EXPERIMENTAL DIABETES MELLITUS AND OXYGEN FREE RADICALS

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ABSTRACT

Oxygen free radicals (OFRs) have been implicated as a causal or contributory factor in various disease states. These oxygen radicals are highly reactive species and cause cellular damage. In diabetes, various sources may lead to an increase in the production of OFRs. These sources include glucose oxidation, autoxidative glycosylation, alteration in sorbitol pathway activity and changes in level of inflammatory mediators. It is hypothesized that the complications in diabetes mellitus could be due to an increase in the levels of OFRs as a result of its increased production and/or its decreased destruction.

To test this hypothesis diabetes was induced in male Sprague Dawley rats by streptozotocin (STZ). The rats were sacrificed after 1st, 2nd, 3rd, 4th, 5th and 6th week (wk) of development of diabetes. The various tissues (liver, heart, pancreas, aorta and kidney) and blood involved either in the development or in the complications were selected for the study. Plasma glucose levels were measured to confirm the diabetic state of the animal. Malondialdehyde (MDA) content (product of lipid peroxidation) and antioxidant enzymes [Catalase (CAT), Glutathione-peroxidase (GSH-Px) and Superoxide dismutase (SOD-Total, Cu-Zn and Mn)] were measured in blood and tissues in control and streptozotocin induced diabetic rats.

An increase of 3-4 fold in plasma glucose levels and

decrease in body weight was observed in diabetic rats as compared to controls at each time interval. MDA levels were increased in all tissues in the diabetic state. With the progression of the disease liver, aorta, kidney and blood showed significant increases in MDA, being maximal on the 6th wk. However, the pancreas showed the highest MDA levels up to the 4th wk. Catalase activity increased significantly in all diabetic tissues as compared to controls at most of the time intervals except the kidney which showed a significant increase on the 1st wk and after that the activity decreased. In aorta and blood the activity increased with time. However, activity decreased in the liver and the heart on the 5th and 6th wk as compared to the 4th wk. Its activity in pancreas decreased with time. GSH-Px activity increased significantly in all the diabetic tissues as compared to controls. Liver, aorta and blood showed increased activity of GSH-Px with progression of disease. Heart and pancreas showed lower activity on the 5th and 6th wk as compared to the 4th wk. The kidney did not show any change. Total SOD and Cu-Zn SOD activity in diabetic tissues showed a significant increase as compared to controls at most of the time intervals. Liver, pancreas and aorta showed decreasing trend after 4 wks; however heart and kidney showed no change. Mn-SOD activity was variable in control and diabetic tissues. While the activity decreased in liver and heart at 4th wk, it increased in heart at 6th wk. No changes were observed in pancreas and

kidney. In general, pancreas and aorta showed the lowest antioxidant enzyme activities and higher MDA content as compared to other tissues.

The results of the present study suggest that oxidative stress is associated with the diabetic state and starts at early onset of disease. The marked changes in MDA and antioxidant enzymes suggest higher production of OFRs in diabetes which may cause oxidative damage to tissues leading to various complications.

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ABBREVIATIONS

$\cdot O_2^-$	superoxide anion
$\cdot OH$	hydroxyl radical
α	alpha
AA	arachidonic acid
ADP	adenosine diphosphate
AGPs	advanced glycosylation endproducts
$AH\cdot$	ascorbic acid free radical
ALX	alloxan
BB	biobreeding
BSA	bovine serum albumin
CAT	catalase
CH_3^+	carbonium ions
CMV	cytomegalo virus
Cu-Zn	copper-zinc
DAG	diacylglycerol
DETAPAC	diethylenetriaminepentaacetic acid
DMTU	dimethylthiourea
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fe_2^+	ferrous ion
Fe_3^+	ferric ion
GSH	glutathione reduced
GSH-Px	glutathione-peroxidase
GSSG	glutathione oxidized

H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
Hb	hemoglobin
HLA	histocompatibility antigens
HOCl	hypochlorous acid
IDDM	insulin dependent diabetes mellitus
IFN-γ	interferon gamma
IL	interleukin
k	first order rate constant
K^+	Potassium ion
KCN	potassium cyanide
LDL	low density lipoproteins
M	transition metal
MDA	malondialdehyde
mg	milligram
MHC	major histocompatibility complex
mM	millimolar
Mn	manganese
MPO	myeloperoxidase
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADH	nicotineamide dinucleotide reduced
NADPH	nicotinamide adenine dinucleotide phosphate reduced
$NaHCO_3$	sodium bicarbonate
NH_4Cl	ammonium chloride

NIDDM	non-insulin dependent diabetes mellitus
NOD	non obese diabetic
O_2	oxygen
OFRs	oxygen free radicals
PGI_2	prostacyclins
PMNL	polymorphonuclear leukocytes
PUFA	polyunsaturated fatty acids
R·	conjugated diene
RBC	red blood cells
RNA	ribonucleic acid
ROO·	lipid peroxide radical
ROOH	lipid hydroperoxide
ß	beta
SDS	sodium dodecyl sulphate
Secs	seconds
SOD	superoxide dismutase
STZ	streptozotocin
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TCA	trichloroacetic acid
TNF	tumor necrosis factor
TXA_2	thromboxane A_2
U	units
Vit-E	α-tocopherol

Vit-C	vitamin-C
wk	week

1. LITERATURE REVIEW

1.1 DIABETES

Diabetes is defined as a complex demonstrable abnormality in metabolism resulting in hyperglycemia (Grodsky *et al.* 1982). Diabetes mellitus is currently classified into "idiopathic diabetes mellitus" and diabetes or glucose intolerance associated with genetic syndromes and other conditions [National Diabetes Data Group International Work Group, 1979; WHO Technical Report on Diabetes mellitus, 1985). The idiopathic category is further subdivided into two major groups:

Type I : insulin dependent (IDDM, Juvenile onset diabetes)

Type II : non insulin dependent (NIDDM, maturity onset diabetes)

This separation is based on family, twin, metabolic, immunologic and HLA associated studies (Tatterall and Fajans, 1975). IDDM is characterized by massive beta cell lesions and necrosis and insulin secretion is either substantially reduced or nonexistent. Although the causes are unclear a direct attack on the beta cell by chemical toxins or auto-immune mechanisms is implicated (Grodsky *et al.* 1982). NIDDM is characterized by significant insulin production varying from less than normal to above normal but always in quantities

insufficient to maintain glucose homeostasis (Craighead, 1988). Genetic influences are a key factor in the occurrence of type II diabetes. It is interesting to note that in NIDDM, administration of insulin corrects blood glucose homeostasis. The defect has been identified as a reduction in response of pancreatic ß-cells to increased glucose load and a deficiency of insulin receptors on all surface of tissues (Gavin *et al.* 1974).

1.2 PATHOGENESIS OF IDDM:

A vareity of causes have been implicated in ß-cell destruction associated with Type I diabetes mellitus. Mononuclear infiltration of the islets is a factor of IDDM suggests that immune, or autoimmune processes, environmental factors, viruses, chemical agents etc. are involved (Gepts and Lecompte, 1981; Nerup and Lernmark, 1981).

1.2.1 HLA : Histocompatibility antigens (HLAs) are proteins found in nucleated cells. The genes that code HLAs are located at chromosome number 6 and have been localized at loci A, B, C, D and DR and are responsible for transplanted tissue rejection. HLA antigens have been associated with IDDM (Nerup, 1978). Type I diabetes has been reported to be associated with HLA-B_8, Bw-15, DR_3 and DR_4. Singal and Blajchman (1973) first found a significant association for HLA-Bw15. Later Nerup *et al.* (1974) confirmed and found an association with HLA-B_8. HLA-D_4 has various subtypes, of which HLA-Dw_4 is associated with Type I diabetes (Platz *et al.*

1981). Studies have shown that autoimmune destruction of ß-cells takes place in man, Biobreeding rat (BB) and NOD mouse. The genetic studies indicate that one diabetogenic gene within the MHC (Major histocompatibility complex) is necessary for development of this disease (Hattori *et al.* 1986).

1.2.2 VIRUSES: In a variety of animal species viral infections can cause diabetes. Some of these include encephalomyocarditis virus (EMC) as in man, mengovirus 2T and reovirus type I and 3 in mice, coxsackie B virus in monkey and rubella virus in hamsters (Yoon *et al.* 1987; Rayfield *et al.* 1986). Yoon and Coworkers (1987) showed that coxsackie B3, coxsackie B4, reovirus type 3 and mumps can infect human ß-cells *in vitro* and destroy them.

1.2.3 AUTOANTIBODIES: The viral infection appears to trigger an immune response directed against ß-cells. Islet cell surface antibodies and insulin antibodies are commonly found in these patients (Ginnberg-Fellner *et al.* 1984). The inclusions of cytomegalo virus (CMV) causing death in children and infants has been reported in islets of Langerhans in 50% of cases (Jensen *et al.* 1980). The viral infection can act as a non specific precipitating factor by causing insulin resistance and increased insulin needs which cannot be met by damaged ß-cells (Palmer and Lernmark, 1990).

1.2.4 CYTOKINES: Cytokines (polypeptides), produced by activated cells of the immune system have been implicated as mediators of islet ß-cell immune injury (Robinovitch *et al.*

1992). Interleukin-1 (IL-1), a cytokine product of activated macrophages and natural killer cells, was the first cytokine reported to inhibit insulin release and to be cytotoxic for islets *in vitro* (Bendtzen *et al.* 1986). Tumor necrosis factor (TNF), cytokine product of macrophages and interleukin-6, and cytokines produced by T-lymphcytes, interferon gamma (INF-γ) and lymphotoxin have been reported to impair insulin secretion and/or destroy islet cells (Mandrup Poulsen *et al.* 1987; Campbell *et al.* 1988; Sandler *et al.* 1990).

1.2.5 CHEMICAL AGENTS: Specific drugs or chemicals like alloxan, streptozotocin, pentamidine and vacor have been reported to be cytotoxic for ß-cells. Alloxan, a uric acid derivative, was the first drug discovered to be cytotoxic for ß-cells and is frequently used to induce IDDM in experimental animals. Streptozotocin is structurally very different than alloxan and is used to induce chemical diabetes. It also causes ß-cell lysis (Mordes and Rossini, 1981). Pentamidine (4-4-diamidino-diphenoxypentane) is a drug now commonly used in the treatment of Pneumocytis carinii pneumonitis and is the recognized cause of drug induced diabetes (Bouchard *et al.* 1981). Vacor (N-3-pyridylmethl N-p-nitrophenyl urea) is a rodenticide whose ingestion in high doses in humans in suicide attempts, causes IDDM (Pont *et al.* 1979). Alloxan and streptozotocin are discussed in detail under the heading Experimentally Induced Diabetes.

1.2.6 DIETARY CONSTITUENTS: Dietary constituents have also

been implicated in the pathogenesis of Diabetes mellitus. Alterations in dietary protein has been reported to decrease the incidence to diabetes. In NOD mouse, diet containing animal proteins especially casein when replaced by hydrolyzed aminoacid base, and in BB-rat, L-amino acids added to laboratory chow reduce the frequency of IDDM (Elliott and Martin, 1984; Elliott *et al.* 1988)

1.3 COMPLICATIONS OF DIABETES

Complications of diabetes are an indirect manifestation of metabolic stress resulting from altered insulin homeostasis and energy metabolism (Baynes, 1991). Every tissue and organ is biochemically and structurally altered as consequence of hyperglycemia of diabetes. Glucose reversibly binds to any free amino groups and can cause structural and functional abnormalities of involved proteins (Baynes, 1991). Modification of long lived extracellular proteins (e.g. crystallins, collagens, elastins, laminin, myelin sheath proteins) and structural changes in tissues rich in these proteins (lens, vascular wall, basement membrane) are associated with complications of diabetes such as retinopathy, microangiopathy, atherosclerosis, cardiomyopathy, neuropathy and nephropathy (Grodsky *et al.* 1982).

1.3.1 Retinopathy : Lesions of small blood vessels have been reported in retina of diabetics, which causes a variety of changes in retinal circulation (Larsen, 1960). Appearance of new blood vessels, proliferation of fibrous tissue in the

retina, basement membrane thickening, dilation of venules and microaneurysyms occur. They are accompanied by haemorrhage and leaking of retinal vessels and patches of retinal edema (Larsen, 1960). Ashton (1963) reported that diabetic retinopathy appears to be the response to retinal ischemia. Because of increased levels of fibrinogen, α-2globulin (Kwaan *et al.* 1972) increased red cell aggregation and platelet aggregation is reported in diabetics which causes sluggish blood flow through capillaries because of elevated levels of von Willebrand factor(VIII) and increased production of thromboxane A-2 by platelets (Coldwell 1975). Formation of water insoluble aggregates in lens crystallins in diabetics has been reported to be responsible for cataract formation (Perry *et al.* 1987).

1.3.2 Angiopathy: This term refers to damage to linings and basement membranes of blood vessel. It increases the risk of coronary heart disease and can lead to retinopathy and nephropathy (Hassen *et al.* 1986; Michael *et al.* 1990).

1.3.3 Atherosclerosis and Cardiomyopathy: The extent and severity of both atherosclerosis and cardiomyopathy increases in diabetics. Thrombotic occlusions of medium and large atherosclerotic vessels are increased in diabetics. Thus myocardial and cerebral infracts are extremely common complications of diabetics. Diabetics accumulate lipoproteins rich in triglycerides and cholesterol. Increasing levels of VLDL and low HDL are reported in diabetics (Hunt *et al.*

1990). These findings are consistent with higher incidence of coronary heart disease, myocardial infarction. In the process of atherogenesis sorbital accumulation in cells of vessels and platelet aggregation plays an important contributory role in endothelial cell injury (Gupta *et al.* 1992; Kinoshita and Nishimura, 1988). These factors result in a major cause of morbidity and mortality in diabetic patients (Baynes, 1991).

1.3.4 Neuropathy: Diabetic neuropathy causes decreased motor and sensory nerve conduction velocities, axonal degeneration and demyelination. Lesions also occur in proliferation of endoneurium and reduplication of basement membrane of nerve fibres, endoneurial blood vessels and the perineurium (Greene *et al.* 1990). Tuck *et al.* (1984) reported reduced blood flow and endoneurial hypoxia in STZ induced diabetic neuropathy in the rat. Endoneurial hypoxia has also been reported in neuropathic patients. The possible causes of impaired nerve perfusion include increased vascular reactivity, deficits in vascular endothelium, production of vasodilator prostacyclin and nitric oxide, increased synthesis of vasoconstrictor prostanoids, and elevated blood viscosity (Greene *et al.* 1990). Microvascular atherogenesis is dependant on advanced glycosylation end products (AGPs). Canon *et al.* (1992) reported that advanced glycosylation end products contribute to the aetiology of early diabetic neuropathy, possibly by acting via a vascular mechanism.

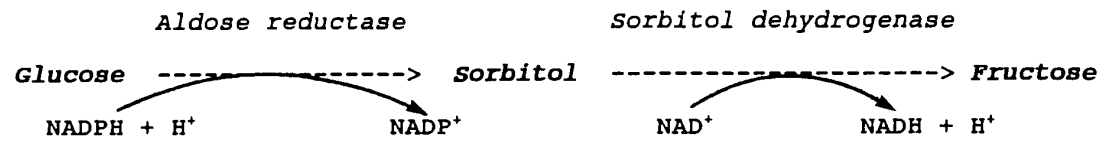
1.3.5 Nephropathy:

Changes in glomerular basement membrane and glomerular filtration rate are detectable in diabetics. Capillary damage is caused by angiopathy, which results in reduction of filtering capabilities of the kidneys (Bank *et al.* 1989). Winegrad (1987) reported that glomeruli in diabetics is injured in two ways : protein denaturation by high glucose levels and adverse effect of intraglomerular hypertension. Hyperfiltration and associated intrarenal hypertension is reported by Hostetter *et al.* (1981). Zatz *et al.* (1985) found that proteinuria was present by 6 months and glomerulosclerosis by 12 months in STZ induced diabetic rats. STZ induced diabetic rats showed increased single nephron glomerular flow and reduced nephron mass. Kidneys showed thickened basement membrane, reduced filtration surface area followed by segmental glomerular sclerosis (Michael *et al.* 1990).

1.4 Biochemical mechanisms for Complications:

Various biochemical mechanisms that occur in these tissues are:

1.4.1 Sorbitol (Polyol) Pathway: The sorbitol or Polyol pathway, which converts glucose to sorbitol, has been implicated in the pathogenesis of many diabetic complications (Cogan, 1984). The metabolism of glucose by the Polyol pathway consists of 2 reactions:



The first reaction is the reduction of glucose to sorbitol by aldose reductase. The reaction uses NADPH as the hydrogen donor and favours a decrease in the NADPH/$NADP^+$ ratio. In the second reaction, the enzyme sorbitol dehydrogenase oxidizes sorbitol to fructose. The reaction uses NAD^+ as a hydrogen acceptor and favours an increase in the NADH/NAD^+ ratio (Morrison *et al.* 1970). Aldose reductase has low affinity for glucose and at normal concentrations in non-diabetics, a very small percentage of total glucose is utilised by this pathway. During hyperglycemia, metabolism of glucose by this pathway has been reported to account for 33% of total glucose utilisation in rabbit lens and 11% in human red blood cells (Kinoshita and Nishimura 1988; Morrison *et al.* 1970; Gonzalez *et al.* 1984). Depletion of NADPH during diabetes leads to the accumulation of the oxidized form of glutathione (GSSG) which inactivates critical membrane proteins. Craighead (1988) reported that excessive accumulation of sorbitol and fructose causes excessive amounts of water entering cells from the extracellular compartment which causes cell swelling and damage (Gonzalez *et al.* 1984). Increased flux through the Polyol pathway is associated with decreased Na^+/K^+ ATPase

activity and increased production of vasoconstrictor prostaglandins in many tissues of hyperglycemic injury (Lee *et al.* 1989; Simmons and Winegrad, 1989; Tesfamarium *et al.* 1991). Decrease in Na^+/K^+ ATPase activity decreases the release of endothelium-derived relaxing factor (NO) or a closely related molecule (Gupta *et al.* 1992). Aldose reductase inhibitors have been reported to correct sorbitol pathway linked biochemical abnormalities (Bank *et al.* 1989). In animals inhibitors of aldose reductase have been shown to prevent microangiopathy (Cohen, 1985; Robinson *et al.* 1985).

1.4.2 Protein Kinase Activity and synthesis of Diacylglycerol: Hyperglycemia is associated with increased cellular protein kinase C activity in cultured endothelial cells, resulting from enhanced synthesis of diacylglycerol (DAG) from glucose (Lee *et al.* 1989). DAG is synthesized by acylation of glycerol -3-phosphate generated as a by product of glycolysis. Increased DAG and associated protein kinase C have been observed in retina and glomeruli of diabetic rats (Lee *et al.* 1989; Craven and DeRubertis, 1989). Protein kinase C is involved in signal transduction, response to hormones, growth factors and neurotransmitters. In vascular cells it has been shown to modify growth rate, DNA synthesis, hormone receptor turnover and contraction in vascular smooth muscle cells (Tesfamarium *et al.* 1991). Williamson *et al.* (1990) showed that in granulation tissue exposed to elevated glucose levels and activation of protein kinase C have been linked to

increased inflammation in vascular wall.

1.4.3 Endothelial Cells and Abnormalities of Haemostasis: Porla *et al.* (1981) reported that in patients with diabetic retinopathy, endothelial cells produce higher amounts of Factor VIII, which promotes microthrombus formation. Prostacyclin (PGI_2) is also produced in endothelial cells and is a powerful vasodilator which antagonises platelet aggregation and platelet adherence to the vascular wall. Carreras *et al.* (1980) reported decreased PGI_2 production from vascular walls in diabetic rats and reduced circulatory PGI_2 in diabetic patients (Jennings *et al.* 1987). Plasminogen activator, which converts plasminogen to plasmin acting to promote fibrinolysis, has been reported to be low in diabetes (Hassen *et al.* 1986). These abnormalities cause altered platelet function. Thromboxane A_2 release is increased in platelets from patients with vascular complications (Butkus *et al.* 1982). It causes platelet aggregation. Platelets also carry mediators of microcirculation such as serotonin and platelet derived growth factors (Sacchni *et al.* 1985). In IDDM, increased platelet aggregation is described by measuring proteins released from platelet aggregates such as β-thromboglobulin and platelet factors (Dallinger *et al.* 1987). The combination of reduced endothelial cell production of PGI_2 activators of fibrinolysis, increased platelet activity with increased factor VIII production produces a thrombotic tendency. This leads to microthrombin formation and small

vessel occlusions and contributes to abnormalities of blood flow in small vessels and target organ into which the vessels are distributed.

1.4.4 Advanced Glycosylation Endproducts (AGPs):

Advanced glycosylation endproducts are a complex group of sugar-derived covalent adducts and crosslinks. Non-enzymatic glycation involves the Malliard reaction (Moonier *et al.* 1991). Condensation of a sugar, with a free amino group results in the formation of Schiff's base, which then undergoes rearrangement to the more stable Amadori product, of which glycosylated hemoglobin is the best example. The Amadori product is also degraded into a variety of highly reactive carbonyl compounds such as 3-deoxyglucosone and sugar fragmentation products, which react again with free amino groups to form a variety of advanced glycosylation endproducts (Uitto *et al.* 1982). AGPs are resistant to degradation and continue to accumulate indefinitely on long lived proteins. They are qualitatively identified by their characteristic "brown pigment fluorescence", "protein browning" and participation in protein crosslinking (Reynolds, 1965). Shilton and Walton (1991) showed *in vivo* glycation of the enzyme alcohol dehydrogenase in man. Inhibition of the glycation process by nucleophilic hydrazine aminoguanidine has been reported to prevent glucose-induced vascular dysfunction in a variety of diabetic tissues (Tilton *et al.* 1990). Tanaka *et al.* (1988) suggested that AGP formation has a

nonlinear dependence on sugar concentration. The rate of formation is 2nd order with respect to concentration of glycated amino groups, suggesting that even modest elevation of glucose significantly increases AGP accumulation. Recent evidence suggests that AGP formation increases by a variety of oxidative processes (Hicks *et al.* 1989; Baynes *et al.* 1991).

1.4.5 Effects of AGPs on Extracellular matrix:

Occlusion of diabetic vessels involves accumulation of plasma protein deposits and extracellular matrix. Low density lipoproteins (LDL) and IgG are covalently trapped by reactive AGP precursors on long lived matrix proteins of blood vessels (Brownlee *et al.* 1985). These cross linked plasma proteins provide additional sites for AGP formation. A variety of structural proteins such as α, β and γ-crystallins can be glycated both *in vivo* and *in vitro* (Steven *et al.* 1978). Srivastava *et al.* (1989) reported that glycation of aldose reductase increases the activity of the enzyme. AGPs formation on matrix proteins interferes with site specific interactions among various components that are important for maintaining normal basement membrane structure and function (Rathi *et al.* 1989). AGP formation on laminin causes decreased polymer self assembly and decreased binding to type IV collagen and decreased binding of heparan sulfate proteoglycan (Charonis *et al.* 1990) to the basement membrane. Permanent loss of basement membrane binding sites for heparan sulfate proteoglycan contributes to irreversible vascular

leakiness and stimulates overproduction of matrix components by cytokines (Ruoslahti and Yamaguchi, 1991). Matrix accumulation of AGPs further accelerates diabetic vascular occlusion by blunting the effect of vasodilatory factors. The endothelium derived relaxing factor nitric oxide (NO) is quenched by AGPs in a dose dependent fashion. In diabetic animals, defects in vasodilatory response to NO correlate with the level of accumulated AGPs and are prevented by inhibition of AGP formation (Bucala *et al.* 1991). Formation of advanced glycosylation products on bone matrix alters its ability to induce bone formation and probably involves alteration of binding sites for extracellular proteins (Fong *et al.* 1993).

1.4.6 Effect of AGPs on cell/matrix interactions:

Alterations in the function of a variety of macrovascular and microvascular cells are induced by interactions with AGP modified matrix proteins. It has been reported that monocytes and macrophages found in atherosclerotic plaques have specific high affinity receptors for AGPs. When these cells interact with AGP modified proteins, they secrete tumor necrosis factor α (TNF), interleukin-1 (IL-1) and insulin like growth factor I (IGF-I) which stimulate glomerular synthesis of Type IV collagen and proliferation of endothelial, mesengial and smooth muscle cells (Vlassara *et al.* 1988). Esposito *et al* (1989) showed that macrovascular endothelial cells express AGP specific receptors. AGP receptor binding causes increased permeability and modulation of cell surface coagulant

properties. It has been shown that modification of cell binding domains of type IV collagen causes decreased endothelial cell adhesion (Tsilibary and Charonis, 1990) and modification of mesengial cell matrix by AGP also decreases cell proliferation by 50% (Crowley *et al.* 1991).

1.4.7 Effects of AGPs on DNA and nuclear proteins :

Diabetes induces increased basement membrane collagen production (Brownlee and Spiro, 1979). Cagliero *et al.* (1991) suggested that euglycemic diabetic animals show continuous increase in matrix component mRNA. The occurrence of this phenomenon suggests a role for irreversible intracellular modifications affecting gene transcription. Glucose-6-phosphate can also react both with amino groups of DNA nucleotides and histones *in vitro*, and thus Amadori products are formed which have been identified histologically in endothelial cells from diabetic patients (Kelly *et al.* 1989). AGPs formed from glucose-6-phosphate and glyceraldehyde-3-phosphate induce strand breakage, base modification, and apurinic/apyrimidinic sites in DNA (Mullokandov *et al.* 1991). Lee and Cerami (1987) showed that in prokaryotic systems, formation of AGPs in DNA has been associated with structural changes, mutations, and altered gene expression. Similarly, when human endothelial cells are cultured in 20 mM glucose, there is an increase in single strand breaks and an increase in DNA repair synthesis. Similar results have been reported in lymphocytes from

chronically hyperglycemic diabetic patients (Lornezi *et al.* 1986).

