DIABETES ASSOCIATED MALE REPRODUCTIVE DYSFUNCTION: ROLE OF OXIDATIVE STRESS AND ITS AMELIORATION

A thesis
submitted to

University of Mysore

For the degree of

Doctor of Philosophy

In the faculty of

Bioscience

by

Shrilatha B, M.Sc.

November 2005

Department of Biochemistry and Nutrition,
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
MYSORE-570 020, INDIA.

ACKNOWLEDGEMENTS

All gratitude and praise to the Lord, for the ocean of blessings He has showered on me

I am sincerely grateful to

My mentor and guide, Dr. Muralidhara, for his invaluable guidance, keen interest and for enabling me to grow with the freedom of thought and expression throughout my stay in his laboratory

Dr. S.G. Bhat, Head Department of Biochemistry & Nutrition for his encouragement

The Director, CFTRI, for giving me an opportunity to work for my PhD programme at CFTRI, Mysore

The ICMR, New Delhi, for the award of Senior Research Fellowship

Dr. P. S. Rajini for her unfailing encouragement, personal advice and affection

Dr. K. Narasimhamurthy, Dr. T.P. Krishnakantha, Dr. V. Baskaran, Dr. B.R. Lokesh, and Dr. K. Srinivasan for their timely help during the course of work

My colleagues at CFTRI, Dr. Rajeshkumar T, Mr. Doreswamy K, Mr. Mahesh MS, Mr. Thyagaraju BM, Mrs. Shinomol George K, Mr. Chandrashekar KN, Mrs. Prasanthi K, Mrs. Nandita Singh, Mr. Vasudev Kamath, and Mr. Kisan for whom words would never suffice to say just thanks

Dr. S Rajendran for having given me an opportunity to work in an Indo-Australian project in which I learnt my first lessons of research

Dr. BK Sharath, HOD, Dept of Biosciences, Hemagangothri, Hassan for his constructive criticism during the doctoral committee proceedings

Dr. S Mahadevamma for her constant support and affection

Miss Ajila, Mrs. Hemalatha, Mrs. Indu, Miss. Reena, Miss. Rajni, Miss. Shyamala, Miss. Smita, for their timely help and their great company

To all my colleagues and staff at the department of Biochemistry and Nutrition for their help received at various times during this investigation.

The staff of Library, Central Instrumentation Facility Services, Stores and Purchase section and Administrative staff for helping me in my accomplishments at CFTRI.

The staff of the Animal house facility at the Dept. of Biochemistry & Nutrition for their constant help in my animal experimentation

Mr. Prasanna KS and Mrs. Asha Devi JS for the excellent DTP work

Mr. Ramaprasad TR, who has stood as a tower of strength during moments of frustration and has goaded me towards the achievement of my goal

My parents, sisters, brother, brother-in-law and grandparents for their love, support, encouragement and constant prayers.

SHRILATHA

.

Dr. Muralidhara
Scientist E II,
Department of Biochemistry and Nutrition

CERTIFICATE

I hereby certify that the thesis entitled '**DIABETES ASSOCIATED MALE REPRODUCTIVE DYSFUNCTION: ROLE OF OXIDATIVE STRESS AND ITS AMELIORATION**' submitted by **Ms. SHRILATHA B** for the degree of **Doctor of Philosophy** in **Biosciences,** University of Mysore is the result of research work carried out by her in the Department of Biochemistry and Nutrition, CFTRI, Mysore under my guidance and supervision during the period of June 2002- June 2005.

Dr. MURALIDHARA
Guide

Place: Mysore
Date:

Shrilatha B
Senior Research Fellow
Dept. of Biochemistry & Nutrition
CFTRI, Mysore

DECLARATION

I hereby declare that the thesis entitled '**DIABETES ASSOCIATED MALE REPRODUCTIVE DYSFUNCTION: ROLE OF OXIDATIVE STRESS AND ITS AMELIORATION**' submitted to the University of Mysore for the award of the degree of **Doctor of Philosophy** in **Biosciences** is the result of research work carried out by me under the guidance of Dr. Muralidhara, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during the period June 2002 to June 2005.

I further declare that these results have not been submitted for any other degree or fellowship.

SHRILATHA B

Place: Mysore
Date:

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

$\cdot O_2$	Superoxide radical
·OH	Hydroxyl radical
$^1O_2$	Singlet oxygen
α	Alpha
β	Beta
°C	Degree Celsius
AA	Ascorbic acid
AO	Antioxidant
AOE	Antioxidant enzyme
BC	Beta-catorene
BHT	Butylated hydrochloride
BSA	Bovine serum albumin
bw	Body weight
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CTR	Control
cHP	Cumene hydroperoxide
CP	Cyclophosphamide
DI	Dead implantation
DLT	Dominant lethal test
DM	Diabetes mellitus
dsDNA	Double stranded DNA
EDTA	Ethylene diamine tetraacetic acid
ES	Epididymal sperms
FA	Ferulic acid
FADU	Flourimetric analysis of DNA unwinding
g	Gram
i.p.	Intra peritoneal
GC	Gas chromatography
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-s-transferase
G6PDH	Glucose-6-phsoaphte dehydrogenase
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
h	Hour(s)
Kg	Kilo gram
KRBS	Krebs ringers bicarbonate solution
µg	Micro gram
µmol	Micromole
µL	Micro litre
LPO	Lipid peroxidation
LI	Live embryos
M	Molar
MDA	Malondialdehyde
min	Minutes
ml	Milliliter
mg	Milligram
MRS	Male reproductive system
ng	Nanogram
nm	Nanometer
N	Normality
OS	Oxidative stress
PCA	Protocatechuic acid
PUFA	Polyunsaturated fatty Acids
Q	Qurcetin
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Standard deviation
SOD	Superoxide dismutase
SDH	Sorbitol dehydrogenase
STZ	Streptozotocin
SFA	Saturated fatty acids
TAU	Taurine
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TI	Total implantations
TCS	Testicular cell suspension
tbHP	Tertiary butyl hydroperoxide
v/v	Volume by volume
w/w	Weight by weight

Preface to the review of literature

In the first part of the thesis, an attempt has been made to review the relevant literature under **three** separate sections.

The **first section** (subsections 1-3) of the review provides a current and general understanding on Diabetes, a disease often described as a metabolic disorder. Further, an overview oxidative stress and its major implications on human health have been presented in greater detail. Additionally, recent developments employing *in vitro* and *in vivo* approaches to understand the role of oxidative stress mechanisms in the development of diabetic complications are also discussed.

The **second section** (subsections 4-7) of the review focuses on the structure, physiology and functional aspects of mammalian male reproductive system and discusses various aspects related to male infertility in humans. Further, a brief account on the role of oxidative stress as an etiological factor in male infertility has been provided. Special emphasis has been given to various aspects related to diabetes and male infertility.

The **third section** (subsections 8 & 9) of the review focuses on the modulatory role of known antioxidants, under conditions of diabetes associated oxidative stress in humans as well as experimental animal models. At the end, a small subsection discusses the possible use of various phytochemicals in modulating the oxidative damage in various somatic organs under diabetic conditions.

1.0 DIABETES MELLITUS: AN OVERVIEW

Diabetes, a disease which dates back to 1500-3000 BC is documented in ancient Hindu and Greek writings and is among the top five causes of death in the world. It is a metabolic disorder that has both juvenile and maturity onset leading to life threatening complications such as hyperglycemia, nephropathy, neuropathy, retinopathy, coma and ultimately death (*Zimmet et al., 2001; Ramachandran et al., 2002*). The β-cells of the pancreas produce insulin, a protein that monitors glucose blood levels in the body. Homeostatic control of normal glucose level is achieved by co-ordinate secretion of insulin and glucagon. If there is an imbalance in glucose homeostasis, hyperglycemia ensues where glucose levels exceed 10mM, a diagnostic feature of diabetes.

The current global estimate of diabetics is 150 million, which is set to rise to 220 million in 2010 and 300 million in 2025 (*Amos et al, 1997*). While T2DM is more prevalent in the general population and is strongly associated with a sedentary life style and obesity (*Zimmet, 1999)*, T1DM diabetes is the most common among children. But, current epidemiological evidences show an increasing prevalence of T2DM in children and adolescents.

Generally, diabetes mellitus can be classified into two types: Insulin dependent diabetes mellitus or type 1 (T1DM), (also known as juvenile onset diabetes) and Non-insulin dependent diabetes mellitus or type 2 (T2DM) based (also known as adult-onset diabetes) on their insulin requirements. While T1DM accounts for 10 to 20% of all the cases of diabetes, T2DM accounts for remaining 80-90% of patients. T2DM is further divided into 'obese' and 'non-obese' types and a third rare form known as 'maturity onset diabetes of the young' (MODY). MODY is manifested by mild hyperglycemia and is transmitted as an autosomal dominant trait.

Type 1 diabetes (T1DM): This form of diabetes results from a severe, absolute lack of insulin caused by a reduction in the β-cell mass. Three interlocking mechanisms are speculated to be responsible for the islet cell destruction: genetic susceptibility, autoimmunity and environmental insult. There is overwhelming evidence implicating autoimmunity, immune mediated injury and environmental factors as causes of β-cell loss in T1DM (*Tisch and McDevitt, 1996; Mathis et al., 2001*).

Type-2 diabetes (T2DM): Though T2DM is the most common type of diabetes, less is known about its pathogenesis. Genetic factors and obesity play important roles in T2DM and there is a correlation between the disease and increasing age. Two metabolic defects characterize this type; (i) a derangement in insulin secretion that is delayed or that is insufficient relative to the glucose load and (ii) an inability of peripheral tissue to respond to insulin resistance. T2DM is a complex multi-factorial disorder involving both impaired insulin release and end organ insensitivity.

Long-Term Complications

Diabetes mellitus, a disease of metabolic dysregulation, is known to be accompanied by characteristic long-term complications such as retinopathy, nephropathy, and neuropathy and sexual dysfunction/s. Patients with all forms of diabetes of sufficient duration, including T1DM and T2DM, are vulnerable to various complications, which cause serious morbidity. Besides the major complications, diabetes is also known to cause substantial increase in atherosclerotic disease of large vessels, including cardiac, cerebral, peripheral vascular disease and sexual dysfunction.

Clinical Descriptions

Retinopathy: Retinopathy occurs in all forms of diabetes and its development depends on the duration of the disease (*Krolewski et al., 1986)*. The first and most common visible lesions are small micro aneurysms arising from the terminal capillaries of the retina. The retinal vessels are abnormally permeable and leak serous fluid, leading to the formation of hard exudates. Further, macular edema occurs when leakage of fluid from abnormal vessels near the maculae disrupts the light path to the maculae and results in the loss of visual acuity (*Nathan et al., 1986*).

Nephropathy: Nephropathy is the diabetes-specific complication associated with the greatest mortality. Although the vast majority of diabetic patients have some degree of retinopathy, nephropathy develops in only 35 to 45 percent of patients with T1DM and less than 20 percent of those with T2DM (*Ballard et al., 1988)*. The natural history of clinically detectable diabetic nephropathy in T1DM begins with the development of microalbuminuria, which may occur as early as five years after the onset of diabetes.

Neuropathy: A peripheral, symmetric sensorimotor neuropathy is the most common form of diabetic neuropathy, whose other forms include cranial and peripheral motor neuropathies and autonomic neuropathy. Although neuropathy is also more common with a longer duration of diabetes, a relatively severe, early-onset polyneuropathy has been described (*Said et al., 1992*). The most common types include distal symmetric sensorimotor neuropathy and autonomic neuropathy. Loss of sensation in the feet, altered foot architecture leading to diabetic ulcers are the symptoms of distal symmetric sensorimotor neuropathy. Autonomic neuropathy can affect gastric or intestinal motility, erectile function, bladder function, cardiac function, and vascular tone.

Impotence is the most common clinical manifestation of autonomic neuropathy, affecting more than 50 percent of men with diabetes. Cardiac autonomic neuropathy may result in resting tachycardia and postural hypotension.

Sexual dysfunction

Diabetes affects an increasingly large number of young men of reproductive age. The fertility of these men many be affected directly or indirectly by the effects of the disease process on erection, ejaculation, spermatogenesis and even embryo development. Erectile and ejaculatory difficulties arise due to vascular and neuropathic problems.

Pathogenesis

In general, the postulated pathogenetic mechanisms are grouped into three categories: *glucose-related*, including abnormalities in polyol (e.g., sorbitol) metabolism and excessive glycation of circulating and membrane-bound proteins (*Brownlee et al., 1988*); *vascular mechanisms*, including abnormalities in the endothelium, supporting cells such as pericytes in the retina, mesangial cells in the glomerulus (*Steffes et al., 1989*), and hyper filtration and intrarenal hypertension in the kidney and other mechanisms, including abnormalities in platelet function , growth factors and genetic influences. Unfortunately, studies of diabetes in both animals and humans are so fragmentary that no coherent pathogenetic mechanism has been established.

2.0 OXIDATIVE STRESS: AN OVERVIEW

Oxygen free radicals or activated oxygen has been implicated in diverse environmental stresses in plants and animals and appears to be a common participator in most, if not all, degenerative conditions in eukaryotic cells (*Marx, 1985)*. Activated forms of oxygen are also

important in the biosynthesis of 'complex' organic molecules, in the detoxification of xenobiotic chemicals and in the defense against pathogens.

There are numerous sites of oxygen activation in the cell, which are highly controlled and tightly coupled to prevent release of intermediate products. Under stress situations, it is likely that this control or coupling breaks down and the process 'dysfunctions' leaking activated oxygen. These uncoupling events are not detrimental provided that they are short in duration and that the oxygen scavenging systems are able to detoxify the various forms of activated oxygen. If the production of activated oxygen exceeds the capacity to detoxify it, deleterious degenerative reactions occur at various levels in the cellular compartments. Thus, it is the balance between the production and the scavenging of activated oxygen that is critical and this imbalance between the oxidants and antioxidants in favor of the former is termed as "Oxidative stress" (*Halliwell and Gutteridge, 1990*).

Reactive oxygen species (ROS) generation

ROS, also called oxygen free radicals, are highly reactive oxidizing agents. A free radical is any compound that contains one or more unpaired electrons. These ROS include superoxide anion, hydrogen peroxide, peroxyl radicals and the highly reactive hydroxyl radicals.

Superoxide anion ($O_2^{\cdot}$)

Superoxide anion is the first reduction product of O_2. It is a negatively charged free radical produced by 1-electron reduction of molecular oxygen either by autooxidation or by various oxidases. Superoxide dismutase (SOD) enzyme removes $O_2^{\cdot}$ via a very short lived intermediate, hydroperoxyl radical ($HO_2^{\cdot}$). As in other tissues, SOD in testicular tissue also exists in multiple forms, e.g., a cytosolic copper-zinc

SOD, mitochondrial manganese SOD and extracellular Cu-Zn-SOD (*Fridovich, 1995*). Any induction in the activities of intracellular oxidases in the testicular tissue or spermatozoa can result in nanomolar levels of O_2· resulting in direct interactions with signaling mechanisms. High levels of SOD will lower O_2· concentrations into the picomolar range by its superoxide anion scavenging effects. The resulting low levels of O_2· can be a source of H_2O_2 in the high picomolar to low nanomolar range that is likely to interact with cell-signaling systems. SOD activity is also associated with high O_2· levels that interact with iron-sulphur moieties at key cellular sites. In semen samples from infertile patients with high O_2· generation, prolonged inhibition of sperms mitochondrial function by the ROS may be the cause of inhibition of sperm motility (*Aitken et al., 1992*).

$$O_2 + \text{electron (e-)} \xrightarrow{\text{Oxidases}} O_2^{\cdot -}$$

Hydrogen peroxide (H_2O_2)

H_2O_2 is the most stable among ROS. H_2O_2 may be generated directly by divalent reduction of O_2 or indirectly by univalent reduction of O_2. It can easily transverse the plasma and nuclear membranes thereby contributing to DNA adduct formation. Peroxide-derived ROS readily react with transition metals such as iron present in biological systems and affect signaling or tissue injury processes. Hydrogen peroxide reacts either with superoxide anion or interacts with free iron to form highly reactive hydroxyl radicals.

Hydroxyl radicals (HO·)

Hydroxyl radical is highly reactive, short-lived and most toxic ROS. Enzymes with peroxidase-like activities., e.g., catalase and lipoxygenase interact with oxidant –related signaling systems (*Brash, 1999; Balazy et al., 1999*). Hydroxyl radicals are known to induce conformational

changes in DNA including strand breaks, base modifications, damage to tumor suppressor gene and enhanced expression of protooncogenes (*Halliwell and Aruoma, 1991; Cerutti et al., 1994*).

Consequences of activated oxygen

The reactions of activated oxygen with organic substrates in biological systems are more complex due to the surface properties of membranes, electrical charges, binding properties of macromolecules and compartmentalization of enzymes, substrates and catalysts. Thus, various sites even within a single cell differ in the nature and extent of reactions with oxygen. The mechanisms by which oxygen radicals damage membrane lipids are well accepted and consequently oxidative damage is often exclusively associated with these peroxidation reactions in membrane lipids.

Oxidative damage to Lipids: The reactions of oxygen free radicals with PUFA have been extensively studied (*Halliwell and Gutteridge, 1989*). The peroxidation of lipids involves three distinct steps: initiation, propagation and termination *(Halliwell and Gutteridge, 1990)*. The initiation reaction between an unsaturated fatty acid and the hydroxyl radical involves the abstraction of an H atom from the methylvinyl group on the fatty acid. In the propagation reactions, this resonance structure reacts with triplet oxygen, which is a biradical having two unpaired electrons and therefore reacts readily with other radicals. This reaction forms a peroxy radical. The peroxy radical then abstracts an H atom from a second fatty acid forming a lipid hydroperoxide and leaving another carbon centered free radical that can participate in a second H abstraction. Therefore, once one hydroxyl radical initiates peroxidation reaction by abstracting a single H atom, it creates a carbon radical product (R) that is capable of reacting with ground state oxygen in a chain reaction. The lipid hydroperoxide (ROOH) is

unstable in the presence of Fe or other metal catalysts because ROOH will participate in a Fenton reaction leading to the formation of reactive alkoxy radicals:

$$ROOH + Fe^{2+} \rightarrow OH^{-} + RO\bullet + Fe^{3+}$$

Therefore, in the presence of Fe, the chain reactions are not only propagated but also amplified. Among the degradation products of ROOH are aldehydes, such as malondialdehyde, and hydrocarbons, such as ethane and ethylene that are commonly measured end products of lipid peroxidation. The peroxidation reactions in membrane lipids are terminated when the carbon or peroxy radicals cross-link to form conjugated products that are not radicals (see reactions below).

$$R\bullet + R\bullet \rightarrow R - R$$

$$R\bullet + ROO\bullet \rightarrow ROOR$$

$$ROO\bullet + ROO\bullet \rightarrow ROOR + O_2$$

Typically, high molecular weight cross-linked fatty acids and phospholipids accumulate in peroxidized membrane lipid samples. Singlet oxygen can react readily with unsaturated fatty acids producing a complex mixture of hydroperoxides. Again, the chemistry of these reactions is based on foods. Oxidation of unsaturated fatty acids by singlet oxygen produces distinctly different products than the hydroxyl radical (*Bradley and Minn, 1992*). Once formed the lipid hydroperoxides will decompose into a variety of products, some of which can produce oxygen free radicals in the presence of metal catalysts.

Oxidative damage to Proteins: Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis (*Berlette and Stadtmann, 1997*). The amino acids in a peptide differ in their susceptibility to attack,

and the various forms of activated oxygen differ in their potential reactivity. Primary, secondary, and tertiary protein structures alter the relative susceptibility of certain amino acids. In spite of this complexity, generalizations can be made. Sulphur containing amino acids, and thiol groups specifically, are very susceptible sites. Activated oxygen can abstract an H atom from cysteine residues to form a thiyl radical that will cross-link to a second thiyl radical to form disulphide bridges. Alternatively, oxygen can add to a methionine residue to form methionine sulphoxide derivatives. Some forms of free radical attack on proteins are not reversible. For e.g. the oxidation of iron-sulphur centres by superoxide destroys enzymatic function. Many amino acids undergo specific irreversible modifications when a protein is oxidized. For e.g. tryptophan is readily cross-linked to form bityrosine products. Histidine, lysine, proline, arginine, and serine form carbonyl groups on oxidation (*Stadtman, 1986*). The oxidative degradation of protein is enhanced in the presence of metal co-factors that are capable of redox cycling, such as Fe. In these cases, the metal binds to a divalent cation-binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidizes an amino acid residue at or near the cation-binding site of the protein (*Stadtman, 1990*). This site-specific alteration of an amino acid usually inactivates the enzyme by destruction of the cation-binding site.

Oxidative damage to DNA: Activated oxygen and agents that generate oxygen free radicals, such as ionizing radiation, induce numerous lesions in DNA that cause deletions, mutations and other lethal and genotoxic effects (*Wiseman and Halliwell, 1996*). Characterization of this damage to DNA has indicated that both the sugar and the base moieties are susceptible to oxidation causing base degradation, single strand breakage, and cross-linking to protein (*Imlay and Linn, 1986*).

Degradation of the base will produce numerous products, including 8-hydroxyguanine, hydroxymethyl urea, urea, thymine glycol, thymine and adenine ring-opened and -saturated products. The principle cause of single strand breaks is oxidation of the sugar moiety by the hydroxyl radical.

Cross-linking of DNA to protein is another consequence of hydroxyl radical attack on either DNA or its associated proteins (*Oleinick et al., 1986*). When these cross-linkages exist, separation of protein from DNA by various extraction methods is ineffective. Although DNA-protein cross-links are about an order of magnitude less abundant than single strand breaks, they are not as readily repaired, and may be lethal if replication or transcription precedes repair.

Endogenous sources of free radicals

Free radicals are produced in cells in general by electron transfer reactions, which can be enzymatically or non-enzymatically mediated. The production of free radicals in cells can happen both accidentally or deliberately. However, the major source of free radicals under normal circumstances is the electron leakage that occurs from electron transport chains such as those in the mitochondria and endoplasmic reticulum to molecular oxygen generating superoxide.

Autooxidation: Autooxidation is a by-product of the aerobic internal milieu and molecules that undergo autooxidation are catecholamines, haemoglobin, myoglobin, reduced cytochrome C and thiol. Autooxidation of any of these molecules in a reaction results in the reduction of the oxygen diradical and the formation of ROS. Superoxide is the primary radical formed. Ferrous ion (Fe II) also, can have its electron stolen from it by oxygen to produce superoxide and Fe III (*Fridovich, 1995)*.

Enzymatic oxidation: A variety of enzyme systems are capable of generating significant amounts of free radicals, including xanthine oxidase, prostaglandin synthase, lipoxygenase, aldehyde oxidase, and amino acid oxidase. The enzyme myeloperoxidase produced in activated neutrophils, utilizes hydrogen peroxide to oxidize chloride ions into the powerful oxidant hypochlorous acid (HOCl) (*Halliwell et al., 1995*).

Respiratory burst: Is a term used to describe the process by which phagocytic cells consume large amounts of oxygen during phagocytosis. Between 70 and 90% of this oxygen consumption can be accounted for in terms of superoxide production (*Baboir, 1984*). These phagocytic cells possess a membrane bound flavoprotein cytochrome-b-245 NADPH oxidase system which on activation initiates a respiratory burst at the cell membrane to produce superoxide. H_2O_2 is then formed from superoxide by dismutation with subsequent generation of ·OH and HOCl by bacteria (*Rosen et al, 1987*).

Sub-cellular organelles such as mitochondria, chloroplasts, microsomes, peroxisomes and nuclei have been shown to generate $O_2^{\cdot-}$ (*Asada and Kiso, 1973*). Mitochondria are the main cellular organelle for cellular oxidation reactions and the main source of reduced oxygen species in the cell. The leakage in mitochondrial electron transport system allows O_2 to accept a single electron forming $O_2^{\cdot-}$ (*Haliwell, 1995*). It has been shown that superoxide production by the mitochondria increases in two conditions: either when the oxygen concentration is greatly increased or when the respiratory chain becomes fully reduced. Microsomes are responsible for 80% of the H_2O_2 produced *in vivo* at 100% hyperoxia sites (*Jamieson et al., 1986*). Peroxisomes are known to produce H_2O_2, but not $O_2^{\cdot-}$, under physiologic conditions (*Chance et al., 1979*). Although the liver is the primary organ

where peroxisomal contribution to the overall H_2O_2 production is significant, other organs that contain peroxisomes are also exposed to these H_2O_2 generating mechanisms.

