number of initiated lymphocytes and macrophages. In other words, the plant extract treated diabetic samples histopathologically approach the corresponding healthy pancreatic samples. The regeneration of the β-cells of the STZ-destructed islets is probably due to the fact that pancreas contains stable (Quiescent) cells which have the capacity of regeneration. Therefore, the surviving cells can proliferate to replace the lost cells (Kumar et al., 1992; Govan et al., 1986).

The study was carried out morphologically by electron microscopes and biochemically in order to detect whether acarbose used in diabetic treatment and *Rumex patientia* used in folk medicine for decreasing blood glucose had an effect on pancreatic β cells.