1.5 OXYGEN FREE RADICALS

Oxygen presents a unique problem for aerobic organisms. It plays a dual role, being essential for life on the one hand and producing a range of free radicals, aldehydes and peroxides on the other which have long term toxic effects (Adelman *et al.* 1988). Harman (1956) first suggested that under normal physiological conditions oxygen consumption generates potentially deleterious free radicals and hydroperoxides.

1.6 Nature of Free Radicals:

Free radicals are defined as atoms or molecules containing an unpaired electron in their outermost orbital (Southorn and Powis, 1988). They are highly reactive species. Both organic (eg. quinones) and inorganic (eg. oxygen) molecules can exist as free radicals and are transient, therefore unstable. Free radicals are formed by gaining an electron (reduction) or by losing an electron (oxidation).

$O_2 + e^- \text{------} > \cdot O_2^-$ (1)

$AH_2 - e^- \text{-----} > AH\cdot - e^- \text{--------} > A$ (2)

$A{:}B \text{----------} > A\cdot + B\cdot$ (3)

Where O_2 is molecular oxygen, $\cdot O_2^-$ Superoxide anion, AH_2 ascorbic acid, $AH\cdot$ ascorbic acid free radical and e^- represents an electron. Molecular oxygen is biradical having one

unpaired electron in each of its two orbitals. These electrons spin in the same direction, thereby reducing its reactivity (Taube, 1965). When molecular oxygen acquires one electron at a time, it forms radicals with greatly increased reactivity. These radicals are termed "oxygen free radicals" or oxyradicals (OFRs). Free radicals are generated as intermediates during many enzymatic and non-enzymatic reactions (Farber *et al.* 1990).

1.7 Sources of Free Radicals: Free radicals can be produced in the body from a variety of sources:

1.7.1 Metabolism of Oxygen: Oxygen free radicals are formed during normal cellular metabolism. During oxidative phosphorylation, the mitochondrial cytochrome oxidase enzyme system causes production of ATP by tetravalent reduction of oxygen to water.

$$O_2 + 4H^+ + 4e^- \text{----------} > 2H_2O \quad (4)$$

In this process the partially reduced oxygen free radical intermediates are tightly bound to the active site of the enzyme and present no threat to the cell (Southorn and Powis, 1988). A small amount of oxygen is reduced by single electron additions: univalent reduction of oxygen generates superoxide ($\cdot O_2^-$), whereas divalent reduction generates hydrogen peroxide (H_2O_2). Trivalent reduction of oxygen, or reaction of $\cdot O_2^-$ with H_2O_2, (Haber-Weiss reaction) generates the highly reactive

hydroxyl radical ($\cdot OH$). By acquiring another electron, the hydroxyl radical is converted into water (Aust *et al.* 1985).

$$O_2 \xrightarrow{e^-} \cdot O^-_2 \xrightarrow{e^- + 2H^+} 2H_2O_2 \xrightarrow{e^- + H^+} \cdot OH \xrightarrow{e^- + H^+} H_2O$$

$$H_2O$$

(Sequential univalent reduction of molecular oxygen)

From various studies it has been shown that ubiquinone cytochrome-b region in the mitochondrial electron transport chain is the major site of $\cdot O_2^-$ production, probably due to autoxidation of ubisemiquinone with NADH dehydrogenase. Dihydro-orotate dehydrogenase also contributes to superoxide anion formation (Freeman and Crapo, 1982).

H_2O_2 so formed is metabolised to H_2O by catalase or glutathione peroxidase (GSH-Px) enzymes.

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2H_2O \qquad (6)$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \qquad (7)$$

Where GSH = reduced form of glutathione;

GSSG = glutathione disulphide (oxidized form of glutathione)

If H_2O_2 is produced at a rate faster than its metabolism, then it may react with $\cdot O_2^-$ to form very reactive and unstable

hydroxyl radicals ($\cdot OH$) by a metal (Me^{n+}) chelate catalyzed Haber-Weiss reaction (reactions 8-9) or iron catalyzed Fenton reaction (reactions 10-11).

$$O_2^{\cdot -} + Me^n \text{ chelate} \text{ ------> } Me^{n-1} \text{ chelate} + O_2 \quad (8)$$

$$Me^{n-1} \text{ chelate} + H_2O_2 \text{ -----> } Me^n \text{ chelate} + OH^- + OH^{\cdot} \quad (9)$$

$$Fe^{2+} + H_2O_2 \text{ ------------> } Fe^{3+} + OH^{\cdot} + OH^- \quad (10)$$

$$Fe^{3+} + O_2^{\cdot -} \text{ ------------> } Fe^{2+} + O_2 \quad (11)$$

The overall reaction can be summarized as follows (reaction 12):

$$O_2^{\cdot -} + H_2O_2 \text{ ------------> } O_2 + OH^- + \cdot OH \quad (12)$$

Iron and copper are the most important metals which catalyze the formation of $\cdot OH$ from $\cdot O_2^-$ and H_2O_2. Both of these metals are present in the body bound to proteins. Iron is present in the body bound to ferritin, hemoglobin, transferrin, and various other hemoproteins. Circulating iron is bound to transferrin in the Fe^{3+} form. The transferrin-Fe^{3+} complex is internalized by endocytosis, iron is released in Fe^{2+} form (Kojima and Bates 1979; Morgan 1979) and is bound to ferritin, a storage protein. Low pH favours release of iron from transferrin. Ferritin also stores iron in the Fe^{3+} form. Release of iron from ferritin requires reduction to the Fe^{2+} form. Superoxide anion radicals generated by xanthine oxidase (Thomas *et al.* 1985) or by activated polymorphonuclear leukocytes (Biemond *et al.* 1984) have been shown to cause release of iron from ferritin. Ferritin also releases iron under acidic conditions. During various pathological

conditions including diabetes, ischemia and hypoxia, acidosis develops which favours release of iron from ferritin. The iron so released can participate in Fenton or Haber-Weiss reaction to form ·OH radicals. Hemoglobin (Sadrzadeh *et al.* 1984), methemoglobin and metmyoglobin (Kanner and Harel, 1985) have been reported to promote lipid peroxidation by $\cdot O_2^-$ dependent ·OH radical formation.

Copper can also catalyze ·OH formation. Copper is present in serum bound to ceruloplasmin. However, free copper concentrations have been found in cerebrospinal fluid, sweat and synovial fluid of rheumatoid arthritis patients. Free radicals have been proposed to cause cytotoxicity in rheumatoid arthritis. Free copper can participate in Haber-Weiss reaction to form OH· and cause cytotoxicity (Morgan, 1979).

1.7.2 Polymorphonuclear Leukocytes (PMNLs): PMNLs on stimulation by soluble (chemotactic factors) or particulate (phagocytizable particles) stimuli, release lysosomal components and exhibit a burst in oxygen consumption (termed respiratory burst) coincident with the generation of reactive oxygen metabolites or radicals (Babior 1978; Badwey and Karnovsky, 1980). As soon as the PMNLs are activated, a membrane bound NADH or NADPH dependent oxidase enzyme is activated and oxidizes cytoplasmic NADH/NADPH to $NAD^+/NADP^+$ and shuttles the resultant electrons to oxygen to form superoxide anion ($\cdot O_2^-$). This radical undergoes dismutation to form H_2O_2.

H_2O_2 so formed is either metabolised to water or it can undergo a Haber-Weiss or Fenton reaction to form hydroxyl radicals. Neutrophils also contain an enzyme myeloperoxidase (MPO) in their azurophilic granules which is released into phagosomes and also into the cytoplasm following stimulation of neutrophils. MPO can transfer two electrons from the halide ions to H_2O_2 to yield highly reactive hypohalous acid (Klebanoff 1968; Harrison and Schultz, 1976).

$$MPO + H_2O_2 + X^- \text{----------} > HOX \qquad (13)$$

Since Cl^- ions are present in most abundant amount, hypochlorous acid (HOCl) appears to be most prevalent hypohalous acid formed by the PMNLs. HOCl so formed can chlorinate amines, amino acids, thioethers, aromatic and other unsaturated carbon groups (Thomas, 1979). HOCl can chlorinate endogenous and exogenous amino acids to N-chloramines which are long lived and very potent oxidizing species (Weiss, 1986; Grisham *et al.* 1984).

1.7.3 Autoxidation of Small Molecules: Intracellular free radical production occurs when a wide variety of soluble cell components undergo oxidation-reduction reactions. These molecules include thiols (Baccanari, 1978), hydroquinones (McCord and Fridovich, 1970), catecholamines (Misra and Fridovich, 1972) and flavins (Ballou *et al.* 1969) When they undergo autoxidation they yield superoxide anion.

1.7.4 Enzymatic Production of Free Radicals: Enzymes like xanthine oxidase, aldehyde oxidase (Rajagopalan, 1980) and

dihydro-orotate-dehydrogenase (Aleman and Handler, 1967) produce free radicals during their catalytic cycles. Of these enzymes, xanthine oxidase is the most important source of free radicals. Human xanthine oxidase exists as NAD^+ dependent dehydrogenase which does not produce free radicals. During ischemia, it is proteolytically converted to the oxidase form (Chambers *et al.* 1985) which catalyzes the univalent oxidation of purine substrates with concomitant formation of $\cdot O_2^-$ and H_2O_2 (McCord and Roy, 1982; Chambers *et al.* 1985).

1.7.5 Non-Enzymatic production of free radicals: Non-enzymatic reactions which produce oxygen free radicals include reactions of oxygen with organic compounds, ionizing radiations, photochemical smog, ozone, pesticides and xenobiotics (Pryor *et al.* 1983; Ames *et al.* 1987).

1.8 Reactivity of Free Radicals: OFRs can attack a wide spectrum of biological substrates.

1.8.1 Superoxide Anion ($\cdot O_2^-$): Superoxide anion can react with a variety of biological substrates as an oxidant or reductant (Halliwell and Gutteridge, 1984). It has been shown to oxidize a number of biological substrates including ascorbic acid, α-tocopherol (Nishikimi and Yagi, 1977), catecholamines (Wolin and Belleni, 1985), hemoproteins and thiols (Halliwell and Gutteridge, 1990). It can also reduce a number of biological substrates including quinones, ferricytochrome-c, and transition metal complexes (McCord and Ray, 1982; Fridovich, 1983; Weiss, 1986). Superoxide anion is reported

to undergo protonation in acid pH and becomes a stronger

$$\cdot O_2^- + H^+ \text{ ----------> } HO_2\cdot \qquad (14)$$

oxidant. The protonated form can oxidize amino acids, fatty acids, α-tocopherol, etc. at significant rates (Weiss and LoBuglio, 1982).

1.8.2 Hydrogen peroxide: Hydrogen peroxide is a strong oxidant but reacts slowly with most of the organic substrates (Fee and Valentine, 1977). H_2O_2 can diffuse far from its site of production and can cross the cell membrane. In intracellular locations its biological effects are greater. It also reacts with transition metal ions and their inorganic or organic complexes to yield highly reactive intermediates.

1.8.3 Hydroxyl Radical (·OH): Hydroxyl radical is an oxidant of comparable high reactivity and most damaging among free radicals. It is an extremely potent oxidant and unlike $\cdot O_2^-$ or H_2O_2, it is indiscriminate in attacking almost all biological substrates (Southorn and Powis, 1988). It is involved in three types of reactions: addition reactions; hydrogen extractions, and electron transfers. The extreme reactive nature of ·OH suggests that this radical reacts with the substrates close to its site of generation. It can not travel larger distances in the biological system. However, it may produce substances which are stable and toxic. These products of ·OH radical may diffuse considerable distances to attack targets distant from their site of generation.

1.9 OFRs and Cellular Damage:

OFR's have been implicated in an increasing number of pathological, physiological and toxicological states (Aust *et al.* 1985; Prasad *et al.* 1988; 1992; Rao *et al.* 1983; Granger *et al.* 1981; McCord and Roy, 1982; Fridovich, 1983; Jolly *et al.* 1984). OFRs are highly unstable and hence, are very reactive moieties produced within cells (Pryor, 1986). They react with almost every molecule available in the cell. Various targets of free radical attack include proteins, nucleic acids and membrane lipids.

1.10 Targets of Free Radical Attack:

1.10.1 Proteins: The susceptibility of proteins to free radical damage depends on protein composition (Pryor, 1986). Proteins containing tryptophan, tyrosine, phenylalanine, methionine, cysteine and histidine can be attacked by free radicals (Freeman and Crapo, 1982). Free radicals, ($\cdot O_2^-$ or H_2O_2) can react with iron of hemoproteins and render them inactive. Examples of this type of reaction include formation of methemoglobin ($Hb\text{-}Fe^{3+}\text{-}O_2$) from oxyhemoglobin ($Hb\text{-}Fe^{2+}\text{-}O_2$) [Weiss and Lo Buglio, 1982] and inactivation of catalase to ferroxy (Compound III) and ferryl (compound II) states (Kono and Fridovich, 1982). Hydrogen peroxide is known to inhibit Cu-Zn superoxide dismutase in a two step reaction. Hydrogen peroxide reduces enzyme bound Cu^{2+} to Cu^{+} and then reacts with Cu^{+} to form hydroxyl radical. This hydroxyl radical then attacks the active site of the enzyme at histidine residue and

renders the enzyme inactive (Hodgson and Fridovich, 1975). Inactivation of SOD by H_2O_2 and of catalase by $\cdot O_2^-$ and oxidation and inactivation of sulphydryl enzymes causes the altered redox state (Aust *et al.* 1985) which may lead to enhanced cytotoxicity of these radicals.

1.10.2 Nucleic Acids and DNA: Lippman (1981) proposed that free radical flux from the respiratory chain can cause transcriptional errors, random RNA errors, cross linking and DNA scissions. Base hydroxylation of nucleic acids, nicking, cross linkage and scissions of DNA have been reported to cause mutations and inhibition of protein, nucleotide and fatty acid synthesis (Southorn and Powis, 1988). Ionizing radiation induced cell mutations and death have been linked to free radical induced damage to DNA (Ward, 1977; Freeman and Crapo, 1982). Hydroxyl radicals have been held responsible for death of more than 80% of cells in radiation induced cell killing in eukaryotic as well as prokaryotic cells. These radicals cause chromosomal aberrations due to nucleic acid base modifications or DNA strand scission (Brawn and Fridovich, 1981).

1.11 Lipid Peroxidation:

Lipid peroxidation has been defined as oxidative deterioration of polyunsaturated fatty acids (PUFA, fatty acids containing more than two double bonds) (Halliwell, 1984). PUFA of subcellular organs and biomembrane phospholipids are susceptible to oxidative attack by free radicals (Mead, 1976). The oxidative attack results in the formation of lipid

peroxides, hydroperoxides, conjugated dienes and aldehydes (Freeman and Crapo, 1982). Peroxidation of fatty acids containing three or more double bonds will produce malondialdehyde (Ohkawa *et al.* 1979).

Lipid peroxidation occurs into three steps:

1.11.1 Initiation : Lipid peroxidation can be initiated by sufficiently reactive oxidizing species (·OH) which extract a hydrogen atom from methylene carbon, since methylene carbon has lowest bond dissociation energy. This results in the formation of lipid carbon radical as one unpaired electron is left on that carbon atom. The resulting lipid radical undergoes rearrangement to form a conjugate diene.

$$RH + {\cdot}OH \longrightarrow R{\cdot} + H_2O$$

$$R{\cdot} + O_2 \longrightarrow ROO{\cdot}$$

Where as RH is a poly unsaturated fatty acid, ·OH is hydroxyl radical, R· is lipid radical.

1.11.2 Propagation: Conjugated diene reacts with O_2 and forms a lipid peroxy radical (ROO·). The peroxy radical has sufficient oxidising potential and attacks the adjacent methylene carbon of PUFA. The reaction results in the formation of lipid hydroperoxides (ROOH) and a new alkyl radical (R·).

$$ROO{\cdot} + RH \longrightarrow ROOH + R{\cdot}$$

1.11.3 Termination: The chain reactions are terminated when two radicals collide to form a non-radical product. This reaction is a termination reaction.

$$R^{\cdot} + R^{\cdot} \text{ ----------> } RR$$

$$ROO^{\cdot} + ROO^{\cdot} \text{ ----------> } O_2 + ROOR$$

$$R^{\cdot} + ROO^{\cdot} \text{ ----------> } ROOR$$

Lipid peroxidation results in loss of cell membrane integrity, permeability, fluidity and ultimately cell death (Barber and Bernheim, 1967). Hydroperoxides formed during lipid peroxidation are toxic and can lead to inactivation of enzymes by oxidation of susceptible amino acids (methionine, histidine, cysteine and lysine) or by a chain polymerisation reaction. Aldehydes formed can diffuse greater distances from their site of generation and can cause toxic effects on distant targets. Malondialdehyde formed by oxidation of PUFA can cause crosslinking of membrane components (Nelson, 1981). Crosslinking of MDA with primary amines yields a fluorescent conjugated Schiff's base products (Dillard and Tappel, 1971). MDA also reacts with RNA, DNA and enzymes leading to intracellular and plasma membrane damage (Fongelman *et al.* 1980).

1.12 Cellular Defence Against Free Radicals: In order to minimize the toxic effect of OFR's, organisms have developed defenses which limit the oxidant load by quenching OFR's

before they damage vital cellular components. Oxidative stress in biological systems originates as a result of imbalance between oxidizing species/cellular antioxidant defenses. The scavengers of these free radicals can be chemical antioxidants or specific enzymes.

1.12.1 Chemical Antioxidants: Cells contain various antioxidants which reduce free radical species to less toxic forms. α-tocopherol (vitamin E), a lipid soluble antioxidant, partitions between cell membranes and prevents radical induced damage (Nishikimi and Yagi, 1977; Halliwell, 1984). It also terminates the chain reaction of lipid peroxidation. Vitamin E reacts with free radicals to form a stable and non-reactive vitamin E radical. Ascorbic acid is a water soluble antioxidant present in the cells. It reacts with free radicals to convert them into non reactive metabolites. Ascorbic acid also serves to maintain tocopherols in a reduced active form (Tappel, 1969). A variety of other compounds including cysteine, cysteamine, methionine etc. are present in the cells. These compounds could act as scavengers of free radicals by donating electrons from their sulfhydryl groups (Halliwell and Gutteridge, 1990).

1.12.2 Enzymes: The removal of damaging oxygen products is catalyzed by antioxidant enzymes. Various enzyme systems act as scavengers of free radicals. Enzymatic scavengers include superoxide dismutase, catalase and glutathione peroxidase. Superoxide radical, the first free radical formed from the

reductive metabolism of oxygen undergoes a spontaneous dismutation reaction to give hydrogen peroxide. Cells have an enzyme, superoxide dismutase (SOD), which enhances the rate of dismutation reaction by 10^9 times (Fridovich, 1978).

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Superoxide dismutase is a metalloenzyme containing either copper and zinc or manganese in its molecule. These metals are essential for the activity of SOD. Manganese bound SOD (Mn-SOD) is present in the mitochondria (membrane bound) while copper zinc SOD (CuZn-SOD) is in the cytosol. The H_2O_2 so formed is metabolized by catalase or glutathione peroxidase (GSH-Px) to water and oxygen. At low concentrations of H_2O_2, GSH-Px appears to be preferably responsible for metabolism of H_2O_2 (Cohen and Hochstein, 1963). H_2O_2 reacts with reduced glutathione (GSH) to form oxidized glutathione (GSSG) and water. This reaction is catalyzed by GSH-Px. The enzyme glutathione reductase (GSH-Rd) regenerates GSH from GSSG by using NADPH formed in the pentose phosphate pathway. GSH-Px also catalyzes the reduction of lipid peroxides by GSH and hence prevents the propagation reaction of lipid peroxidation (Chance *et al.* 1979).

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

These enzyme systems act in a cooperative manner. CuZn-SOD is inhibited by high concentrations of H_2O_2 (Bray *et al.* 1975; Blech and Borders, 1983). Catalase and GSH-Px, by metabolizing H_2O_2, prevent its accumulation and hence the activity of SOD is preserved. Superoxide anion is reported to inhibit glutathione peroxidase (Blum and Fridovich, 1985) and catalase (Kono and Fridovich, 1982).

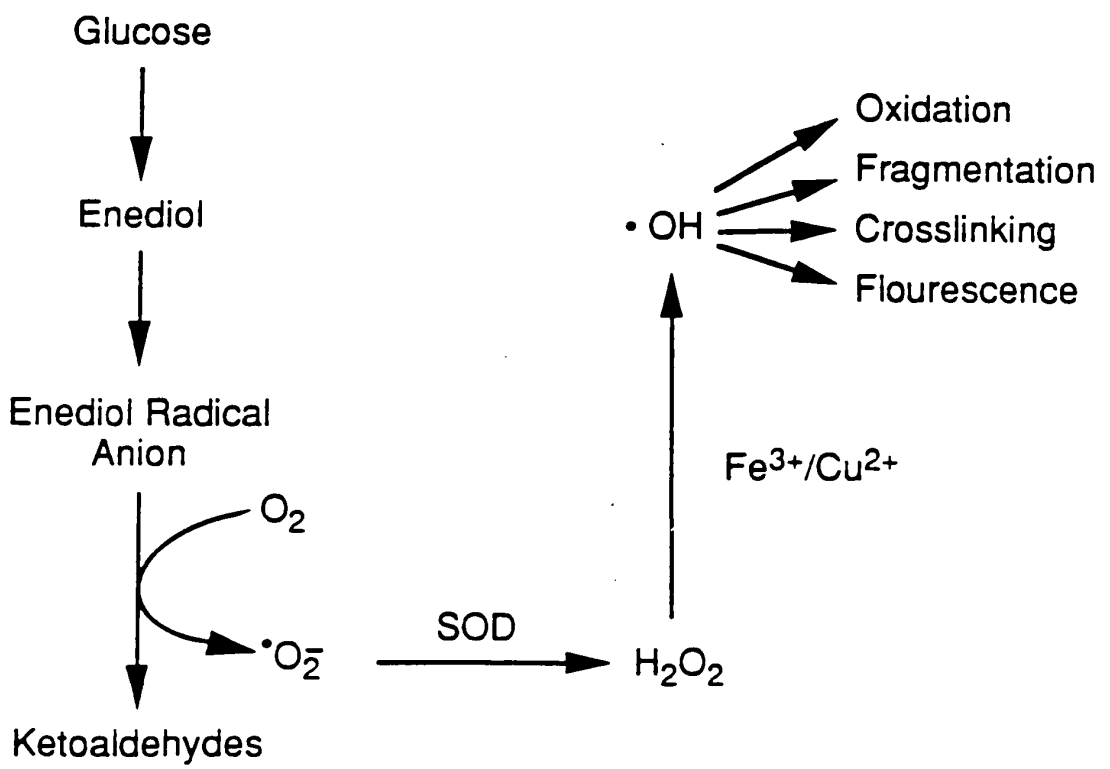
1.13 OFRs and Pathophysiology of Various Diseases: OFRs have been implicated in a number of disease states including: heart failure (Prasad *et al.* 1992); ischemia-reperfusion injury to heart (Prasad *et al.* 1992; Bolli, 1991; Rao *et al.* 1983), intestine (Granger *et al.* 1981; Parks *et al.* 1982), liver (Adkison *et al.* 1986), and kidney (Paller *et al.* 1984); catecholamine induced cardiomyopathy (Singal *et al.* 1982); atherosclerosis (Prasad and Kalra, 1989; 1992); inflammatory diseases (Petrone *et al.* 1980); adriamycin induced cardiomyopathy (Lee *et al.* 1991) and streptozotocin induced diabetes (Gandy *et al.* 1982).

1.14 ROLE OF OXYGEN FREE RADICALS IN DIABETES

Oxidative stress can be defined as the production of free radicals at rates or sites exceeding those consistent with the normal cellular function (Wolff *et al.* 1986). It may be a contributory factor for pathogenesis and complications of diabetes (Hunt *et al.* 1990). It has been postulated that mechanism of complications of diabetes involves oxidative stress perhaps as a result of

hyperglycemia. The increased plasma and tissue glucose is primary source of increased oxidative stress. Various mechanisms that contribute to increased oxidative stress in diabetes include glucose oxidation, formation of OFR's by glycated proteins or auto-oxidative glycosylation, alteration in sorbitol pathway activity, changes in level of inflammatory mediators, lipid peroxidation and antioxidant defence system status (Baynes, 1991; Hunt *et al.* 1990; Wolff *et al.* 1987).

1.14.1 Glucose Oxidation and Oxidant production : It has been hypothesized that glucose under physiological conditions produces oxidants.



Scheme for the production of reactive oxygen metabolites from glucose.

Oxidation of glucose to protein reactive dicarbonyls (ketoaldehydes) is rate limited by enediol formation rather than presence of transition metal (M) (Hunt *et al.* 1988). Superoxide anion ($\cdot O_2^-$) is produced during formation of dicarbonyl compounds. $\cdot O_2^-$ undergoes dismutation to H_2O_2. In the presence of metals such as copper and iron, H_2O_2 leads to production of extremely reactive hydroxyl radical (Hunt *et al.* 1988).