Transition metals ions: Iron and copper play a major role in the generation of free radical injury and the facilitation of lipid peroxidation. Transition metal ions participate in the Haber-Weiss reaction that generates ·OH from O_2·- and H_2O_2

$$H_2O_2 + Fe^{2}- \rightarrow \cdot OH + OH- + Fe^{3}-$$

The Haber-Weiss reaction accelerates the non-enzymatic oxidation of molecules such as epinephrine and glutathione that generates O_2·- and H_2O_2 and subsequently ·OH.

Ischemia reperfusion injury: Ischemia confers a number of effects all contributing to the production of free radicals. Normally xanthine oxidase is known to catalyse the reaction of hypoxanthine to xanthine and subsequently xanthine to uric acid. This reaction requires an electron acceptor as a cofactor. During ischemia two factors occur, first the production of xanthine and xanthine oxidase are greatly enhanced. Second, there is a loss of both antioxidants superoxide dismutase and glutathione peroxidase. The molecular oxygen supplied on reperfusion serves as an electron acceptor and cofactor for xanthine oxidase causing the generation of the O_2·and H_2O_2.

Exogenous sources

Drugs: A number of drugs can increase the production of free radicals in the presence of increased oxygen tensions. They are *antibiotics* that depend on quinoid groups or bound metals for activity (nitrofurantoin), antineoplastic agents (e.g., bleomycin, anthracyclines (adriamycin) and methotrexate, which possess pro-oxidant activity (*Gressier et al., 1994*).

Radiation: Radiotherapy may cause tissue injury that is caused by free radicals. Electromagnetic radiation (X rays, gamma rays) and particulate radiation (electrons, photons, neutrons, alpha and beta particles) generate primary radicals by transferring their energy to cellular components such as water. These primary radicals can undergo secondary reactions with dissolved oxygen or with cellular solutes.

Tobacco smoking: Oxidants in tobacco exist in sufficient amounts to suggest that they play a major role in injuring the respiratory tract. It has been shown that tobacco smoke oxidants severely deplete intracellular antioxidants in the lung cells *in vivo* by a mechanism that is related to oxidant stress (*Georgellis et al., 1987*).

Antioxidant defenses

Enzymic antioxidants

Cells have developed enzymatic systems, which convert oxidants into non-toxic molecules to protect themselves, from the deleterious effects of oxidative stress. Endogenous sources of antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione transferase (GST).

Superoxide dismutase (SOD): SOD is an endogenously produced intracellular enzyme essentially present in every cell. Cellular SOD is actually represented by a group of metalloenzymes with various prosthetic groups. The prevalent enzyme is cupro-zinc (CuZn) SOD, which is a stable dimeric protein (32,000 D). SOD appears in three forms: (i) Cu-Zn SOD in the cytoplasm with two subunits, (ii) Mn-SOD in the mitochondrion and (iii) extracellular SOD recently has been described contains Copper (CuSOD).

$$2O_2^{\cdot}- + 2H+ + SOD\ (H_2O_2) + O_2$$

SOD is considered fundamental in the process of eliminating ROS by reducing (adding an electron) superoxide to form H_2O_2. Catalase and the selenium-dependent glutathione peroxidase are responsible for reducing H_2O_2 to H_2O. The respective enzymes that interact with superoxide and H_2O_2 are tightly regulated through a feedback system. Excessive superoxide inhibits glutathione peroxidase and catalase to modulate the equation from H_2O_2 to H_2O. Likewise, increased H_2O_2 slowly inactivates CuZn-SOD. Catalase and glutathione peroxidase by reducing H_2O_2, conserve SOD; and SOD, by reducing superoxide, conserves catalases and glutathione peroxidase. Through this feedback system, steady low levels of SOD, glutathione peroxidase, and catalase, as well as superoxide and H_2O_2 are maintained, which keeps the entire system in a fully functioning state. SOD also exhibits antioxidant activity by reducing $O_2 \cdot^-$ that would otherwise lead to the reduction of Fe^{3+} to Fe^{2+} and thereby promote $\cdot OH$ formation. When the catalase activity is insufficient to metabolize the H_2O_2 produced SOD will increase the tissue oxidant activity.

Glutathione peroxidase (GPx): The glutathione redox cycle is the central mechanism for reduction of intracellular hydroperoxides. GPx is a tetrameric protein 85,000-D and has 4 atoms of selenium (Se) bound as seleno-cysteine moieties that confers the catalytic activity. One of the essential requirements is glutathione as a co-substrate. Glutathione peroxidase reduces H_2O_2 to H_2O by oxidizing glutathione (GSH) (Equation A). Reduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase (Equation B). These enzymes also require trace metal cofactors for maximal efficiency, including selenium for glutathione peroxidase; copper, zinc, or manganese for SOD; and iron for catalase (*Halliwell, 1995*).

H_2O_2 + 2 GSH → GSSG + 2 H_2O (equation A)

GSSG + NADPH + H^+ → 2 GSH + $NADP^+$ (equation B)

Catalase: Catalase is present in all body organs and is especially concentrated in the liver and erythrocyte while, the brain, heart, skeletal muscle contain only low amounts. Catalase and glutathione peroxidase seek out hydrogen peroxide and convert it to water and diatomic oxygen. An increase in the production of SOD without a subsequent elevation of catalase or glutathione peroxidase leads to the accumulation of hydrogen peroxide, which gets converted into the hydroxyl radical.

Non-enzymic antioxidants molecules

Glutathione (GSH): GSH is synthesized intracellularly from cysteine, glycine, and glutamate. In addition to its role as a substrate in GSH redox cycle, GSH is also a scavenger of hydroxyl radicals and singlet oxygen. It is capable of either directly scavenging ROS or enzymatically via glutathione peroxidase, as described previously. In addition, GSH is crucial for the maintenance of enzymes and other cellular components in a reduced state. GSH also has an important role in xenobiotic metabolism and leukotriene synthesis. It is found in millimolar concentration in all human cells (*Halliwell, 1994*). The majority of GSH is synthesized in the liver, and approximately 40% is secreted in the bile. The biologic role of GSH in bile is believed to be defence against dietary xenobiotics and lipid peroxidation in the lumen of the gut and protection of the intestinal epithelium from free radicals.

***Taurine and hypotaurine*:** Taurine and its precursor hypotaurine are β-amino acids that are derived from cysteine metabolism (*Malmezat et al, 1998*). These molecules are implicated in the cellular mechanisms of defense against oxidative stress. While hypotaurine is shown to

scavenge hydroxyl radical and to inhibit lipid peroxidation *in vitro* (*Tadolini et al., 1995),* taurine does not really have an antioxidant activity (*Aruoma et al., 1988*). However, taurine supplementation decreases lipid peroxidation *in vivo* in diabetic rats. Additionally concentrations of taurine in specific cerebral areas such as the striatum, cortex, nucleus accumbens and cerebellum are known to diminish during aging (*Benedetti et al., 1991*).

***α-lipoic acid*:** α-lipoic acid exists in cells as lipoamide, which is covalently linked to different cytoplasmic protein complexes by dihydrolipoamide dehydrogenase (*Sen et al.,1997*). This enzyme can reduce (using NADH) exogenous lipoate to dihydrolipoate, a potent reductant. Lipoate can also be reduced by glutathione reductase or thioredoxin reductase (*Packer et al., 1995*).

Oxidative stress and diseases

Acute and chronic free radical pathology occurs under conditions of extraordinary radical flux such as inflammation, radiation, metabolism of specific xenobiotics, or overload of autooxidation of catalyzing agents such as transition metals (manganese, copper, or iron). Some of the disease states that are reported to be associated with the involvement of oxidative stress are atherosclerosis, inflammatory responses, rheumatoid arthritis, cancer, reperfusion injury, diabetes, cataract and eye injury, CNS syndromes or diseases (Parkinson's and Alzheimer's) etc.

Diabetes: In diabetic situations, oxidative stress seem to occur primarily due to both an increase in the plasma free radical concentration and a sharp reduction in antioxidant defenses (*Cross et al.,1987*). Among the causes of enhanced free radical production, hyperglycemia and hyperinsulinemia seem to play a major role (*Giugliano et al., 1996*). There are many ways by which hyperglycemia may increase the

generation of free radicals. Four main hypotheses linking the hyperglycemia and diabetic complications are increased polyol pathway, increased advanced glycation end product formation, activation of protein kinase C isoforms and increased hexosamine pathway flux (*Brownlee, 2001*). The unifying hypothesis linking these four mechanisms and oxidative stress is the activation of the mitochondrial superoxide over production (*Du et al., 2000*).

Male infertility: Recent evidences have implicated oxidative stress as a major causative factor in male infertility (*Agarwal et al., 2004*). The major cause of oxidative stress appears to be by the generation of high rate of ROS associated with the retention of excess residual cytoplasm in the sperm mid-piece (*Aitken et al., 1996*). Other possible causes include the redox cycling of xenobiotics and antioxidant depletion or apoptosis. Oxidative stress is shown to induce peroxidative damage in the sperm plasma membrane and DNA damage in both the mitochondrial and nuclear genomes.

3.0 OXIDATIVE STRESS MECHANISMS IN DIABETES

Both T1DM and T2DM are characterized by chronic hyperglycemia leading to the development of various complications. Increasing body of evidence in both experimental and clinical studies suggests that oxidative stress plays a key role in the pathogenesis of both types of diabetes mellitus (*Baynes, 1991*; B*aynes and Thorpe, 1999*).

Evidence for oxidation in diabetes: Glucose oxidation is believed to be the main source of free radicals under diabetic situations. In its enodiol form, glucose is oxidized in a transition-metal dependent reaction to an enodiol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by

catalase or glutathione peroxide and in presence of transition metals, can lead to the production of extremely reactive hydroxyl radical (*Jiang et al., 1990*). Interaction of glucose with proteins resulting in the formation of amadori adducts (altering the charge, conformation and molecular recognition of protein) is another source of free radicals.

Diabetes is followed by an increased generation of free radicals which are generated disproportionately by glucose oxidation, non-enzymatic glycation of proteins and the subsequent oxidative degradation of glycated proteins. Abnormally high levels of free radicals and the simultaneous decline of antioxidant defense mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation and development of insulin resistance. These consequences of oxidative stress can promote the development of complications of diabetes mellitus. In addition to their ability to damage macromolecules, ROS can indirectly induce damage to tissues by activating a number of cellular stress-sensitive pathways (Fig 1) which include protein kinase, hexsoamines, glucose autoxidation, polyol, and others that cause cellular damage and are ultimately responsible for the complications of diabetes (*Brownlee, 2001*).

Hyperglycemia and stress-activated pathways: Recently, hyperglycemia has been implicated in the activation of additional biochemical pathways that play a significant role in the etiology of diabetic complications which include advanced glycation end product (AGEs) and protein kinase C (PKC) activation, polyol and hexosamine pathway (*Brownlee, 2001*). These biochemical pathways are dependent on the ROS generation by the mitochondrial electron transport chain (*Du et al., 2000*).

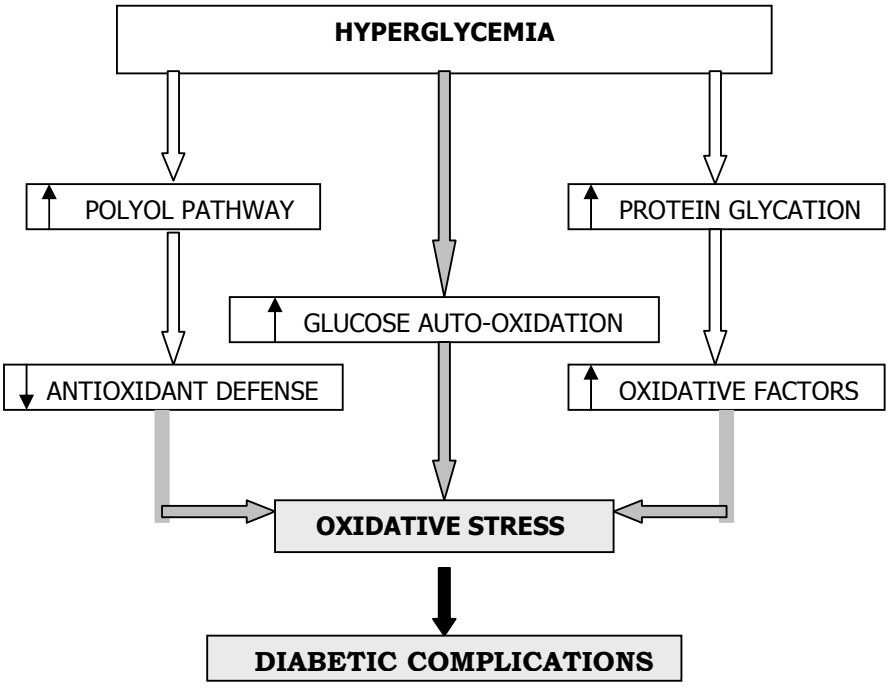


Fig 1: Schematic representation of hyperglycemia induced oxidative stress

Increased polyol pathway flux: In some tissues, which do not require insulin for glucose transport, hyperglycemia leads to an increase in intracellular glucose. The excess glucose is metabolized to sorbitol, a polyol by the enzymes aldose reductase and eventually to fructose. The accumulated sorbitol and fructose leads to increased intracellular osmolarity and influx of water and eventually to osmotic cell injury.

Recent studies have proposed that oxidation of sorbitol by NAD^+ increases the cytosolic NADH: NAD^+ ratio thereby inhibiting the activity of the enzyme glyceraldehyde -3-phosphate dehydrogenase (GAPDH). It is also been proposed that reduction of glucose by NADPH consumes NADPH. In cells where aldose reductase activity is sufficient to deplete reduced glutathione (GSH), oxidative stress is augmented (*Lee and Chung, 1999)*.

Increased advanced glycation end product formation: The term 'autoxidative glycosylation' describes the suggested role of reducing sugars as catalysts of oxidative chemical modification and cross-linking of proteins. This process is initiated by the oxidation of an aldose or ketose to a more reactive dicarbonyl sugar, which would then react with a protein. The reduced oxygen products formed in the autoxidation reaction include superoxide, hydroxyl and hydrogen peroxide, which in the presence of metal ions would cause oxidative damage to neighboring molecules. Sequential glycation followed by free radical-mediated oxidation will generate early glycosylation products. Some of these products undergo further chemical derangements to form irreversible, advanced glycosylation end products (AGEs), which accumulate with aging and duration of diabetes in structural proteins. Intracellular hyperglycemia is speculated to be the primary initiating event in the formation of both intracellular and extracellular AGE's (*Degenhardt et al., 1998*). AGE formation damages target cells by altering cellular functions, modifications of extra cellular matrix and binding of AGE precursors to AGE receptors inducing receptor mediated ROS leading to pathological changes in gene expression.

Hexosamine pathway flux: Evidences suggest that the increased flux of glucose through the hexosamine pathway may contribute to insulin resistance leading to diabetic complications (*Nerlich et al., 1998*). The rate limiting enzyme in this pathway glutamine: fructose-6-phosphate amidotransferase which catalyses the conversion of fructose-6-phosphate to glucosamine-6-phosphate which is metabolized to UDP-N-acetyl glucosamine. Thus, increased glucose flux activates the hexosamine pathway, which may result in raised glucosamines that may cause insulin resistance (*Marshell et al., 1991)*, changes in gene

expression (*Goldberg et al., 2000)* and protein function, which together contribute to the pathogenesis of diabetic complications.

Increased activation of Protein kinase C: Of the different forms of PKC, and isoforms are activated by the lipid second messenger diaclglycerol (DAG). Hyperglycemia increases DAG content by altering the glycolysis wherein there is an increased formation of α-glycerol-3-phosphate, which is a precursor of DAG. Increased de novo synthesis of DAG is reported to activate PKC (β-isoform) in retina and glomeruli of diabetic animals. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial NO synthatase (*Kuboki et al., 2000)*, endothelin-1 (*Glogoswski et al., 1999*), vascular endothelial growth factor, transforming growth factor TGF- β and by activation of NF-kB (*Yerneri et al., 1999*).

Mitochondrial superoxide production: Hyperglycemia derives electron donors from the TCA cycle (NADH derived from both cytosolic glucose oxidation and mitochondrial TCA cycle activity donates electrons complex I) by pumping protons across the mitochondrial inner membrane. This inhibits electron transport complex III, increasing the half-life of free radical intermediates of coenzyme Q (ubiquinone), which reduce O_2 to superoxide. This hyperglycemia-induced mitochondrial superoxide activates four pathways of hyperglycemic damage. Excess superoxide partially inhibits the glycolytic enzyme GAPDH, thereby diverting upstream metabolites from glycolysis into pathways of glucose over utilization. This results in increased flux of dihydroxyacetone phosphate (DHAP) to DAG, an activator of PKC and of triose phosphates to methylglyoxal, the main intracellular AGE precursor. Increased flux of fructose-6-phosphate to UDP-N-acetlyglucosamine increases modification of proteins by O-linked N-

acetlyglucosamine and increased glucose flux through polyol pathway consumes NADPH and depletes GSH.

4.0 MALE REPRODUCTIVE SYSTEM

The male reproductive system comprises of 1) *the testis*, where the continuous process of gamete production (spermatogenesis) occurs; 2) the *excurrent ducts* for further maturation, storage and transport of gametes and 3) *accessory glandular organs* that secrete complex fluids into the final ejaculate. The integration and control of these diverse components and functions necessitates specialized cellular interactions and hormonal control mechanisms (*Creasy and Foster, 1991)*.

The Hypothalamic-Pituitary-Gonadal Axis

Of the three components of the HPG axis, the hypothalamus and the pituitary gland have solely regulatory functions, which are mediated by the hormones they produce and secrete. The third component the testes also produces key hormones, including testosterone, which control male sexual characteristics and behavior. In addition, the testes are responsible for sperm production. The hypothalamus produces luteinizing hormone-releasing hormone (LHRH), which is released in pulses into a system of blood vessels that connect the hypothalamus and the pituitary gland. In response to the LHRH signal, the pituitary gland produces two protein hormones called gonadotropins.

These two gonadotropin hormones-luteinizing hormone (LH) and follicle-stimulating hormone (FSH)-are then released into the body's general circulation and act primarily at the level of the gonads. In males, LH stimulates testosterone production from specialized cells called Leydig cells. FSH is important to sperm maturation in another compartment of the testes, the epididymis. Testosterone circulates in

the blood back to the hypothalamic-pituitary unit and regulates the further production and secretion of LHRH and LH as shown in Fig 2.

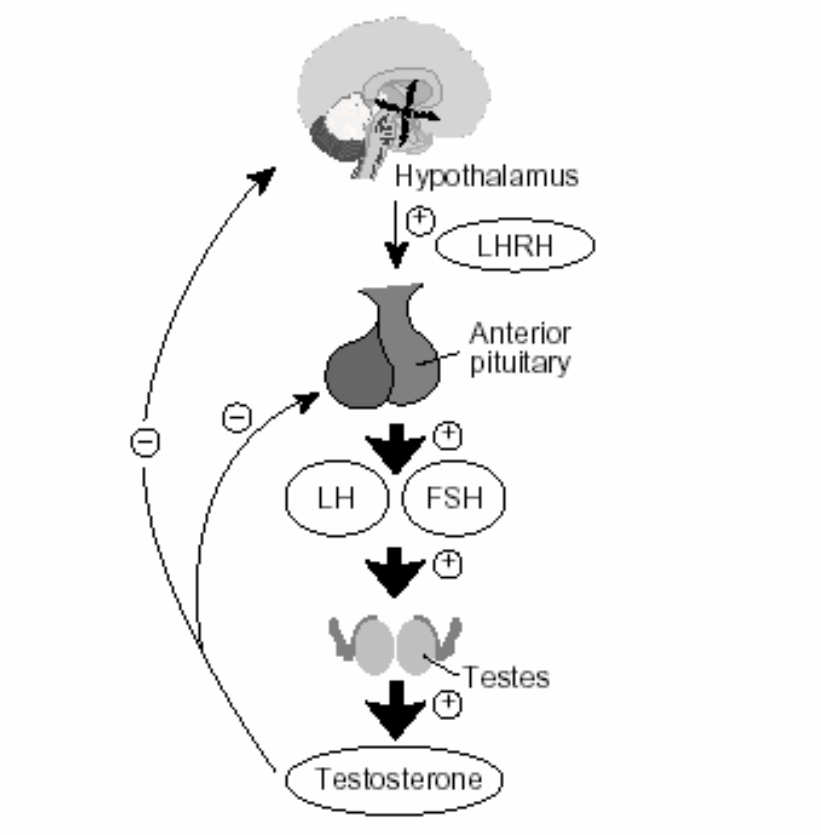


Fig 2: The hypothalamic-pituitary-gonadal axis. The hypothalamus produces luteinizing hormone releasing hormone (LHRH), which is released to the pituitary gland. In response to the LHRH signal, the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In males, LH stimulates testosterone production and FSH is important to sperm maturation. Testosterone circulates in the blood back to the hypothalamic-pituitary unit and regulates the further production and secretion of LHRH and LH. NOTE: + = stimulatory effect; – = inhibitory effect.

Under normal conditions, a lowered testosterone level results in a rise in pituitary gonadotropins. Prolactin, a third reproductive hormone synthesized in the pituitary gland, is important to normal LHRH synthesis and secretion. Low levels of testosterone (i.e., hypogonadism) in adult men have been associated with a variety of medical problems including accelerated osteoporosis, decreased muscle and prostate

function, anemia, altered immune function, and decreased reproductive ability (*Klein and Duwall, 1994*). Each of these conditions can cause significant health problems. These effects of low testosterone are greater in adult men who have had low testosterone levels since adolescence compared with adult men who experience diminished testosterone levels only in adulthood. An adolescent or teenager who experiences short-term, intermittent decreases in testosterone or permanent hypogonadism is predisposed to experience these problems later in life. As testosterone levels decrease, levels of LH and FSH are expected to increase to stimulate the production of more testosterone. The hypothalamic cells which produce LHRH do not function correctly when the feedback normally provided by testosterone is removed (i.e., when testosterone levels decrease) (*Yen and Jaffe, 1991*).

Spermatogenesis

Spermatogenesis is a complex process whereby primitive stem cells or spermatogonia, either divide to reproduce themselves for stem cell renewal or they divide to produce daughter cells that will later become spermatocytes. The spermatocytes eventually divide and give rise to mature cell lines that eventually give rise to spermatids. The spermatids then undergo a transformation into spermatozoa. This transformation includes nuclear condensation, acrosome formation, loss of most of the cytoplasm, development of a tail and arrangement of the mitochondria into the middle piece of the sperm. Groups of germ cells tend to develop and pass through spermatogenesis together. This sequence of developing germ cells is called a generation. There are fourteen stages of seminiferous epithelium development. The progression from stage one through stage fourteen constitutes one cycle. In rat, the duration of the entire spermatogenic cycle in rat with a cycle time of 12-13.3 days equals 48-53 days.

Hormonal Control of Spermatogenesis

A structural and functional relationship exists between the two separate compartments of the testis, i.e. the seminiferous tubule (ST) and the interstitium between the tubules. LH affects spermatogenesis indirectly and stimulates androgenous testosterone production. FSH targets Sertoli cells. Therefore, testosterone and FSH are the hormones that are directed at the ST epithelium. Androgen-binding protein, which is a Sertoli cell product, carries testosterone intracellularly and serves as a testosterone reservoir within the ST in addition to transporting testosterone from the testis into the epididymal tubule. The physical proximity of the Leydig cells to the ST and the elaboration by the Sertoli cells of androgen-binding protein, cause a high level of testosterone to be maintained in the microenvironment of the developing spermatozoa.