1.14.2 Formation of OFR's by glycated proteins or Autoxidative glycosylation: During diabetes mellitus, increased concentration of glucose molecules bind to free amine radicals of proteins, both in tissue and plasma forming aldimines which are rearranged into "fructosamine" derivatives of proteins. The latter compound contains a ketol group which makes it a candidate for formation of $\cdot O_2^-$, when it comes in contact with oxygen molecule which in the presence of metal ions, would produce $\cdot OH$ and will cause oxidative damage to neighbouring molecules (Gillery *et al.* 1989). Exposure of proteins to glucose also causes conformational alterations, loss of recognition, loss of hydrogen binding capacity, loss of cellular recognition and formation of complex products capable of inter-/intra molecular cross linking. Production of free radicals during autooxidative glycosylation leads to fragmentation of proteins (Wolff, 1987) and oxidation of associated lipids (Hunt *et al.* 1990) during glycation reactions. On the other hand, autoxidative glycosylation

products and pentosidine are sugar derived autoxidation products formed by the radical oxidations, also participate in initiation and propagation of damaging free radical reactions. Hunt *et al.* (1988) reported that protein fragmentation and conformational changes induced by the exposure of proteins to glucose are dependent on hydroxylating/oxidizing agents generated in the presence of transition metal from H_2O_2 produced by glucose autooxidation or autoxidative glycosylation. Albumins, crystallins, ribonuclease lysozyme and myoglobin are prone to oxidative fragmentation in the presence of glucose. Hunt *et al.* (1990) reported that in the presence of glucose LDL glycosylation is also accompanied by peroxidative modification. LDL exposed to glucose results in decreased clearance from rabbit plasma (Steinprecher and Witztum, 1984).

1.14.3 Glycoxidation products: Fluorescent crosslinking pentosidines are formed by sequential glycosylation and oxidation reactions between reducing sugars and proteins and these compounds are termed glycoxidation products. These products are biomarkers of more extensive glycative and oxidative damage to protein and are present in trace concentrations (Baynes, 1991).

1.14.4 Alterations of sorbitol pathway activity: Increased utilization of NADPH in polyol pathway results in less NADPH being available for conversion of antioxidants back to their free radical scavenging reduced form (Barnett *et al.* 1986)

which will render tissue less able to deal with oxidative stress. Carroll *et al.* (1986) reported that significant depletion of NADPH leads to deplete reduced form of glutathione (GSH) that protects against oxidation inactivation of critical membrane proteins i.e. Na^+, K^+-ATPase (Carroll *et al.* 1986).

1.14.5 Changes in levels of inflammatory mediators: Pancreatic islet ß-cell destruction in IDDM as a consequence of immune/inflammatory cell mediated processes has been reported both in humans and rodents (Barbosa and Bach, 1987; Charlton *et al.* 1988). Oxygen free radicals have been implicated in cell killing by cytokines and other toxic molecules (Mandrup-poulsen *et al.* 1987; Ruddle *et al.* 1987; Robinovitch *et al.* 1992). The cytokines, IL-1, TNF and IFN-γ have been reported to stimulate OFR production during the respiratory burst in a variety of cell types, such as macrophages (Nathan and Tsunawaki, 1986) and PMNL (Berton *et al.* 1986) in association with host defence against microorganisms and tumors. The stimulation of OFR production in pancreatic islet cells, may have a lethal consequence to islets. In many cell types cytokine-induced production of OFR are primary product of arachdonic acid metabolism and inhibitors of AA metabolism can protect islet cells from cytotoxic effects of these free radicals (Robinovitch *et al.* 1992). Sumoski *et al.* (1989) reported that free radical scavengers dimethyl urea and citiolone inhibited the cytotoxic

effects of t-butylhydroperoxide, IL-1, TNF, IFN-γ and alloxan. Robinovitch *et al.* (1992) reported that cytokines produce significant increase in islet malondialdehyde content and islet necrosis and showed that lazoroid U78518E protects ß-cells from necrosis.

1.14.6 Lipid Peroxidation (LP)

High levels of lipid peroxides have been related to a number of degenerative diseases, such as aging, atherosclerosis and retinal degeneration and are toxic to living organisms (Cutler, 1984). Free radical intermediates of lipid peroxidation react with proteins causing denaturation and polymerization. Over the past decade a considerable body of evidence has been accumulated which indicated that peroxidation of lipids may lead to an impairment of cellular integrity.

In diabetes, significant changes in lipid metabolism and structure occur. Structural changes are oxidative in nature and oxidation of lipids in plasma lipoproteins and in cellular membranes is associated with vascular disease in diabetes (Sato *et al.* 1981; Stringer *et al.* 1989). Increased LP in diabetic rats is also associated with hypertriglyceridemia. Administration of lipophilic antioxidants inhibited the oxidation and resultant toxicity of oxidized lipoproteins (Morel and Chisolm, 1989). Lipid peroxidation may cause browning or crosslinking of collagen, and contribute to development of fluorescence in plasma proteins and possibly

collagen in diabetes (Jones and Lunec, 1987; Pokorny *et al.* 1990; Tsuchida *et al.* 1985)., Jain *et al* (1990) reported higher levels of lipid peroxides in red blood cells (RBC) of diabetic rats than in controls. Low and Nickander (1991) observed severe hyperglycemia, increased conjugated dienes and reduced hydroperoxides in streptozotocin induced male rats. Sato *et al.* (1981) reported first increased lipid peroxide levels in plasma of diabetic patients. A recent work reported a significant relationship between amount of RBC membrane LP and hyperglycemia in diabetic patients (Jain *et al.* 1989). Nishimura and Kuriyama (1985) reported that enhanced nonenzymatic LP is not a causal factor in galactosemic or diabetic cataractogenesis, although some alterations of lens redox status and oxidant accumulation which might be deleterious if present. Hayaishi and Shimizu (1982) observed that aspirin and salicylate inhibited the increase in tail collagen crosslinking in diabetic rats and reported that administration of anti-inflammatory agents cause a significant decrease in lipid peroxidation in rabbit plasma.

Although there are numerous reports of changes in the lipid peroxide level in the diabetic state, but the relative importance of its enzymatic and nonenzymatic sources is unknown. At what stage of diabetes it starts is not clear. It is difficult to conclude whether increased LP is a cause or complication in diabetes.

1.14.7 Antioxidant Enzymes in Diabetes

Various changes in the level of antioxidant enzymes in different disease states have been reported. Recently Taniguchi (1992) reported that human erythrocyte Cu-Zn SOD is inactivated under hyperglycemic conditions. Glycated proteins produce $\cdot O_2^-$ and the Cu-Zn SOD undergoes glycation and inactivation by H_2O_2. These processes will result in accumulation of $\cdot O_2^-$, H_2O_2 and $\cdot OH$ resulting further in oxidative damage.

Grankvist *et al.* (1979) reported that SOD, CAT and hydroxyl radical scavengers prevent the damage to isolated pancreatic cells exposed to the diabetogenic agent alloxan. Cu-Zn SOD is known to protect ß-cells from damage of alloxan (Tharte *et al.* 1985). Exogenous pancreatic Cu-Zn SOD reproductivity protected the morphological features of pancreatic ß-cells against damage by alloxan, determined by light microscopic and ultrastructural examinations. ß-cells appear unaffected with a combination of alloxan and Cu-Zn SOD. Tharte *et al.* (1985) showed that mucosa of the small intestine and colon show decreased Cu-Zn SOD in diabetic rats and insulin treatment reversed the SOD response. Hagglof *et al.* (1983) studied the activities of Cu-Zn SOD, Mn SOD, catalase and $GSHP_x$ in lymphocytes and erythrocytes of children with IDDM and reported that these activities in erythrocytes were significantly lower in IDDM as compared to control.

Lammikeefe *et al.* (1984) observed no change in SOD but increased activity of catalase in cardiac skeletal muscles. Nath *et al.* (1984) reported a reduction in mitochondrial and cytoplasmic SOD and increased level of superoxide anion ($\cdot O_2^-$) in polymorphonuclear leukocytes (PMNL) from diabetic subjects as compared to normal. Findings of Errikson and Borg (1991) suggest that free radicals are involved in glucose induced embryonic dysmorphogenesis. Normalized embryo can be developed *in vitro* by retarding the concentrations of glucose and by increasing free oxygen radical scavenging capacity of conceptus. Asplund *et al.* (1984) reported that SOD coupled to polythene glycol reduces the hyperglycemic response in mice injected with a single large dose of STZ, however, blood glucose levels after multiple low doses of STZ were not altered by SOD polythene glycol. The findings suggest that superoxide radicals may play a diabetogenic action of STZ injected as a high dose. SOD, catalase and hydroxyl free radical scavengers protect against toxicity of alloxan. Similarly diabetogenic effect of STZ has been modified by SOD and dimethylurea. These observations suggest that oxygen containing free radicals may play a role in determining the cytotoxicity of these drugs (Watala *et al.* 1986). Chari *et al.* (1984) reported decreased levels of reduced glutathione (GSH) and glutathione peroxidase and no change in glutathione reductase activity in polymorphonuclear leukocytes (PMNL) in diabetic state. Similar changes in GSH content in diabetic

erythrocytes were observed by Gandhi and Roy Chowdhary (1979) and attributed this change to marked defect in glutathione metabolism. Gupta *et al.* (1990) observed changes in $GSHP_x$ and GR activities in erythrocytes of alloxan induced diabetic rats. Insulin treatment reversed the $GSHP_x$ activity however no change in glutathione reductase activity was observed during diabetes.

1.15 ANIMAL MODELS

Spontaneous diabetes is a common occurrence in many animal species. In addition, using variety of experimental techniques animals can be rendered diabetic. Animal models are advantageous to study as they can be regarded as models of disease in man. Despite the presence of hyperglycemia as a common feature there are a wide variety of pathophysiological differences occurring in animals as compared to human subjects. The most commonly used spontaneously diabetic animals include BB rat, sand rat, yellow mouse, KK mouse, C57BL/6J db/db mouse, spiny mouse, chinese hamster, (Macaca nigra) monkey, and dog, humans etc. Animals provide a powerful tool to study interactions of heredity and environmental factors such as diet, drugs, toxins and infectious agents (Mordes and Rossini, 1981).

In the present study male Sprague Dawley rats were used because of its suitable size, longevity, availability, cost and maintenance. The diabetic state in rat is metabolically similar to that observed in human subjects with juvenile onset

diabetes. There is marked deficiency of insulin and increase in plasma glucagon, somatostatin, glucose, free fatty acids and branched chain amino acids (Like, 1977). So study of IDDM in rats may provide useful data to study the etiologies, complications and prevention of diabetes.

1.16 EXPERIMENTAL INDUCED DIABETES

Various experimental techniques are used for the creation of diabetic syndromes. Chemicals that produce hyperglycemia fall into three categories :

(i) First are cell specific toxins which destroy ß-cells and cause a primary insulin deficiency state. (Recovery of ß-cells is slow and requires regeneration of new ß-cells).

(ii) Second are agents which act on ß cells and do not destroy it, they cause transient inhibition of insulin production or insulin secretion.

(iii) A third class impair insulin's metabolic efficiency in target tissues (increases endogenous insulin requirements in pancreas) and secondarily producing diabetes (Mordes and Rossini, 1981).

Insulin is synthesized as a single large chain precursor, preproinsulin, which is processed in the cell to pro-insulin and then to the biologically active molecule, insulin (Steiner and Oyer, 1967). It is synthesized in the ß-cells of the islets of Langerhans of pancreas. The function of these cells is to synthesize insulin, to store it and release the required

amount at the appropriate time into blood (Cahil, 1973). Epinephrine, glucagon, glucocorticoids and growth hormone all have an antagonistic effect on insulin. Metabolic derangement, glucose tolerance is reduced and hyperglycemia is induced.

For the present study chemicals in the first category were of particular interest because they more closely duplicate lesions which occur during ß-cell destruction in IDDM and provide a permanent diabetic state suitable for long term experimental studies. The most commonly used agents are Alloxan and Streptozotocin. They selectively damage pancreatic ß-cells and exhibit most potent diabetogenicity and are free of non specific toxic effects (Hunt *et al.* 1990). Their effect varies with species, age and metabolic state of animals and it is usually 4-5 times less than lethal dose.

1.16.1 ALLOXAN

Alloxan is a highly unstable pyrimidine with structural similarities to uric acid and glucose (Dulin and Soret, 1977). Alloxan was first noted to have diabetogenic activity by Dulin and Soret, 1977; Fischer and Rickert 1975. Although the exact mechanism of action is not known, evidences indicates that its action is mediated through some membrane interaction on the surface of ß-cell. Autoradiographic studies with [^{14}C] alloxan revealed that it has high affinity for islet cell membranes (Fischer and Rickert, 1975) and causes rapid *in vitro* and *in vivo* inhibition of insulin secretory

mechanisms. Approximately after 2 hours of its injection, mild hyperglycemia, massive hypoglycemia and insulin release from dying ß-cells occur within 1-2 days and permanent hyperglycemia is established. A number of hydroxyl and superoxide radical scavengers (e.g. superoxide dismutase (SOD), catalase, NADPH, and metal chelators) block alloxan toxicity. So it is assumed that these radicals may be involved for its toxicity. Certain metabolizable and non metabolizable sugars (including 2-Deoxy-glucose, mannose and 3-0 methyl glucose) when provided before alloxan administration neutralize its effectiveness. Alloxan is not entirely specific and lesions occur in other organs and may produce metabolic changes unrelated to ß cell lesions and diabetes.

Several investigators have suggested that toxic oxygen free radicals are involved in the diabetogenic action of alloxan (Wilson *et al.* 1984; Grankvist *et al.* 1981). Tharte *et al.* (1985) reported that auto-oxidation of dialuric acid generates H_2O_2 and more toxic ·OH (hydroxyl) radicals are generated from metal catalyzed Haber-Weiss reaction. Lienzen and Gottingen (1991) reported that both alloxan and the sugar mannoketoheptose inhibit glucose induced insulin secretion through inhibition of pancreatic ß-cell glucokinase but only alloxan is ß-cell toxic. Alloxan binds thiol groups of glucokinase sugar binding site and inhibit this enzyme and also yield dialuric acid though reduction of alloxan. The

toxification of alloxan is due to redox cycling between alloxan and dialuric acid with resultant generation of cytotoxic free radicals, which has been detected using luminal chemiluminescence in the presence of transition metals also in cell free systems (Grankvist *et al.* 1979; Asayama *et al.* 1984). Takasu *et al.* (1991) demonstrated that alloxan generated H_2O_2 which causes DNA strand breaks and then diabetes.

DNA

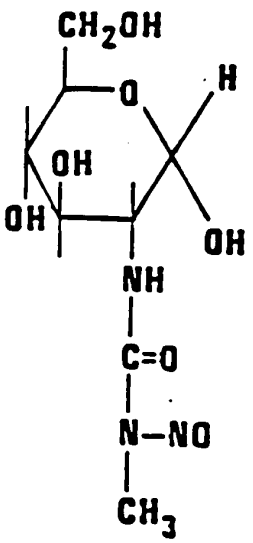
Alloxan -------> H_2O_2 -------> *Strand Breaks* -------> *Diabetes*

Generation mellitus

They suggested that H_2O_2 acts as an intermediate for DNA strand breaks and other radicals like hydroxyl ($^{\cdot}OH$) and superoxide ($^{\cdot}O_2^-$) may also be involved in alloxan induced diabetes.

1.16.2 STREPTOZOTOCIN (STZ)

STZ is a nitrosourea compound from *streptomyces achromogenes and* possesses three distinct biological activities:



STRUCTURE OF STREPTOZOTOCIN

(i) Antimicrobial (Vavra *et al.* 1959), (ii) Antitumoral (Evans *et al.* 1965) and diabetogenic (Rakieten *et al.* 1963). Rakieten *et al.* (1963) first reported the development of diabetes and attributed it to the disruption of pancreatic islets in rats and dogs treated with streptozotocin. Junod *et al.* (1967) reported that STZ has a highly specific ß-cell toxicity similar to alloxan but more specific and with wider margin of safety than alloxan. Arison *et al.* (1967) showed that rats treated with STZ showed complications of chronic diabetes.

Streptozotocin is a 2-deoxymethyl nitrosourea pyranose that has a specific toxic effect on beta cells. Presumably the first step in its pathologic process is cell membrane binding. The nitrosourea moiety provides the cytotoxic effect and the 2-deoxyglucose moiety confers specificity of ß-cell. It has also been suggested that the glucose component of STZ enhances its uptake into ß-cell in which cytotoxicity of nitrosourea moiety can be concentrated (Agius and Gidari, 1985). Removal of the glucose moiety renders compound much less specifically toxic for ß-cell and even galactose for glucose also decreases its effectiveness (Fischer and Rickot,1975 and Hales and Kennedy, 1964). The metabolic effects induced by STZ include insulinopenia and are increased gluconeogenesis, decreased glycolysis, subnormal levels of hepatic glycogen, increased catabolism and hyperlipidemia, which are similar to those as described for alloxan. STZ

produces three kinds of distinct syndromes:

1. A single large dose administration causes acute ß-cell necrosis and major hypoglycemia within 2-72 hrs. In case of alloxan, it causes an inhibition of insulin release followed within a few hours by hypoglycemia and rapid release of insulin from dying cells. Effective and lethal doses are sensitive to strains, species, age, sex, nutritional state. Young male and fasted animals are more sensitive (Gold *et al.* 1981). In rodents a large dose of STZ destroys ß-cell and may impair amino acid stimulated glucagon release. STZ is unstable at biological pH like alloxan (Riley *et al.* 1981).
2. Multiple low doses of STZ (no one of which could be diabetogenic) for 4-5 days causes progressive diabetes beginning shortly after the final dose and increasing within several days to cause complete ß-cell destruction and severe diabetes (Mordes and Rossini, 1981). Alloxan induced similarly produces none of these effects.
3. The administration of a single low dose (50% of standard single dose) can result in a slow and gradual development of severe diabetes over a period of 10-60 days as seen in young and adult mice (Riley *et al.* 1981). Within ß-cell STZ is

believed to decrease levels of nicotinamide adenine dinucleotide (NAD) by decreasing its synthesis and increasing its degradation. NAD protects animals against alloxan and streptozotocin toxicity. Whish *et al.* (1975) suggested primary action of STZ may be to stimulate poly ADP ribose polymerase which would alter regulation of DNA synthesis, repair and cell differentiation. However a further exploration is required to know how a change in DNA could result in ß-cell necrosis.

Wilson *et al.* (1984) studied the effects of both scavengers of oxygen free radicals and inhibitors of poly (ADP ribose) synthetase on STZ or alloxan induced ß-cell damage in islet cell monolayer cultures of neonatal rat. Results showed that superoxide radical scavenger, SOD did not protect against toxic effects of STZ at concentrations up to 5000 U/ml; however, scavengers attenuated alloxan toxicity. The hydroxyl radical scavenger 1-1 dimethylurea, significantly reduced the toxic effects of STZ and alloxan in a dose dependent manner up to concentration of 50 mg/ml. Results also suggest that poly ADP ribose synthetase inhibitors nicotinamide, 3-aminobenzamide and 1-1 dimethyl urea also scavengers of $^{\cdot}OH$ radical partially protect ß-cells against toxic effects of alloxan and STZ.

Differing results have been obtained from studies to elucidate the mechanism of action of these two toxins.

Uchigata *et al.* (1982) proposed that alloxan and STZ induce ß-cell damage by initiating biochemical events which cause DNA strand breaks. During repair of lesions nuclear poly ADP-ribose synthetase is activated to form poly ADP-ribose utilizing NAD as a substrate. This reaction leads to depletion of NAD resulting in functional impairments and then leading to cell death. It has been reported that alloxan exerts its initial effect by generating oxygen free radicals and it is supported by above results and elsewhere utilizing superoxide scavengers SOD and hydroxyl scavengers 1-1 dimethylurea (Grankvist *et al.* 1979; Fischer and Hamburger, 1980; Tibaldi *et al.* 1980) which block the toxic effect of alloxan. Also it has been shown that alloxan generates oxygen free radicals in presence of islets (Grankvist *et al.* 1979). The $^{\cdot}OH$ radical scavenger 1-1 dimethyl urea partially protects ß-cells against diabetogenic effect of STZ support the hypothesis that STZ induces the generation of oxygen free radicals (Wilson *et al.* 1984; Marklund and Grankvist, 1980; Robins *et al.* 1980). Margison and O'Conner (1979) reported that STZ exerts its initial biochemical effect by generating carbonium ions (CH_3^+) formed during decomposition of diethylurea which is N-nitrogen moiety of STZ. These ions are capable of alkylating DNA bases at various positions and to repair them poly ADP-ribose system is activated and NAD is depleted. This hypothesis is supported by observation that both nicotinamide and 3-aminobenzamide inhibit activation of

poly ADP-ribose synthetase and reduce diabetogenic effects of STZ and alloxan (Fischer *et al.* 1983).

The exact sequence of events where these toxins interact with ß-cells to cause functional impairments and cell death remains to be elucidated.

1.17 Hypothesis: It is hypothesized that the complications in diabetes mellitus could be due to an increase in the levels of OFR's as a result of its increased production and/or decreased destruction. If OFR's are increased then one would expect an increase in the levels of malondialdehyde (MDA, lipid peroxidation product) in various organs and a change in the antioxidant enzymes.

1.18 Objectives : The main objectives of the research proposal are to investigate:

(i) If changes in OFR precedes development of diabetes

(ii) Tissue specific changes in MDA and antioxidant enzymes in control and diabetic rats

(iii) Time dependent changes in oxidative stress in different tissues

(iv) Ultimately the role of OFR in the pathogenesis and complications of diabetes.

To achieve these objectives the following studies were undertaken:

The male Sprague Dawley rats were divided into two groups. One control and other group was induced diabetes with

Streptozotocin. Periodic testing of glycosuria and body weight changes were recorded. To study the time course changes control and diabetic animals were used at the interval of 1st wk, 2nd wk, 3rd wk, 4th wk, 5th wk and 6th wk. To confirm the diabetic state of the animal plasma glucose levels were measured. Tissues (liver, heart, pancreas, aorta and kidney) and blood which are involved in pathogenesis and complications of diabetes were selected for the study and following parameters were measured in these tissues:

(i) To determine the level of free radical induced damage the lipid peroxidation product, MDA was measured in various tissues at different intervals.

(ii) To study the defence against oxidative stress: antioxidant enzymes viz., Catalase, Glutathione peroxidase and SOD (total, Cu-Zn SOD and Mn SOD) were measured.

2.0 MATERIALS AND METHODS:

2.1 Animals : Male Sprague Dawley rats weighing between 230-280 gms were used for the experiments. They had free access to food and water ad libitum. The animals were fasted overnight prior to the experiment.

2.2 Chemicals : Streptozotocin, bovine serum albumin, 2-thiobarbituric acid, phosphotungstic acid, glutathione (reduced form), glutathione reductase, nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), nitrobluetetrazolium, diethylenetriaminepentaacetic acid, bathocuprosulfonic acid, sodium cyanide and deoxycholic acid were obtained from Sigma Chemical Company, St. Louis, MO, USA. Xanthine oxidase was procured from Boehringer Mannheim GmbH, Germany. All other chemicals used were of analytical grade.

2.3 Induction of Diabetes :

Male Sprague Dawley rats were fasted for 16h before inducing diabetes with Streptozotocin (STZ). Animals were anesthetized with ether and injected intraperitonealy (IP) with 0.5ml of freshly prepared solution of STZ (80 mg/kg) dissolved in citrate buffer (0.1M, pH 4.5). Control rats were injected IP with the buffer alone, the same volume. STZ treated animals were allowed to drink 5% glucose solution overnight to overcome drug induced hypoglycemia. Then control and treated animals were allowed access to food and water ad libitum. Periodic testing (every 3 days) for glucosuria with Ames Multistix (Miles Canada Inc. Etobicoke Ontario) was done. Control and diabetic animals were sacrificed at intervals of

1st wk, 2nd wk, 3rd wk, 4th wk, 5th wk and 6th wk after onset of diabetes using anesthetising ether. Body weight changes were recorded at these intervals.

2.4 Tissue Collection and Processing : At the end of 1st wk, 2nd wk, 3rd wk, 4th wk, 5th wk and 6th wk rats were anaesthetized with ether and blood was obtained by cardiac puncture and collected into heparinized tubes and the animals were sacrificed by cardiac excision. The tissues (liver, heart, pancreas, aorta and kidney) were removed, washed in 0.9% saline, and then in 50 mM phosphate buffer (pH 7.4) to remove blood contaminants. All the tissues were homogenized in 10 volumes of buffer using a Polytron (Brinkmann Instruments, Westburry, N.Y.) at 4°C for 30 seconds (2 x 15 with 15 sec cooling intervals). Homogenate was filtered through cheese cloth and then centrifuged at 3000 rpm (705 x g) for 5 minutes and the supernatant was used for various assays.

2.5 Plasma Glucose :

Blood was collected in vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, New Jersey USA) containing EDTA. It was immediately centrifuged at 3000 rpm (705 x g) for 15 minutes and the plasma was collected for estimation of glucose. Plasma glucose was measured by oxygen rate method of Kadish *et al.* (1968) using a Beckman Oxygen electrode on the Synchron CX3 instrument. The method is based on the principle that the reaction of β-D-glucose with oxygen in the presence

of glucose oxidase generates H_2O_2.

Glucose oxidase

β-D-glucose + O_2 ----------> Gluconic acid + H_2O_2

By the oxygen electrode, the rate of oxygen consumption is measured. Oxygen is consumed at same rate as glucose reacts to form gluconic acid. The rate of oxygen consumption is directly proportional to the concentration of glucose.

10μl of plasma was aspirated into the analyzer reaction cup. To this glucose reagent [glucose oxidase (590 U/ml), ethanol (10%) and potassium iodide (0.04 mol/L)] was added. The oxygen electrode senses the rate of oxygen consumed in reaction mixture and the Synchron CX3 system electronics determines the glucose concentration as mmol/L.

2.6 Tissue MDA :

Blood and tissue MDA were estimated by the method of Yagi (1984). MDA reacts with thiobarbituric acid (TBA) to give a fluorescent product which is measured on a fluorescence spectrophotometer. 200μl of anticoagulated blood was added to 2ml freshly prepared isotonic (0.9%) saline. It was centrifuged at 3000 rpm (705 x g) for 10 minutes and the supernatant was used for assay. 750μl of blood supernatant and 200μl of each tissue supernatant were taken in different glass test tubes. 4 ml of 0.08N H_2SO_4 was added to each tube

and then 600μl of 10% phosphotungstic acid. It was vortexed and centrifuged at 3000 rpm (705 x g) for 6 minutes. The supernatant was decanted and to the pellet 2ml of 0.08N H_2SO_4 and 300μl of 10% phosphotungstic acid were added again and was vortexed and centrifuged at 3000 rpm (705 x g) for 6 minutes. The supernatant was discarded. In other set of test tubes 0, 25 and 50μl of 100μM tetraethoxypropane (TEP) standard solution was added. To each tube distilled water was added to make the final volume of 4 ml. Then to all tubes, 1.0 ml of 50:50 TBA : glacial acetic acid - pH 3.4 was added. The contents of each tube were mixed and incubated for one hour at 95°C in a polyethylene glycol bath. At the end of the incubation period, tubes were cooled in tap water and 5.0 ml of n-butanol was added to each tube. Each tube was vortexed for 40 sec. and the butanol layer was separated by centrifugation at 3000 rpm for 15 minutes. Fluorescence intensity of the n-butanol layer was taken at emission of 553 nm and 515 nm excitation using the Hitachi F-2000 fluorescence spectrophotometer. The tissue MDA content was calculated by the formula:

$$MDA = Cs \times f \times df / F$$

where Cs = concentration of TEP standard (nM)

f = fluorescence intensity of sample

F = fluorescence intensity of TEP standard

df = dilution factor

Tissue MDA is expressed as nmol/mg protein and blood MDA as nmol/ml blood.

2.7 Antioxidant Enzymes :

2.7.1 Catalase Activity :

Catalase activity in tissue supernatants was estimated by the method of Aebi (1974) and as described by Prasad *et al.* (1992). Catalase catalyzes the conversion of H_2O_2 to H_2O. In the UV range, H_2O_2 shows a continuous increase in absorbance with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by measuring a decrease in absorbance at 240nm. The difference in absorbance per unit time is a measure of catalase activity.

Tissue supernatant or erythrocyte lysate samples were added to a cuvette containing 50 mM phosphate buffer (pH 7.0) to make final volume 3ml. The solution was mixed by inverting the cuvette three times and the instrument blank was adjusted to zero absorbance. The cuvette was washed and to this cuvette tissue supernatant and phosphate buffer was added to final volume of 2ml. The reaction is started by adding 1.0 ml of 30 mM H_2O_2. The absorbance was recorded every 15 seconds for 30 seconds. The enzymatic decomposition of H_2O_2 is a first order reaction and its rate is proportional to peroxide

concentration present. H_2O_2 decomposition from the period of 0-30 sec at the concentration of 0.01 to 0.05M follows 1st order reaction. The first order rate equation is :

$$k = 2.303/\Delta t * \log (A_1/A_2)$$

(where k is the first order rate constant, Δt is the measured time interval, A_1 and A_2 are the initial and final absorbance) was used to calculate catalase activity. Tissue catalase activity was expressed as k/sec/mg protein and blood CAT activity as k/sec/gm Hb.

2.7.2 Glutathione Peroxidase (GSH-Px) Activity :

GSH-Px activity in the tissue samples was determined by the method of Paglia and Valentine (1967) modified by Lawrence and Burk (1976) and as described by Prasad *et al.* (1992). In this method, the rate of oxidation of the reduced form of glutathione (GSH) by H_2O_2 is measured, as catalyzed by the glutathione peroxidase (GSH-Px) present in the sample. Instead of measuring the progressive loss of GSH, this substrate is maintained at a constant concentration by addition of exogenous glutathione reductase (GSSG-R) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH immediately converts any oxidized glutathione to the reduced form.

$$2GSH + H_2O_2 \xrightarrow{\text{GSH-Px}} H_2O + GSSG \xrightarrow[\text{NADPH}\ \rightarrow\ \text{NADP}]{GSSG\text{-}R} 2GSH$$

where GSH = Reduced form of Glutathione;

GSSG = Oxidized form of glutathione;

GSH-Px = glutathione peroxidase;

GSSG-R = glutathione reductase;

The rate of GSSG formation is measured by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP.

An assay mixture was prepared by mixing 50 ml of 75 mM phosphate buffer (pH 7.0), 1.25 ml each of 60 mM glutathione solution and 120 mM sodium azide solution, and 2.5 ml each of 15 mM ethylene diamine tetra acetic acid (EDTA), 3.0 mM NADPH, and 30 U/ml glutathione reductase solutions. 2.4 ml of assay mixture was taken in a test tube. To this solution 0.5 ml water and 0.1 ml of 7.5 mM H_2O_2 was added, mixed, and the absorbance at 340 nm was continuously recorded using PU 3000 UV/Vis Spectrophotometer. The rate of change of absorbance per minute was calculated (blank). Similarly, the rate of change of absorbance per minute was recorded for tissue sample by taking tissue supernatant sample, 2.4 ml assay mixture, 0.4 ml distilled water, and 0.1 ml H_2O_2. The GSH-Px activity in tissue samples is expressed as (μmoles/min/mg protein) and in blood as μmoles/min/gm Hb. The samples activity was calculated by the following formula:

$$A = 0.868 * Vi * ([NADPH]s - [NADPH]b)/[GSH]_0 * t * Vs \text{ (P or Hb)}$$

where A = GSH-Px activity in μmoles/min/mg protein

[NADPH]s = Rate of decrease in absorbance at 340 nm in the presence of sample

[NADPH]b = Rate of decrease in absorbance at 340 nm in the absence of sample

Vi = Total volume in cuvette

Vs = Sample volume (Protein or Hb)

$[GSH]_0$ = Concentration of glutathione (taken as unity at stead state)

t = Time in minutes

P = Protein content of the sample

Hb = Hemoglobin

The extinction coefficient of NADPH is taken as $6.22 \times 10^3 l. M^{-1} cm^{-1}$ (Paglia and Valentine 1967).

2.7.3 Superoxide Dismutase (SOD) Activity :

SOD activity in the samples was estimated by the method of Sun *et al.* (1989) and as described by Prasad *et al.* (1992). Superoxide anions reduce nitroblue tetrazolium (NBT) to dark coloured formazone. SOD, by dismutating superoxide anions, inhibits the reduction of NBT. This property is utilized in estimating the SOD activity of the sample. Superoxide anions were generated by the reaction between xanthine, and xanthine oxidase.

2.7.3.1 Sample Preparation :

0.5 ml of tissue supernatant and 0.25ml of erythrocyte lysate were taken in different test tubes and distilled water

was added to make final volume of 2 ml. To this solution 0.8 ml of chloroform ($CHCl_3$) : ethanol (C_2H_5OH) (3:5 v/v) was added, and the tube was vortexed for 90 seconds. The $CHCl_3$: C_2H_5OH layer was separated by centrifugation for 25 minutes at 3000 rpm (705 x g) at 0 - 4° C, and used for the estimation of enzymatic activity.

2.7.3.2 Procedure :

The assay mix was prepared by thoroughly mixing 60 ml of 0.3mM xanthine, 30 ml of 0.6 mM diethylenetriamine-pentaacetic acid (DETAPAC) (prepared in 50mM phosphate buffer, pH 7.0), 30 ml of 0.15 M NBT solution, 18 ml of 0.4 M sodium carbonate/bicarbonate buffer (pH 10.2), and 9 ml of 1g/L bovine serum albumin (BSA). The total SOD activity was determined by adding 2.35 ml of this assay mix to each tube followed by 0.1 ml of 1.67 mM bathocuproinedi-sulfonic acid (BCS). 0.5 ml of distilled water was added to blank and reagent blank tubes. In other tubes, 10, 20, 50, 100, 250, and 500 μl of $CHCl_3$:C_2H_5OH extract layer was added, followed by sufficient water to make sample + water = 0.5 ml. For the determination of Mn-SOD, 100μl of sodium cyanide (15 mM) was added to another set of tubes. 50 μl (0.833 U/ml) xanthine oxidase solution was added to each tube except the reagent blank tube. Xanthine oxidase was added to the tubes at an interval of 20 seconds so that it could be added to all the tubes in less than 20 minutes. Immediately after adding the xanthine oxidase, the tubes were vortexed and incubated in a

water bath at 25°C for 20 minutes for total-SOD activity and 45 minutes of incubation for Mn-SOD activity. The reaction was stopped by addition of 1 ml of 0.8 mM cupric chloride. The absorbance of the developed colour was measured spectrophotometrically at 560 nm against the reagent blank. The value obtained in the presence of sodium cyanide represents Mn-SOD activity. The activity of Cu-Zn SOD was obtained by the difference in the activities determined in the absence (Total SOD) and in the presence of sodium cyanide.

2.7.3.3 Calculations :

Percentage inhibition caused by the standard or the sample was calculated by the formula:

$$\% \text{ Inhibition} = 100(Abs_{blank} - Abs_{sample}) / Abs_{blank}$$

Percent inhibition was plotted against protein content of the sample. From this plot, the value of SOD was calculated in terms of units, defined as the amount of SOD that inhibits the reduction of NBT by 50%.

2.8 Protein: The protein content of the tissue homogenate and supernatant was determined by the Biuret method (Gornell *et al.* 1948). A 200 µl sample was diluted to 800 µl with distilled water and solubilized with 200 µl of 0.2% w/v deoxycholic acid by incubation at 37°C for 5 minutes (Jacob *et al.* 1956). The biuret reagent (4 ml) was added and samples were further incubated at 37°C for 30 minutes. At the end of

the incubation period, the samples were vortexed and the absorbance was measured at 540 nm. The standard curve was plotted with bovine serum albumin (10 mg/ml). The protein content was estimated using absorbance of sample from the standard curve.

2.9 Blood Hemoglobin :

For measurement of hemoglobin 2-3 ml of blood was collected in vacutainer tubes (Becton Dickinson Vacutainer System, Rutherford, New jersey USA) containing potassium EDTA. The hemoglobin content was measured by the cyanmethemoglobin method of Eilers (1967) using the Technicon H 6000 system. A diluted blood sample was combined with hemoglobin diluent (One litre contains : water, 0.6 g potassium ferricyanide, 0.42g potassium phosphate monobasic and 0.15g potassium cyanide). The ferricyanide component in the hemoglobin diluent oxidizes the hemoglobin iron from the ferrous to ferric state to form methemoglobin. The methemoglobin reacts with the potassium cyanide to produce stable cyanmethemoglobin and its absorbance is measured at 550nm. The values are expressed as g/L.

2.10 Statistical Analysis :

The results are expressed as Mean $\pm$ standard error of the mean (S.E.M.). Control values are n = 5, diabetic n = 6. The comparison between groups and at various weeks is done by unpaired Student's "t" test (BMDP statistical software, University of California, Berkeley). The difference was considered significant if p was less than 0.05.

3.0 Results :

3.1 General Characteristics of diabetic rats : Male Sprague Dawley rats were made diabetic by IP injection of STZ. More than 85% of animals injected with STZ developed diabetes. It was confirmed by the presence of glycosuria after 36 hrs of induction of diabetes. Controls did not showed any change in urinary glucose levels. However, in the diabetic group the levels were greater than 56 mmol/L on dipstick. The STZ induced animals, which did not show any change were excluded from the study. Plasma glucose levels were measured at interval of 1st wk, 2nd wk, 3rd wk, 4th wk, 5th wk and 6th wk. Plasma glucose levels are expressed as mmol/L and the changes are summarized in Fig.1. The changes in plasma glucose levels within the control group were not significant. However, in diabetic group, the levels were higher at the 4th wk as compared to 1st wk and at 6th wk it was higher than 1st, 2nd and 3rd wk. A positive correlation was found between time and diabetic plasma glucose levels ($r=0.85$, $p<0.05$). Diabetic animals showed 4-5 fold increase in plasma glucose as compared to controls at each time interval.

The body weight changes in control and diabetic rats are summarized in Fig.2. Control animals gained significant weight. In the diabetic group, there was a progressive decrease in weight. The decrease on the 5th wk and the 6th wk

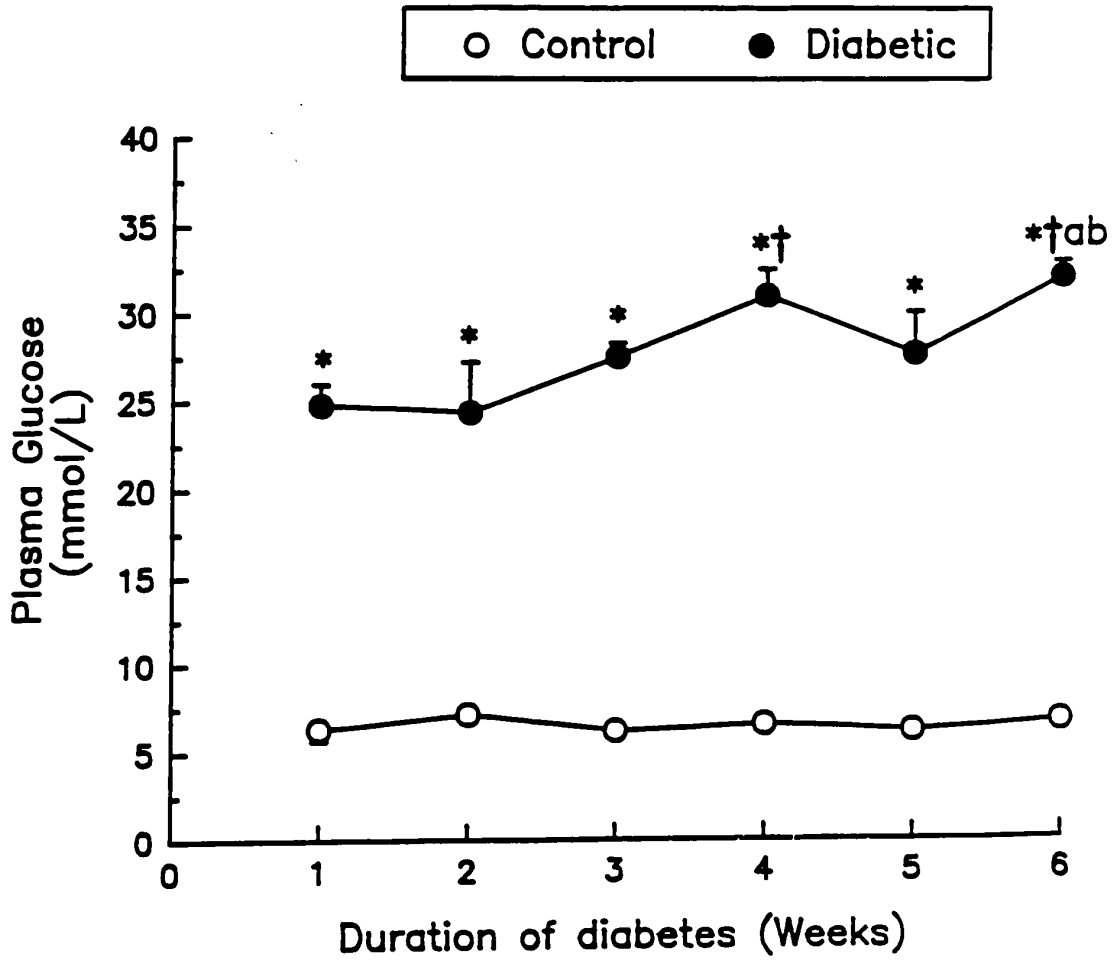


Fig.1: Changes in plasma glucose levels in control and diabetic rats. The results are expressed as Mean ± S.E.M. * P<0.05, control vs diabetic group; † p<0.05, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a p<0.05, 2nd week vs 3rd, 4th, 5th and 6th week; b p<0.05, 3rd week vs 4th, 5th and 6th week.

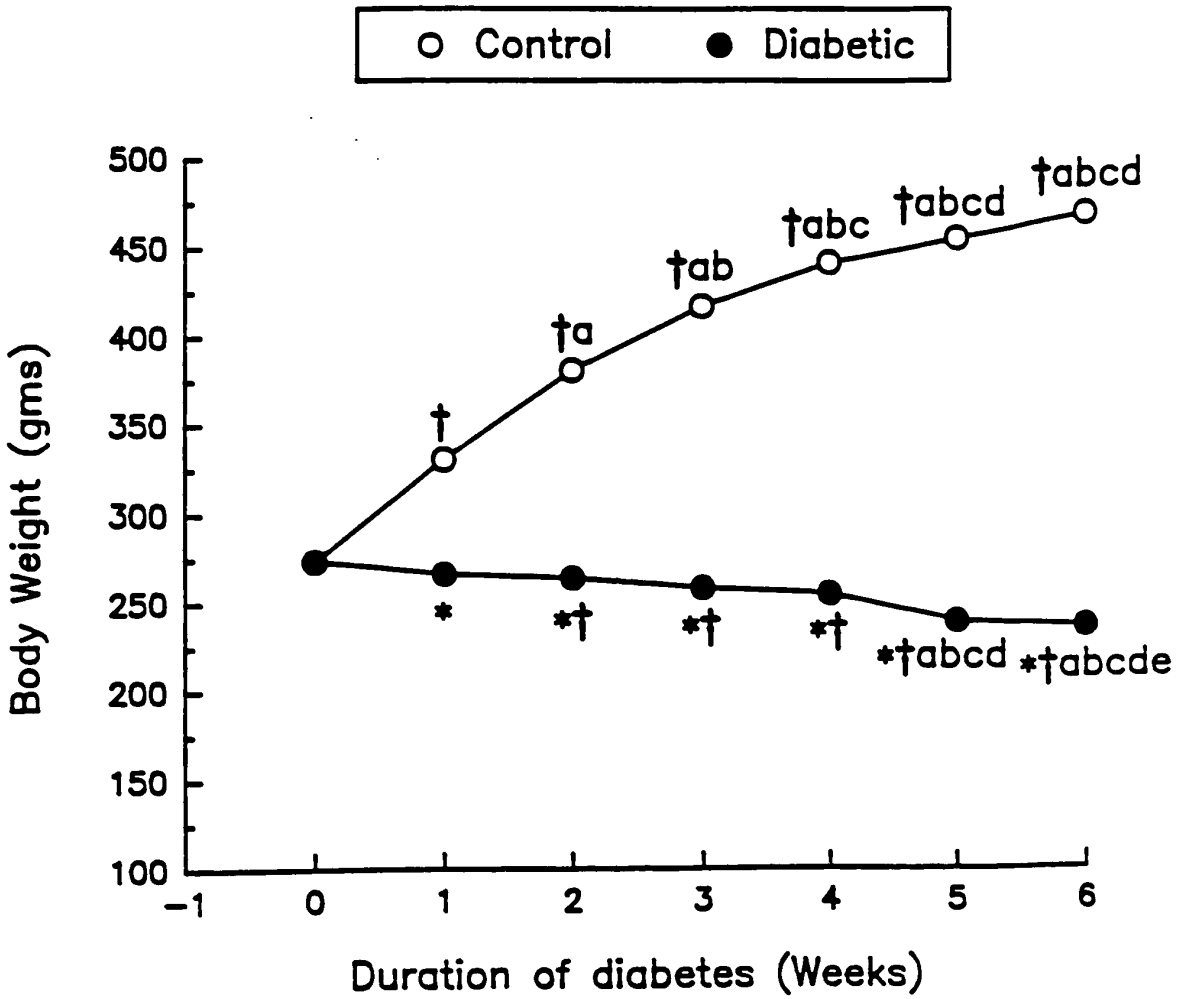


Fig.2: Changes in body weight in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 0 week vs 1st, 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; b $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; c $p<0.05$, 3rd week vs 4th, 5th and 6th week; d $p<0.05$, 4th week vs 5th and 6th week; e $p<0.05$, 5th week vs 6th week.

was significant as compared to all previous intervals. A negative correlation was found between time and body weight of diabetics ($r=0.96$, $p<0.05$). In comparison to control the diabetic rats showed a significant decrease in weight throughout the protocol.

3.2 Biochemical parameters:

3.2.1 Liver

3.2.1.1 Malondialdehyde Content : The time course of changes in MDA content in liver of control and diabetic rats is shown in Fig.3. The results are expressed as nmol MDA/mg protein. The MDA content remained unchanged in the control group except at the 4th wk it was slightly lower than the 2nd wk. In the diabetic group, there was significant increase at the 3rd wk (31.14%) and at the 6th wk (41%) as compared to the 1st wk. An increase of 38.7% and 36.5% was also observed at the 6th wk as compared to the 4th and the 5th wk respectively. Diabetic rats showed a significant increase ($p<0.05$) in MDA content as compared to controls at all time intervals except at the 2nd wk. During the 1st wk of diabetes there was a significant increase of 24.5%. The increase at 3rd wk (63.2%) and at 6th wk (68.6%) was almost similar being maximum in the later.

3.2.1.2 Catalase : The time course of changes in liver catalase activity in control and diabetic rats is shown in Fig.4. The activity in the control group at the 3rd wk and the 6th wk was lower as compared to 2nd wk. In the diabetic group there was a significant decrease at the 2nd wk and the

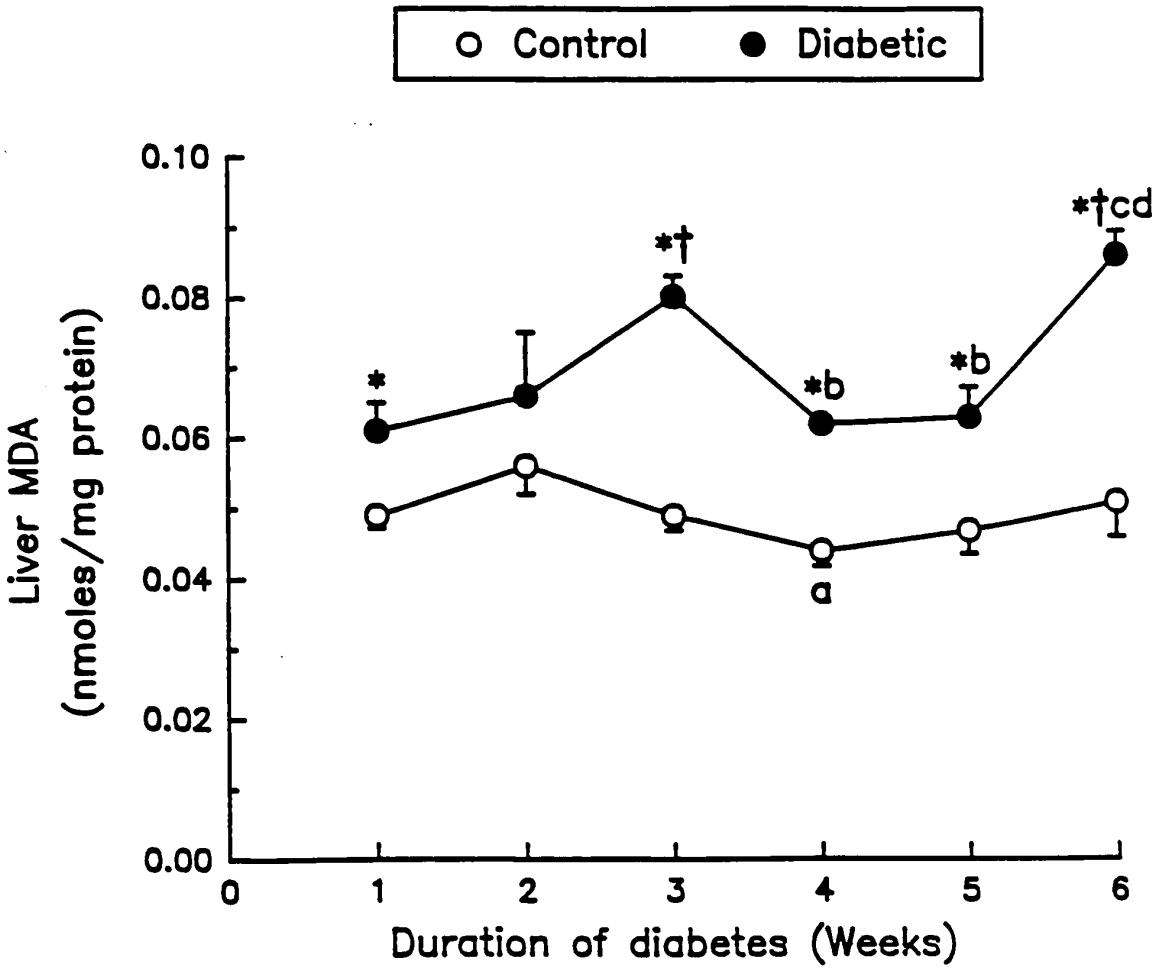


Fig.3: Changes in lipid peroxide (MDA) level of liver in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week.