Transport-Maturation-Storage of Sperm

Although the testis is responsible for sperm production, the epididymis is intimately involved with the maturation, storage and transport of spermatozoa. Testicular spermatozoa are non-motile and are incapable of fertilizing ova. Spermatozoa gain progressive motility and fertilizing ability after passing through the epididymis. The coiled seminiferous tubules terminate within the rete testis, which in turn coalesces to form the ductuli efferentes. These ductuli efferentes conduct testicular fluid and spermatozoa into the head of the epididymis. The epididymis consists of a fragile single convoluted tubule, which is divided into the cauda, cauput and corpus. It is during the period of maturation in the epididymis that the sperm develop the increased capacity for progressive motility and also acquire the ability to penetrate oocytes during fertilization. The epididymis also serves as a reservoir or storage organ of sperm. The sperm that are stored in cauda epididymis enter the vas deferens and are propelled by peristaltic

motion into the ejaculatory duct. Sperms are then transported to the outside of the male reproductive tract by emission and ejaculation.

5.0 MALE INFERTILITY: CAUSES AND TYPES

Infertility affects approximately 15% of all couples trying to conceive, and male factor is the sole or contributing factor in roughly half of these cases (*WHO, 1999a*). The testicular disorders worldwide have increased over the last two generations. An increasing incidence of germ-cell derived cancer of the testis is accompanied by increase in the prevalence of cryptoorchidism and hypospadias. These trends correlate well with an apparent decline in mean sperm count for fertile men from 113 million/ml in 1940 to 66 millions/ml in 1990 (*Carlsen et al., 1992*).

Causes of Infertility

Infertility has a wide range of causes stemming from three general sources: physiological dysfunctions, preventable causes, and unexplained issues. Anatomical, genetic, endocrinological and immunological problems can all cause or contribute to infertility (*Daar and Merali, 2001*). Male factors include issues with sperm counts, motility, and quality; and ejaculatory dysfunctions. Current estimates suggest about 6% of men between the ages of 15 and 50 are infertile (*Purvis and Christiansen, 1992*). Most causes of male infertility reflect an abnormal sperm count or quality. Unfortunately, in about 90% of cases, the cause for the decreased sperm formation cannot be identified and the condition is labeled "idiopathic oligospermia or azoospermia" (*Wyngaarden et al., 1992*).

Male infertility may be caused by a number of factors, including problems associated with sperm production, sperm transport, and sperm motility as well as anatomical problems, blockage of the vas deferens and infection. Problems with sperm production result in a

reduced sperm count (Table 1). A reduced sperm count may be caused by a number of factors, including hormonal changes, medications, drug use, alcohol use, excessive caffeine, cigarette smoking and testicular injury. In men, hormone disorders, illness, reproductive anatomy trauma and obstruction, and sexual dysfunction can temporarily or permanently affect sperm and prevent conception.

Table 1: Causes of male infertility (*Lammarrone et al., 2003*)

Varicocele
Infections: a. acute: smallpox, mumps, other viral infections b. chronic: Tuberculosis, leprosy, prostatitis
Sexually transmitted diseases
Idiopathic - cause unknown
Injury a) direct: testicular or pelvic trauma, heat, irradiation b) indirect: radiotherapy, chemotherapy, environmental toxins, drugs, marijuana, tobacco, alcohol
Undescended testes (cryptorchidism)
Previous surgery: inguinal, scrotal, retroperitoneal, vasectomy
Obstructions: congenital (aplasia), vasectomy, post-infective
Systemic illnesses esp. hepatic, renal
Immunologic : infection, obstruction
Ejaculatory disturbances
Spinal cord lesions
Genetic, endocrine & familial disorders: Klinefelter's syndrome, Young's syndrome, cystic fibrosis, adrenal hyperplasia
Sexual dysfunctions

Table 2: Causes of male infertility based on the male reproductive physiological dysfunctions

1). Pre-testicular causes	
Hypothalamic disease	Pituitary disease
Isolated gonadotropin deficiency (Kallmann's syndrome)	Isolated LH deficiency ("Fertile eunuch")
Isolated FSH deficiency	Pituitary insufficiency (tumors, infiltrative processes, operation, radiation)
Congenital hypogonadrotropic syndromes	Hyperprolactinemia Hemochromatosis
	Exogenous hormones (estrogen-androgen excess, glucocorticoid excess, hyper and hypothyroidism)
2). Testicular causes of infertility	
Chromosomal abnormalities (Klinefelter's, XX disorder, XYY syndrome)	
Noonan's syndrome (male Turner's syndrome)	
Myotonic dystrophy	
Bilateral anorchia (vanishing testes syndrome)	
Sertoli-cell-only syndrome (germinal cell aplasia)	
Gonadotoxins (drugs, radiation)	
Orchitis; Trauma	
Systemic disease (renal failure, hepatic disease, sickle cell disease)	
Defective androgen synthesis or action	
Cryptorchidism; Varicocele	
3. Post-testicular causes	
Disorders of sperm transport	Congenital, acquired & functional disorders
Disorders of sperm motility or function	Congenital defects of sperm tail; Maturation defects; Immunologic disorders; Infections
Sexual dysfunction	Erectile dysfunction; premature ejaculation; failure of intromission

6.0 OXIDATIVE STRESS: AN ETIOLOGICAL FACTOR IN MALE INFERTILITY

Recently, oxidative stress has become the focus of interest as a potential cause of male infertility (*Agarwal et al., 2004*). In the context of human reproduction, a balance normally exists between ROS production and antioxidant scavenging activities in the male

reproductive tract. As a result of such balance only minimal amount of ROS remain and they are used for the regulation of various normal sperm functions (*Aitken, 1999)*. However, production of excessive amounts of ROS in semen can overwhelm the antioxidant defense mechanisms in spermatozoa and seminal plasma and cause OS (*Sikka, 2001; Agarwal and Saleh, 2002*).

There is clear evidence suggesting that human spermatozoa can produce ROS (*Hendin et al., 1999; Gil-Guzman et al., 2001*). High levels of ROS are reported in the semen of 25-40% of infertile men (*de Lamirande et al., 1995*; *Pardon et al., 1997*). Production of high levels of ROS in the reproductive tract is detrimental not only to the fluidity of sperm membrane but also affect the integrity of DNA in the sperm nucleus (*Aitken,* 1999). Evidences indicate that DNA fragmentation commonly observed in the spermatozoa of infertile men is mediated by high levels of ROS (*Kodama et al., 1997; Sun et al., 1997*). ROS production is reported to be highest in immature sperms with abnormal head morphology and cytoplasmic retention and lowest in mature sperm and immature germ cells (*Gil-Guzman et al., 2001)*. Further, the levels of ROS products by immature sperm were directly associated with sperm nuclear DNA damage and inversely correlated with motility. Some of these findings have led to the hypothesis that oxidative damage of mature sperm by ROS-producing immature sperm during their co-migration from seminiferous tubules to the epididymis may be an important cause for male infertility (*Agarwal and Saleh, 2002*).

ROS and sperm toxicity

ROS has a special penchant to attack PUFA and lipid peroxidation affects the fluidity of sperm plasma membrane with subsequent loss of the ability to oocyte-fusion. Increased formation of ROS has been correlated with reduction in sperm motility (*Iwasaki and Gagnon, 1992*).

Spermatozoa are particularly susceptible to damage induced by ROS because the plasma membrane contains large quantity of PUFA and their cytoplasm contains low concentration of scavenging enzymes (*Alvarez and Storey, 1995; Storey et al., 1998)*. In addition to structural lipids and proteins, the spermatozoa DNA is also prone to oxidative insult. In sperm, DNA damage has been reported as an indicator of alternations in the regulation of spermatogenesis and poor pregnancy outcome (*Sakkas et al., 1998*).

The increased sensitivity to DNA damage in abnormal spermatozoa is probably due to failed chromatin condensation, which makes the DNA more accessible to damage (*Sakkas et al., 1998; Twigg et al., 1998*). Presence of DNA fragmentation in ejaculated spermatozoa has also been suggested to correlate with defects in spermatogenesis (*Gandini et al., 2000*). Studies have shown that exposure of sperms to artificially produced ROS causes DNA damage in the form of modification of all bases, production of base free sites, deletions, frame shift, DNA cross-links and chromosomal rearrangements (*Duru et al., 2000*). Oxidative stress is also associated with high frequencies of single and double DNA strand breaks (*Sun et al., 1997*). Further, studies have indicated that spermatozoa with significantly damaged DNA retain the capacity for fertilization (*Aitken, 1999*).

Semen analysis shows that fertile males have higher antioxidant capacity than infertile controls, indicating that impaired antioxidant capacity may play a key role in infertile disorders (*Potts et al., 1999*). Seminal plasma and spermatozoa possess several AOE: glutathione peroxidase, glutathione reductase, superoxide dismutase, in addition to containing high concentrations of thiol groups, ascorbic acid, glutathione and a-tocopherol (*Yeung et al., 1988*). Epididymis is known to synthesize specific quantities of all the AOE in addition to catalase

activity, which may be potentially protective for spermatozoa from oxidative attack during storage (*Potts et al., 1999*).

Antioxidant therapy and semen quality

It is abundantly clear that a definite conclusion cannot be drawn from the available literature on the effectiveness of antioxidants in male infertility due to multitude of factors. Current evidences support the use of variety of antioxidants was used in the treatment of infertile males and different regimens have been employed. Numerous experimental evidences (*in vitro* and *in vivo*) are available with regard to the efficacy of various antioxidants in humans as discussed below (Table 3 and 4).

Table 3: Effect of selected antioxidants on sperms under *in vitro* conditions

ANTIOXIDANT	OBSERVATION	REFERENCE
Vitamin E	Reduction in LPO; enhances motility; reduces H_2O_2-induced ROS; reduction in H_2O_2 induced DNA damage	*Aitken et al., 1989; Donnelly et al., 1999; Sierens et al., 2002*
Vitamin C	Reduction in LPO; enhances motility; reduces H_2O_2-induced ROS; reduction in H_2O_2 induced DNA damage	*Verma and Kanwar, 1998; Sierens et al., 2002*
Glutathione	Reduces DNA damage induced by ROS; reduces PMA-induced ROS	*Lopez et al., 1998*
N-Acetyl-L-cysteine	Improves motility; reduces ROS production; reduces DNA damage	*Oeda et al., 1997; Lopes et al., 1998*
SOD	Reduces loss of motility and LPO	*Aitken et al 1993*
Catalase	Reduces loss of motility and LPO and DNA damage	*Lopez et al., 1998;*

In vitro studies: Exogenous addition of antioxidant such as SOD, vitamin E in the seminal plasma are known to effectively prevent the increase in sperm plasma membrane LPO and enhance the motility characteristics (*Jones et al., 1978; Aitken et al, 1989; Kobayashi et al., 1991; Verma and Kanwar, 1998; Bolle et al., 2002)*. Addition of different concentrations of

vitamin C and E to sperm preparation medium significantly reduced H_2O_2-induced ROS (*Dounelly et al., 1999; 2000)*. Protective roles of vitamin E and C on sperm DNA damage induced by H_2O_2 were also demonstrated in both normozoospermic and asthenozoospermic samples (*Donnelly et al., 1999*). Further, exogenous SOD significantly reduced the loss of motility and MDA concentration (*Kobayashi et al., 1991*) and addition of catalase reduced the generation of ROS (*Gagnon et al., 1991*).

In vivo studies: Based on recent studies, it has been presumed that antioxidants may be useful as therapeutic agents for male infertility (*Rolf et al., 1999*), although the results of clinical trials have been controversial (*Tarin et al., 1998; Ford and Whittington, 1998; Lenzi et al., 1998*). The beneficial effects of oral administration of vitamin C on sperm quality have been reported in smokers. A significant positive correlation was observed between serum, seminal plasma vitamin C concentrations and sperm quality for different sperm parameters (*Dawson et al., 1992*). In addition, the combination of vitamin E, C and GSH treatment in infertile patients resulted in a significant improvement in sperm concentration. Combination of vitamin E and selenium supplementation in oligoastheno-teratozoospermic patients resulted in an increase in seminal plasma concentrations of selenium and vitamin E along with significant improvement in sperm motility, viability, and morphology (*Vezina et al., 1996*). Improved sperm motility in infertile patients using a combination of vitamin E and selenium supplementation (*Keskes-Ammar et al., 2003)* were also reported.

Table 4: Summary of studies (*in vivo*: semen parameters) using oral antioxidants in the treatment of male infertility

ANTIOXIDANT	OBSERVATIONS	REFERENCE
Glutathione	Enhanced motility; morphology	*Lenzi et al., 1993*
L-carnitine	Increased sperm conc. and morphology	*Costa et al., 1994*
Vitamin E	Improved motility; decreased MDA conc.	*Suleiman et al., 1996*
Selenium	Improved conc. motility; morphology	*Iwanier and Zachara, 1995*
Vitamin E + Selenium	Improved motility; viability; morphology	*Vezina et al., 1996*
Vitamins E & C, GSH	Reduced MDA conc.; DNA damage	*Kodama et al., 1997*
Coenzyme Q_{10}	Improved fertilization rate	*Lewin & Laven, 1997*
NAC + Vitamin E	Improves sperm conc.; motility & morphology	*Comhaire et al., 2000*

7.0 DIABETES AND MALE INFERTILITY

The impact of diabetes upon male fertility although well known, appears to be limited to only a small number of patients who seeks reproductive assistance. Traditionally, T2DM was later in onset than T1DM, but according to recent estimates, the annual incidence of younger people between the ages 40 to 60 years developing T2DM with its associated complications is 26 per 1000 men in the general population (*Meuleman, 2002*). The fertility of these men may be affected directly or indirectly by the effects of the disease process on erection, ejaculation, spermatogenesis and even embryo development.

Pathogenesis

Basically, diabetes can affect the MRS in multiple ways which can be categorized based on their physiology: (i) Pre-testicular (hypothalamic-

pituitary-gonadal axis: endocrine control), (ii) Testicular (spermatogenesis) and (iii) Post-testicular (ejaculation). The most common abnormalities encountered in infertile diabetic patients are due to overt ejaculatory dysfunction. Calcification of the vas deferens is also another abnormality, which occurs six times as frequent as in diabetics as in normal men.

Clinical trials

Endocrine control: Diabetes can affect the hypothalamic-pituitary-gonadal axis in many ways. A direct effect upon the leydig cells within the testicular interstitium may produce primary testicular failure, which is detected by the low testosterone levels and high gonadotropin levels. Diabetic neuropathy affecting the hypothalamus or adenohypophysis or abnormalities of the hormone receptors may cause secondary testicular failure (*Dinulovic and Radonjic, 1990*).

Testicular biopsies of male diabetics (oligospermia or impotence) revealed numerous abnormalities including decreased tubule diameter, hyalinized tubule walls, occluded lumia form epithelial encroachment or cellular debris, exfoliated germ cells, altered sertoli cell connections with spermatids, degenerating sertoli cell apical membranes, vacuolization of both sertoli and leydig interstitial cells and invasion of the interstitial compartment with a collagen rich extra cellular matrix (*Cameron et al., 1985*). Clinical trials also revealed mild to moderate abnormalities in semen parameters, which appear to be dependent upon the degree of metabolic control and presence of neuropathy (*Pardon et al., 1984*).

Ejaculatory disorders: Ejaculation involves three integrated components: seminal emission, bladder neck closure and antegrade ejaculation. Diabetes causes autonomic neuropathy affecting the sympathetic

fibers (*Fisher et al., 1984*). This neuropathic changes lead to partial or complete retrograde ejaculation and also develop failure of emission.

Animal studies

The hormonal profile in spontaneously diabetic rats is characterized by low levels of total and free testosterone and an elevation in luteinizing hormone (LH) (*Howland and Zebrowski, 1980; Murray et al., 1983; Seethalakshmi et al., 1987*). Serum levels of LH were initially low in spontaneously diabetic BB-wistar rats, but they later became nearly two times higher than that of the controls (*Murray et al., 1983*). Concomitantly, a progressive increase in concentrations of lipid within leydig cells of diabetic rats have been reported suggesting impaired steroidogenesis and primary leydig function (*Orth et al., 1979*). Accessory sex gland changes, morphologic disturbances and spermatozoal abnormalities are also reported in diabetic rat models which appear to be reversible with either testosterone or insulin administration (*Seethalakshmi et al., 1987*).

Significantly lower testicular sperm counts (2.5million vs. 5.7 million) and epididymal sperm motility (14.3% vs. 46.8%) are reported in diabetic rats compared with normal controls. Only insulin restored motility to normal levels, suggesting either a direct effect of faulty carbohydrate metabolism on spermatozoa or an indirect effect on spermatozoa from lack of energy substrate (*Seethalakshmi et al., 1987)*.

8.0 MODULATION OF DIABETES: ROLE OF ANTIOXIDANTS

Diabetes associated hyperglycemia is known to significantly impair various endogenous antioxidant defense system. Consequently, numerous workers have examined the role of antioxidants in hindering oxidation, thereby delaying or preventing oxidative stress and associated diabetic complications. An antioxidant has been defined

as 'any substance when present at low concentrations compared with those of an oxidizable substrate (*Halliwell and Gutteridge, 1999*), significantly delays or prevents oxidation of that substrate'. Intracellular antioxidant defense is primarily provided by AOE, which catalyze decomposition of ROS through different mechanisms (Fig 3).

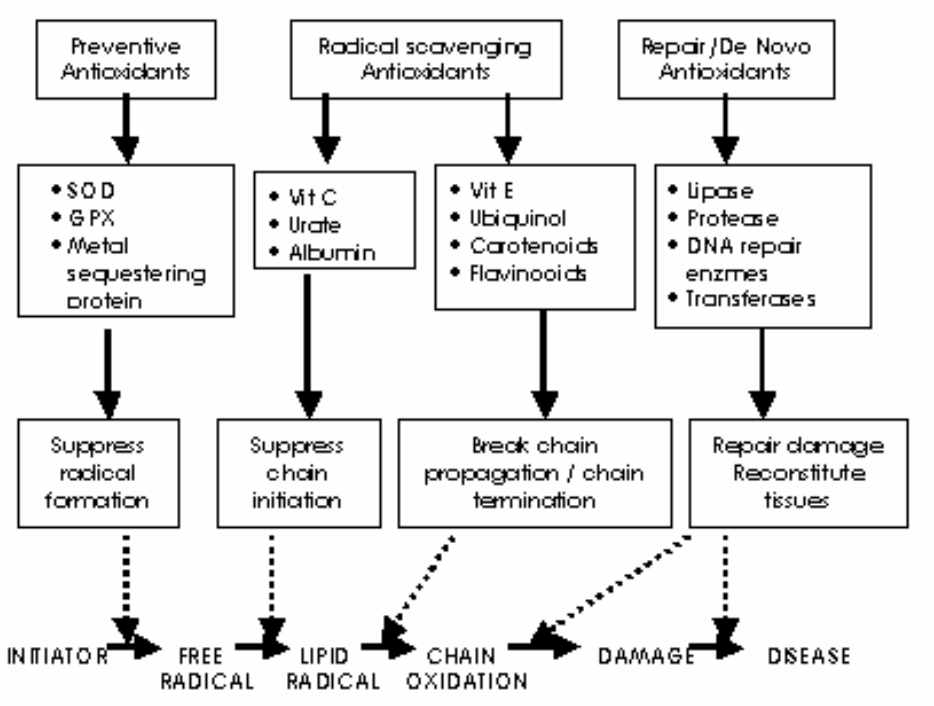


Fig. 3: **Antioxidants: types and actions** (*Willcox et al., 2004*); *(dotted lines: suppression)*

As a first line of defense, the preventive antioxidants such as peroxidases and metal chelating proteins suppress the generation of free radicals. Next, the radical-scavenging antioxidants such as vitamin C and E scavenge radicals to inhibit the oxidation chain initiation and prevent chain propagation as a second line of defense. This may also include the termination of a chain by the reaction of two radicals. The repair and de novo enzymes act as the third line of defense by

repairing damage and reconstituting membranes. These include lipases, proteases, DNA repair enzymes and transferases (*Niki, 1997*).

Modulation of OS biomarkers of diabetes in vivo

Extensive studies on pharmacological interventions based on biological antioxidants have been carried out and exhaustively reviewed (*Oberley, 1988; Maritim et al., 2003*). Decreased levels of GSH, elevated concentrations of TBARS have been consistently reported in diabetes. However, discrepancies in various biomarkers of OS especially in the activities of SOD, CAT and GPx are reported in experimental diabetic animals. The increase in TBARS associated with diabetes is known to be prevented by various modulants as shown in Table 5. Further, many modulants are known to affect the GSH concentrations in various somatic organs in chemically induced diabetic animals. Many of the modulants are known to restore the altered GSH concentrations.

Diabetes is known to induce significant perturbations (increase or decrease) in the activities of various AOE in various somatic organs (as reviewed by *Maritim et al., 2003*). There is no agreement about the effects of diabetes on the activities of these enzymes. However, some of these effects are known to be significantly altered by treatment with various modulating agents as shown in Table 6. The protective effects of exogenously administered antioxidants in animal models provides useful insights into the relationship between free radicals, diabetes and its various complications.

Clinical trials: Studies in humans have demonstrated beneficial effects of various antioxidants in one of the complications, diabetic neuropathy. One such study supplemented type-1 and 2 diabetics with α-lipoic acid, vitamin E or selenium for 12 weeks.

Table 5: Effect of selected modulants on the inhibition of increased LPO, restoration of GSH and AOE in experimental diabetic rodents

Inhibition of LPO (measured in terms of TBARS) in somatic tissues	
Nicotinamide	*Melo et al., 2000*
Melatonin	*Maritim et al., 2002*
Piperine	*Rauscher et al., 2000*
Aminoguanidine	*El-Khatib et al., 2001*
α-lipoic acid	*Sailaja Devi and Das, 2000*
Quercetin	*Obrosova et al., 1999*
Vitamins C, E and β-carotene	*Mekinova et al., 1995*
Reduced GSH in somatic tissues (e.g. liver, kidney)	
Vanadyl SO_4	*Thompson and Mc Neil, 1993*
Isoeugenol	*Rauscher et al., 2000*
Quercetin	*Sanders et al., 2001*
Nicotinamide	*Melo et al., 2000*
Vitamins C, E and β -carotene	*Mekinova et al., 1995*
Taurine	*Obrosova et al., 1999*
α-lipoic acid	*Borenshtein et al., 2001*
GPx	
Probucol	*Kaul et al., 1996*
Vitamins C, E and β -carotene	*Mekinova et al., 1995*
Desferroxamine	*El-Khatib et al., 2001*
Aminoguanidine	*Kedziova-Kornatowska et al., 1998*
Melatonin	*Maritim et al., 1999*
α-lipoic acid	*Kocak et al., 2000*
SOD	
Captropril	*Kedziova-Kornatowska et al., 1998*
Aminoguanidine	*El-Khatib et al., 2001*
Stobadine	*Stefek et al., 2000*
Melatonin	*Maritim et al., 1999*
Piperine	*Rauscher et al., 2001*

Results showed improvement in neurological symptoms and decreased lipid peroxidation. In a dietary intervention, 10 stable type-2 diabetic

patients were placed on flavonol (quercetin) rich diet for two weeks, after following a low flavonol diet for preceding two weeks.
Lymphocytes were subjected to an oxidative challenge *ex vivo* and DNA damage was measured. DNA damage was significantly reduced following consumption of high flavonol diet, compared to the low flavonol diet (*Lean et al., 1999*).

A randomized, double-blind, placebo-controlled clinical trial supplemented 28 type-1 diabetic patients with a flavonoid based antioxidant medication while following a standardized 1,800-2,000 calorie diet (*Keenoy et al., 1999*) and assessed after 3 months. Glycated hemoglobin (HbA1c) values decreased slightly but significantly whereas the decline was non-significant in the placebo group. There were significant differences in the supplemented group as seen by increase glutathione reductase activity, increased plasma protein thiol content and increased lag time of *ex vivo* copper induced LDL oxidation (*Keenoy et al., 1999*).