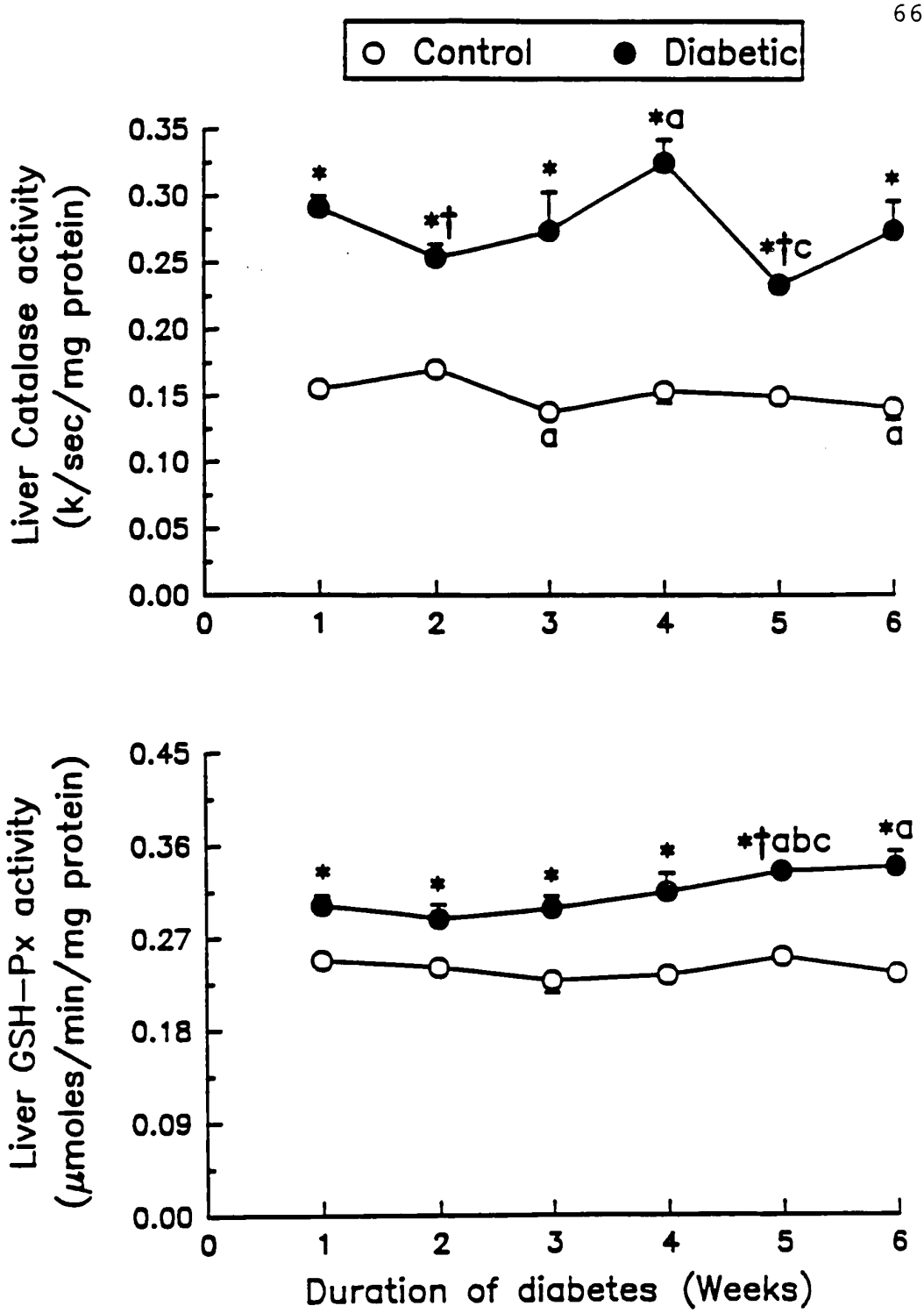


Fig.4: Changes in Catalase and Glutathione peroxidase activities of liver in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

5th wk as compared to the 1st wk. CAT activity was significantly higher at the 4th wk (28.3%) as compared to the 2nd wk and significantly lower at the 5th wk (28.2%) as compared to the 4th wk. Diabetic liver showed a significant increase at all times as compared to controls. The activity was maximum at the 4th wk and was two fold higher than the 4th wk control.

3.2.1.3 GSH-Px : The changes in GSH-Px activity in control and diabetic rats are shown in Fig. 4. The values are expressed as μmol/min/mg protein. No significant difference in GSH-Px activity was observed within control group. However, in the diabetic group there was a significant increase at the 5th wk as compared to the 1st, 2nd, 3rd and 4th wk. An increase was also observed at the 6th wk as compared to the 2nd wk. GSH-Px activity of diabetic liver was significantly higher than that of control at all the times. At the 1st wk there was an increase of 21.8%. The activity gradually increased from the 2nd wk onwards attaining a maximum at the 6th wk (44.9%).

3.2.1.4 SOD : Time course of changes in total SOD, Cu-Zn SOD and Mn-SOD in control and diabetic liver are summarized in Fig. 5. The results are expressed as U/mg protein.

Total SOD : Total SOD activity remained unchanged in the control group. However in the diabetic group the activity at the 4th wk was significantly higher as compared to the 1st wk. The activity was maximum at the 3rd wk . After that there

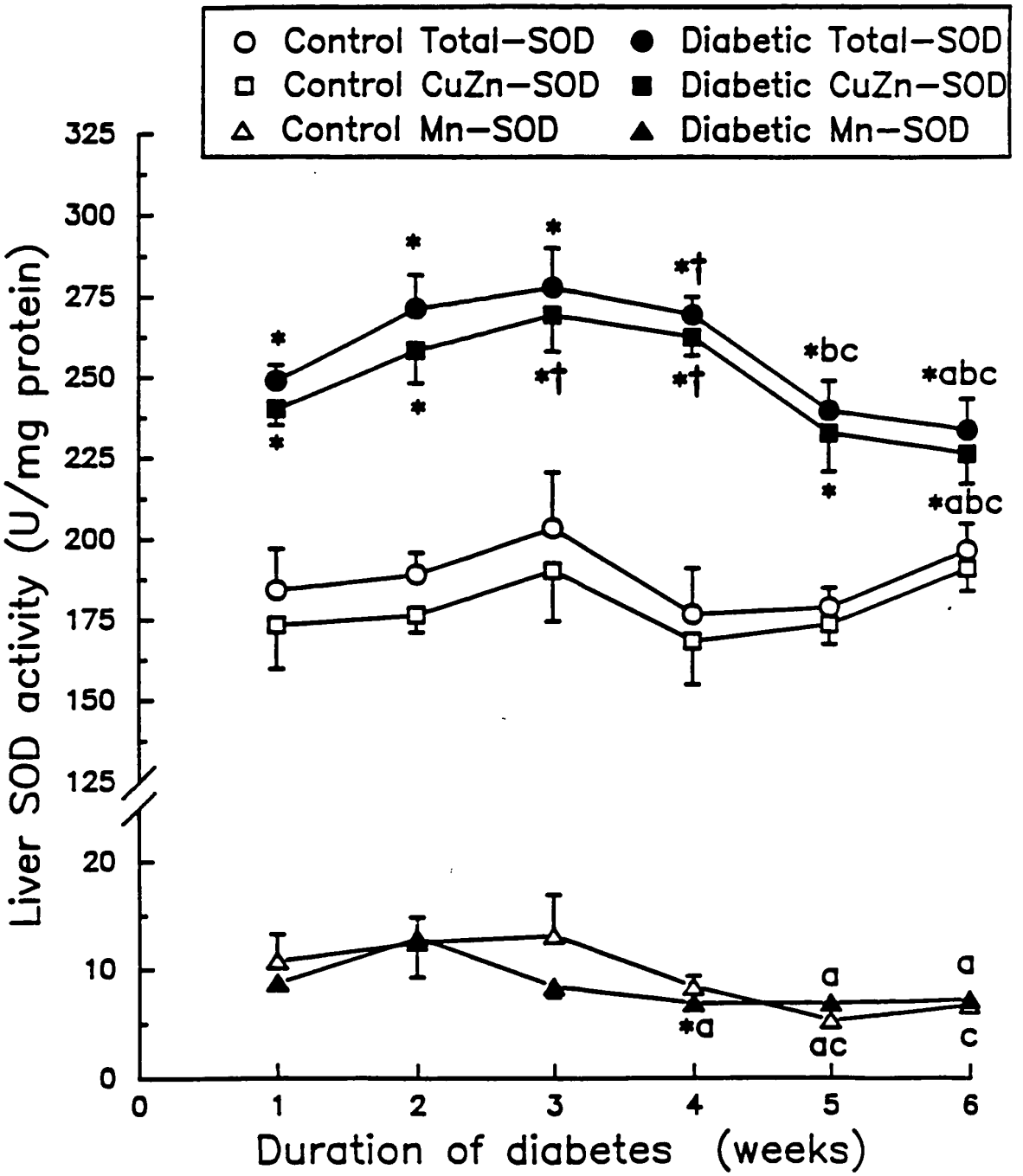


Fig.5: Changes in Superoxide dismutase activities of liver in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

was a decreasing trend, at the 5th and 6th wks. The decrease was significant as compared to the 3rd and 4th wks. The total SOD activity in the liver of diabetics showed significant increases as compared to controls at all times. The maximal increase was observed in the 1st wk, being 64.7% higher than its respective control values.

Cu-Zn SOD : Cu-Zn SOD activity showed no difference in the control group. In the diabetic group, the trend was similar to total SOD. Activity at 3rd and 4th wks was significantly higher as compared to the 1st wk. After that there was a decreasing trend: the decrease was significant on the 6th wk as compared to 2nd wk (12.3%), 3rd wk (15.9%) and 4th wk (13.7%). The Cu-Zn SOD activity in diabetic liver was higher at all times as compared to the control group. The activity was maximum at the 3rd wk, but the percentage increase as compared to control was maximum at the 4th wk (58.8%).

Mn-SOD : The trend in Mn-SOD was variable in the control group. In the diabetic group, the activity was lower in the 4th wk (43.88%), 5th wk (43.88%) and 6th wk (44.6%) as compared to the 2nd wk. Only at the 4th wk, Mn-SOD showed a significant decrease (17.8%) as compared to control. The changes at other intervals were not significant.

3.2.2 Heart

3.2.2.1 Malondialdehyde Content : The changes in MDA content in the heart are summarized in Fig. 6. The MDA content remained unchanged in the control and diabetic groups during

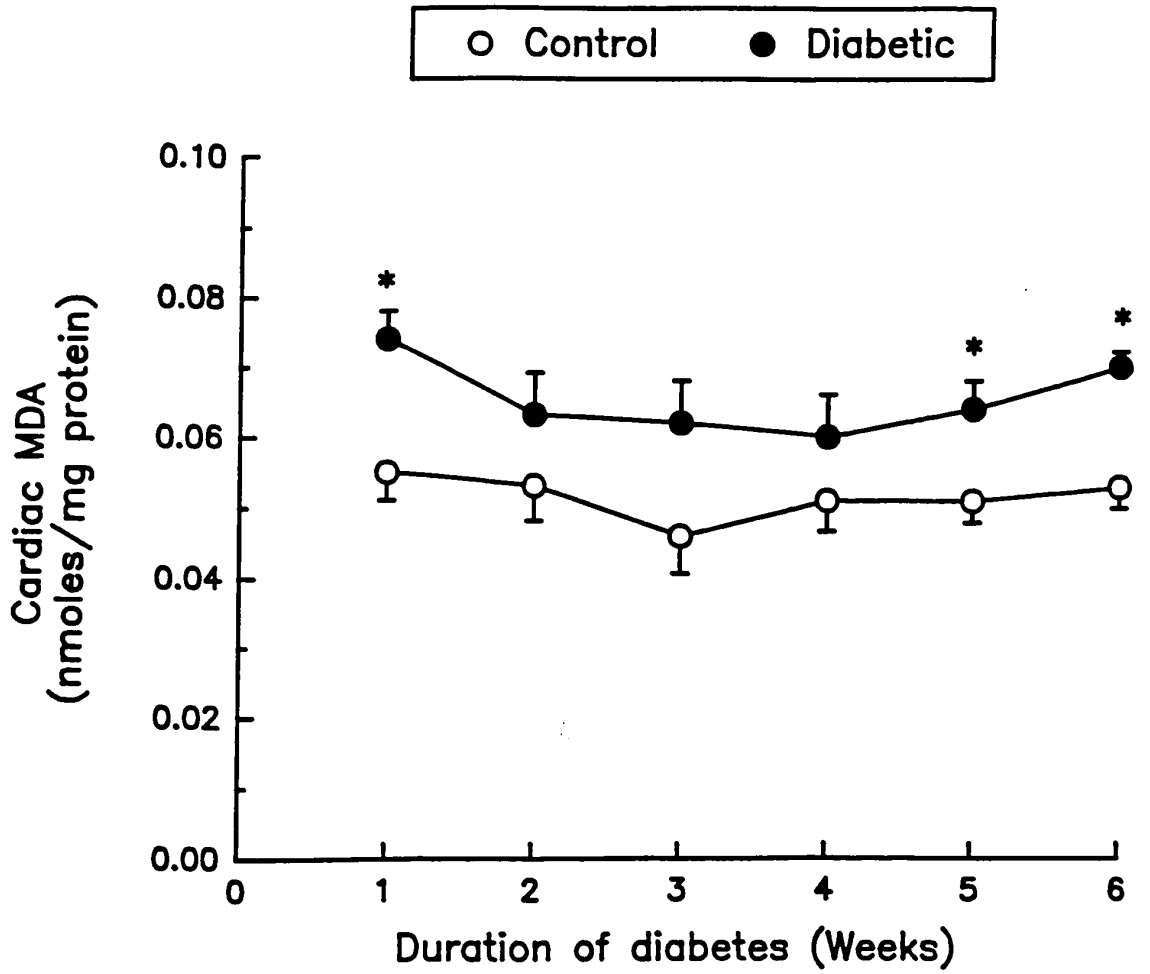


Fig.6: Changes in lipid peroxide (MDA) level of heart in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group.

the course of 6 wks. However, the diabetic rats showed a significant increase on the 1st wk (34.5%), 5th wk (25.5%) and 6th wk (32.1%) as compared to the respective control intervals.

3.2.2.2 Catalase : Changes in catalase activity of the heart are summarized in Fig. 7. The activity in the control group remained unchanged. In the diabetic group, the activity at the 4th wk and 6th wk was higher as compared to the 1st wk. Increases of 28.75%, 17.6% and 21.03% were observed at the 4th, 5th and 6th wks respectively in comparison to the 2nd wk. Catalase activity of the diabetic heart was greater at all times as compared to respective controls. The activity was maximal at the 4th wk, being 125.6% higher than its control value.

3.2.2.3 GSH-Px : The changes in GSH-Px activity of the heart are shown in Fig. 7. In the diabetic group, the activity at the 4th wk was higher as compared to the 2nd and 3rd wk. However, the activity at the 5th wk decreased (18.5%) in comparison to the 4th wk. The sixth wk showed an increase as compared to 2nd wk. The control group showed no difference in activity during the six wk course. Diabetic heart GSH-Px activity showed a significant increase at all times except in the 2nd wk. The activity was a maximum at the 4th wk, 65.3% higher than its control value.

3.2.2.4 SOD : Changes in the total SOD, Cu-Zn SOD and Mn-SOD of the heart are summarized in Fig. 8.

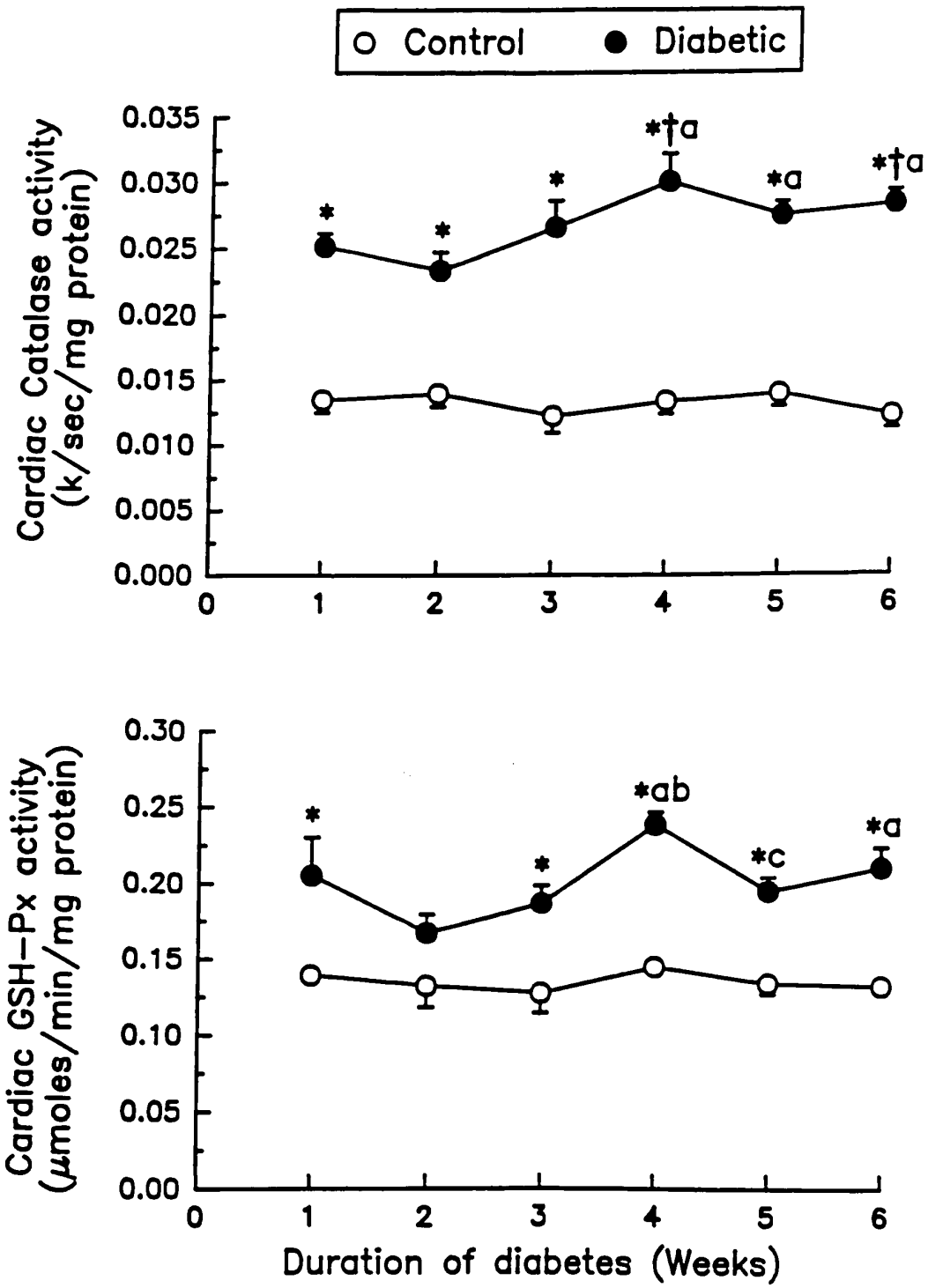


Fig.7: Changes in Catalase and Glutathione peroxidase activities of heart in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

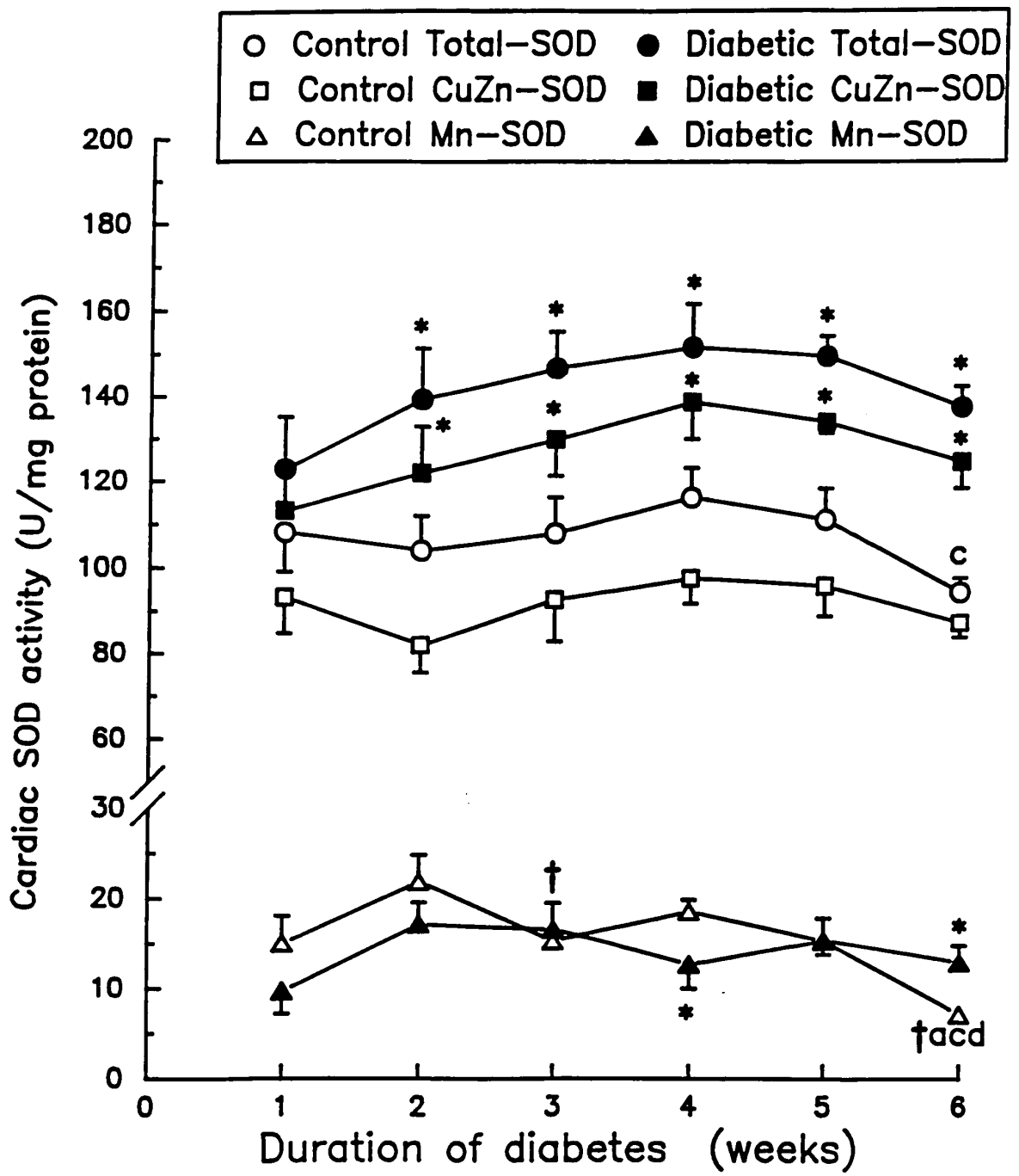


Fig.8: Changes in Superoxide dismutase activities of heart in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week

Total SOD : In the control group, the activity at the 6th week was lower as compared to the 4th wk, however there was no difference at other intervals. The diabetic group showed no change at all intervals. The changes in diabetic group were significant ($p<0.05$) as compared to the control from the 2nd wk onwards. The activity increased gradually up to the 4th wk to a maximum.

Cu-Zn SOD : Cu-Zn SOD activity remained unaltered in the control group. The trend of change in the diabetic group was similar to total SOD. The diabetic heart Cu-Zn SOD showed a significant increase from the 2nd wk onwards in comparison to respective controls. The activity was at a maximum at the 4th wk, 41.9% higher than its control value.

Mn-SOD : The trend of change in Mn-SOD was variable. The control group showed a decrease at the 6th week in comparison to the 1st, 2nd, 4th and 5th wks. The diabetic group, showed significant increase at 3rd wk in comparison to 1st wk. Mn-SOD activity of diabetic heart decreased at the 4th wk (31.7%) and increased at the 6th wk (81.7%) as compared to 4th and 6th wk controls.

3.2.3 Pancreas :

3.2.3.1 MDA : The changes in MDA content of the pancreas are summarized in Fig.9. The control group showed no changes in MDA content. In the diabetic group, there was a decrease at 5th wk as compared to 1st wk, 3rd wk and 4th wk. During the course of 6 wks, the MDA content of the pancreas increased

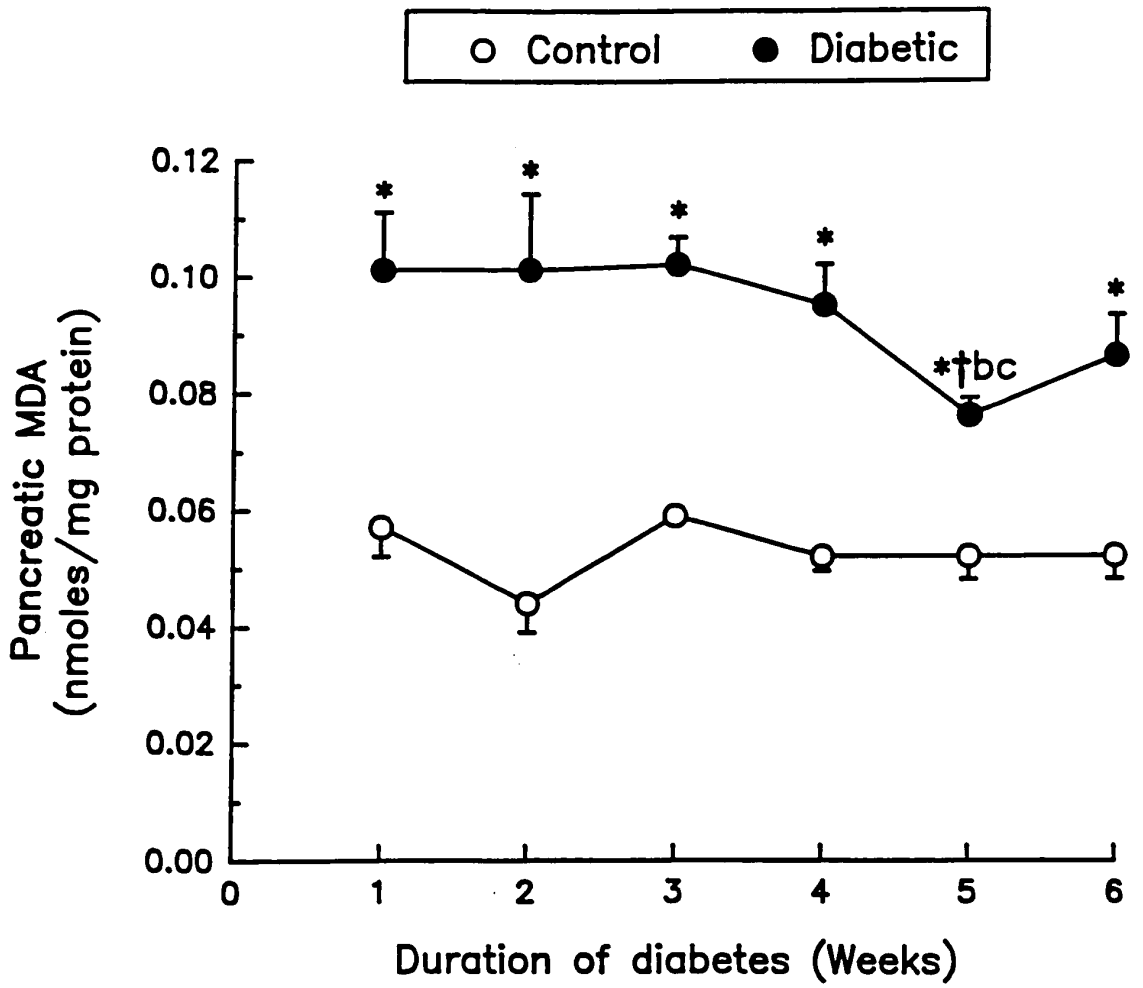


Fig.9: Changes in lipid peroxide (MDA) level of pancreas in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

significantly in diabetics in comparison to controls. The magnitude of the increase up to 4th wk was similar.

3.2.3.2 Catalase : The time course of changes in catalase activity of the pancreas are summarized in Fig.10. The control group showed no change in catalase activity. However, in the diabetic group, the activity decreased at the 5th wk in comparison to 1st and 2nd wk. The decrease at the 6th wk was also significant in comparison to the 1st wk, 2nd wk, 3rd wk and 4th wk. The CAT activity in diabetic pancreas increased significantly in comparison to control at all times. The activity was maximum (108% higher of its control value) at 1st wk. After that it decreased gradually. The decrease was significant only at 5th and 6th wk.

3.2.3.3 GSH-Px : The changes in GSH-Px activity of pancreas are summarized in Fig.10. The control group showed no changes in the activity. In the diabetic group, the activity at the 4th wk was 43.4% and 32.8% higher in comparison to the 2nd wk and the 3rd wk, respectively. The decrease in activity at the 5th and the 6th wk in comparison to the 4th wk was of a similar magnitude. GSH-Px activity of the diabetic pancreas increased significantly at all times in comparison to respective controls. The activity was maximum at the 4th wk (217.6%) as compared to the respective control.

3.2.3.4 SOD : The time course of changes in Total SOD, Cu-Zn SOD and Mn SOD in pancreas are summarized in Fig.11.

Total SOD : Total SOD activity remained unchanged in the

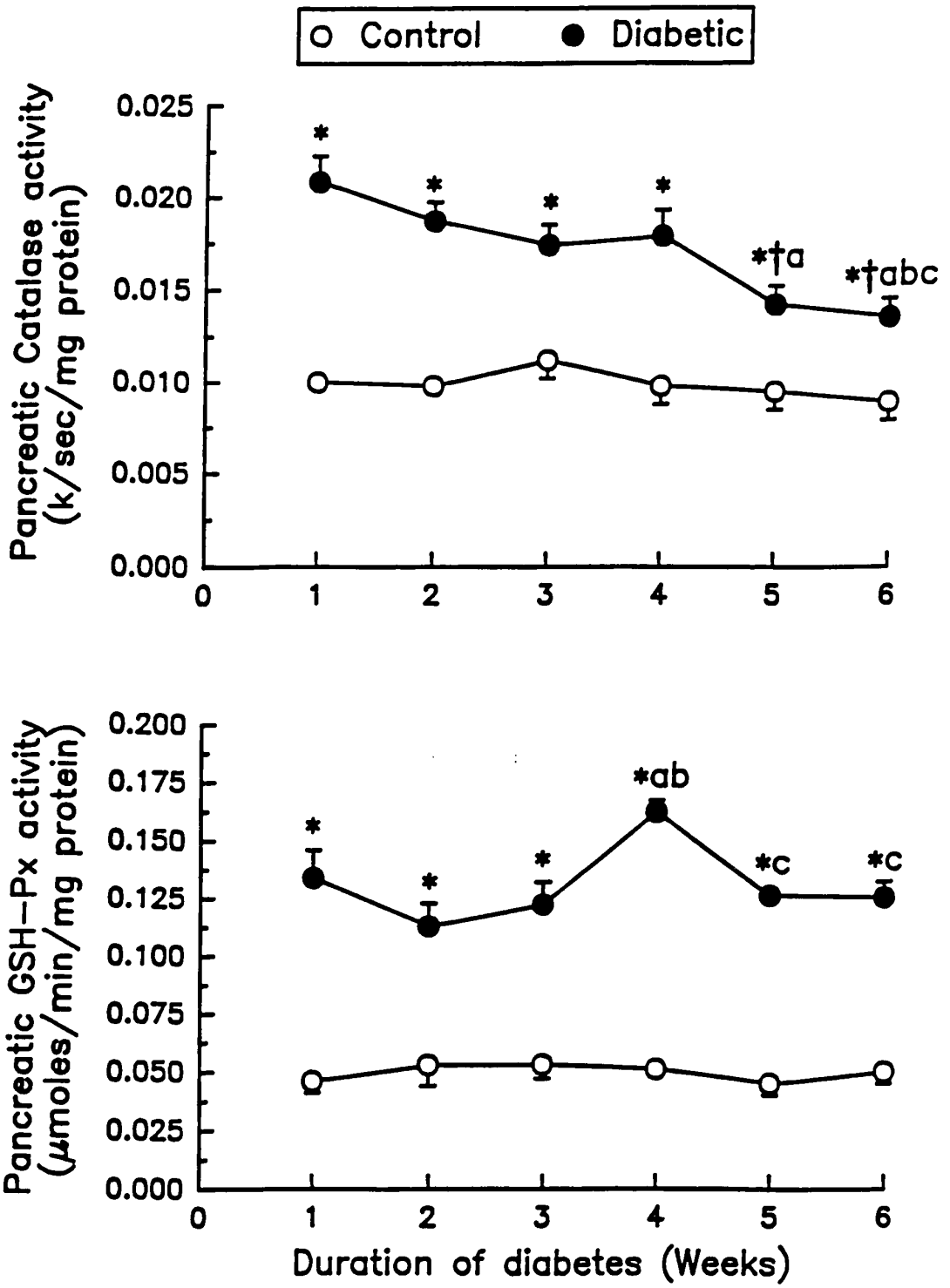


Fig.10: Changes in Catalase and Glutathione peroxidase activities of pancreas in control and diabetic rats. The results are expressed as Mean + S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

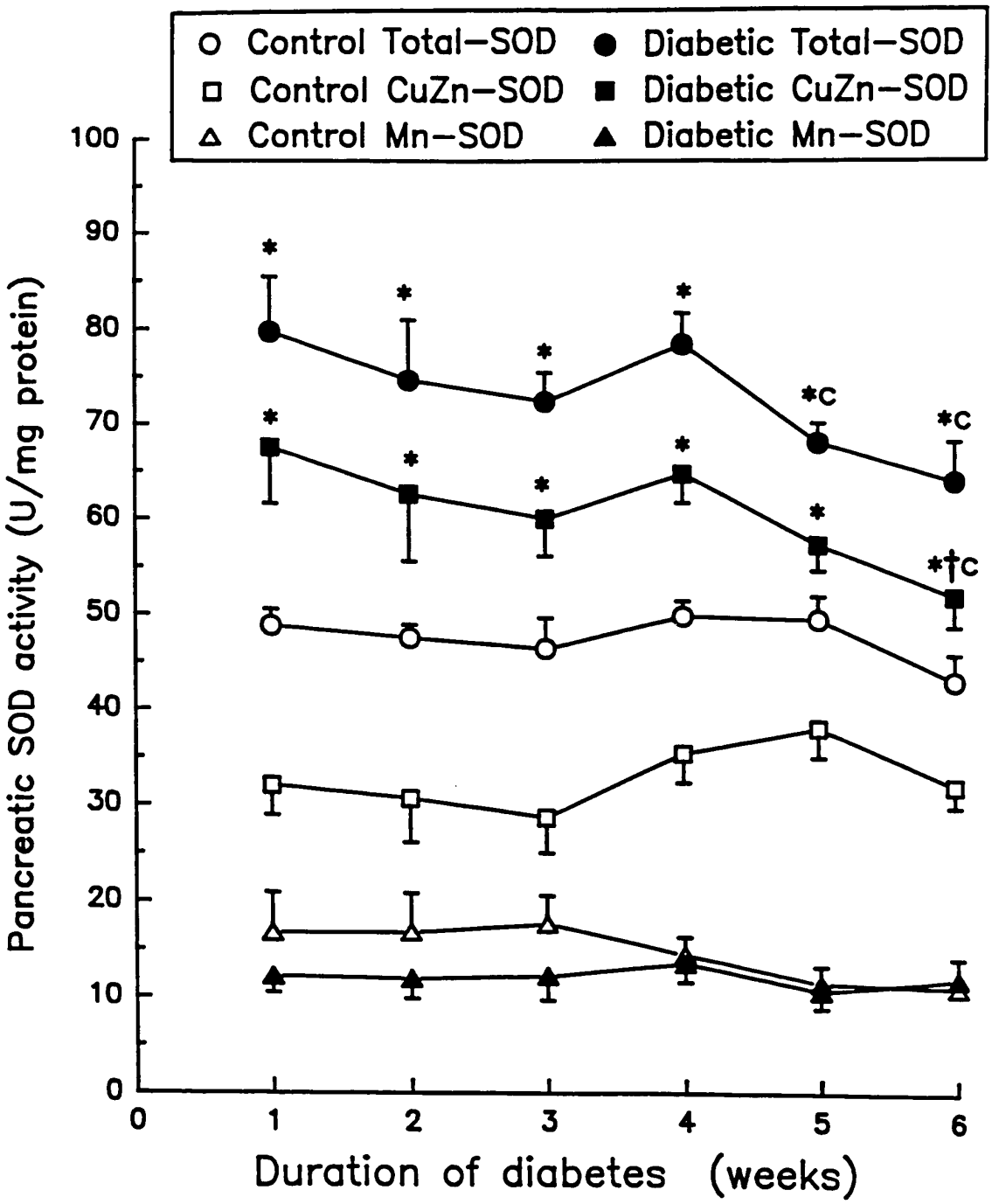


Fig.11: Changes in Superoxide dismutase activities of pancreas in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week.

control group. However in the diabetic group, the activity decreased at the 5th and the 6th wk in comparison to the 4th wk. The diabetic pancreas showed significant increase in activity at all times in comparison to respective controls. The activity was maximal at the 1st wk, being 63.18% higher than control value.

Cu-Zn SOD : The control group showed no changes in activity during the course of the 6 wks. The trend of change in Cu-Zn SOD in diabetic group was similar to that of total SOD. There was significant decrease at 6th wk in comparison to 1st and 4th wk. The activity in diabetic pancreas was significantly higher as compared to controls. The activity was maximum at 1st wk, 63.2% higher than its control value.

Mn-SOD : There was no difference in activity in control and diabetic group. Mn SOD activity of diabetic pancreas had lower values as compared to controls, but the changes were not significant.

3.2.4 Aorta :

3.2.4.1 MDA : The time course of changes in MDA content, in aortic tissue are summarized in Fig.12. The control group showed no changes in MDA. In the diabetic group the levels at 1st wk were higher as compared to 2nd, 3rd and 5th wk. MDA content at the 5th wk showed an increase of 36.2% and 51.9% as compared to the 2nd and 3rd wk respectively. Sixth wk showed maximum MDA content, the levels were greater than 2nd, 3rd, 4th and 5th wk. Diabetic aorta showed significant increase in

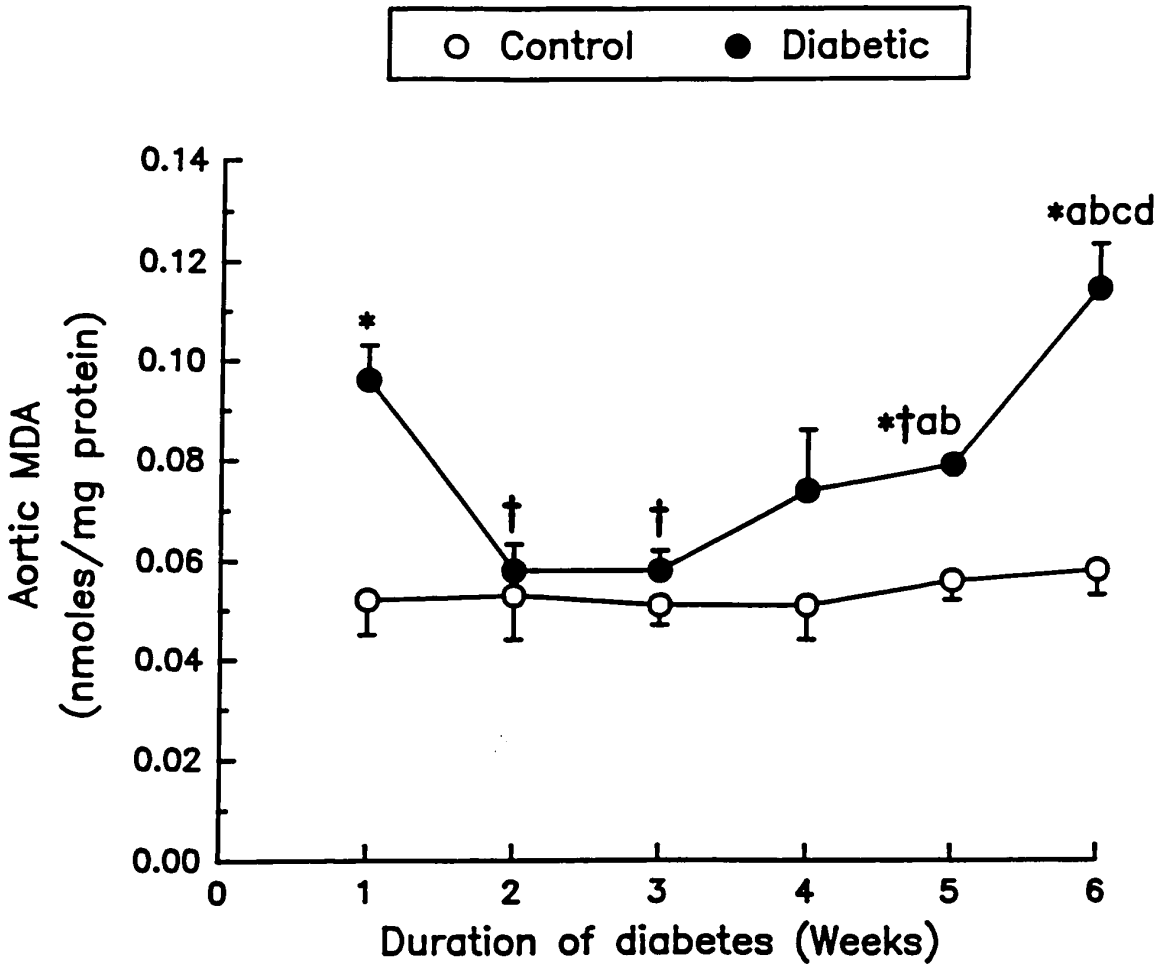


Fig.12: Changes in lipid peroxide (MDA) level of aorta in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week.

MDA content at 1st wk (84.6%), 5th wk (41.1%) and 6th wk (96.6%) as compared to respective controls. The increase was maximum at 6 wks.

3.2.4.2 Catalase : The changes in catalase activity are summarized in Fig.13. The activity remained unchanged in the control group. In the diabetic group, the activity at 5th wk and 6th wk was significantly higher as compared to the 1st wk, 2nd wk and 4th wk. The activity at sixth wk was higher than that at 3rd wk. CAT activity increased significantly ($p<0.05$) at all times as compared to controls. The activity was similar at 1st and 2nd wk, 3rd and 4th wk, after which, it increased gradually and was maximum at 6th wk, being 83.5% higher than its control value.

3.2.4.3 GSH-Px : The changes in GSH-Px activity of aorta are summarized in Fig.13. The GSH-Px activity remained unaltered in the control group. In the diabetic group, the activity at 6th wk was greater by 59.7% from 1st wk, 35.6% from 4th wk and 33.8% from 5th wk. The aortic tissue GSH-Px activity during the progression of diabetes increased significantly at 4th wk (37.7%), 5th wk (51.02%) and 6th wk (120%) as compared to respective controls. The activity was almost similar from 2nd wk to 5th wk, being maximum at 6th wk.

3.2.4.4 SOD : The changes in SOD activity of aortic tissue are summarized in Fig.14. There was no change in activity of the control group. However in the diabetic group the activity at all the intervals was higher as compared to 1st wk. The

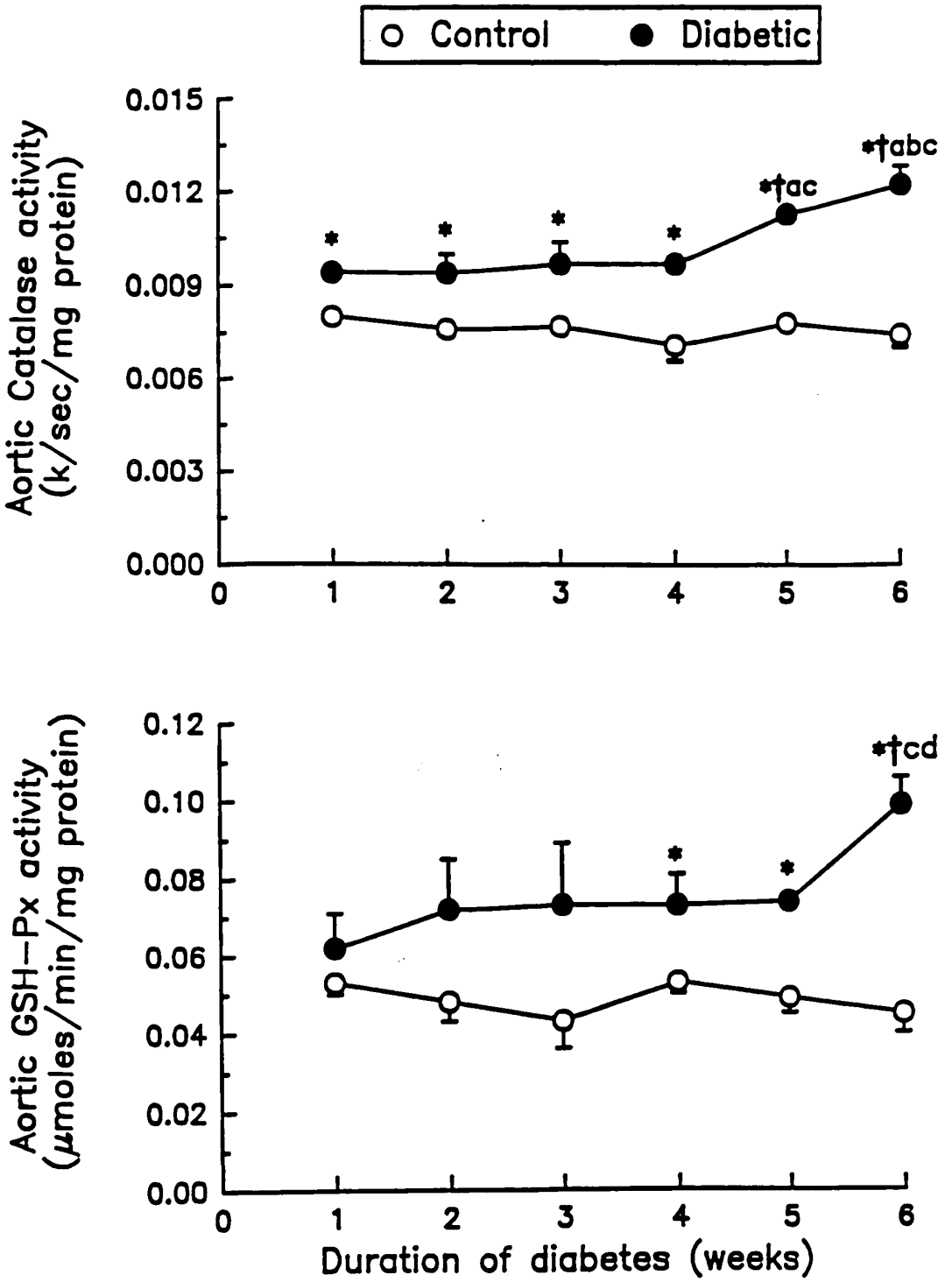


Fig.13: Changes in Catalase and Glutathione peroxidase activities of aorta in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week.

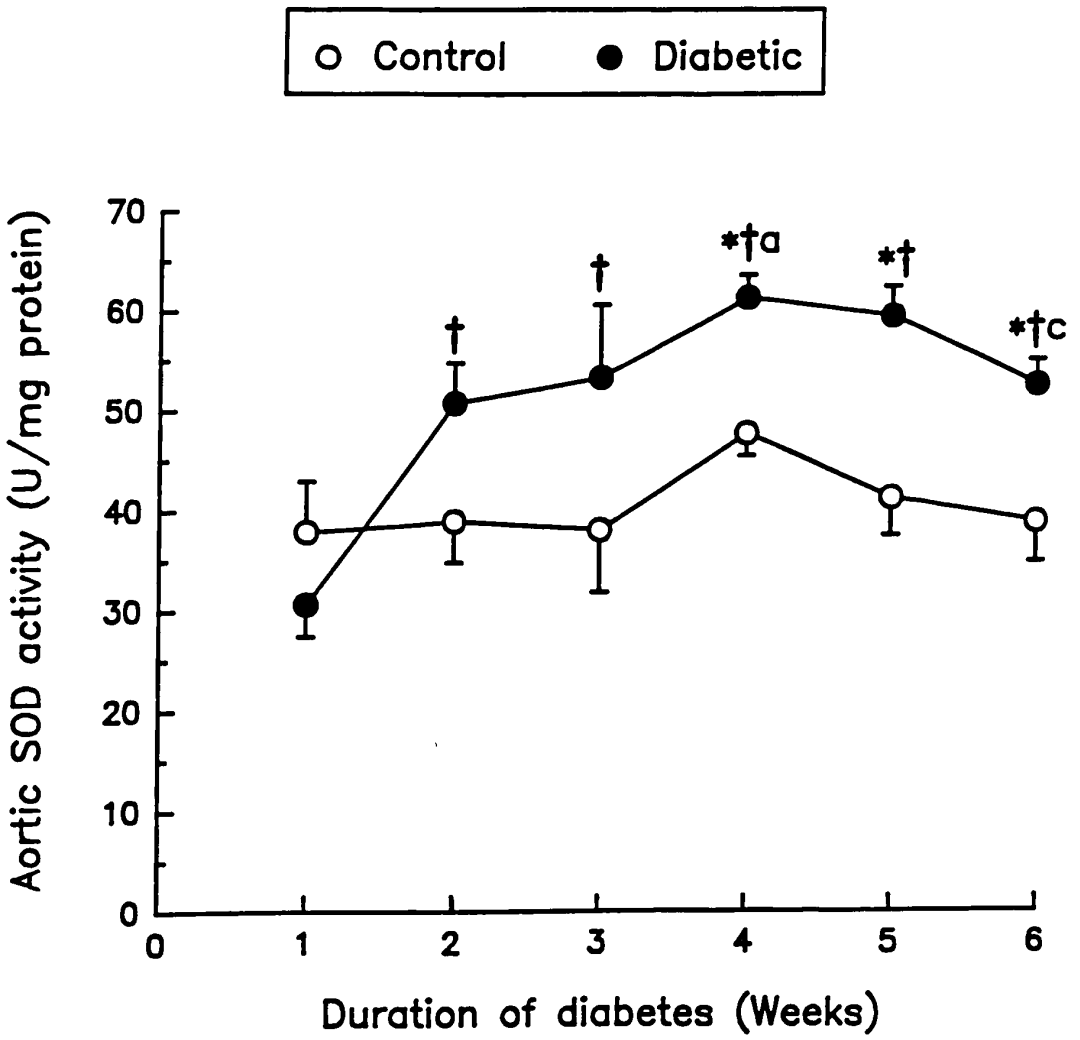


Fig.14: Changes in Superoxide dismutase activity of aorta in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

activity at 4th wk was higher as compared to 2nd wk and 6th wk showed lower activity as compared to 4th wk. In diabetic aortic tissue, the activity increased significantly from 4th wk onwards as compared to respective controls. The activity being maximum at 4th wk.

3.2.5 Kidney :

3.2.5.1 MDA : The changes in MDA content of kidney is summarized in Fig.15. The activity remained unchanged in the control group. In the diabetic group, the MDA levels were lower in 2nd and 4th wk as compared to the 1st wk. In the sixth wk it showed higher content as compared to 2nd, 3rd, 4th and 5th wk. The diabetic kidney showed a significant increase in MDA content at 1st wk (67.2%), 5th wk (50%) and 6th wk (60.6%) as compared to respective controls. MDA content was similar at 1st and 6th wk.

3.2.5.2 Catalase : The changes in catalase activity of kidney are summarized in Fig.16. There was no change in activity in the control group, where as in the diabetic group, the CAT activity was significantly lower at all the times except 3rd wk, as compared to 1st wk. The activity at 5th and 6th wk was lower as compared to the 2nd, 3rd and 4th wk. In the first wk, diabetic kidney showed a significant increase (19.9%) as compared to the 1 wk control, however at other times, it showed decreases which were significant at 5th (27.8%) and 6th wk (20.6%).