9.0 PHYTOCHEMICALS AS MODULATORS OF OXIDATIVE STRESS IN DIABETES

Over the last decade, there is a growing body of evidence on biologically active compounds present in various plants, which are known to impart health benefits (*Oomah and Maaza, 2000*). These potentially active biomolecules which are called 'phytonutrients' or 'phytochemicals' refer to all naturally occurring chemical substance present in plants (*Caragay, 1992*). Many major phytonutrients have been reported to possess antioxidant, anticarcinogenic properties and a wide spectrum of health promoting activities (*Craig, 1997*). The most common components in the plants, which act as health promoters, are polyphenols, epigallacatechin, gallate, isoflavonols, vitamins etc (*Karakaya and Kavas, 1999*). The pharmacological action of

phytochemicals may be an additive or synergistic effect of several components present which sometimes when isolated are ineffective. The plausible mechanisms with which the phytochemicals prevent a disease complication are by regulating the antioxidant-prooxidant homeostasis, endocrinal homeostasis or by reducing the DNA damage or being an immunomodulator (*Wise, 2001*). These phytochemicals are used as therapeutics or dietary supplements in treating or alleviating various disease conditions such as cancer, cardiovascular diseases, diabetes, etc (*Yeh et al., 2003*).

Plant derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems around the world viz., Indian ayurvedic system of medicine, Chinese, Mexican, Jewish, etc. Many modern pharmaceuticals used in conventional medicine today also have natural plant origins. Among them, metformin (oral hypoglycemic drug) was derived from the flowering plant, *Galega officinalis* which was a common traditional remedy for diabetes (*Oubre et al., 1997*).

The ethnobotanical information reports about 800 plants that possess anti-diabetic potential (*Alarcon-Aguilara et al., 1998*). Out of an estimated 250,000 higher plants, less than 1% have been screened pharmacologically and a few in the management of diabetes. Some of the active ingredients studied include alkaloids, glycosides, galactomannan gum, polysaccharides, peptidoglycans, hypoglycans, guanidine, and steroids. Recent observations strongly suggest that phytonutrients, which can slow the absorption of ingested carbohydrate and suppress the glycemic index, may be effective for diabetes prevention (*Willett et al., 2002*). The recent findings that heavy consumption of coffee markedly lowers the risk for diabetes (*Isogawa et al., 2003; Rosengren et al., 2004*) has not yet been adequately

explained, and there is suggestive evidence that the chief polyphenolic compound in coffee chlorogenic acid, may slow carbohydrate absorption by inhibiting intestinal glucose transport.

Table. 6: List of selected plants employed in clinical trials for their hypoglycemic and other attributes (*Grover et al., 2002*)

Plant	Part used	Beneficial properties
Acacia arabica	Seed	increased glucose tolerance, hypolipidemic, normalized diabetes-induced histopathological alterations
Allium sativum	Bulb	antioxidant, hypolipidemic,
Azadirachta indica	Fruit, leaves	increases glucose uptake
Aloe vera	Gum	anti-inflammatory, increased glucose tolerance
Ficus bengalensis	Bark	hypolipidemic, antioxidant
Gymnema sylvestre	Leaf	reduced glucose tolerance, hypolipidemic
Momordica charantia	Fruit pulp, seed, leaves	antioxidant, increased beta cell number, Hypocholesterolemic, improved glucose tolerance
Musa sapentum	Flower	antioxidant, hypolipidemic
Mucuna pruriens	Seed	stimulates insulin release
Ocimum sanctum	Leaves	antioxidant
Punica granatum	Flowers	antioxidant
Trigonella foenum gracecum	Seed	antiglycosuric, antioxidant, hypocholesterolemic, hypolipidemic
Tinospora cordifolia	Root	antioxidant, hypolipidemic

Since, several evidences, suggest that diabetic complications are amenable to antioxidant therapy, hypoglycemic medicinal plants with antioxidative properties are being employed extensively to mitigate the development of diabetic complications (Table 6). Only few such plants have been studied for their efficacy and safety. Although, oral hypoglycemic agents are the mainstay of treatment of

diabetes and are effective in controlling hyperglycemia, they have prominent side effects such as hematological, cardiovascular diseases, gastrointestinal reactions, hypoglycemic coma, damage to vital organs and fail to significantly alter the course of diabetic complications. As the knowledge of heterogeneity of this disorder increases, there is a need to look for more efficacious agents with lesser side effects.

SCOPE OF THE PRESENT INVESTIGATION

Diabetes mellitus (DM), long considered a disease (or metabolic disorder) of minor significance to world health, is now taking its place as one of the main threats to human health in the 21st century. The past two decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide. While T2DM is numerically more prevalent in the general population, T1DM is the most common chronic disease of children. Recent evidences show an increasing prevalence of T2DM in children, teenagers and adolescents. The socioeconomic and public health impact of this '*downward shift in disease onset'* on society is much greater through its *negative impact on fertility and reproduction* and through effects on work force, premature morbidity and mortality.

Increased oxidative stress (OS) is a widely accepted participant in the development and progression of diabetes and its complications. Diabetes is usually accompanied by increased products of free radicals and impaired antioxidant defenses. Mechanisms by which increased OS is involved in the diabetic complications are largely unknown. Nevertheless hyperglycemia, auto-oxidation of glycated proteins, increased production of reactive oxygen species (ROS), decreased antioxidant defense, increased lipid peroxidation and associated membrane degeneration are implicated as main causes of cellular apoptosis or necrosis, which are common in diabetes.

Reproductive disturbances in diabetic patients are well known and in males, diabetes is reported to cause testicular degeneration and impotence. In experimental diabetic animals, seminiferous tubules have been reported to show increased tubular wall thickness, severe germ-cell depletion, sertoli cell vacuolization, decreased testicular volume, sperm motility and semen volumes. Further, decreased numbers of

Leydig cells and testosterone secretion have also been reported. However, the pathophysiological mechanisms of testicular dysfunctions in diabetic situations are largely unknown. More importantly the possible involvement of OS mechanisms and their contribution towards diabetes associated male reproductive dysfunctions are poorly understood.

In the male reproductive system, ROS and free radicals have been ascribed certain regulatory roles as they can modulate some of the structural and morphological changes that the mammalian spermatozoa undergo during their maturation and development. Mammalian testis is anatomically and physiologically more prone for OS. Although, testicular cells are endowed with various antioxidant defenses their efficiency is restricted due to their limited concentration and varied distribution. Sperms are even more vulnerable to OS as they are endowed with more of PUFA, superoxide anion generating system in their membranes, less antioxidants and less repair capacity. An elevated production of ROS in testis either of exogenous or endogenous origin can lead to alterations in tissue physiology or cause oxidative damage to DNA, which is of potential risk to the offspring.

In view of the above, currently, OS has become the focus of interest as a potential cause of male infertility. There is paucity of data on the involvement of OS mechanisms in testis under diabetic situations has not been comprehensively investigated. Further, under different degree/duration of diabetic state, data on the perturbations of antioxidant levels in testis and the physiological/biochemical implications that may lead to testicular dysfunctions is lacking. Hence there is a need to understand the occurrence and role of oxidative damage in testis during different phases of diabetes (early and progressive), impairments of antioxidant defenses, susceptibility of sperms to OS, and their genotoxic consequences. Only such an

understanding will aid in developing therapeutic strategies to ameliorate the reproductive dysfunctions in diabetic situations.

Hence, it is hypothesized that "the testis under diabetic situations is likely to be subjected to significant oxidative stress and this mechanism may be partly or wholly responsible for the testicular dysfunctions". Accordingly, the scope of the present investigation is (1) to understand the involvement of oxidative stress mechanisms under conditions of diabetes associated male reproductive dysfunction and determine its implications on physiology and function of testis (2) to investigate the occurrence of early oxidative damage in testis, its progression and its genotoxic consequences and (3) to examine the propensity of biomolecules to protect testicular tissue from diabetes-induced oxidative damage.

CHAPTER 1:

MATERIALS AND ASSAY METHODS

SECTION A

MATERIALS

Chemicals

β-carotene, β-D-fructose, α-ketovaleric acid, α-tocopherol, 1,1,3,3-tetraethoxy propane, 1-chloro-2,4-dinitro benzene, 2, 4-dinitrophenylhydrazine, 5,5-dithio-bis(2-nitrobenzoic acid), adenosine diphosphate, ascorbic acid, boron trifluoride in methanol, bovine serum albumin, caffeic acid, cetyltrimethylammonium bromide, cholesterol, collagenase, cumene hydroperoxide (80% aqueous CAS # 80-15-9), dipalmitoylphosphatidyl choline, diphenyl-β-picryl hydrazyl (DPPH), cyclophosphamide, 2',7'-dichlorofluorescein, 2',7'-dichlorofluorescin diacetate, DL-Isocitrate, ethidium bromide, fatty acid methyl esters, ferrulic acid, ferricytochrome-C, gallic acid, glucose-6-phosphate, glutaraldehyde, glutathione (GSH and GSSG), glutathione reductase, hydrogen peroxide, mercaptoethanol, NADH, NADP, NADPH, oxidized and reduced glutathione (GSH and GSSG), protocatechuic acid, quercetin, t-butyl hydroperoxide (70% aqueous CAS # 75-91-2), thiobarbituric acid, triolein, tryphan blue, trypsin, urea, xanthine and xanthine oxidase were procured from M/s. Sigma Chemical Co., (St. Louis, MO, USA). Ferric chloride, ferrous sulphate, Folin's reagent, o-pthalaldehyde, potassium ferricyanide, sodium dodecyl sulfate and tricholoro acetic acid were procured from M/s. Sisco Research Laboratories, Mumbai, India. All other chemicals used were of analytical grade.

Diabetogenic agent:

Streptozotocin (N- (methyl-nitrosocarbamoyl)-α-D-glucosamine) was obtained from M/s. Sigma-Aldrich Chemical Co., USA.

Animals and Care

Experiments were carried out using adult/growing male rats (CFT-wistar strain) or adult mice (male/female; CFT-swiss strain). Animals were fed on commercial pellet diet (Gold Mohur, M/s. Lipton India Ltd, Mumbai, India) *ad libitum* and had free access to tap water.

Rats: Adult male rats of 8-10 wk old (weighing 180-200g) and growing male rats of 5 weeks old (weighing 70-80g) were drawn from the 'animal house facility' of our Institute. Generally they were acclimatized for a week before use. They were housed in polypropylene cages with 12:12h light/dark cycles, at control atmosphere of temperature and humidity.

Mice: Adult male mice of 8-10 wk old (weighing 35-40g) were used throughout the studies. They were randomly drawn from the stock colony of our animal house facility. They were placed in groups of five in polypropylene rectangular cages (27" long, 20" wide and 14" high) with sawdust as the bedding material. The cages were kept on racks built of slotted angles and were housed in a controlled atmosphere with a temperature range of 25 ± 2°C and mean humidity of 50% ± 5. Mice were acclimatized for one week by feeding on commercial pellet *ad libitum* and had free access to tap water.

For dominant lethal studies, adult virgin females (10 wk old) were used. They were acclimatized for a week and were housed in groups of five in polypropylene cages. At the time of experiment, adult virgin females were caged in pairs with adult proven males for a stipulated period of time.

Ethical considerations: All the experiments were carried out in accordance with the protocols approved by the 'Ethical committee' of the Institute (CFTRI). Animal handling, sacrifice were conducted strictly in conformation with standard guidelines provided thereof.

Preparation of tissue samples

Isolation of plasma

Samples of blood were collected either by cardiac puncture or from retro-orbital plexus in tubes coated with sodium fluoride (0.1%). Blood was centrifuged at 1500 rpm for 10min at 4°C to obtain plasma.

Tissue homogenates

Tissues (Liver and Testis) excised from adult male rats or mice were blotted free of blood, rinsed in ice-cold saline and homogenized (10% w/v) in 1.15% KCl. The homogenates were centrifuged at 1500 rpm at 4°C for 5 min and the supernatants were used.

Cytosol

Tissues were homogenized in ice-cold sodium phosphate buffer (0.1 M, pH 7.4) and centrifuged at 10,000g for 30min. Supernatants were used for assessing the enzyme activities.

Mitochondrial fractions *(Trounce et al., 1996)*

Tissues were homogenized (10% w/v) in ice-cold homogenizing buffer (2mM Tris-HCl, 250mM sucrose, pH 7.4) and the homogenates were centrifuged at 2400 rpm for 10 min at 4°C. The mitochondrial pellet was washed twice with ice-cold MSH buffer (200mM Mannitol, 70mM Sucrose, 10mM HEPES, 0.1mM EDTA) and was finally suspended in a known volume of MSH buffer. Protein was estimated in the mitochondrial suspension according to the method of *Lowry et al., 1951*.

Microsomal fractions *(Hanna et al., 1994)*

Microsomes were prepared from rat/mice tissues (liver/testis) according to the modified method of *Hanna et al., 1994*. Tissues were homogenized in ice-cold KCl (1.15%; pH 7.4) and the homogenates were centrifuged at 9,000g for 15 min at 4°C. The resulting supernatant was centrifuged at 1,05,000g for 45 min at 4°C. The microsomal pellet was suspended in a known volume of homogenizing buffer. Protein was

estimated in microsomal suspension according to the method of *Lowry et al., 1951*.

Epididymal sperm isolation *(Sokol et al., 1994)*

Cauda epididymis was excised from rats and rinsed in saline. Each cauda epididymis was then placed in a Petri dish with 2 ml of phosphate buffered saline. With the help of forceps and fine needles, the mucus membrane over the cauda was removed and the exposed tubules were gently teased with the help of needle to release the sperms into PBS. The preparation was later filtered through a stainless steel sieve (100µ) to remove the particulate matter. The whole process was performed on an ice bath and was completed within 20 min after excising the epididymis. The suspension was then centrifuged at 2000rpm for 5min at 4°C and resultant sperm pellet was resuspended in known volume of Kreb ringer bicarbonate solution (KRBS). The sperm count was determined using hemocytometer.

Preparation of testis explants *(Jacobson and Miller, 1997)*

The method adopted was essentially similar to that described by Jacobson and Miller, 1997 with minor modifications. After perfusion with saline, testis was excised, rinsed in saline and decapsulated by making small incision on tunica albuginea from caudal pole and the seminiferous tubules were gently expressed. 50mg of testis explants (TE) (seminiferous tubules with associated interstitium) were dispensed into each well of a sterile tissue culture plate (12wells of 24mm diameter). Tubules were suspended in small volume of media, teased apart, and were cut into 3-5mm fragments. Finally, the TE was suspended in a required volume of KRBS. All the above processes were carried out on an ice bath and completed within 30min after excising the testis.

Testicular cell suspension *(Romrell et al., 1976)*

Rats were anesthetized and perfused with 20ml saline *via* the descending thoracic aorta to remove blood cells from testis. The apex

of the heart was punctured to provide an outlet to the perfusate. Immediately after perfusion, the testis was excised, decapsulated by making a small incision on tunica albugenia from caudal pole and the seminiferous tubules were gently expressed. The aggregate of tubules were incubated with KRBS containing collagenase (0.5mg/ml) for 15min on a shaker at 31-33^{o}C. Trypsin (0.5mg/ml) was added during additional shaking period of 15min. The resulting cell suspension was gently pipetted over 2-3min using a Pasteur pipette, filtered through a stainless steel sieve (100µ), washed twice with fresh KRBS by centrifuging at 200g for 5 min and resuspended in KRBS adjusting the cell number to 5 x 10^5 cells/ml. The number of cells was determined by hemocytometer.

Suspending media

Kreb's ringer bicarbonate solution (KRBS) was used for suspending the sperm pellet and during *in vitro* experimentation. The composition of the KRBS was: 120.1mM NaCl, 4.8mM KCl, 25.2mM $NaHCO_3$, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.3mM $CaCl_2$, 11mM glucose. The solution was filtered, sterilized and the pH was adjusted to 7.2.

Preparation of stock solutions

Stock solutions of quercetin, protocatechuic acid and caffeic acid were prepared in dimethylsulphoxide (DMSO). The working stock solutions were prepared by diluting the stock solution using DMSO such that final concentration of DMSO in the incubation volume was within 1% (We observed no effect at 1% level).

Preparation of *Ficus bengalensis* seed powder

Fresh fruits of *Ficus bengalensis* (FB), which were available locally, were collected and dried to separate the seeds. The seeds thus obtained were finely powdered. The powder was stored in airtight containers at 4^{o}C.

Preparation of seed extract

0.1g of FB seed powder was homogenized with 2 ml of distilled water and then centrifuged at 2000 rpm for 5min at room temperature to obtain the supernatant. The supernatant was used for the assay.

Preparation of experimental diets (FB as a dietary supplement)

Commercially available pellet diet (M/s. Lipton India Ltd., Mumbai, India) was powdered and was supplemented with 2% FB (W/W) seed powder for studying its potency to attenuate diabetes induced oxidative damage.

SECTION B

ASSAY METHODS

Blood glucose estimation

The plasma glucose level was estimated using a commercial kit based on *Trinders* method in which glucose oxidase (GOD) and peroxidase (POD) are used along with phenol and 4-aminoantipyrine (M/s. Dr. Reddy's laboratories, India).

Pathology *(Lee, 1960)*

Testis and epididymis of mice or rats were excised and fixed in Bouin's fluid. The fixed tissues were then dehydrated through series of alcohol, embedded in paraffin, processed by standard histological techniques, stained with hematoxylin and eosin and examined by light microscopy.

Protein estimation *(Lowry et al., 1951)*

Tissue homogenate or supernatant fraction (1mg equivalent) was made upto 1ml with lowry's reagent (2% Na_2CO_3 in 0.1N NaOH with 1% copper sulfate and 2% sodium potassium tartarate) and the total volume was made up to 2 ml with distilled water. 0.1ml of Folin-Ciocalteu (1N) was added, cyclomixed and allowed to stand for 20 min at room

temperature. After 20min, the color developed was measured at 750nm by using a spectrophotometer (Shimadzu 1601A). The total protein concentration was determined from the standard curve obtained under the same conditions with BSA as the standard.

Determination of lipid peroxidation *(Ohkawa et al., 1979)*

Lipid peroxidation was quantified in tissue homogenates or supernatant fractions by measuring the thiobarbituric acid reactive substances (TBARS). To 0.2ml of the 10% homogenate or supernatant fractions (0.5-1mg protein equivalent) 0.2ml of 8% SDS, 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% thiobarbituric acid aqueous solution were added. The mixture was heated for 1hr in a boiling water bath. After cooling, 3ml of n-butanol was added and mixed vigorously. The color extracted into butanol layer was read at 532nm using a spectrophotometer. 1,1,3,3-tetramethoxypropane was used as an external standard.

Reactive oxygen species *(Driver et al., 2000)*

Determination of reactive oxygen species (ROS) was based on the modified method of *Driver et al*. Homogenates were diluted 1:10 in ice-cold Locke's buffer to obtain a concentration of 5mg tissue/ml. The homogenates were then pipetted into tubes and allowed to warm to room temperature (21°C) for 5 min. at that time, 5µl of DCFH-DA (10µM final concentration) was added to each well and the plates were pre-incubated for 15min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group cleaved by esterases. After 30min, the conversion of DCFH to the fluorescent product DCF was measured using a fluorescence spectrophotometer with excitation at 485nm and emission at 530nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS

production was quantified from a DCF standard curve and results were expressed as *p*mol DCF formed/mg protein/min.

Antioxidant molecules

Glutathione estimation *(Mokrasch and Teschike, 1984)*

Homogenized tissue samples (10-50mg tissue, 10%) were added to 2ml of formic acid (0.1M) and centrifuged at 10,000rpm for 20min. To 100µl of the supernatant, 100µl buffer A (1:4 (v/v) 37% formalin: 0.1M Na_2HPO_4) was added and after 5min, 1ml of buffer B (0.1M sodium phosphate, 5mM EDTA, pH 8.0, for reduced glutathione or 0.1M NaOH for oxidized glutathione) was added along with 100µl of o-pthalaldehyde (1mg/ml). This mixture was vortexed and incubated for 45min at room temperature and the absorbance was read at an excitation of 345nm and an emission of 425nm using a spectrofluorimeter (Shimadzu 5301 PC). The amount of total glutathione was determined from the standard curve simultaneously obtained under the same conditions with standard solutions of GSH and GSSG.

Vitamin E analysis *(Zaspel and Csallany, 1983)*

Testicular tissue and epididymal sperms were homogenized in 20 volumes of acetone. The homogenates was centrifuged and an aliquot of the supernatant was transferred to a tube and dried to approximately 0.5ml under N_2 at 30-40^{o}C. The pellet was extracted twice with acetone. All the supernatant fractions were pooled and concentrated to 1ml under N_2 and vortexed. Suitable aliquot of this filtrate was injected into reverse phase HPLC using C18 (4.6mm x 15cm) column. Tissue α -tocopherol was detected by UV detector at 280nm with a flow rate of 1.5 ml/min with mobile phase of acetonotrile: methanol (1:1 v/v). A standard curve was prepared by plotting peak area versus concentration of vitamin E. The amount of vitamin E present in tissues was calculated using a standard curve.

Determination of Protein carbonyls (*Levine et al., 1990*)

Tissues were homogenized in a homogenizing buffer (20mM Tris-HCl, 0.14M NaCl, pH 7.4) and centrifuged at 10, 000 rpm for 10min at 4°C. Aliquots of supernatants containing 0.5-1mg protein were precipitated with 0.5ml of 20% trichloroacetic acid (v/v) and centrifuged. The pellet was resuspended in 1ml of 2, 4-dinitrophenylhydrazine (10mM) in 2M HCl and allowed to stand at room temperature for 1h, vortexing every 10min. Proteins were then precipitated with 0.5ml of 20% (v/v) trichloroacetic acid, centrifuged and the pellet was washed thirce with 1ml of acetone. The final pellet was dissolved in 1ml of 2% (v/v) sodium dodecyl sulfate prepared in 20mM Tris-Hcl and 0.4M NaCl buffer (pH 7.4). Carbonyl content was calculated from the maximum absorbance (360nm) using a molar extinction coefficient of $22mM^{-1}cm^{-1}$. Results were expressed as nmol carbonyls/mg protein.

Antioxidant enzymes

Catalase *(Aebi, 1984)*

The enzyme activity was determined by measuring the change in absorbance in 1 ml reaction mixture using final concentration of 8.8mM H_2O_2 and 50mM phosphate buffer (pH 7.0) at 240nm, 25°C after addition of sample equivalent to 100µg protein. The enzyme activity was expressed as µmol of H_2O_2 oxidized/min/mg protein.

Glutathione peroxidase *(Flohe and Gunzler, 1984)*

The assay mixture contained 500µl 0.1M phosphate buffer (pH 7.0), 100µl of enzyme sample, 100µl of glutathione reductase (0.24U/ml), 100µl of 10mM GSH and 100µl NADPH. The final volume of the reaction mixture was 1ml. The reaction was started by the addition of 100µl 12mM t-butyl hyroperoxide. Conversion of NADPH to NADP+ was monitored continuously in a spectrophotometer at 340nm for 3 min. GPx activity was expressed as *n*moles of NADPH oxidized/min/mg protein using an extinction coefficient ($6.22 \times 10^3\ M^{-1}cm^{-1}$).

Glutathione transferase *(Guthenberg et al., 1985)*

To 0.85- 0.9ml of phosphate buffer (0.1M, pH 6.5), 0.05ml of 20mM glutathione, 0.05ml of 20mM 1-chloro-2,4-dinitrobenzene (CDNB) were added and mixed. 0.05ml of supernatant (enzyme sample) was then added to the above mixture and the formation of the adduct of CDNB (S-2,4-dinitrophenly glutathione) was monitored by measuring the net increase in absorbance at 340nm. The enzyme activity was calculated based on the absorption coefficient of $9.6mM^{-1}cm^{-1}$. Results were expressed as µmols adduct formed/min/mg protein.

Glutathione Reductase *(Carlberg and Mannervick, 1985)*

The enzyme activity was measured by NADPH coupled assay. 1ml of reaction volume consisted of 900µl of phosphate buffer (0.2M, pH 7.0, 2mM EDTA), enzyme sample (~150µg protein), 50µl of NADPH (2mM) and 50µl of 20mM oxidized glutathione. The rate of decrease in absorbance was monitored at 340nm at 37°C. The enzyme activity was expressed as nmol of NADPH oxidized/min/mg protein.