3.2.5.3 GSH-Px : The changes in GSH-Px activity in kidney

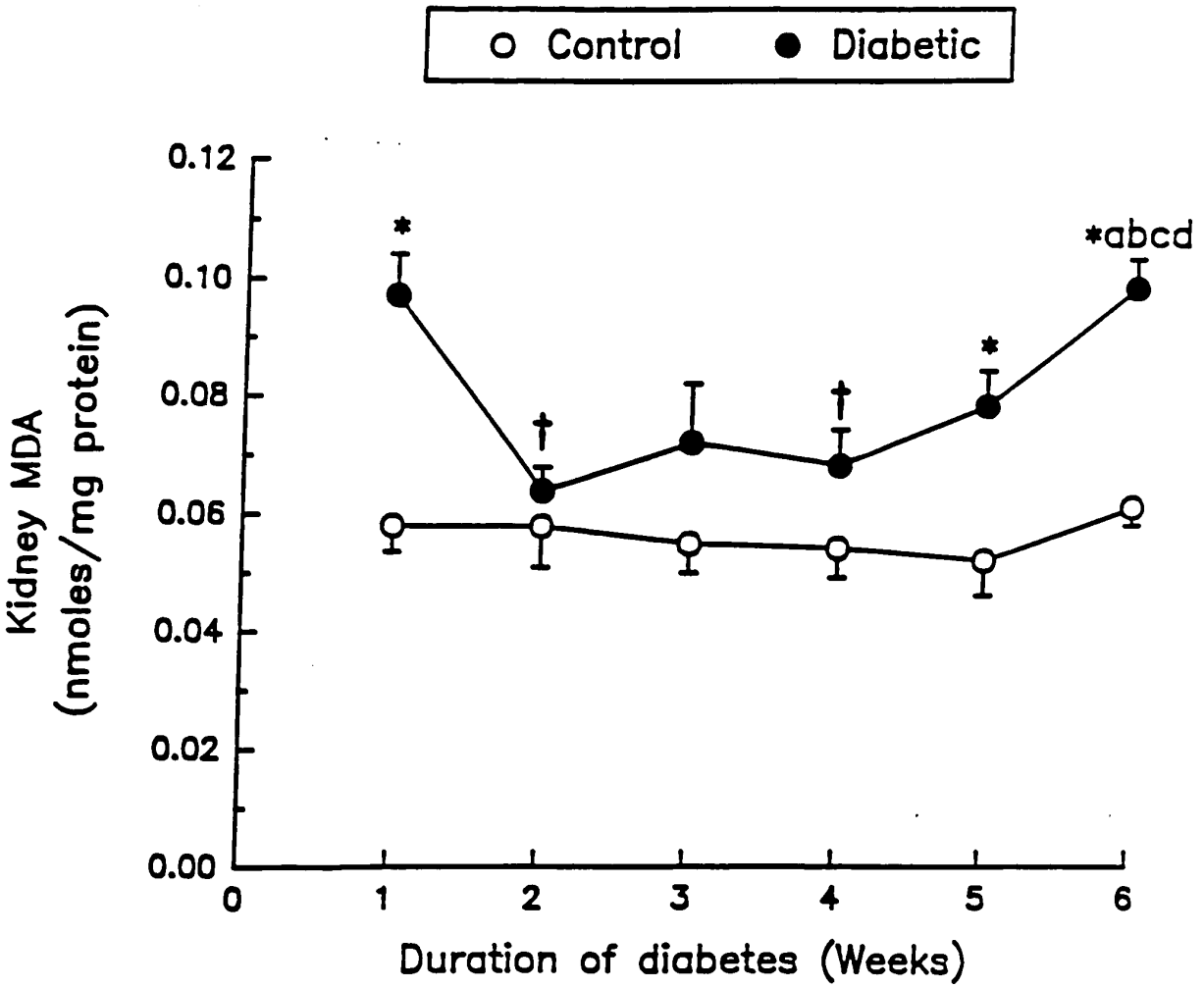


Fig.15: Changes in lipid peroxide (MDA) level of kidney in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week.

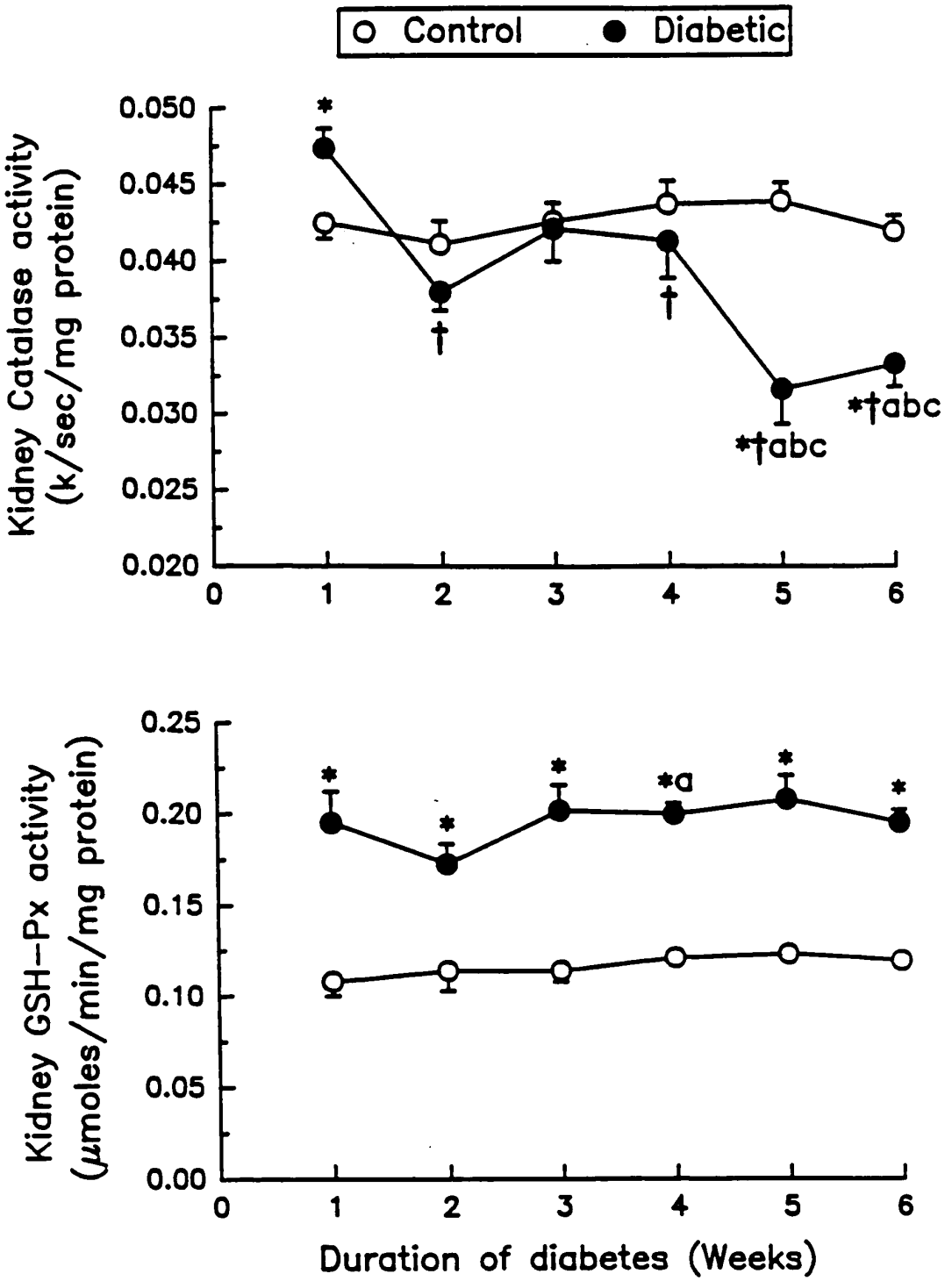


Fig.16: Changes in Catalase and Glutathione peroxidase activities of kidney in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

are shown in Fig.16. In the control group, the activity at different time intervals was almost similar. However in the diabetic group, there was no significant change, except that the 4th wk had higher activity as compared to 2nd wk. GSH-Px activity increased at all times during the course of diabetes as compared to respective controls. The magnitude of increase was almost similar in 1st wk (80.5%), 3rd wk (77.2%) and 5th wk (69.1%) however the maximum activity was observed at 5th wk.

3.2.5.4 SOD : The changes in SOD activity of kidney are summarized in Fig.17.

Total SOD: The total SOD activity remained unaltered in control and diabetic group. However, the diabetic group showed increased activity at all times as compared to respective controls. The magnitude of the increase was almost similar at all times. The maximum activity was observed at 1st wk however the increase was maximal at 3rd wk, 43.04% higher than its control value.

Cu-Zn SOD : Cu-Zn SOD activity was not altered in the control and diabetic group. However in the diabetic kidney the activity increased significantly as compared to controls at all times. Cu-Zn SOD activity showed a similar trend like total SOD. The activity was almost similar upto 3rd wk, after that there is a decrease which was not significant. The maximal increase in activity was observed at 3rd wk, 49.5% being higher of its control value.

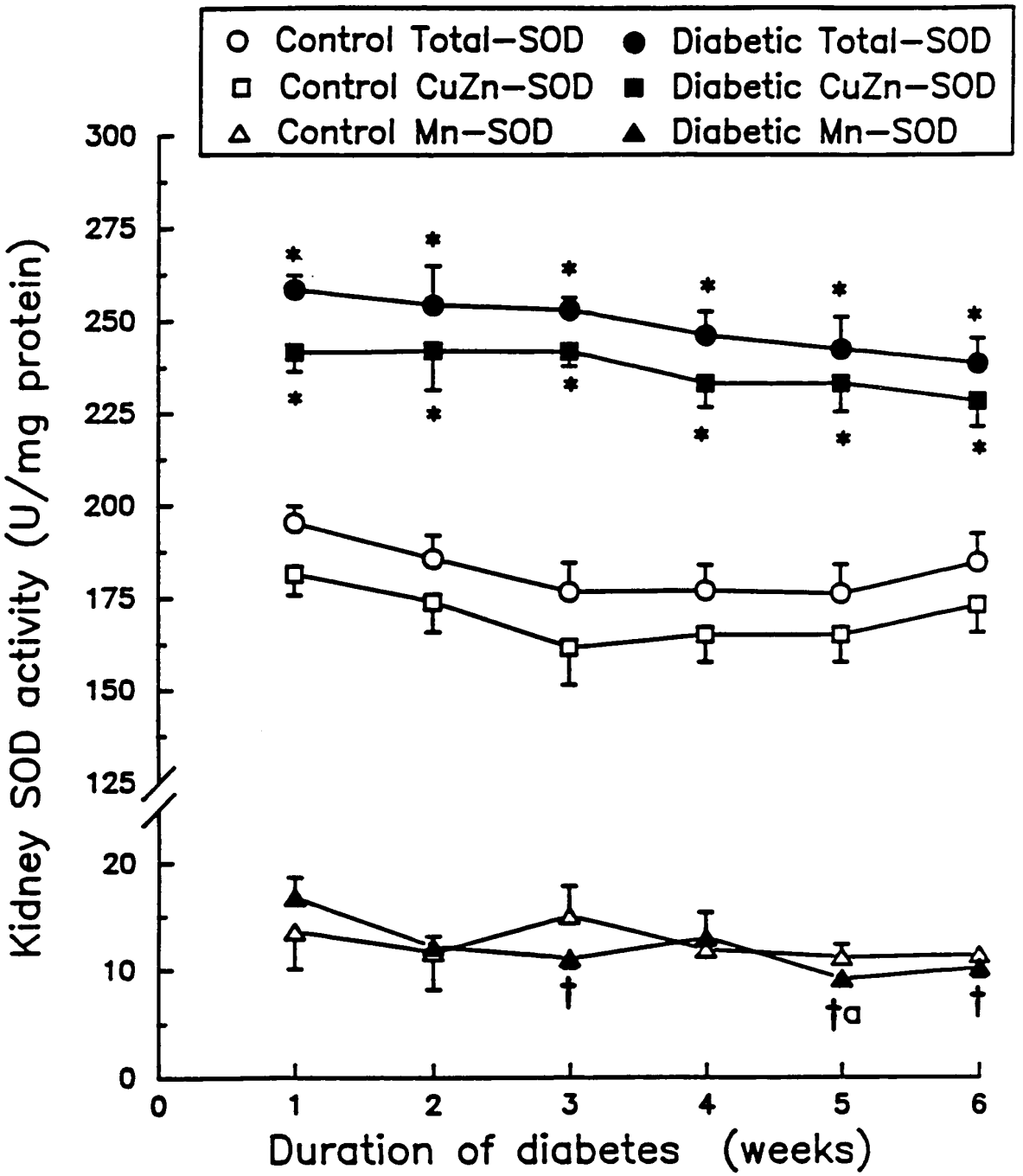


Fig.17: Changes in Superoxide dismutase activities of kidney in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week.

Mn SOD : The trend of changes in Mn SOD activity in control and diabetic groups was variable. In the diabetic group, a significant decrease was observed at the 3rd, 5th and 6th wk as compared to 1st wk. A significant decrease at the 5th wk was also observed as compared to 2nd wk, however no changes were observed in the control group. Comparison of control and the diabetic group showed no significant difference.

3.2.6 Blood :

3.2.6.1 MDA : The changes in MDA content of blood are summarized in Fig.18. MDA content is expressed as nmol/ml blood. The level remains unaltered in the control group. In the diabetic group, the 3rd wk showed an increase (39.6%) as compared to 2nd wk and after that a decrease was observed at 4th wk as compared to 3rd wk. The increase at 6th wk was significant compared to all previous intervals. The MDA content of diabetic blood was higher at the 3rd wk (28.2%), 5th wk (53.6%) and 6th wk (117.8%) as compared to respective controls. Maximum MDA content was observed at the 6th wk.

3.2.6.2 Catalase : The changes in catalase activity are summarized in Fig.19. The activity is expressed as k/sec/gm Hb. CAT activity was unaltered in control group. However in the diabetic group, 5th and 6th wk showed gradual significant increase as compared to 1st, 2nd, 3rd and 4th wk. The diabetic blood CAT activity showed an increase at 4th wk (31.8%), 5th wk (65.3%) and 6th wk (97.5%) as compared to respective controls.

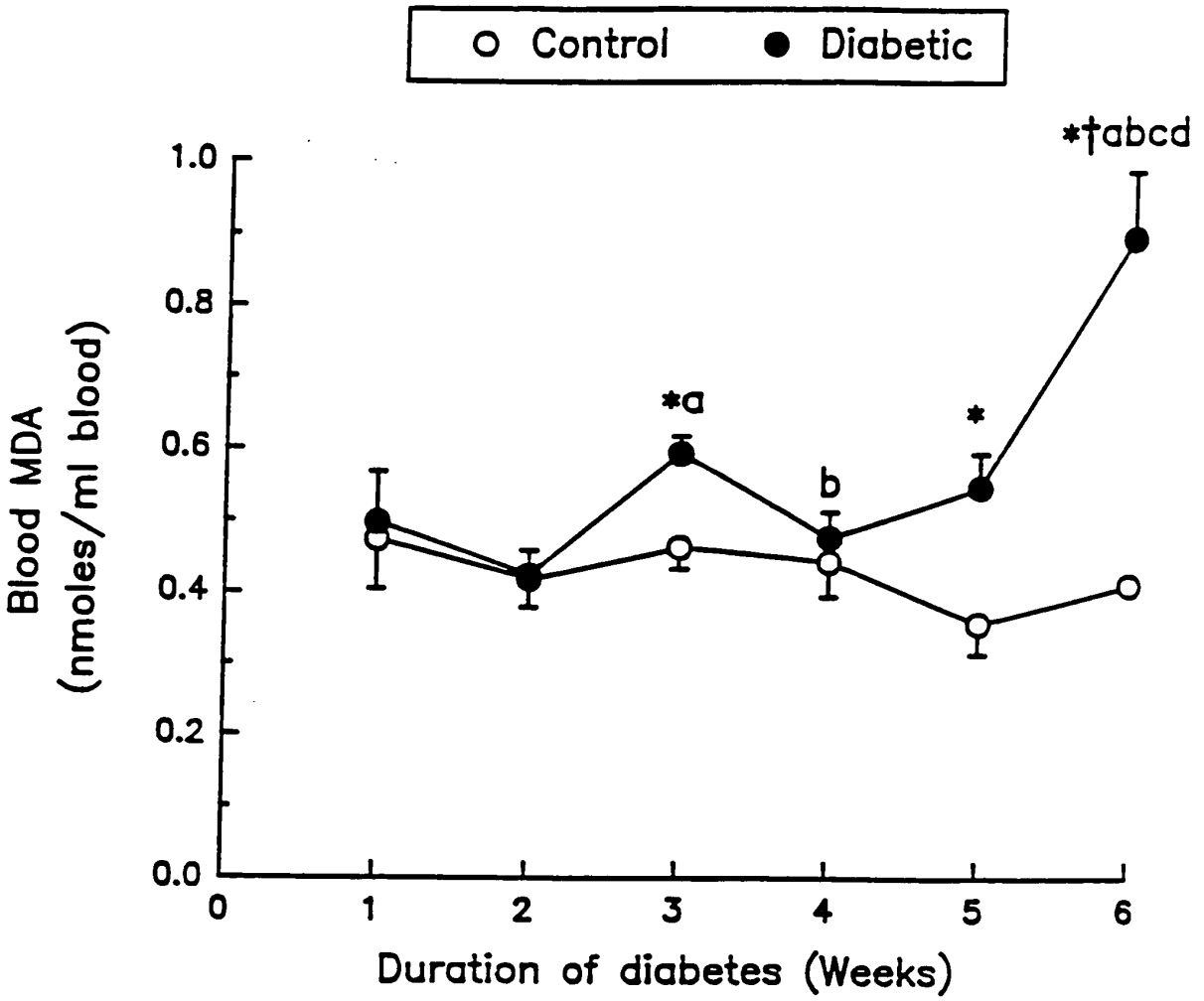


Fig.18: Changes in lipid peroxide (MDA) level of blood in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week; d $p<0.05$, 5th week vs 6th week.

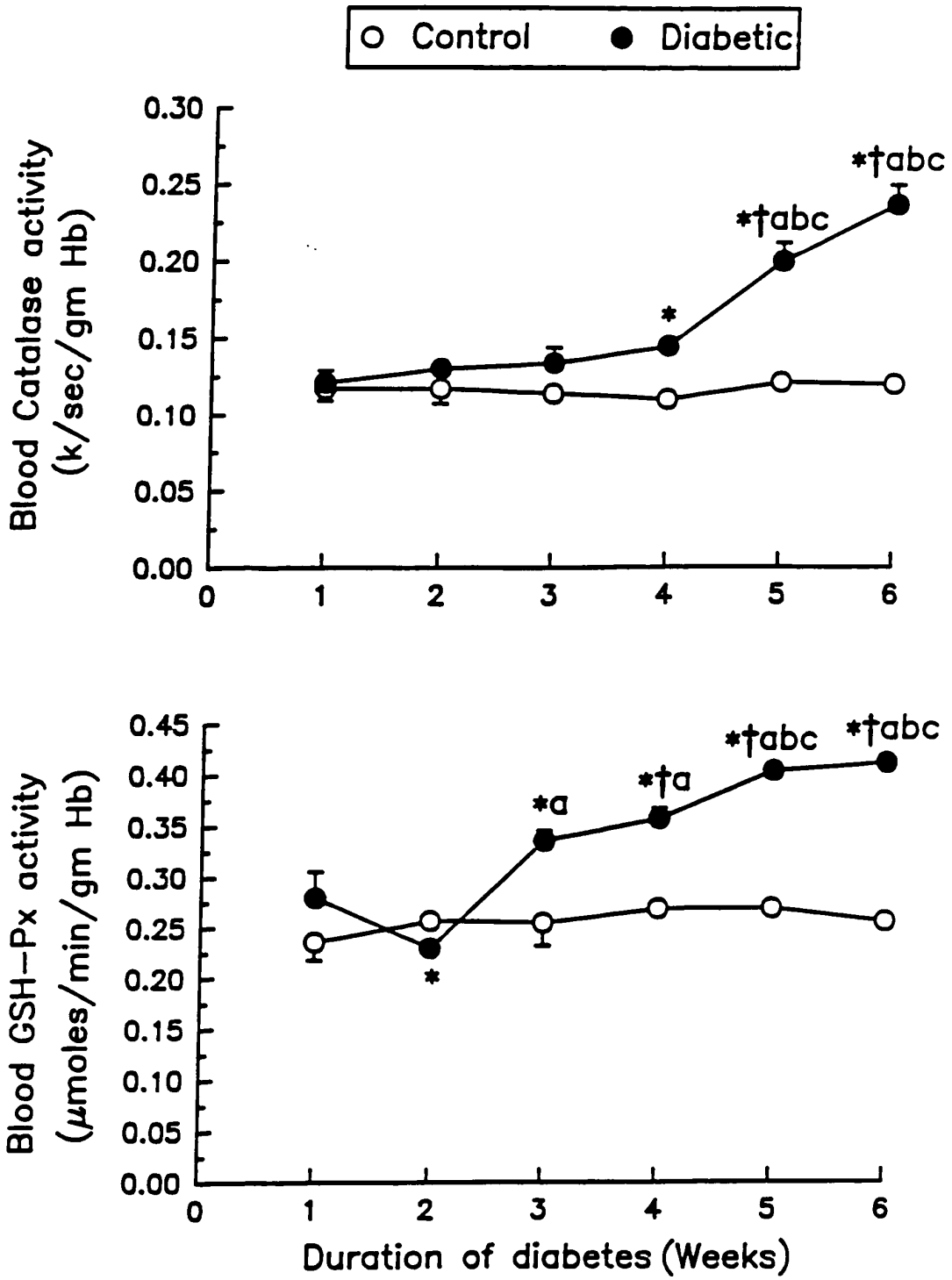


Fig.19: Changes in Catalase and Glutathione peroxidase activities of blood in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week; a $p<0.05$, 2nd week vs 3rd, 4th, 5th and 6th week; b $p<0.05$, 3rd week vs 4th, 5th and 6th week; c $p<0.05$, 4th week vs 5th and 6th week.

3.2.6.3 GSH-Px : The changes in GSH-Px activity of blood are summarized in Fig.19. No change in activity was observed in control group. However, in the diabetic group the activity at 3rd wk was higher as compared to 2nd wk and at 4th wk it was higher as compared to 1st and 2nd wk. The activity increased gradually from 3rd wk onwards and at the 5th and 6th wk it was significant as compared to all previous intervals. GSH-Px activity of diabetic blood showed a small decrease at 2nd wk and after that an increase was observed at 3rd wk (31.7%), 4th wk (33.2%), 5th wk (50.4%) and 6th wk (61.2%) as compared to respective controls. The maximum activity was observed at 6th wk.

3.2.6.4 SOD: The changes in total SOD activity of blood are summarized in Fig. 20. The total SOD activity remains unaltered in control group, however in the diabetic group the activity at 4th wk was significantly higher as compared to 1st wk. The activity showed a significant increase from 3rd wk onwards as compared to respective controls. The maximum activity was observed at 4th wk, 69.8% higher than its control value.

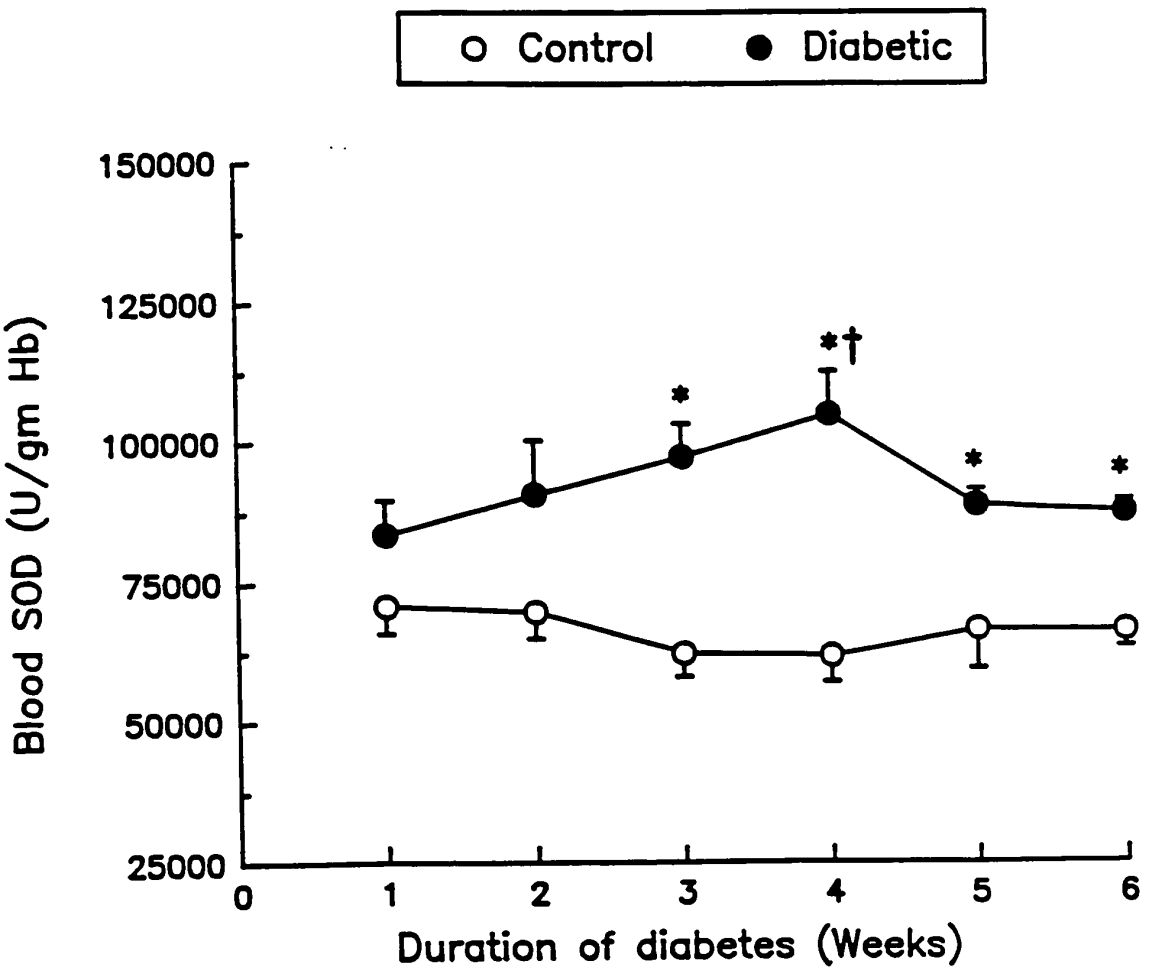


Fig.20: Changes in Superoxide dismutase activity of blood in control and diabetic rats. The results are expressed as Mean ± S.E.M. * $p<0.05$, control vs diabetic group; † $p<0.05$, 1st week vs 2nd, 3rd, 4th, 5th and 6th week.