Superoxide dismutase *(Flohe and Otting, 1984)*

To a semi micro cuvette were added 2.9ml of solution A (5µl xanthine in 0.01N NaOH + 2µl Cytochrome-c + 50mM phosphate buffer in 0.1mM EDTA) and 0.1ml of solution B (an equal volume of xanthine oxidase in 0.1mM EDTA). Reaction mixture without enzyme was used as blank. After adding various volumes of enzyme sample, inhibition of cytochrome-c reduction was monitored for 5min at 560nm. Results were expressed as units of SOD/mg protein. One unit was defined as the amount of enzyme that decreases the initial rate of cytochrome-c reduction to 50% of its maximal value for the particular sample being analyzed.

Functional enzymes

Sorbitol Dehydrogenase *(Gerlach, 1974)*

The enzyme activity was determined as the amount of fructose reacted per unit time, measured by the decrease in absorbance due to oxidation of NADH. The reaction was carried out in a 1ml cuvette to which 0.75ml of Tris buffer (111mM, pH 7.5), 0.05ml of NADH (1.8mM) and sample equivalent to 200µg protein were added. The solutions were mixed and incubated for about 5min at 25°C until the absorbance was constant. The reaction was started by the addition of 0.1ml of fructose (66.6mM). The solutions were mixed thoroughly, waited for 2-3min until reaction became linear, followed by recording the absorbance at 1min interval for 3-5min at 340nm.

Glucose-6-Phosphate Dehydrogenase *(Deutsch, 1974)*

G6PDH was assayed in the supernatants of testicular and sperm homogenates by following the rate of reduction of NADP. The reaction volume of 1ml contained 0.1ml of 3.8mM of NADP, 0.1ml of 63mM $MgCl_2$, 0.1ml of 33mM glucose-6-phosphate and 0.5ml of distilled water. Solutions were mixed, incubated for 5min at 30°C and the reaction was started by adding the enzyme sample (150µg protein). The rate of increase in absorbance was recorded for 5min at 340nm.

DNA damage

FADU assay *(Birnboim and Jevcak, 1981)*

Testicular cell suspensions were prepared in PBS by following the procedure as described earlier (section 1.1.3). Aliquots of these suspensions were distributed in to 9 tubes designated as **T** (total fluorescene), **P** (partial fluorescence) and **B** (basal fluorescence) in triplicates. To each tube, 100µl of solution C (9M urea-10mM, NaOH-0.1% SDS) was aged and the contents were incubated at 0°C for 10min. To P and B tube 100µl each of solution D (0.45 vol of solution C in 0.2N NaOH) and solution E (0.40 volume of solution C in 0.2N NaOH) were

added. To tube T, 400µl of solution F (1M glucose-14mM mercaptoethanol) was added prior to the addition of solution D and E. The tubes were kept at 0°C for 30 min at the end of which, the contents of B tubes were placed on ice and sonicated for 60sec at 40W. The tubes were then incubated at 15°C for 30min, chilled to 0°C, and 400µl of solution F was added to P and B tubes. To each tube, 3ml of solution G (6.7µg ethidium bromide/ml in 133mM NaOH) was added and the contents were mixed gently. Appropriate blanks and controls were included to eliminate non-specific fluorescence. The extent of DNA damage was calculated as follows

% double stand DNA= D=100 x P-B/T-B

% damage = 100-D=A

% damage induced by alkali = basal damage = C

% absolute damage = D = A-C

Biochemical constituents in serum and testis

Total lipid extraction *(Folch et al, 1957)*

Serum: 0.8ml of serum was taken in a stoppered tube and 2ml of methanol was added and mixed well for 30 seconds and to that mixture, 1ml of chloroform was added and mixed well for 30 seconds. Further, 1ml each of chloroform and water was added and mixed well for 30 seconds. The extract was filtered using Whattman (no 1) filter paper. Filtrate was allowed to settle for phase separation. Aqueous methanolic upper layer was removed by aspiration and the lower chloroform layer with total lipid was used for further analysis after drying on anhydrous sodium sulphate.

Testis: 1g of tissue was homogenized with 1.0ml of 0.74% potassium chloride in a homogenizer. To the extract, 20ml of chloroform: methanol (2:1 v/v) was added and homogenized. The mixture was left overnight and filtered through a Whattman no.1 filter paper. 3ml of 0.74%

potassium chloride was added and mixed well by vortexing. The solution was allowed to stand at room temperature. The upper aqueous layer was removed carefully and then lower phase was washed with 3ml of 0.74% potassium chloride and twice with 3ml of chloroform: methanol: water (3:48:47 v/v) mixture. The chloroform layer was used for lipid analysis.

Total cholesterol estimation *(Rudel and Morris, 1976)*

An aliquot of the chloroform extract was dried under a stream of nitrogen followed by the addition of 1.5ml of ferric chloride - acetic acid reagent. The reagent was prepared by diluting the stock containing 504mg/ml anhydrous $FeCl_3$ in 10ml of glacial acetic acid to 1:100 dilution with glacial acetic acid. After mixing thoroughly, it was left at room temperature for 15 min. 1ml of concentrated sulphuric acid was added, mixed immediately on a vortex mixer and left at room temperature in dark for 45 min. The color intensity of the clear solution was measured by spectrophotometer at 540 nm. Cholesterol levels in testis/serum samples were estimated from the cholesterol (30-150µg) reference standard.

Phospholipid estimation *(Stewart, 1980)*

Phospholipids were analyzed by ferrous ammonium thiocyanate method using dipalmitoylphosphatidyl choline (10-100µg) as reference standard. The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 2ml of chloroform. 2ml of ferrous ammonium thiocyanate was added and vortexed for 1 min. Following the phase separation, absorbance of chloroform phase was measured at 488 nm by a spectrophotometer.

Triglyceride estimation *(Fletcher, 1968)*

The lipid extract in chloroform was evaporated under a stream of nitrogen and then dissolved in 4ml of isopropanol. Two grams of activated alumina was added, mixed well and centrifuged.

Supernatant was transferred to another test tube, saponified with 0.6ml of 5% potassium hydroxide in isopropanol: water (40:60 v/v) at 60°C for 15 min. After cooling, 1 ml of sodium metaperiodate prepared from the stock solution of 0.025M in 1N acetic acid (sodium metaperiodate (12ml) and 20ml of isopropanol and made up to 100ml with 1N acetic acid) was added, mixed and 0.5ml of acetyl acetone was added, stoppered and incubated at 50°C for 30 min. After cooling to room temperature, the color intensity was read at 405 nm by spectrophotometer. Triglycerides were estimated by using triolein as a reference standard (30-300µg).

Fatty acid analysis *(Morrison and Smith, 1963)*

Fatty acids were analyzed as methyl esters prepared using boron trifluoride in methanol as described by Morrison and Smith (1963) and analyzed by gas chromatography (Shimadzu 14B, fitted with FID) using fused silica capillary column 25m x 0.25mm (Parma bond FFAP-DF-0.25: Machery-Nagel Gm BH co. Duren, Germany). The operating conditions were: initial column temperature 160^0C, injector temperature 210^0C and detector temperature 250^0C; column temperature was programmed to rise at 6^0C per min to the final temperature of 240^0C. Nitrogen gas was used as the carrier. Individual fatty acids were identified by comparing with retention times of standards (NU-Check prep. Inc., Elysian Minnesota, USA) and were quantified by online chromatopack CR-6A integrator.

Genotoxicity studies

Epididymal sperm counts *(Muralidhara and Narasimhamurthy, 1996a)* ***and sperm morphology assay*** *(Wyrobeck and Bruce, 1975)*

Epididymal sperm count was determined by using haematocytometer as described earlier. Fresh epididymis was freed from the testis and the cauda was separated from the caput and corpus. Cauda was held in 0.9% NaCl and gently teased to remove the sperms out and aliquots of

these sperm suspensions were used for counting using a phase contrast microscope.

For examining the abnormal sperms, aliquots of sperm suspensions were stained with 0.1% eosin Y and the smears were examined for abnormal sperms as described by *Wyrobeck and Bruce*. A minimum of 1000 sperms per mice was scored for abnormal head morphology.

Dominant lethal mutation assay *(Muralidhara and Narasimhamurthy, 1996)*

The treated males were mated with virgin females (1:2 ratio) each week sequentially for a period of 7 weeks. Successful mating was ascertained by the presence of vaginal plugs and all the pregnant females were humanely killed 16-17 days post detection of plugs. To assess the degree of post-implantation embryo lethality, both the uterine horns were longitudinally cut open and the uterine contents were analyzed for total implantation sites, resorptions and fetal deaths. Simultaneously, the induction of dominant lethal response of a positive mutagen cyclophosphamide (CP) was also studied along with control mating.

Scanning electron microscopy (*Woolley, 2003*)

The washed sperm pellets obtained after teasing were fixed in glutaraldehyde (4%) for 2hr at room temperature followed by washing twice with Tris buffer (0.1%, pH 6.8). The sperm pellet was suspended in a known volume of Tris buffer. An aliquot of the suspension was allowed to stand on ploy-lysine coated glass slides for 30min. The slides were drained, rinsed in Tris buffer, dehydrated in alcohol (30 – 100%) for 15 min each. The sperms were mounted on an aluminum stub (100 – 200Å) using double-sided tape and sputter-coated with gold (Polarum E 5000, SEM coating system) and samples were viewed using a scanning electron microscope (model no. LEO 435 VP, Electron microscopy Ltd.,

Cambridge, UK) with an acceleration voltage of 20KV. Electron photomicrographs were taken with VP 120 negative film (Kodak USA).

Determination of antioxidant activity

Total polyphenol estimation *(Shetty et al., 1995)*

An aliquot of FB seed powder extract with volume adjusted to 3ml with distilled water was incubated with 0.5ml of 95% ethanol and 250µl of Folin's reagent (1:1 dilated with distilled water) for 5 min at room temperature. To this, 0.5ml of sodium carbonate (5%) solution was added, mixed and the mixture was incubated for 60 min at room temperature. The absorbance of the solution was read at 720nm against a reagent blank. Gallic acid (0.1mg/ml) was used as the standard and the polyphenol content in the extract was expressed as mg Gallic acid equivalent (GAE)/g powder.

Reducing power *(Yen and Chen, 1995)*

The reducing power of the extract was estimated by a modified method of *Yen and Chen* (1995). Briefly, 1ml of reaction volume containing FB seed powder (1.25-10mg) in phosphate buffer (0.2M, pH 6.6) was incubated with potassium ferricyanide (1% v/v) at 50°C for 20 min. The reaction was terminated by adding tricholoro acetic acid (TCA) solution (10% v/v) and the mixture was centrifuged at 3000 rpm for 10min. 2.5ml of the supernatant was mixed with 2.5ml of distilled water and ferric chloride (0.1% w/v) solution. The absorbance of the mixture was measured at 700nm against blank. Increased absorbance of the reaction mixture indicated increased reducing power.

Determination of radical scavenging activity *(Ohnishi et al., 1994)*

The total free radical scavenging capacity of the aqueous extract of FB seed powder was estimated according to the method described by *Ohnishi et al.,* (1994). 1.5ml of DPPH solution (0.1mM in 95% ethyl alcohol) was incubated with the FB extract (0.5 - 5mg) and the final volume was adjusted to 1.75ml with 95% ethyl alcohol. The reaction mixture was

shaken well and incubated for 20min at room temperature and the absorbance of the resulting solution was read at 517nm against blank. The radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation

$$\text{Scavenging effect (\%)} = \left(1 - \frac{\text{A sample}}{\text{Control}} \right) \times 100$$

Statistical analysis

Experimental data obtained in adult and growing rats (Chapter 2) were analyzed using student 't' test a *P*-value less than 0.05 was set as the minimum level of significance.

Experimental data on oxidative damage related parameters in adult mice (Chapter 3) were also analyzed by student 't' test. Data on the response of antioxidants on EOD was analyzed by ANOVA (one-way), However, data on epididymal sperm count, sperm abnormalities and three variables of DL assay, viz., pregnancy, total implantations (TI) and post-implantations (TI) and post-implantation deaths were analyzed employing a non-parametric test, the Mann-Whitney *U*-test (*Snedecor and Cochran, 1971*). Experimental data on the attenuation of testicular oxidative damage by antioxidants were also analyzed employing ANOVA (one-way).

(*All the statistical calculations were done through SPSS for Windows: Version 10.0.1(SPSS. 1999. SPSS Inc, New York). Scheffe's post-hoc test was used as one of the test whenever F value was significant)*.

CHAPTER 2:

EVIDENCES OF OXIDATIVE STRESS IN MALE REPRODUCTIVE SYSTEM UNDER STZ–INDUCED DIABETES: RAT MODEL

INTRODUCTION

Diabetes mellitus (DM) is the most common endocrine disease, which is known to have significant impact on male fertility. Recent epidemiological surveys have shown an explosive increase in the number of diabetics (*Zimmet et al., 2001*). The global figure of people with diabetes is set to rise from the current estimate of 150 million to 220 million in 2010, and 300million in 2025. The increasing incidence of T2DM in children, teenagers and adolescents, is likely to have an adverse impact on male reproductive health. According to a recent estimate, there are nearly 32 million diabetics in India (*Ramachandran et al., 2002)*. Epidemiological data from different parts of India show a rising prevalence of diabetes in the urban areas. A recent national study showed that the prevalence in urban adults aged $\geq$ 20 years was 12.1%. Onset of diabetes is known to occur at a younger age in Indians giving ample time for development of various chronic vascular complications.

The potential contribution of increased oxidative stress to the development of various diabetes complications is well known (*Zimmet, 2000)*. Increased cellular oxidative stress (OS) and altered antioxidant pool have been implicated in both clinical and experimental T1DM (*Baynes, 1991; Vincent et al., 2002)*. Hyperglycemia, auto-oxidation of glycated proteins, increased production of reactive oxygen species (ROS), decreased antioxidant defense, increased lipid peroxidation and associated membrane degeneration are implicated as the main causes of cell death -apoptosis or necrosis, which are common in diabetes (*Desco et al., 2002*).

Reproductive disturbances in diabetics are well known and in males, diabetes is reported to cause testicular degeneration and impotence (*Orth et al., 1979; Paz and Homonnai, 1979; Sanguinetti et*

al., 1995; Sexton and Jarrow, 1997). Animal models with spontaneous and induced diabetes have attempted to provide some insight into the potential adverse effects of diabetes upon human male reproductive dysfunctions. In experimental diabetic animals, seminiferous tubules have been reported to reveal increased tubular wall thickness, severe germ-cell depletion, sertoli cell vacuolization, reduced testicular and semen volumes, decreased sperm motility and decreased leydig cell numbers along with diminished testosterone level (*Anderson and Thliveris, 1987*). However, the pathophysiological mechanisms of testicular dysfunctions in diabetic situations are largely unknown. More importantly the possible involvement of OS mechanisms, and their contribution to the development of male reproductive dysfunctions are poorly understood.

Male infertility has become a common disease, which is increasing with industrialization and is the major cause for conception failure in 25% of all infertile couples (*Cates et al., 1985; Hull et al., 1985; Sharlip et al., 2002; Agarwal et al., 2004)*. The situation is further alarming since there is an apparent decline in the mean sperm count of fertile men, from 113 million per ml in 1940 to 66 millions per ml in 1990 (*Carlsen et al., 1992)*. Several researchers speculate that chronic exposure to OS induced by varied life styles (smoking and alcohol), increase in environmental pollution or socioeconomic state may be a major contributing factor in these rising tides of male reproductive pathologies. In the male reproductive system, ROS and free radicals have been ascribed certain regulatory roles as they can modulate some of the structural and morphological changes which the mammalian spermatozoa undergo during their maturation and development (*Riley and Behrman, 1991*). Current epidemiological evidences indicate that OS is a significant contributory factor in human

infertility (*Aitken, 1995; Sharma and Agarwal, 1996; Pasqualotto et al., 2001; Agarwal and Saleh, 2002; Saleh and Agarwal, 2002; Agarwal et al., 2004)*.

Mammalian testis is anatomically and physiologically more prone for OS (*Cummins et al., 1994*). Although, testicular cells are endowed with various antioxidant defenses, their efficiency is restricted due to their limited concentration and varied distribution. Sperms are even more vulnerable to OS as they are endowed with more of PUFA, superoxide anion generating system in their membranes, less antioxidants and less repair capacity. Despite the above findings, the involvement of OS mechanisms in testis under diabetic situations has not been comprehensively investigated. Further, under different degree/duration of diabetic state, data on the perturbations of antioxidant levels in testis and the physiological/biochemical implications that may lead to testicular dysfunctions is lacking.

This chapter describes studies which focus on understanding whether male reproductive system (testis and epididymal sperms) is subjected to significant OS in an experimentally induced diabetic model. The results are presented under two sections, A and B. **Section A** comprises of studies pertaining to diabetes induced oxidative stress in testis and epididymal sperms of adult rats following administration of an acute dose of STZ. Data on the incidence of pathological alterations in testis, progression of oxidative damage, perturbations in antioxidant defenses and associated biochemical implications have been presented. **Section B** describes the pattern of testicular oxidative damage occurring in sexually immature rat with emphasis on testicular weights and pathology. Data on the reduced GSH, protein carbonyls and effects on the activity of GST and lipid profile are also presented.

SECTION A

OXIDATIVE STRESS IN TESTIS AND EPIDIDYMAL SPERMS OF ADULT RATS

EXPERIMENTAL DESIGN

Preliminary investigations

Adult male rats (8-10 wk old, 180-200g) housed individually were used for the investigations. With an objective of determining the optimum dosage of Streptozotocin (STZ) that would induce consistent hyperglycemia and low mortality in our rat strain, preliminary studies were conducted. STZ was dissolved (conc. 75mg/ml) in freshly prepared citrate buffer (0.05M; pH 4.5). Initially, groups of male rats (n=4) were administered (i.p.) acute dosages of STZ (ranging from 30-120 mg/kg bw). Animals were provided with glucose water (5% w/v) for 48h following STZ treatment to prevent initial drug induced hyperglycemic mortality. Fasting blood glucose levels were monitored by drawing blood from retro-orbital plexus of both control and STZ –treated rats. Mortality was recorded as and when it occurred.

Determinative studies

All investigations presented in this section were conducted in adult male rats. Based on the mortality data and the hyperglycemic response, a dosage of 60 mg /kg bw was considered as the optimum dosage.

Hyperglycemic response: frequency and magnitude

To assess the induction of hyperglycemia, plasma glucose levels in both control and STZ treated rats were monitored at various sampling weeks.

Food consumption and growth pattern

Rats from both control and STZ groups were provided with pellet diet (weighed) and water. Daily food consumption was recorded by weighing the residual diet. To examine the effect of STZ treatment on growth pattern, weekly body weights were monitored during the experimental period of eight weeks.

Testis/epididymal weights and pathological lesions

At each sampling week, both control and treated animals were sacrificed under mild ether anesthesia and testes and epididymis were excised, trimmed, rinsed in ice-cold physiological saline, blotted and weighed. Testis was then fixed in Bouin's fluid, processed according to standard histopathological techniques and pathological lesions were investigated.

Evidences of oxidative damage in testis and epididymal sperms (ES)

Status of lipid peroxidation (LPO) in testis

Following STZ administration, the degree of oxidative damage in testicular tissue (homogenates, mitochondrial and microsomal fractions) was measured in terms of lipid peroxidation. In order to obtain a comparative picture, LPO was also determined in both serum and hepatic samples at all sampling times.

Perturbations in enzymic and non-enzymic antioxidants

At all sampling intervals, the activities of AOE viz., CAT, SOD, GST, GPx and GR and levels of non-enzymic antioxidants viz., GSH and vitamin E were determined in testicular homogenates of both control and STZ-treated rats.

Oxidative damage to proteins

Fresh samples of testicular homogenates of both control and treated groups were processed for the determination of total protein carbonyl content at all sampling intervals.

Susceptibility of ES to lipid peroxidation

To investigate the degree of STZ-induced oxidative damage in the epididymal milieu, fresh sperm suspensions were prepared from the cauda and were subjected to quantification of TBARS at all sampling intervals.

Antioxidant enzyme activities and dehydrogenases

Activities of AOE viz., CAT, GST, GPx, GR and dehydrogenases viz., SDH, G6PDH were determined at all sampling intervals.

Protein carbonyls in ES

Levels of protein oxidation in ES were quantified in terms of protein carbonyls at all sampling intervals.

Oxidative damage associated biochemical alterations in testis

Effects on biochemical constituents

The excised testis from both control and diabetic groups were processed for quantification of total cholesterol, phospholipids, triglycerides and fatty acid profile at various sampling weeks (two, four, six and eight).

Influence on dehydrogenases

Cytosolic fractions prepared from whole homogenates of testis from control and treated rats were used for determining the activities of dehydrogenases viz., sorbitol dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (G6PDH).

SECTION B

OXIDATIVE DAMAGE IN TESTIS OF GROWING RATS

Preliminary studies

Growing rats (4-5 wk old) randomly housed in pairs were used for all studies. With an objective of determining the optimum dosage of STZ, that would induce consistent hyperglycemia and low mortality in growing rats preliminary studies were conducted. For this, groups of growing rats (n=4) were administered (i.p) STZ at acute dosages ranging from 30-120mg/kg bw.

Determinative studies

Investigations presented in this section were conducted in immature rats. Based on the mortality data and the hyperglycemic response, a dosage of 90 mg/kg bw was considered as the optimum dosage.

Induction of hyperglycemia and weekly progression

Plasma glucose levels were estimated after 3-5 days of STZ administration and were further monitored in both control and STZ-treated rats at weekly intervals.

Food intake and growth characteristics

Daily food intake was monitored by providing a known amount of diet and weighing the residue each day. In order to obtain the effect of STZ treatment on growth, weekly body weights were recorded.

Testicular weights and pathological alterations

At each sampling week, testicular weights were determined and one testis was fixed in Bouin's fluid and processed for histopathological investigations.

Quantification of oxidative damage in testis and liver

Lipid peroxidation profile

At weekly intervals, both control and treated rats were sacrificed. Testis and liver were excised and subjected to various biochemical analyses. Fresh homogenates were used for assessment of oxidative damage in terms of TBARS levels.

Generation of ROS

Tissue homogenates from both control and treated groups were assayed for the generation of ROS levels (employing DCF-DA as the probe) wherein the fluorescent product (DCF) formed was measured.

Redox status

The amount of reduced glutathione in both testis and liver was determined in both control and treated groups at all sampling weeks.

Response of testicular antioxidants

As a measure of oxidative stress, activity of selected antioxidant enzymes viz., CAT, GPx and GST were determined in cytosolic fractions of testicular homogenates.

Total protein carbonyl content

The total protein carbonyl content was quantified in both testis and liver cytosol at all sampling periods.

Perturbations in testicular physiology

Effect on lipid profile

For these measurements, frozen (-80°C) testicular tissue was used. Testis from both control and treated groups were processed as per the requirement and selected biochemical constituents viz., total cholesterol, phospholipids and triglycerides were quantified at all sampling times.

RESULTS

SECTION A

OXIDATIVE STRESS IN TESTIS AND EPIDIDYMAL SPERMS OF ADULT RATS

STZ treatment and Mortality

In our preliminary study, STZ at the lowest dosage (30mg/kg bw) failed to induce any consistent increase in the plasma glucose in adult rats measured on day 3 of post treatment. At the highest dosage (90 mg/kg bw), the percent mortality was as high as 70% and was accompanied by an elevated (4-5 fold) plasma glucose levels (data not shown). However, the dosage of 60 mg/kg bw caused a consistent, significant increase in the plasma glucose levels (3-4 fold) and at this dosage, nearly 30% mortality occurred over a period of six-eight weeks.

Hence, based on the mortality data and the hyperglycemic response, 60mg/kg bw was considered optimum dosage and was deployed for all further investigations.

Hyperglycemic response: frequency and magnitude

Pooled (two independent experiments) hyperglycemic response following administration of STZ at the dosage of 60mg/kg bw, has been illustrated in a scattered diagram (Fig 2.1). Nearly a 3-fold increase in the plasma glucose levels was observed in diabetic rats at various weeks of post administration. While the glucose level in controls was in the range of 124-136 mg%, those of STZ rats ranged from 406-539mg%. The data in Table 2.1 clearly shows that the hyperglycemic response was consistent and progressive.