4.0 Discussion

The aim of the study was to determine the oxidative stress in experimental diabetes mellitus that might be responsible for complications in diabetes. Streptozotocin used for induction of diabetes in male Sprague Dawley rats was given intraperitoneally. The dose of STZ to produce diabetes in various species varies between 25-200 mg/kg. The dose of 80 mg/kg selected for the present study was similar to that used by others (Lammikeefe *et al.* 1984; Asplund *et al.* 1984). The STZ treated animals showed glycosuria and hyperglycemia. The increase in plasma glucose levels could be due to the decreased levels of insulin. Insulin is known to stimulate glucose and amino acid uptake into cells (Howard *et al.* 1979; Kletzien *et al.* 1976). Intravenous injection of STZ, which destroys ß-cells of pancreas (Evans *et al.* 1965), results in decreased levels of insulin which in turn would decrease utilization of blood glucose by peripheral tissues and would lead to hyperglycemia. The decrease in body weight in diabetic animals could be due to the changes in cellular metabolism. Glucose and amino acids are essential for normal metabolism. The lack of their utilization by the tissues affects cellular processes. The prolonged decrease of nutritional availability in diabetic animals can lead to reduction in body weight. The time course selected for our study was 1-6 wks after induction of diabetes. Wong and Tzeng

(1993) reported that after STZ administration it takes one wk to have all the diabetic symptoms.

4.1 Tissue MDA:

MDA (an indirect measure of levels of OFR) is a breakdown product of polyunsaturated fatty acids. It is an index of free radical induced damage (Halliwell, 1984).

Liver:

In the present study the diabetic liver showed a significant increase in MDA with respect to control group at all time intervals except at 2nd wk. This suggests that the levels of OFRs increase with the duration of disease. The lower levels observed at 4th and 5th wk as compared to 3rd wk may be due to higher activity of antioxidant enzyme GSH-Px. Due to high phospholipid content in polyunsaturated fatty acids liver is susceptible to peroxidative agents, free radical generating compounds and lipid peroxides (Hammer and Willis, 1978).

Heart: The increase observed in MDA at the 1st, 5th and 6th wk in heart tissue, suggests an increase in OFR levels. With the progression of diabetes, no change was observed in MDA levels and the increase was less marked as compared to other tissues which suggests that the heart seems to possess increased resistance against oxidative stress. Parinandi *et al.* (1990) reported that diabetic heart contains high levels of glutathione which offers protection from OFRs. In the present study significant increases were observed in CAT, GSH-Px and SOD enzymes in heart which may be responsible for lower

MDA levels.

Pancreas: Pancreas is the target tissue which is damaged first with STZ. The pancreas showed an increase in MDA as compared to controls at all intervals. Lower levels of antioxidant enzymes were observed in the pancreas as compared to liver, heart and kidney which may make it susceptible to lipid peroxidation. The other possible reason for increased MDA in pancreas could be due to STZ itself. Pancreatic cells rapidly take up STZ, which may mediate its action by generation of free radicals (Grankvist *et al.* 1979; Mordes and Rossini, 1981; Asplund *et al.* 1984). The decrease observed in MDA levels at 5th wk as compared to other intervals is unexplained. However, it could be due to decreased levels of oxidative stress at that interval. Armstrong and Al-Awadi (1991) reported similar results and suggested that it could be due to depletion of substrates for thiobarbituric acid reactive substances (TBARS). But in the present study we found increased MDA content at 6th wk and hence that argument does not hold. Decreased glutathione levels reduce the redox potential of the cell and offer less protection against free radicals. The present findings are in agreement with those of Malaisse *et al.* (1982) who showed that islets exposed to alloxan and STZ show low levels of GSH and hence increased susceptibility of membranes and macromolecules to lipid peroxidation (Barnett *et al.* 1986).

Aorta: The increase in MDA in aorta at the 1st, 5th and 6th

wk could be due to higher levels of OFR production. Aorta possesses the lowest antioxidant enzyme activities which makes it susceptible to free radical attack. Increased lipid peroxidation in aorta may be an important factor in development of atherosclerosis (Hunt *et al.* 1990). This could also have adverse effects on normal organs into which vessels are distributed (Yagi, 1987). The present increase in MDA in this study is in agreement with the findings of Uzel *et al.* (1988) and Jennings *et al.* (1987) who showed that in diabetic patients lipid peroxidation may be involved in the pathogenesis of degenerative and microangiopathic complications. Our findings also support the findings of Hunt *et al.* (1990) who reported that low density lipoproteins (LDL) exposed to high glucose levels show increased production of hydroperoxides. Oxidized LDL is cytotoxic to endothelial cells and is taken up by macrophages to stimulate foam cell formation (Morel *et al.* 1983). Foam cells are found in the majority in atherosclerotic plaques. They prolong the half life of LDL by impairing its catabolism and also increase the chances of LDL oxidation by $\cdot O_2^-$ (Hiramatsu *et al.* 1987). Oxidized LDL is considered to be a major atherogenic factor (Morel *et al.* 1983). Therefore increased OFRs as observed by increased MDA levels can promote atherosclerotic process by peroxidation of LDL.

Kidney : The increase in MDA observed on the 1st, 5th and 6th wk in the kidney could be due to higher oxidative stress. The

diabetic kidney on the 6th wk showed a significant increase as compared to other time intervals, suggesting an increase in OFRs with time. In the present study we observed a decrease in CAT activity which would lead to higher H_2O_2 levels. H_2O_2 can react with $\cdot O_2^-$ to form $\cdot OH$ and result in increased lipid peroxidation and hence higher MDA.

Blood MDA: Blood MDA increased on the 3rd, 5th and 6th wk. The increase in MDA suggests an increased production of OFRs with progression of diabetes. Even with increased CAT and GSH-PX the MDA levels were high. This suggests that oxidative stress was in excess of the capacity of the antioxidative enzymes to scavenge OFRs. The present findings are consistent with those of Sato *et al.* (1979), Jain *et al.* (1990) and Young *et al.* (1992) who reported an increase in MDA in plasma and erythrocytes of diabetic patients. Increased MDA observed in blood is due to release of MDA from cell membranes (Woolard *et al.* 1990). Increase in MDA can alter properties of red blood cells (RBCs). Jones and Peterson (1981) reported increased viscosity, adhesiveness and reduced life span of RBC, which can lead to rheological impairment in certain tissues of diabetics. Decreased vitamin E, increased lipid peroxidation and increased lipofuscin formation in erythrocytes of diabetic rats have been reported by Jain *et al.* (1990). This study suggests that increased MDA formation could result in its crosslinking with membrane phospholipids and proteins impairing microvascular flow.

In general, the increase in MDA in all the tissues suggests that hyperglycemia appears to induce lipid peroxidation in diabetic rats. In diabetes, OFRs are produced through mitochondria when the increased load of glucose causes increased flow through electron transport chain (Morris and Dat, 1979). Glucose oxidation and glycosylation of enzymatic proteins results in generation of $\cdot O_2^-$, H_2O_2 and other free radicals which will promote lipid peroxidation (Ahmed *et al.* 1986; Wolff *et al.* 1987; Hunt *et al.* 1990). These oxygen metabolites are not particularly toxic but in the presence of transition metals like iron or copper, they produce hydroxyl radicals by the Haber-Weiss reaction (Southorn and Powis, 1988). Transition metals like iron and copper can act as mediators of lipid peroxidation. Hunt *et al.* (1990) reported increases in levels of copper in diabetes. Iron is found in many enzymes, hemoglobin and storage proteins such as ferritin. During diabetes increased $\cdot O_2^-$ production occurs during glucose oxidation (Hunt *et al.* 1988).

In the present study, increased SOD levels in all the tissues suggest increased production of superoxide anion. Thomas *et al.* (1985) demonstrated that $\cdot O_2^-$ can promote the release of iron from ferritin which can cause lipid peroxidation. The above mentioned effects can cause membrane damage by changing fluidity, permeability and loss of membrane integrity and inactivation of membrane receptors which can effect cellular functions (Halliwell and Gutteridge, 1985).

Moreover it has been shown that products of crosslinking formed between MDA and macromolecules exert their toxic effects far from their site of production (Vaca and Ringdahl, 1989).

Cardiomyopathy, angiopathy and nephropathy and other complications in diabetes may be due to high levels of lipid peroxides in the tissues and blood.

4.2 Catalase:

Liver:

Catalase activity increased significantly in diabetic liver. The increase during the 1st wk could be due to increased H_2O_2 levels. In diabetes insulin deficiency promotes the ß-oxidation of fatty acids, with resulting H_2O_2 formation (Horie *et al.* 1981). The increase at the 4th wk as compared to the 2nd wk suggests that with the duration of diabetic state, enzyme activity increases. The decrease observed at the 5th wk could be due to inactivation or depletion of enzyme due to free radicals. The general trend seen for catalase activity in control tissues was that highest activity was found in liver followed by kidney, heart, pancreas and aorta.

Heart: Catalase activity in heart increased significantly at all times. The higher activity at the 4th, 5th and 6th wks as compared to 1st and 2nd wk suggests higher levels of oxidative stress with progression of disease. The elevation of cardiac CAT activity may be a compensatory increase due to an increase in endogenous H_2O_2 production in heart.

Pancreas: A significant increase in catalase activity was observed in the diabetic pancreas. The increase was maximal at the 1st wk after which it decreased becoming significant at 5th and 6th wks only. Our findings in control animals that the pancreas contains a relatively low CAT activity as compared to liver, heart and kidney agrees with Wohaeib and Godin (1987) and Grankvist *et al.* (1979) in rat and mouse tissues, respectively. The highest increase was observed in the 1st wk of diabetes, which suggests that quite high amounts of H_2O_2 are produced in the begining of diabetic state due to insulin depletion. Another possible reason is that STZ action could be mediated through production of H_2O_2. Asayama *et al.* (1984) reported that the ability of STZ to induce diabetes was associated with cellular metabolic oxidative patterns producing peroxidative phenomenon, similar to that producing oxygen free radicals. Recently, Takasu *et al.* (1991) reported that STZ and alloxan stimulate H_2O_2 generation in pancreatic ß-cells which causes damage to DNA by $\cdot O_2^-$ and $\cdot OH$ radicals. The decreasing trend in pancreatic CAT activity may be due to depletion or inactivation of enzyme.

Aorta: Catalase activity of diabetic aorta showed a significant increase. Increased activity at the 5th and 6th wks may be due to oxidative stress as evidenced by enhanced lipid peroxidation. Aorta had lowest enzyme activity as compared to all other tissues which may make it more susceptible to oxidative attack. Our findings do not agree

with Dohi *et al.* (1988) who reported no change in CAT activity in diabetic aorta but decreased GSH-Px activity. The lowest CAT activity found in control aorta as compared to other tissues suggests that weakness in the antioxidative defence system may be the biochemical background for the pathogenesis of endothelial dysfunction associated with diabetes.

Kidney: The significant increase in CAT activity observed on the 1st wk was followed by a decrease in its activity. Srivastava *et al.* (1993) also reported a decrease in catalase activity in the kidney of alloxan induced diabetic rats. Superoxide anion has been reported to inhibit catalase activity (Hodgson and Fridovich 1975; Kono and Fridovich 1982). The decrease in catalase activity in pancreas and kidney could be due to increased production of superoxide anion.

Blood: Diabetic blood catalase activity on the 4th, 5th and 6th wks was higher as compared to controls. The higher activity at 5th and 6th wk as compared to previous intervals suggests higher oxidative stress with progression of the disease.

Catalase is located in peroxisomes (Herzog and Fahimi 1974). Its increased activity may reflect increased activity of fatty acyl CoA oxidase that initiates ß-oxidation of fatty acids in peroxisomes resulting in increased production of H_2O_2 (Osumi and Hashimoto, 1978).

4.3 Gluthanione Peroxidase:

Liver: GSH-Px has a key role in enzymatic defence systems and removes peroxides (H_2O_2, lipid or organic peroxides) [Meister and Andersen, 1983]. GSH-Px activity of the liver showed a significant increase as compared to controls. The levels at 5th and 6th wks were higher as compared to other intervals suggesting that the duration of diabetes affects GSH-Px activity. During diabetes there is increased formation of oxidized glutathione due to depletion of NADPH. This can result in higher level of peroxides. The increased GSH-Px activity may be to maintain a redox balance in the system. In general, the highest GSH-Px activity was found in liver followed by heart, kidney, pancreas and aorta.

Heart: GSH-Px activity of heart showed a significant increase at all times except on the 2nd wk. The decrease on the 5th wk as compared to the 4th wk could be due to higher level of oxidative stress at that interval. The increasing trend in activity was observed up to the 4th wk after which it decreased. This suggest its declining capacity to cope with free radicals. The slight increase at 6th wk could be an adaptive increase.

Pancreas: GSH-Px activity of pancreas increased at all times. Activity was maximum up to the 4th wk and after that it decreased. The increase in GSH-Px activity was similar to that reported by Godin *et al.* (1988). There are other reports (Grankvist *et al.* 1981) showing decreases in GSH-Px

activity in alloxan induced diabetic rats. Increased GSH-Px activity in the pancreas suggests increased H_2O_2 levels. Takasu *et al.* (1976) suggested that STZ accumulates in islets and generates H_2O_2. The decreasing trend was observed in GSH-Px activity after 4 wks. Haemenegildo *et al.* (1993) also reported decreased GSH-Px activity in sciatic nerve of diabetic mice. The decrease in activity could be due to glycosylation of the enzyme or inactivation by increased oxidative stress (Stadtman 1992; Wolff 1990). Haemenegildo *et al.* (1993) reported inactivation of GSH-Px from bovine erythrocytes in presence of glucose. The decrease in pancreatic GSH-Px activity may make pancreas more sensitive to H_2O_2. Another possibility is that iron located at that site of H_2O_2 production can result in $\cdot OH$ radicals which are deleterious for the cell (Grankvist *et al.* 1979) who reported that diethylenetriaminepentaacetic acid (DETAPAC) binds and inactivates iron and is found to protect ß-cells against action of alloxan. The reason that pancreatic ß-cells are affected by STZ could be a deficiency in antioxidant enzymes (Tjalve *et al.* 1976).

Aorta: Aortic GSH-Px activity showed a significant increase from the 4th wk onwards as compared to controls. With progression of diabetes, the changes at the 6th wk were significant. Our findings of increased GSH-Px activity do not agree with those of Dohi *et al.* (1988) who reported decreased GSH-Px activity in aorta after 8 months of induction of

diabetes. This suggests that the duration of disease might affect the GSH-Px activity. GSH-Px activity was lowest in control aorta as compared to all other tissues suggesting that it has reduced ability for defence against oxidative attack.

Kidney: GSH-Px activity of the diabetic kidney showed an increase as compared to controls at all times. However the activity at the 4th wk was higher as compared to the 2nd wk. The decreased catalase activity and increased GSH-Px activity in kidney suggests there may be compensatory mechanisms among the antioxidant enzymes in response to increased oxidative stress. Catalase and GSH-Px enzymes are responsible for detoxification of H_2O_2. In the present study decreased CAT and increased GSH-Px activity also suggest that tissues lacking significant catalase activity may be critically dependent on activity of GSH-Px. Christophersen (1969) reported that GSH-Px has broader protective spectrum than catalase in catalyzing the reduction of both H_2O_2 and other hyroperoxides including lipid peroxides.

Blood: Blood of diabetic rats showed a significant increase in GSH-Px activity from the 2nd wk onwards as compared to controls. Similar results were obtained by Matkovics *et al.* (1982) in RBC of diabetic patients. Hagglof *et al.* (1983) reported decreased GSH-Px activity in erythrocytes of diabetic children and these findings are contradictory to ours. There was a gradual increase in GSH-Px activity with progression of diabetes.

The protective effect of GSH-Px depends not only on its activity but also on the availability of GSH.

$$H_2O_2 + 2GSH \text{ ----------> } 2H_2O + GSSG$$

The present findings of increased GSH-Px activity are similar to those of Godin *et al.* (1988) who reported that addition of NADPH protects RBC against oxidation of GSH. Increased glucose concentration in RBC increases the hexose monophosphate shunt (HMP) activity and increase in production of NADPH which could be utilized by glutathione reductase to regenerate GSH from its oxidised form under conditions of oxidative stress. That may be the possible reason for increased GSH-Px activity due to lesser inactivation by OFRs.

In the present study the diabetic state was associated with significant increases in GSH-Px activity in all tissues. Elevation of GSH-Px activity is associated with small increases in oxidative stress (Frank and Messaro 1980; Oei et al 1982). Recently Shull *et al.* (1991) reported induction of SOD, catalase and GSH-Px with differing forms of oxidative stress. Oxidative stress is reported to increase GSH-Px activity which in turn would protect SOD inactivation from H_2O_2 and hence increase SOD activity. H_2O_2 is known to inactivate SOD (Bray *et al.* 1974). Catalase and GSH-Px are inactivated by $\cdot O_2^-$ (Kono and Fridovich 1982; Blum and Fridovich 1985). Thus, an increase in SOD would protect GSH-Px against inactivation by $\cdot O_2^-$. The net effect would be an increase in activity of these enzymes. The increase in CAT and GSH-Px in

diabetic tissues suggests that endogenous production of H_2O_2 may be increased in diabetes (Horie *et al.* 1981).

4.4 Superoxide dismutase (SOD):

There are several sources of production of $\cdot O_2^-$ and H_2O_2 in the cell (Bors *et al.* 1974; Fridovich,1983). Superoxide dismutase catalyzes the conversion of $\cdot O_2^-$ anions into H_2O_2 in cell cytosol and mitochondria (Salin *et al.* 1974).

Liver: The record of change in total SOD and Cu-Zn SOD was similar in liver. Both showed significant increases as compared to controls at all times. The activity increased till 3rd wk after which it tended to decrease. The increase in SOD activity may be a compensatory response to increased production of $\cdot O_2^-$ anion. Increased CAT and GSH-Px in liver and other tissues suggest increased H_2O_2 production which has also been reported to be an inducer of SOD (Matkovics, 1977).

Heart: Heart total SOD and Cu-Zn SOD showed similar changes with significant increase in diabetics as compared to controls. The decreasing trend observed after 4th wk was not significant. Wohaeib and Godin (1987) reported no change in SOD activity in diabetic heart. The increase observed could be a compensatory response to higher oxidative stress.

Pancreas: SOD activity in pancreas was highest in the 1st wk of diabetes after which it tended to decrease. Pancreatic SOD activity in controls was lower as compared to liver, heart and kidney. With the progression of diabetes the activity declined further making it vulnerable to free radical attack

and inflammatory damage. Most inflammatory cells PMNL, monocytes and macrophages during diabetes produce increased amount of $\cdot O_2^-$ and H_2O_2 and $\cdot OH$ when activated and cause tissue damage (McCormick *et al.* 1981; Nath *et al.* 1984). Metals like Cu, Zn and Mn upon which SOD activity depends can alter its activity (Halliwell and Gutteridge 1986). Rest and Sptznagel (1977) reported a reduction in Cu, Zn and Mn contents in diabetic PMNL, which may contribute to decreased activity of SOD.

Aorta: Aortic tissue SOD showed significant increase from 4th wk onwards as compared to controls. The activity within the diabetic group was higher at 4th wk as compared to 1st and 2nd wk and there was a decreasing trend thereafter, the decrease being significant at 6th wk. The decrease in SOD activity in present study suggests increased production of OFRs. In the blood vessels higher OFR levels can cause inactivation of endothelial cell function and impaired prostacyclin synthesis (Porla *et al.* 1981). The functional and metabolic changes in the blood vessels can cause microangiopathy as observed in retina during diabetes (Porla *et al.* 1981).

Kidney: Kidney showed significant increase in total SOD and Cu-Zn SOD activity in diabetics as compared to controls. Decreased CAT activity observed in kidney and increased SOD levels reflects higher H_2O_2 and $\cdot O_2^-$ production. Toxicity of $\cdot O_2^-$ and H_2O_2 is lesser than that of $\cdot OH$ radical, but in kidney their increased levels can cause increased production of $\cdot OH$

radicals which can damage kidney cells in contrast to other tissues.

Blood SOD: SOD activity in blood increased from 3rd wk onwards. The activity was maximum till 4th wk after which it decreased but the decrease was not significant. The present results are in agreement with the findings of Matkovics *et al.* (1982) who also reported a decrease in erythrocyte SOD.

Decreasing trend of SOD observed in erythrocytes, liver, pancreas and aorta could be due to glycation of the enzyme. Recently, Taniguchi *et al.* (1992) and Arari *et al.* (1987) reported increased glycation of Cu-Zn SOD in erythrocytes of IDDM patients. Glycated Cu-Zn SOD contributes to oxidative damage through formation of $\cdot O_2^-$ and H_2O_2 (Hodgson and Fridovich, 1975; Misra and Fridovich 1972; Taniguchi *et al.* 1992).

The results of present study are in agreement with that of others (Watala *et al.* 1986; Nath *et al.* 1984) who reported increased $\cdot O_2^-$ in PMNL of diabetic patients and decreased cytoplasmic and mitochondrial SOD. Crouch *et al.* (1978 and 1981) reported that STZ directly inhibits SOD activity of retina and erythrocytes and the Islets of Langerhans. The complex alterations observed in antioxidant enzymes in the present study could be primarily due to the diabetic state and secondarily due to the effects of diabetogenic agent STZ. Loven *et al.* (1982) reported that STZ has half life of 6h, and is readily metabolized. So it appears that at the period

of 1wk it will not exert any direct effects and therefore the depressed SOD activity observed in the present study in some tissues cannot be the result of STZ present in tissues. Another possible reason for the decrease in liver and pancreas SOD activity could be the reduction in GSH and NADPH levels (Mukherjee *et al.* 1978) which can lead to accumulation of H_2O_2 in diabetic tissues as H_2O_2 has been shown to inhibit Cu-Zn SOD (Hodgson and Fridovich, 1975).

Increased SOD activity in blood was observed up to 4th wk after which it tended to decrease. Tho *et al.* (1988) reported that OFRs and other factors which can affect enzyme activity in diabetic state in RBC include erythrocyte aggregation and microviscosity, increased glycated hemoglobins with increased oxygen affinity, decreased 2,3-diphosphoglycerate, aggregation of platelets, accelerated production of prostaglandin derivatives and enhanced blood clotting factors (Hagglof *et al.* 1983). In hyperglycemic state these factors can lead to low oxygen tension and over a prolonged period of time and can also affect enzyme activity (Tho *et al.* 1988).

Mn SOD: Mn-SOD activity was variable in different tissues. No significant change in diabetic liver was observed except at 4th wk at which it was lower as compared to control. In the diabetic group the activity decreased at 4th, 5th and 6th wk as compared to 2nd wk. The decreased Mn-SOD activity of liver and heart at 4th wk and increase at 6th wk in case of

heart could be due to differential levels of oxidative stress. Mn-SOD activity is dependent on Mn levels. Brandt and Schramm (1986) reported that different response in liver and heart could be due to levels of Mn availability. Increased Mn-SOD activity at 6th wk could be due to increased production of $\cdot O_2^-$. Wong *et al.* (1989) reported that $\cdot O_2^-$ has been shown to have stimulatory effect on synthesis of Mn-SOD activity in human embryonic lines. Bond *et al.* (1983) reported accumulation of Mn in liver in 3-10 days period following STZ injection. The decrease in SOD activity in liver could be due to accumulation of Mn. Another possible reason could be that liver unlike other organs contains enzymes like pyruvate carboxylase, PEPCK and arginase which utilize Mn for their activation as a cofactor. The decrease at 3rd wk and 6th wk in control group is unexplained. Mn SOD activity in aorta was not detectable with present assay technique. Pancreas showed no change however kidney showed decreasing trend in diabetic group. The decrease observed in kidney could be due to changes in Mn content with time in STZ induced diabetes (Thompson *et al.* 1992).

In general most of the tissues (liver, pancreas and aorta) showed an increase in SOD activity up to 3rd or 4th wk and thereafter it tended to decrease. Increased level of SOD suggest enhanced production of $\cdot O_2^-$ which can lead to inactivation of CAT and GSH-Px (Kono and Fridovich 1982; Blum and Fridovich 1985) and hence elevation of H_2O_2 which will

inactivate SOD leading to decreased SOD activity (Bray *et al.* 1974). The changes in MDA content and antioxidant enzymes during diabetes could also be due to changes in the levels of insulin, glucagon and status of ketosis.

5.0 Conclusions

The present study was undertaken to investigate the role of oxygen free radicals in experimental diabetes which may be responsible for complications in diabetes mellitus. The experimental results show that:

1. There was an increase in plasma glucose and decrease body weight in streptozotocin-induced diabetes.
2. There was an increase in MDA content of all the tissues.
3. There was a progressive increase in MDA content in liver, aorta, kidney and blood which suggests oxidative damage.
4. There was a significant increase in the activities of CAT and GSH-Px in all tissues suggesting oxidative stress. The decrease in some tissues after an initial increase was also observed.
5. There was an increase in SOD activities in all the tissues. Total SOD and Cu-Zn SOD activities showed a decreasing trend in liver, pancreas and aorta after 3-4 wks of induction of diabetes. This suggests higher OFR production and decreased antioxidant defence system which can make these tissues more susceptible to oxidative attack.

The results of this study suggest that the diabetic state is associated with higher oxidative stress, which starts at an early stage of the disease. The oxidative

stress might cause damage to various tissues including vascular endothelium resulting in complications in diabetes.

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