Food consumption and growth pattern

A significant decrease (50%) in food intake was evident in the STZ treated rats during the first week of STZ administration. However, there

was an increased (~40-45%) food intake in diabetic rats when compared to the control rats during the rest of the experimental period (data not shown). In general, a decrease in the body weight gain was evident among the STZ treated rats beyond week two. Body weights in the diabetic group (179.7 ± 29.8) were significantly reduced than those of controls (230.9 ± 28.1) at week four (25%) and six (30%). Similarly, at the end of the eight-week there was 35% decrease in the absolute body weight (191.7 ± 27.5 vs. 296.0 ± 18.7).

Absolute / relative weights of testis and pathological lesions

Data on the testicular and epididymal weights in control and diabetic rats is presented in Table 2.2. A significant decrease (49%) in testicular weights was evident among the diabetic rats from week four onwards. However, epididymal weights among diabetic rats were significantly decreased 26, 32 & 64% at two, four and six weeks of sampling respectively.

Histopathological lesions were studied in the testis at various sampling weeks (1-6). There were no obvious pathological alterations during the first three weeks (data not shown). However, significant and varying degrees of pathological lesions were discernable in the diabetic rats. Some of the typical lesions in the testis of diabetic rats sampled at weeks 4, 5 and 6 have been presented in Plate 1-3. Beginning from week four onwards, there was obvious shrinkage of seminiferous tubules with reduced tubule diameter, sertoli cell vacuolization and absence of sperms in the lumen (Plate 1 & 2). Further desquamation of germinal epithelium, prominent vacuolization in sertoli cells and degenerating spermatids were evident beyond fifth week. The incidence of tubules showing various lesions gradually increased from fifth week onwards. Most of the tubules from the testis sampled at sixth week, showed marked atrophy with varying degree of

spermatogenetic arrest with total absence of sperms in the lumen (Plate 3). The number of leydig cells was reduced from fourth week onwards and was associated with reduced seminiferous tubule size and thickening of seminiferous epithelium.

Evidences of oxidative damage

Status of Lipid peroxidation in Serum

The lipid peroxidation (LPO) status measured as MDA levels in serum of both control and STZ treated rats at different weeks of post administration is presented in Fig. 2.2. In general, a marked elevation (50-135%) in LPO was evident in serum of diabetic rats at various sampling intervals (Table 2.3). While the increase in LPO in diabetic rats over control was 87% in the second week, the extent of LPO was relatively lower in the first, third and fourth weeks.

Status of Lipid peroxidation in testis

In the testicular homogenates, a marginal but significant increase (29%) in MDA levels was evident during the first week of sampling among treated animals (Table 2.3). However, elevated MDA levels in testis were a regular feature from second week onwards. The increase ranged from 35-90% over the basal levels suggesting the progressive nature of oxidative damage from week two onwards upto week eight of STZ post administration.

The extent of oxidative damage in the mitochondrial and microsomal fractions of testis of STZ treated rats is depicted in Fig. 2.3. Diabetes induced significant elevation (31%) in the MDA levels when compared to the controls at week two of post administration. The response in the fourth, sixth and eighth week was relatively higher (90-160%) compared to the second week. A similar pattern of response was evident in testicular microsomal fractions (35-130%) at all the sampling intervals.

In order to obtain a comparison, MDA levels of hepatic tissue (homogenates) were also determined at all sampling times. This data clearly showed significantly increased (29-149%) MDA levels in liver of diabetic rats over the basal levels at various weeks of sampling (Table 2.3).

Perturbations in antioxidant enzymes and molecules

Data on the activity of selected AOE in testis measured at various sampling periods following STZ treatment are presented in Figures 2.4 and 2.5. Significant perturbations were commonly observed in the cytosolic fractions. While there was a marginal to moderate increase (10-31%) in the activity of GST, activities of other enzymes were decreased (CAT: 17-44%; GPx: 15-35%; GR: 14-39% and SOD: 10-28%) at different sampling times.

Testicular GSH levels marginally increased (17%) at week two and decreased (12%) at week four of post administration and a significant decrease (27%) in GSH levels was evident at week six after STZ treatment (Table 2.4). A significant decrease in vitamin E levels were also evident in the diabetic rats compared to the controls (Table 2.4) at various weeks of sampling which ranged from 11% to 32%.

Oxidative damage to proteins

Protein carbonyl levels monitored in testes of the control and STZ rats are also presented in Table 2.4. In general, the carbonyl levels were consistently elevated (20-50%) in the diabetic testis compared to basal control levels. Significant increases in protein carbonyl levels were found in diabetic animals at week two (63.83 ± 6.09 vs. 52.91 ± 5.62), four (92.99± 6.57 vs. 59.61 ± 2.46) and six (78.10 ± 1.93 vs. 54.24 ± 2.32) of post administration.

Evidences of oxidative damage in epididymal sperms

Susceptibility of ES to lipid peroxidation

The status of lipid peroxidation determined in ES sampled at various weeks is presented in Table 2.5. While there was a marginal increase in MDA among the diabetic rats during the first week of sampling, moderate to marked increases in MDA levels were evident during week two (69%), four (73%) and week six (134%) of post administration.

Data obtained on the protein carbonyls levels in ES (Table 2.5), also showed a significant increase among diabetic rats. The increase ranged between 28-58% over the basal levels at various weeks of sampling.

Effect on antioxidant enzymes and dehydrogenases

The levels of selected antioxidant enzymes determined in epididymal sperms of control and STZ treated rats are shown in Table 2.6. In general, significant decreases in CAT, GPx and GR activities were evident at all weeks of sampling except GST activity. The CAT activity was significantly decreased (10-50%) at various weeks, while the activities of both GPx and GR were decreased uniformly throughout in sperms of diabetic rats (15 % at week two; 21% at week four; 27% at week six; and 35-40% at week eight). Interestingly, the GST activity was enhanced significantly (30-40%) and a similar trend was evident at all sampling weeks.

In ES, the activity of dehydrogenases such as G6PDH and SDH were found to be significantly altered in diabetic animals compared to those of controls (Table 2.7). The activity of G6PDH levels was significantly increased (~40%) at all weeks of sampling. While there was a significant increase (27%) in SDH at week two, the enzyme activity was found to be significantly decreased (19 and 28%) during week four and six.

Oxidative damage associated biochemical alterations in testis

Effects on biochemical constituents-Lipid /fatty acid profile

Data on testicular biochemical constituents measured among the control and diabetic rats at various weeks of sampling is shown in Table 2.8. During the first two weeks, testis of diabetic rats showed no significant alterations in any of the lipid parameters. However, at week three and four, the testis showed a significant increase in cholesterol (~40%), triglycerides (23-34%) and phospholipids (~20%). Similar degree of alterations in total cholesterol (42%), triglycerides (39%) and phospholipids (17%) in diabetic rats were evident even at week eight.

Table 2.9 shows the percent distribution of fatty acids in lipids of testis from controls and diabetic rats. No significant changes were observed in the concentration of various fatty acids among the rats rendered diabetic over a period of time. However, the concentration of arachidonic acid 20:4 (n-6) in testis of diabetic rats was marginally lower in week four and eight compared to controls.

Influence on Testicular dehydrogenases

Effects of STZ-induced diabetes on the testicular dehydrogenases are presented in Table 2.7. Significant decreases (30-50%) in G6PDH were found in the testis of diabetic rats compared to the controls (at week four and six). Similarly, significant decreases in SDH activity (14% at week two, 27% at week four and 42% at week six) were observed.

SECTION B

OXIDATIVE DAMAGE IN TESTIS OF GROWING RATS

Mortality following STZ administration

STZ administered at the dosages of 30 and 60mg/kg bw failed to induce any clinical signs of toxicity or mortality. While STZ at a dosage of 90mg/kg bw produced only a 20% mortality at the end of four weeks of post administration. The highest dosage (120mg/kg bw) induced 60% mortality within a week of post administration (data not shown).

Induction of hyperglycemia and its weekly progression

There was no significant increase in glucose levels at lower dosages (30 and 60mg/kg bw) of STZ (data not shown). However, the plasma glucose levels, measured, among treated rats (STZ; 90mg/kg bw) were significantly elevated during four weeks of sampling (Fig. 2.6). The mean plasma glucose levels among the control rats ranged between 121-139mg%. At first week, the mean plasma glucose levels in the STZ-treated rats were 533mg%. Further, the plasma glucose levels were higher at all sampling times and the mean levels ranged between 461-533 mg% (461.01± 61.18).

Food intake and growth characteristics

There was a significant reduction (85%) in daily food consumption in rats administered STZ during the first week of post administration, which eventually normalized. The food intake was increased (25-30%) in the diabetic animals from week two onwards (data not shown).

The average gain in body weight among the controls over the four-week experimental period was 71.3 ± 5. In contrast, there was a marginal (23%) decrease in body weight among STZ-treated rats calculated based on their initial body weights at the end of four weeks.

Testicular weights and pathological alterations

In general, the testis weights were reduced in the diabetic rats at all sampling week (data not shown). In most of the diabetic animals, the testis had a reddish-purple appearance during the third and fourth week of post administration. The testicular weights of the diabetic animals were reduced by 12, 34, 45 and 55% at week one, two, three and four respectively.

Pathological alterations in testis of growing rats were monitored for a period of four weeks at weekly intervals. Following STZ administration, no significant changes were evident during the first two weeks (data not shown). However, the testis showed varying degrees of pathology at weeks three and four and some of the typical lesions are presented in Plate 4 (a-d). The growing testis appeared to be more susceptible since we observed marked shrinkage of seminiferous tubules with reduced tubule diameter at third week itself. The salient lesions were: reduced interstitial cells, thickening of seminiferous epithelium, high incidence of sertoli cell vacuolization, moderate to severe necrotic changes and absence of spermatogenesis.

Status of oxidative damage in testis and liver

Lipid peroxidation profile (LPO)

The effect of STZ-induced diabetes on LPO in both testicular and hepatic tissue measured at weekly sampling weeks is shown in Fig. 2.7. Testicular MDA levels of growing diabetic rats showed a significant elevation (30%) when compared to controls at week one of STZ-treatment. Interestingly, an increase in MDA by 85, 62 and 38% in STZ treated rats were evident in week two, three and four respectively. Likewise, in the hepatic homogenates, there was significant increase (35%) in MDA at week one of post administration. However, only

marginal increases of 23 and 15% were evident during the second, third and fourth week respectively.

Generation of ROS levels in testicular and hepatic tissues

STZ treatment resulted in a significant elevation in ROS levels in both testicular and hepatic homogenates at all weeks of sampling (Fig. 2.8). Interestingly, the pattern of elevation was similar to the LPO response among both the tissues. Testis of treated animals showed an elevation of 23, 72, 48 and 55% in ROS levels compared to controls at week one, two, three and four respectively. However, the ROS levels in liver homogenates of STZ-treated rats showed a range of 13-55% elevation over the basal levels.

Further, ROS levels measured in mitochondrial fractions of both testis and liver also showed significant elevations among the STZ treated rats (Fig. 2.8). While the mitochondrial fractions of testis showed elevations of 25-44% over the basal levels, the percent increase in hepatic mitochondrial fractions was 32-70% over the basal levels.

Reduced GSH in testis

The GSH contents in testicular tissue were significantly increased by 49 and 34% in STZ-treated group as compared to their respective controls at weeks two and four (Fig. 2.9).

A significant increase of 61% in hepatic GSH levels at week one and 8% at week four and a decrease of 16% was evident at week two in diabetic animals when compared to the controls (no data was obtained at week three).

Total protein carbonyl content

Significant increases of 15, 34 and 25% were found in the testicular protein carbonyl levels of diabetic animals at week one to four (Fig 2.10). Likewise, the hepatic protein carbonyl contents were also increased by 20-25% at various sampling points.

Response of testicular GST

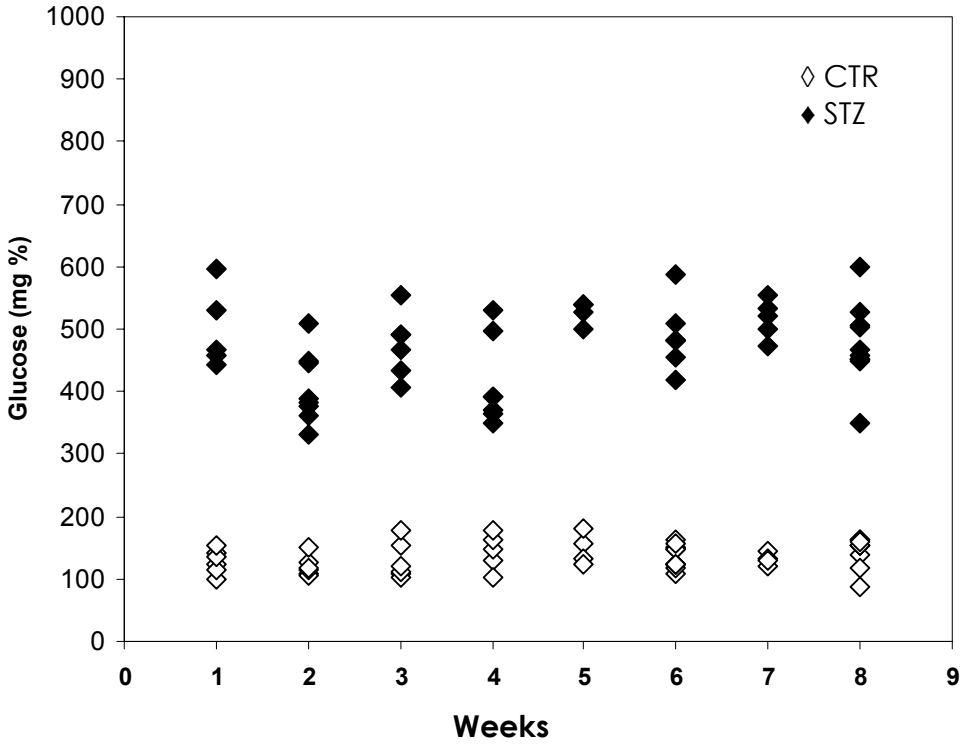
Alterations in the activities of testicular and hepatic GST are illustrated in Fig. 2.11. Testicular GST was significantly increased (~3.3-fold) at week one but marginal increases of 44 and 24% were evident in treated animals when compared to the controls at week two and four.

A significant increase of 45 and 39% was found in hepatic GST levels of diabetic animals compared to controls at week two and four of sampling.

Effect on lipid profile

Significant alterations were evident in the testicular biochemical constituents of growing rats measured at week-four (Fig. 2.12). Marked increases of 33% in the total cholesterol, 27% in triglycerides levels among the STZ-induced diabetic rats compared to controls. There was a marginal decrease (17%) in the testicular phospholipid levels in STZ-induced diabetic rats.

Fig. 2.1: Plasma glucose levels measured at weekly intervals in adult rats administered an acute dose of STZ (60mg/kg bw)



Data pooled from two independent experiments (n=8)
(All measurements were made on the day of sacrifice)

Table 2.1: Plasma glucose levels measured at weekly intervals in adult rats rendered diabetic by an acute dose of STZ (60mg/kg bw)

Plasma glucose (mg %)								
	Weeks							
Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
CTR	128 ± 18	120 ± 15	129 ± 27	125 ± 27	129 ± 11	136 ± 18	124 ± 22	124 ± 24
STZ	498** ± 57	406** ± 54	474** ± 47	414** ± 65	539** ± 35	459** ± 106	424** ± 61	476** ± 52

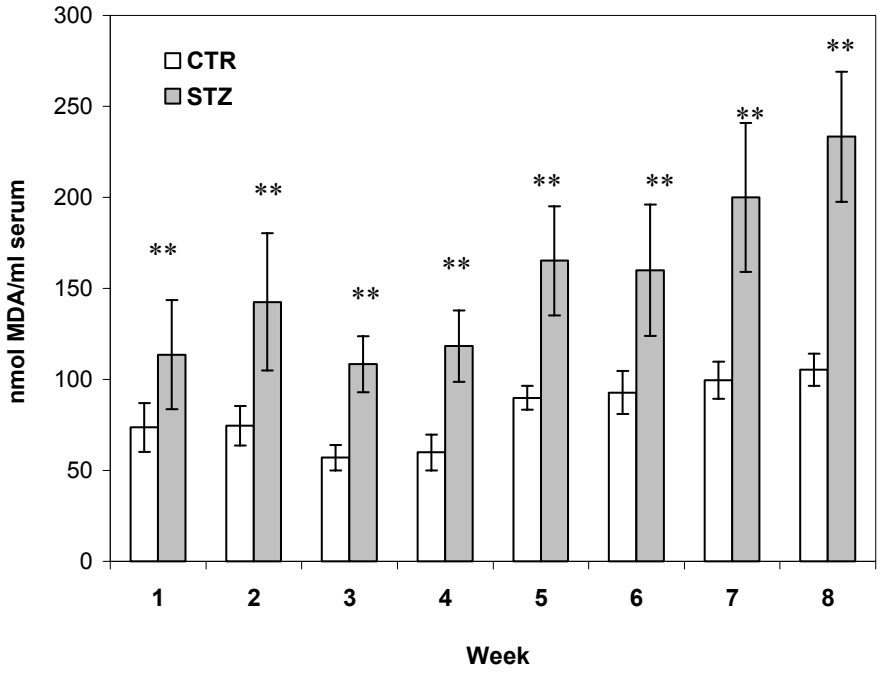
Values are mean ± SD (n=8)
Data analyzed by student's 't' test; ***P <0.001*
(Data pooled from two independent experiments)

Table 2.2: Effect of STZ (60mg/kg bw) administration on the testicular and epididymal weights of adult rats

Week	Group	Final body weight (g)	Testis		Epididymis (g)
			Absolute (g)	Relative (g/100g bw)	
2	CTR	184.4 ± 10.65	2.71 ± 0.24	0.86 ± 0.08	0.35 ± 0.07
	STZ	171.3* ± 4.66	1.99** ± 068	1.54 ± 0.03	0.26** ± 0.07
4	CTR	230.9 ± 9.87	2.77 ±0.08	1.05 ± 0.03	0.38 ± 0.03
	STZ	179.7** ± 12.21	1.40** ± 0.21	0.95 ± 0.16	0.20** ± 0.04
6	CTR	259.5 ± 10.05	2.82 ± 0.14	1.03 ± 0.07	0.39 ± 0.02
	STZ	182.5** ± 14.52	1.66** ± 0.09	1.22 ± 0.02	0.14** ± 0.01

Values are mean ± SD (n=8)
Data analyzed by student's 't' test; **P<0.01; **P<0.001*
(Data pooled from two independent experiments)

Fig. 2.2: Progression of LPO in serum of adult rats administered an acute dose of STZ (60mg/kg bw)



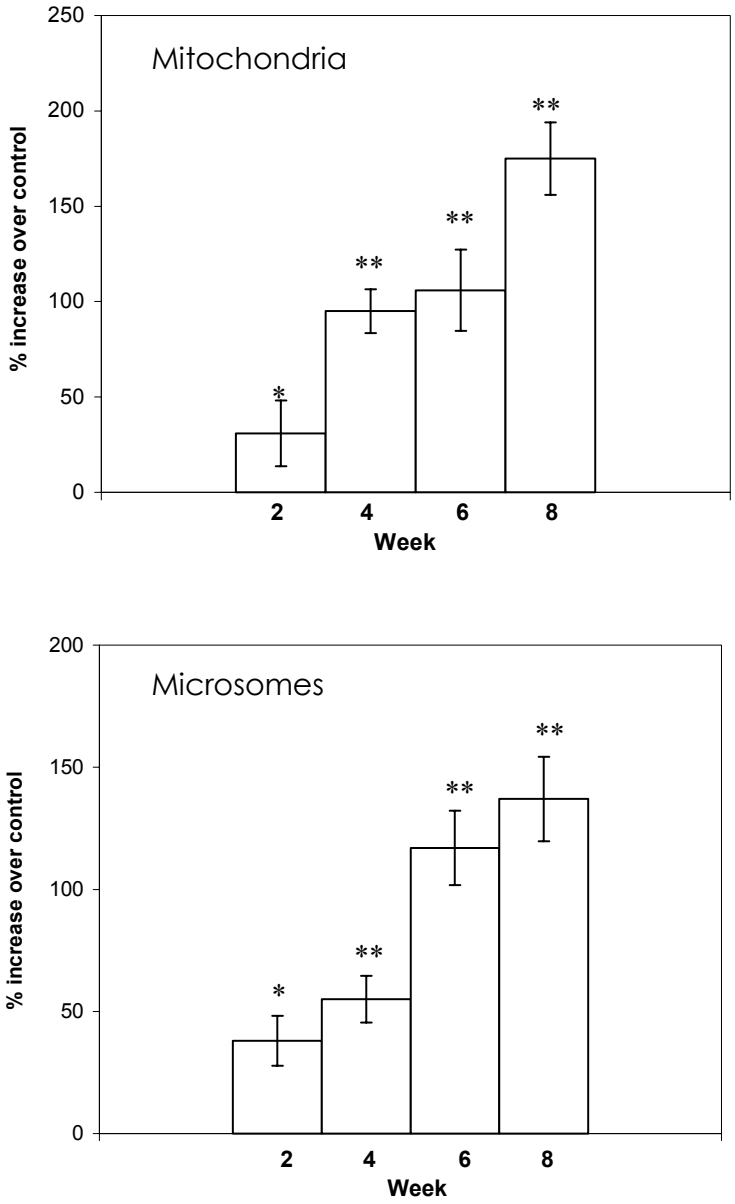
Values are mean ± SD (n=8)
Data analyzed by student's 't' test; **P<0.001*
(Data pooled from two independent experiments)

Table 2.3: Malondialdehyde levels in testis and liver of adult rats administered an acute dose of STZ (60 mg/kg bw)

Malondialdehyde (nmol/mg tissue)						
		Week				
Tissue	**Group**	**1**	**2**	**4**	**6**	**8**
Testis	CTR	0.348 ± 0.02	0.372 ± 0.08	0.336 ± 0.01	0.360 ± 0.01	0.354 ± 0.09
	STZ	0.414* ± 0.02	0.503** ± 0.02	0.619** ± 0.01	0.612** ± 0.02	0.674** ± 0.03
Liver	CTR	0.494 ± 0.03	0.514 ± 0.01	0.507 ± 0.09	0.582 ± 0.01	0.521 ± 0.01
	STZ	0.638** ± 0.05	0.724** ± 0.07	0.889** ± 0.03	1.379** ± 0.01	1.298** ± 0.03

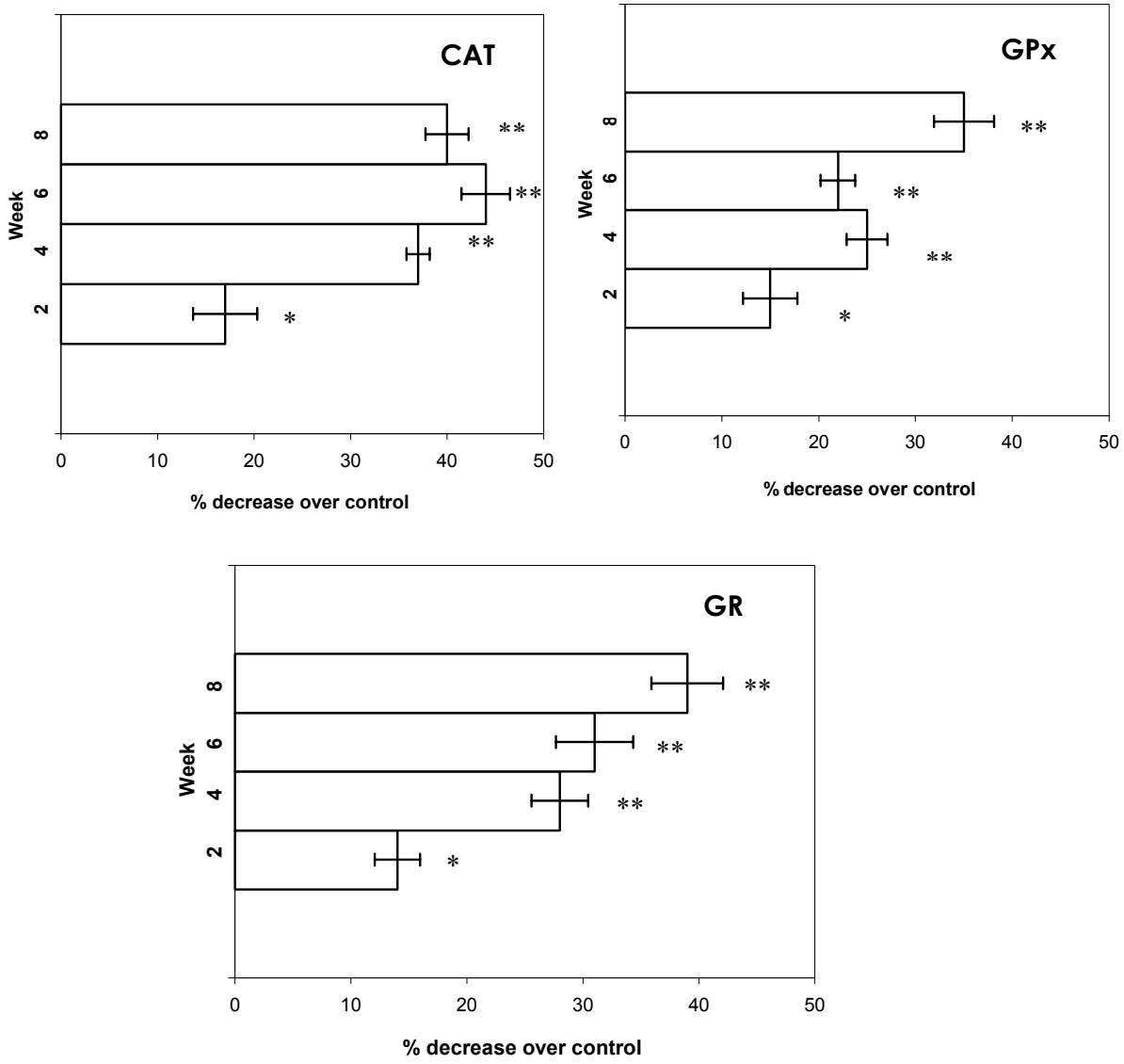
Values are mean ± SD (n=8)
Data analyzed by student's 't' test; **P<0.01; **P <0.001*
(Data pooled from two independent experiments)

Fig. 2.3: Percent increase in MDA levels in testicular mitochondria and microsomal fractions of adult rats administered an acute dose of STZ (60mg/kg bw)



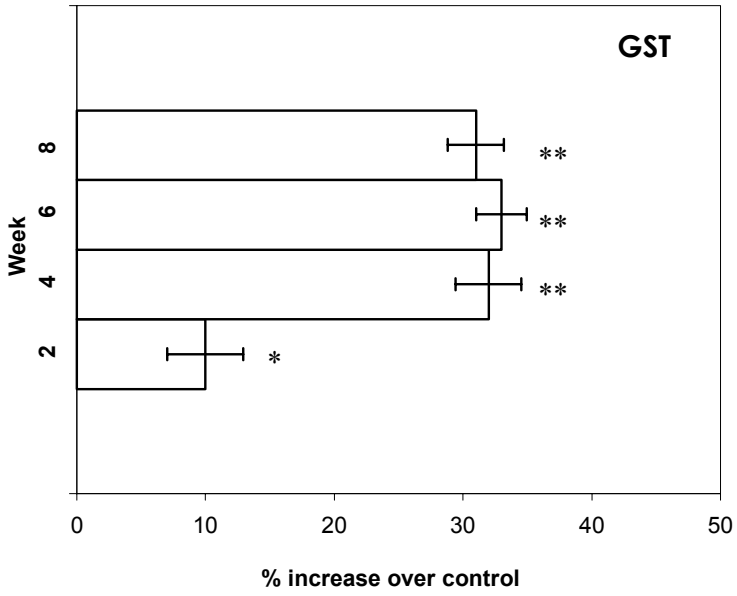
Values are mean ± SD (n=8)
Data analyzed by student's 't' test; **P<0.01; **P <0.001*
(Data pooled from two independent experiments)

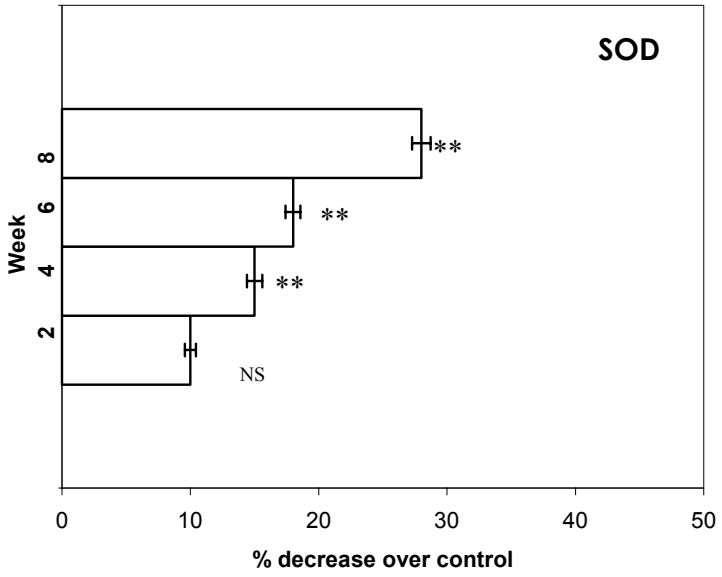
Fig. 2.4: Alterations in testicular antioxidant enzyme activities of adult rats administered an acute dose of STZ (60 mg/kg bw)



Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.01; **P <0.001*

Fig. 2.5: Alterations in testicular antioxidant enzyme activities of adult rats administered an acute dose of STZ (60 mg/kg bw)





Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.01; **P <0.001*

Table 2.4: Non-enzymic antioxidants and protein carbonyl (PC) levels in testis of adult rats administered an acute dose of STZ (60mg/kg bw)

Antioxidant	Group	Week		
		2	4	6
GSH [1]	CTR	2.98 ± 0.14	3.13 ± 0.35	3.29 ± 0.23
	STZ	3.48* ± 0.23	2.75** ± 0.14	2.40** ± 0.28
Vit E [2]	CTR	34.8 ± 0.12	31.25 ± 0.22	32.56 ± 0.31
	STZ	30.90 ± 1.40	25.31* ± 1.25	22.14** ± 2.08
PC [3]	CTR	52.91 ± 3.62	59.61 ± 4.46	54.24 ± 2.32
	STZ	63.83* ± 6.09	92.99** ± 5.57	78.10** ± 1.93

1: µg/mg protein; 2: µg/g tissue; 3: nmol carbonyls/mg protein
Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.01; **P<0.001*

Table 2.5: Progression of lipid peroxidation and protein carbonyl content in epididymal sperms of adult rats administered an acute dose of STZ (60mg/kg bw)

	Malondialdehyde (ηmol/mg protein)			Protein carbonyl (ηmol carbonyls/mg protein)		
Group	**2**	**4**	**6**	**2**	**4**	**6**
CTR	7.98 ± 0.37	7.81 ± 0.91	7.72 ± 0.58	27.51 ± 2.77	27.64 ± 2.53	26.40 ± 1.09
STZ	13.45** ± 2.69	13.55** ± 2.28	18.04** ± 3.95	35.34* ± 3.71	38.12* ± 1.70	41.71** ± 3.87

Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.01; **P<0.001*

Table 2.6: Activities of AOE measured in epididymal sperms of adult rats rendered diabetic and sampled at various weeks

Enzyme	Group	Week			
		2	4	6	8
CAT	CTR	2.16 ± 0.39	2.22 ± 0.24	2.53 ± 0.35	2.23 ± 0.30
	STZ	1.92^{NS} ± 0.23	1.53* ± 0.27	1.43** ± 0.26	1.02*** ± 0.17
GST	CTR	3.02 ± 0.11	2.81 ± 0.22	2.76 ± 0.24	3.45 ± 0.19
	STZ	3.96** ± 0.14	4.09** ± 0.59	3.37* ± 0.13	4.72** ± 0.30
GPx	CTR	1.38 ± 0.15	1.54 ± 0.23	1.68 ± 0.16	1.59 ± 0.21
	STZ	1.17* ± 0.11	1.21* ± 0.21	1.23** ± 0.21	1.04** ± 0.22
GR	CTR	1.35 ± 0.15	1.42 ± 0.22	1.39 ± 0.17	1.54 ± 0.28
	STZ	1.16* ± 0.22	1.12* ± 0.26	1.03** ± 0.21	0.92** ± 0.22

CAT: µmol H_2O_2 oxidized/min/mg protein
GST: µmol product formed/min/mg protein
GPx: nmol NADPH oxidized /min/mg protein
GR: nmol NADPH oxidized /min/mg protein

Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.05; **P<0.01; * **P<0.001*

Table 2.7: Activities of dehydrogenases measured in testis and Epididymal sperms of adult rats sampled during the progressive phase

Enzyme	Group	Testis			Epididymal sperms		
		Week			Week		
		2	4	6	2	4	6
G6PDH‡	CTR	13.06 ± 0.70	13.84 ± 1.10	13.82 ± 0.44	5.93 ± 0.50	5.68 ± 0.14	6.12 ± 0.22
	STZ	12.20^{NS} ± 0.49	9.84** ± 1.80	6.04*** ± 1.41	8.16** ± 0.99	8.06** ± 0.60	8.60** ± 0.50
SDH‡	CTR	3.36 ± 0.25	3.27 ± 0.27	3.09 ± 0.20	8.38 ± 0.37	8.71 ± 0.77	8.09 ± 0.39
	STZ	2.89^{NS} ± 0.15	2.38* ± 0.41	1.79** ± 0.30	10.64* ± 0.60	7.05* ± 0.51	5.82** ± 0.67

‡µmol of product formed /min/mg protein
Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.05; **P<0.01; * **P<0.001*

Table 2.8: Biochemical constituents in testis of adult rats sampled during the early and progressive phase

Week	Group	mg/g tissue		
		Cholesterol	Triglyceride	Phospholipid
1	CTR	3.02 ± 0.21	4.98 ± 0.22	6.36 ± 0.04
	STZ	3.30^{NS} ± 0.02	4.98^{NS} ± 0.36	6.09^{NS} ± 0.08
2	CTR	2.96 ± 0.12	4.82 ± 0.19	6.41 ± 0.04
	STZ	3.11^{NS} ± 0.13	5.10^{NS} ± 0.06	6.34^{NS} ± 0.06
3	CTR	3.26 ± 0.16	4.63 ± 0.13	6.57 ± 0.15
	STZ	4.54** ± 0.25	5.71* ± 0.26	7.66* ± 0.15
4	CTR	3.23 ± 0.13	4.71 ± 0.36	6.52 ± 0.09
	STZ	4.52** ± 0.32	6.31** ± 0.36	7.75* ± 0.13
8	CTR	3.32 ± 0.13	4.31 ± 0.22	6.66 ± 0.12
	STZ	4.70** ± 0.25	6.00** ± 0.24	7.81* ± 0.22

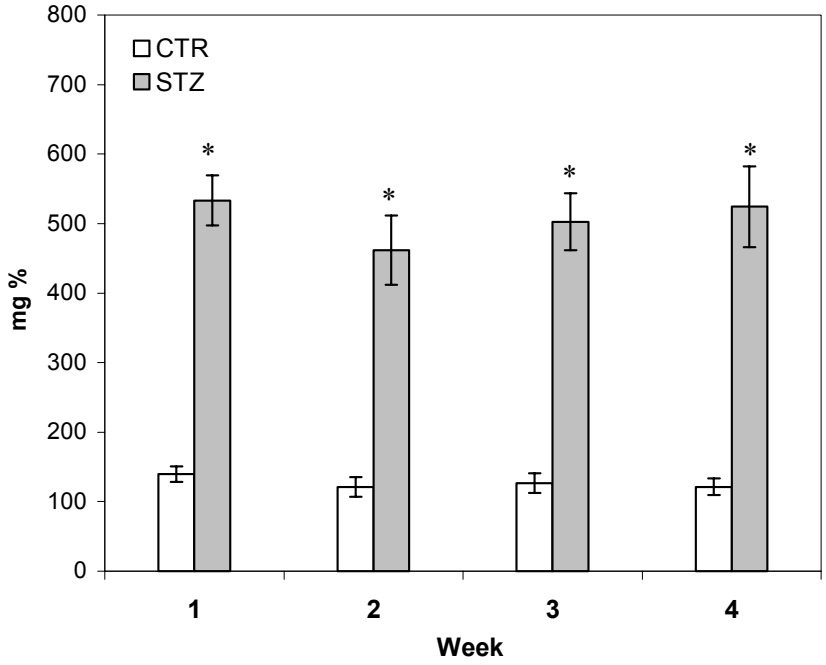
Values are mean ± SD (n=4); NS: not significant
Data analyzed by student's 't' test; **P<0.05; **P0.01*

Table 2.9: Fatty acid composition (mg %) of testis of adult rats sampled at various weeks of STZ administration

Fatty acid	Week									
	1		2		3		4		8	
	CTR	STZ	CTR	STZ	CTR	STZ	CTR	STZ	CTR	STZ
16:0	35.2 ± 0.73	35.4 ± 0.75	37.4 ± 1.26	33.7 ± 2.6	32.3 ±1.55	34.98 ±1.78	35.75 ± 0.68	31.33 ± 0.58	36.48 ± 0.46	33.61 ±1.02
16:1	0.90 ± 0.45	0.76 ± 0.05	0.82 ± 0.05	0.66 ± 0.06	1.03 ± 0.06	0.98 ± 0.11	0.69 ± 0.05	0.70 ± 0.09	1.07 ± 0.3	0.40 ± 0.1
18:0	7.13 ± 0.08	7.35 ± 0.06	7.03 ± 0.26	7.34 ± 0.12	7.79 ± 0.89	7.58 ± 1.9	6.99 ± 0.38	6.18 ± 0.77	6.72 ± 0.06	4.95 ± 0.81
18:1	14.43 ± 0.59	14.34 ± 0.15	13.78 ± 0.37	13.67 ± 0.31	17.08 ± 1.39	16.46 ± 1.63	12.93 ± 0.59	12.69 ± 0.50	13.0 ± 1.22	13.21 ± 2.43
18:2	5.60 ± 0.52	5.30 ± 0.33	4.13 ± 0.04	4.40 ± 0.06	5.31 ± 0.81	6.10 ± 0.38	4.25 ± 0.11	4.13 ± 0.19	5.92 ± 0.1	6.26 ± 0.31
20:4	15.40 ± 0.35	14.45 ± 0.18	14.94 ± 0.98	14.97 ± 0.02	12.25 ± 1.77	15.7 ± 0.94	15.74 ± 0.14	10.96 ± 1.48	14.04 ± .91	19.62 ± 0.64
22:5	21.57 ± 0.34	22.93 ± 0.89	21.67 ± 0.58	22.38 ± 0.59	18.77 ± 1.78	20.57 ± 2.48	19.91 ± 1.28	20.41 ± 2.47	20.51 ± 1.48	22.95 ± 1.01
SFA (S)	42.33	42.75	44.43	41.04	40.09	42.56	42.74	37.51	43.2	38.56
PUFA (P)	42.57	42.68	40.74	41.75	36.33	42.37	37.9	35.5	38.47	40.87
P/S	1.00	0.99	0.91	1.01	0.9	0.99	0.88	0.94	0.89	1.05

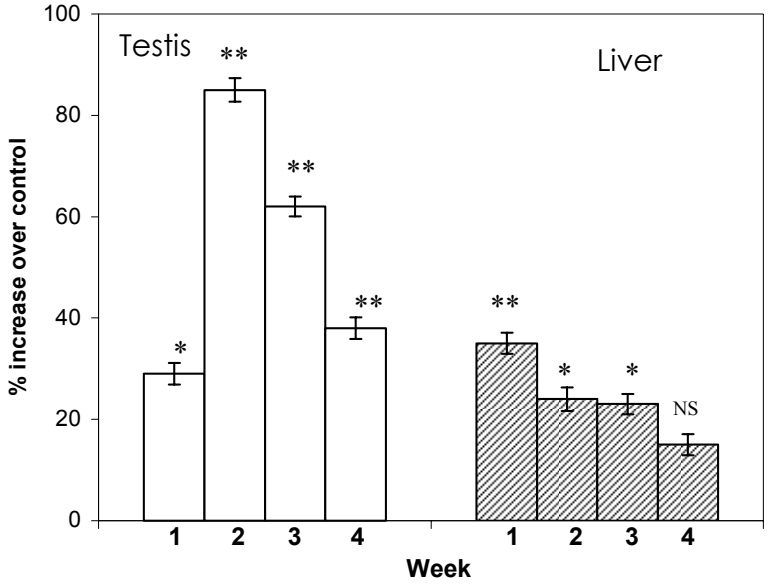
Values are mean ± SD (n=4)

Fig. 2.6: Plasma glucose levels in growing rats administered an acute dose of STZ (90mg/kg bw)



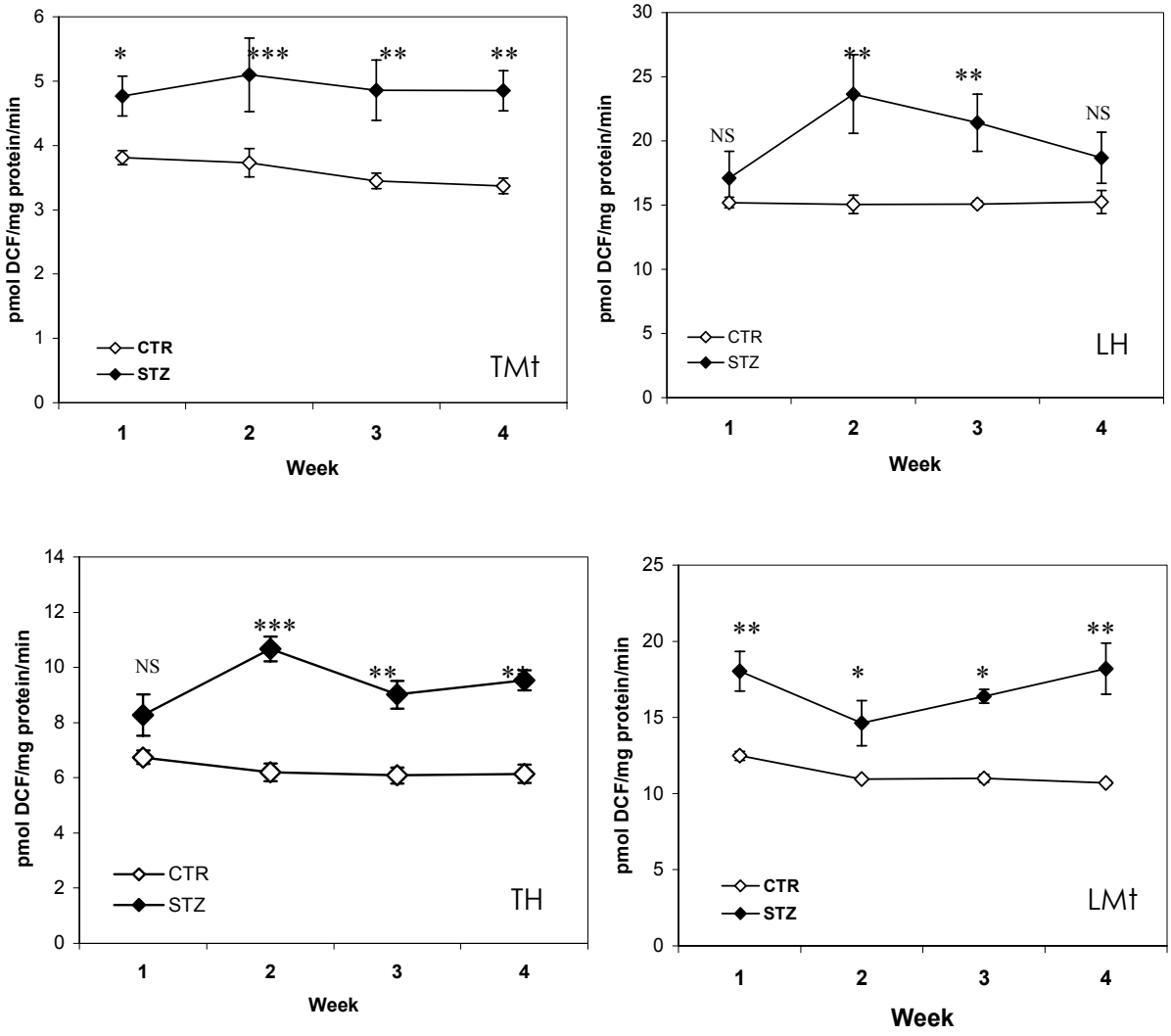
Values are mean ± SD (n=4); NS: not significant
Data analyzed by student's 't' test; **P<0.001*

Fig. 2.7: Increase in lipid peroxidation in testis and liver of growing rats administered an acute dose of STZ (90mg/kg bw)



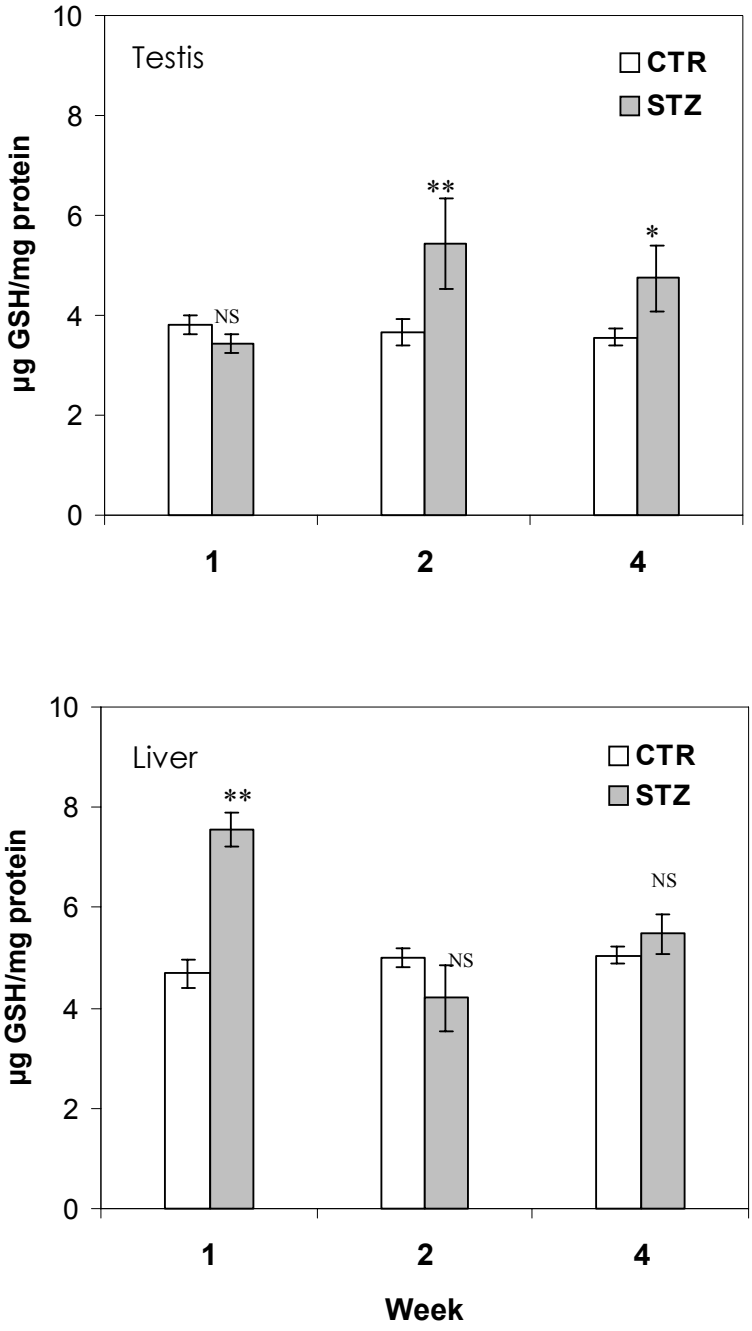
Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.01; **P<0.001*

Fig. 2.8: Pattern of ROS generation in testis, liver homogenates (TH & LH) and mitochondrial fractions (TMt & LMt) of growing rats administered an acute dose of STZ (90mg/kg bw)



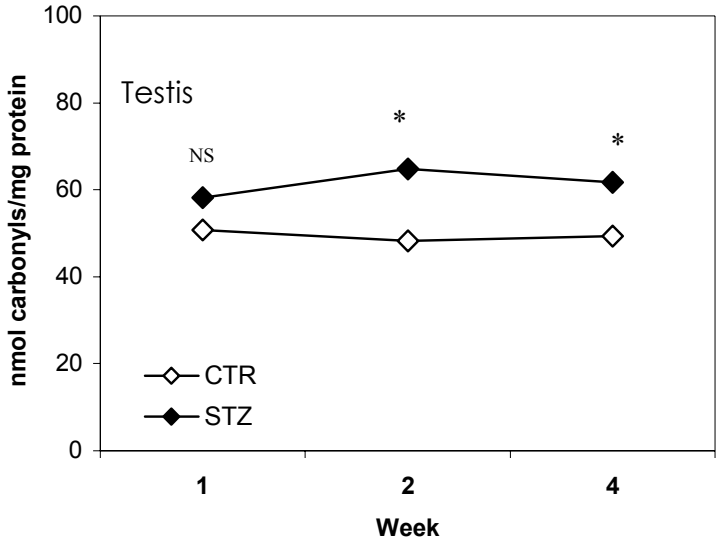
Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.05; **P<0.01; * **P<0.001*

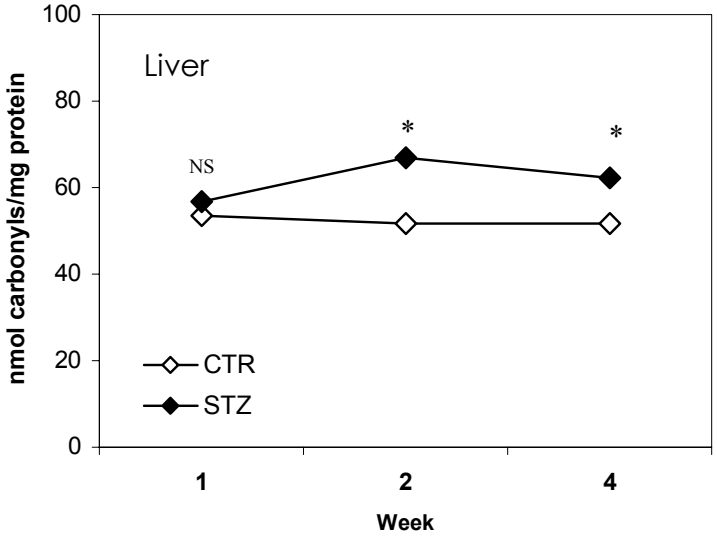
Fig. 2.9: Testicular and hepatic GSH status of growing rats administered an acute dose of STZ (90mg/kg bw)



Values are mean ± SD (n=4); NS: not significant
Data analyzed by student's 't' test; **P<0.05; **P<0.01*

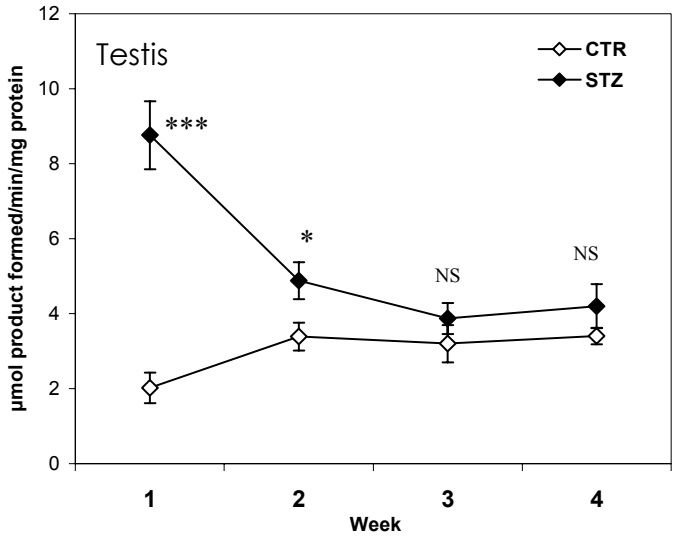
Fig. 2.10: Protein carbonyl content in testicular and hepatic homogenates of growing rats administered an acute dose of STZ (90mg/kg bw)

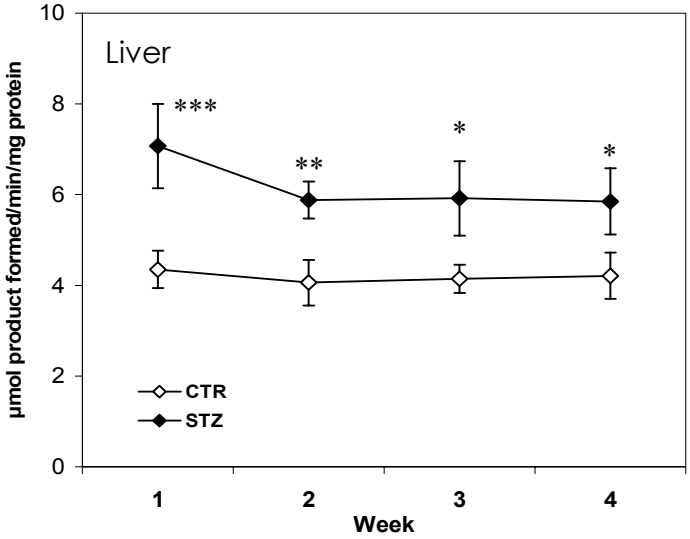




Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.05*

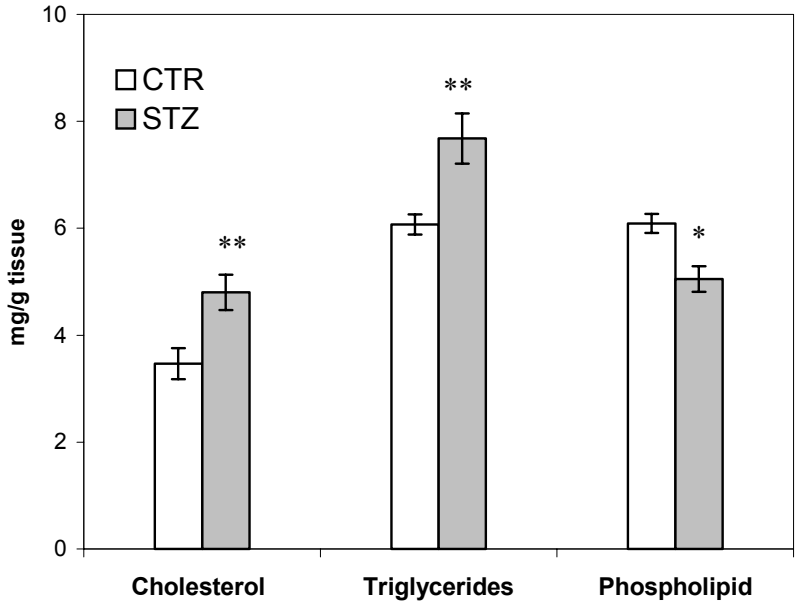
Fig. 2.11: GST activity in testicular and hepatic tissues of growing rats administered an acute dose of STZ (90mg/kg bw)





Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.05; **P<0.01; ***P<0.001*

Fig. 2.12: Biochemical constituents in testis of growing rats rendered diabetic by STZ



Values are mean ± SD (n=4)

Data analyzed by student's 't' test; **P<0.05; **P<0.01*

DISCUSSION

Oxidative stress in testis and epididymal sperms of adult rat

The primary objective of the first series of investigations in adult rats was to examine whether the male reproductive milieu is subjected to oxidative stress under experimentally induced diabetes. The hypothesis was also tested in growing (sexually immature) rats with an objective of understanding if any difference exists in the vulnerability of developing testis to oxidative damage under diabetes.

Animal models are often used to investigate the pathophysiology and treatment of diabetes. Most of the available models are based on rodents, which are rendered diabetic by administration of chemical diabetogenic agents such as streptozotocin (STZ) or alloxan (Alx). In our studies, we have employed STZ, which was administered at an acute dose of 60mg/kg bw to adult rats. The criterion for the selection of this dose was based on our preliminary studies. Since the dosage of 60mg/kg bw induced a consistent and significant increase in the glucose levels and produced only 30% of mortality during the observation period of eight weeks, we considered this as the optimum dose for adult rats.

There is currently a great interest in understanding the potential contribution of increased oxidative stress (OS) to the development of various complications in diabetes (*Baynes and Thorpe, 1999*). Although, varying degree of OS and ensuing oxidative damage are unequivocally demonstrated in somatic organs (e.g. liver, kidney, brain) of STZ-induced diabetic rats, comprehensive data on the occurrence, progression and associated biochemical/genotoxic consequences in the male reproductive system are limited (*Sexton, and Jarrow, 1997; Cai et al., 2000*). In the present study, induction of OS in testis and liver were

ascertained by quantification of MDA levels in homogenates and mitochondria. Significant increase in MDA levels of testicular homogenates was observed during the first two weeks of STZ post administration. Although moderate (19-35%) during the first two weeks, the degree of damage progressed and enhanced markedly during the subsequent sampling weeks (week 4 onwards). While the degree of oxidative damage was similar in the hepatic tissue during the first four weeks (29-75%), the increase was dramatic during the subsequent weeks. The status of LPO measured in both mitochondria and microsomes in testis also showed a similar trend. These data indicate that the OS differentially affects the cytosolic, mitochondria/microsomal components in the testis. Our data is consistent with increased cellular OS and accumulation of lipid peroxides, 4-HNE and MDA demonstrated in diabetic patients and also in experimental T1DM (*Santos et al., 2001; Traverso et al., 2002*).

Further evidence to show that testis is subjected to OS under diabetic situations was obtained in terms of significant perturbations in antioxidant defenses (enzymic and non-enzymic) and higher protein carbonyls. Significant depletion of the major non-enzymic antioxidants such as GSH, vitamin E was evident in the testis of diabetic rats. In general, the imbalance between the antioxidants and prooxidants produces a state of OS and a change in AOE activity is frequently used as an important indicator of OS *in vivo*. In diabetic testis, significant reduction in the activities of CAT, GPx, GR, SOD and concomitant increase in GST levels, which were a common feature at all sampling periods are suggestive of the ongoing oxidative disturbances. Decline in the activities of these AOE might be due to their inactivation caused by excessive ROS production (*Pigeolet et al., 1990*). A decrease in SOD activity has been shown to increase the level of superoxide, which is

known to inactivate GPx. Similarly, when GPx fails to eliminate H_2O_2 from the cells, the accumulated H_2O_2 has been shown to cause inactivation of SOD. Thus a balance of the enzyme system is very critical to dispose the superoxide anion and peroxides generated. The consistent reduction in the activities of testicular AOE, viz., SOD, GPx, CAT and increase in LPO is clearly indicative of the fact that testis is subjected to OS under STZ-induced diabetic situations.

GSTs are a group of primary phase II detoxifying enzymes, which provide protection against products of OS (*Hayes and Pulford, 1995*). The abundance of GSTs in germ cells and their protective role under OS situations have been studied *in vitro* (*Hemachand and Saha, 2003*) and it is opined that GSTs form a part of an adaptive response of germ cells to OS. Significant enhancement in GST activity observed beyond second week in our study indicates the major role played by this enzyme in regulation of OS products in diabetic testis. Earlier *in vitro* findings in testicular cell suspensions report similar enhancement of GST under OS conditions (*Aravinda et al., 1995; Rao and Saha, 2000*). These observations are also consistent with increased cytosolic and mitochondrial ROS, lipid peroxidation, accumulation of 4-HNE in various somatic tissues of STZ-induced diabetic rats (*Raza et al., 2004*). Induction of GST A4, the isozyme that metabolizes 4-HNE is shown to be elevated in cytosol and mitochondria in different tissues of diabetic rats (*Raza et al., 2004*). Although we did not measure the activity of GST isozyme in testis, the consistent elevated activity of the cytosolic GST is probably related to increased LPO products such as 4-HNE.

Additionally, the reduced GSH pools in the cytosol were also significantly depleted in the testis suggesting the protective role of glutathione and related enzymes. GSH is important in the regulation of cellular redox state and a decline in its cellular level in diabetes has

been considered to be indicative of OS (*Mc Lennan et al., 1991)*. The decrease in testicular GSH levels in STZ-treated rats might be in part attributed to the inhibition of GR activity, which is responsible for regeneration of GSH from its oxidized form. GR is known to be inactivated by superoxide anion (*Blum and Fridovich, 1985*) and hence, testis showing reduced SOD activity might have enhanced flux of superoxide radicals that potentially could damage GR and decrease GSH content in testis. Furthermore, a regression of the antioxidant recycling mechanism due to a decrease in vitamin E level in diabetic rats might also contribute to a decline in GSH level in testis.

Protein carbonyls are employed as a biomarker of ROS mediated protein oxidation (*Stadman, 1990; Isabella et al., 2003*) and elevated levels of oxidized protein have been reported in animal tissues and cell models under various conditions of OS e.g. hyperoxia, forced exercise, ischemia-reperfusion, chronic alcoholics, X-irradiation, cigarette smoking etc (*Schuessler and Schilling, 1984*). ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross linkage and oxidation of protein back bone resulting in protein fragmentation. Protein carbonyls are introduced into the protein by reaction with aldehydes (4-HNE, MDA) produced during LPO (*Uchida and Stadman, 1993)* or with reactive carbonyl derivatives (ketoamines, ketoaldehydes) generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins (*Baynes and Thorpe, 1996)*. Consistently higher amounts of protein carbonyls in testis and liver of diabetic rats at various sampling times observed in this study reflect a high rate of protein oxidation, which is consistent with high LPO state. Further, it also reflects a very low rate of oxidized protein degeneration and or low repair activity since oxidized forms of some proteins (e.g. cross linked protein) and proteins

modified by LPO products are not only resistant to proteolysis, but also can inhibit the ability of proteases to degrade the oxidized forms of other proteins (*Suarez et al., 1995)*.

In the present study, we observed significant elevations in lipid constituents in testis at week 3 and beyond (cholesterol, 40%; triglycerides, 23-34%; and phospholipids, ~20%) and these data are consistent with earlier reports, which describe altered lipid metabolism in several tissues under experimentally induced diabetes. Abnormalities closely related to fatty acid metabolism such as hypercholesterolemia and hypertriglyceridemia are reported to occur in diabetic patients (*Wester et al., 1983) and rats (Bar- on et al., 1976; de Catalfo et al., 1998; Tanaka et al., 2001*). Impairments in PUFA biosynthesis in diabetic testis owing to the inhibition of destaturases have reported earlier (*Pelufo et al., 1970; Huang et al., 1984*; *de Catalfo et al., 1998*). Few investigators have demonstrated a strong inhibition of Δ^5 desaturaation process in testis which is known to control the arachidonate flow as well as the production of oxygenated metabolites regulating a variety of pathological and phsyiological processes (*Brenner et al., 1968; Reddy et al., 1992*). Although speculative, it is likely that impairment in arachidonic biosysnthesis observed at week 3 and beyond in the present study, is responsible for the altered leydig cell /sertoli cell functions and the consequent effects on spermatogenesis.

The present findings also showed decreased activities of sorbitol dehydrogenase (SDH) and glucose-6-phosphate dehydrogenase (G6PDH) in testis activity at all sampling times. In general, our observation on the decreased activities of SDH is consistent with the reported acceleration of polyol pathway under conditions of hyperglycemia (*Nishinaka and Nishimura, 2001*). In the polyol pathway, sorbitol is oxidized to fructose by the enzyme SDH, with NAD+ reduced

by NADH. Sorbitol does not diffuse easily across the cell membranes and induces osmotic stress and damage. Under conditions of hyperglycemia, it is speculated that the acceleration of polyol pathway leads to an overflow of the products and the depletion of NADPH and NAD^+ in the cells where insulin-independent uptake of glucose takes place (*Brownlee, 2001*). Depletion of NADPH caused by acceleration of the polyol pathway attenuates GR activity, and the level of GSH declines. This results in reduced GPx activity and an increase in the level of H_2O_2.

G6PDH enzyme is part of pentose phosphate pathway, which is involved in the regeneration of NADPH. While the activity of this enzyme in testis was consistently decreased in the present study, a significant increase was evident in epididymal sperms at all sampling times. This increase in sperm G6PDH activity can prove detrimental since it generates high NADPH, which apart from serving as a co-factor for GPx, can also act as a co-factor for NADPH oxidase, which is involved in production of superoxide. This is consistent with the hypothesis that speculates a strong correlation between ROS generation and presence of residual cytoplasm (associated with high levels of G6PDH) in human spermatozoa (*Sikka, 2001*).

In the present study, the susceptibility of spermatozoa in the epididymal milieu to OS under diabetic conditions was increasingly evident beyond two weeks. Significant increases in MDA levels were also accompanied by marked alterations in the activity of AOE (CAT, GST, GPx, GR), and increased protein carbonyls. While sperms produced in the testis are reasonably well protected by the microenvironment of sertoli cells, they are less protected against the oxidant environment in the epididymis owing to the slow transit time and prolonged storage (*Cummins et al., 1994*). Additionally, it is well

known that spermatozoa are extremely susceptible to damage by ROS and ROS generated products due to the preponderance of oxidation-prone unsaturated fatty acids on the plasma membrane (*Lenzi et al., 2000; Ball et al., 2001*). The production of abnormal levels of ROS are now believed to be involved in many aspects of human male infertility where spermatozoa are rendered dysfunctional by LPO, altered membrane function and impaired metabolism (*Ong et al., 2002)*.

Accordingly, it is quite likely that under diabetic condition ES are subjected to marked repeated OS, which can probably lead to genotoxic implications. Employing mice models, we have earlier demonstrated the susceptibility of ES to prooxidant induced OS (*Rajeshkumar et al*., 2002; *Doreswamy et al., 2004; Doreswamy and Muralidhara, 2005)*. Numerous other researchers have also unequivocally demonstrated that oxidative damage to sperms can lead to DNA damage, altered membrane functions, impaired motility and sperm-oocyte fusion, loss of capacity to undergo acrosome reaction and fertilization (*Mazilli et al., 1994; Lopez et al., 1998*). Interestingly, these data are consistent with recent epidemiological evidences (*Saleh and Agarwal, 2002*), which have reported significantly high levels of various ROS in infertile patients, compared with fertile normospermic males clearly suggesting that a similar situation could prevail in diabetic males.

Taken together, these data indicate that diabetes induction is associated with significant OS in rat testis from first week onwards and it progresses with time. Since the oxidative impairments are evident both in testicular/epididymal milieu, it is likely to contribute towards the development of testicular dysfunctions. However, further investigations are necessary to understand the specific effects on various cell types and their interaction in terms of its implication on steriodogenesis. More

importantly, it would be more interesting to characterize the genotoxic and fertility implications, during the early phase of diabetes induction in rodent models.

Oxidative damage in testis of growing rats

The principle objective of the second set of studies was to examine the pattern of susceptibility of developing testis in growing rats to oxidative damage *in vivo*. To induce hyperglycemia in growing rats, a higher dose (90mg/kg bw) was employed since the dose used for adult rats (60mg/kg bw) failed to elicit a consistent higher hyperglycemic response in these animals. The specific reasons as to why the growing rats do not develop marked hyperglycemia at lower dose as adult rats is not clear. However, it is quite likely to be related to the metabolic capacity of the young males and probably the decreased susceptibility of β-islets of pancreas in young ones. Further, it may also be related to the induction of higher protective mechanisms in young males.

Although T1DM remains the main form of diabetes in children globally, it is speculated that T2 DM will be the predominant form within 10 years in specific ethnic groups (*Zimmet et al., 2001*). T2DM in children, teenagers and adolescents is a serious new aspect to the epidemic and is considered as an emerging public health problem of significant proportions (*Kitagawa et al., 1998; ADA 2000; Fagot-Campagna et al., 2000*). Recent epidemiological evidences have documented the occurrence of OS in children with T1 DM (*Martin-Gallan et al., 2003; Varvarovska et al., 2003*). It is in this context, the present study assumes relevance.

In the present study, we determined the status of oxidative damage in developing testis and liver in terms of MDA levels and ROS generation in homogenates and mitochondria at weekly intervals of

sampling (1-4wk). Higher levels of MDA (35, 85%) observed during the first two weeks provide direct evidence for increased LPO occurring in the testis and possibly increased tissue damage by free radicals. During weeks three and four, testis still showed significant (60, 38%)increase in MDA levels although the damage appeared to be repaired. Measurement of ROS generation in testis revealed a similar trend of results in both homogenates as well as mitochondrial fractions. In contrast, the pattern and degree of oxidative damage in liver was quite different. In liver, although initially (wk 1) there was a significant increase (30%) in LPO, the extent of increase gradually reduced. Excepting for the first week, the degree of oxidative damage in hepatic tissue was significantly lesser compared to that found in testis at all further weeks. ROS levels in hepatic homogenates, which were marginally increased initially (wk 1) and terminally (wk 4), were found to be higher during second and third week indicating significant oxidative perturbations occurring in liver. These differential patterns of oxidative damage and OS in testis and liver may in part be related to the differences in the antioxidant complements in liver and testicular tissues.

The role of OS in the development of clinical complications in diabetic patients has scarcely been reported. The clinical findings in newly diagnosed child and young diabetic patients, have suggested that the increased OS may not be due to the complications but rather contribute to their development (*Dominguez et al., 1999*). Hence, currently few workers have assessed various biomarkers of diabetes associated OS such as LPO, GSH status/GPx, protein oxidation and changes in status of antioxidant defenses in blood samples (*Fagot-Campagna, 2000; Martin-Gallan et al., 2003; Varvarovska et al., 2004*). Few epidemiological studies have also confirmed increased OS along with impaired antioxidative defenses in diabetic children as well as a

similar tendency in their non-diabetic siblings. Our principle findings in developing testis viz., increased LPO and ROS levels, alterations in GSH and protein carbonyl status during the second and fourth week are indicative of the extent of diabetes associated oxidative impairments. This correlated well with the development of significant pathological lesions in testis from third week onwards. To the best of our knowledge, the occurrence of STZ-induced oxidative damage in growing testis has not been reported so far. However, further studies are essential to understand the mechanisms underlying the induction of oxidative damage and more importantly their implications on the fertility potential of such males.

The role of GST as phase II detoxifying enzymes in testis is fairly well understood (*Raza et al., 2004*) and the pattern of its response in adult testis under experimentally induced diabetes has been discussed above. Interestingly, in growing rats there was nearly a four-fold increase in testicular GST activity (cytosolic) during first week of post administration suggesting the extent of elevation of various LPO products (such as 4-HNE). Marginal increases in MDA and ROS levels and no alterations in GSH and protein carbonyls observed during the first week are indicative of the protective role played by enhanced GST activity in the cytosol. Marginal elevations in the activity of testicular GST levels in diabetic rats at two weeks and beyond suggested a compromised antioxidant status.

Taken together, all these data in developing testis in growing rats demonstrate the susceptibility to diabetes induced OS and oxidative impairments. However, further studies are needed to understand the role of GSH and GSH related enzymes in protecting the developing testis against OS during the vulnerable period of testicular growth, maturation, spermatogenesis and androgenesis. More importantly, the

genotoxic implications that may ensue in diabetic young males under experimentally induced diabetes (uncontrolled) need to be investigated in greater detail.

SUMMARY

1. Administration of an acute dose of STZ (60mg/kg bw, i.p) in adult rats produced nearly a 3-fold increase in plasma glucose levels indicating induction of diabetes condition

2. Our experimental data in adult rats clearly suggest that testis is indeed subjected to significant oxidative stress under STZ-induced diabetes

3. STZ administration produced a marked oxidative impact as evidenced by consistent and progressive increase in lipid peroxidation in testis beginning from first week onwards

4. Perturbations of major non-enzymic antioxidants viz., GSH and vitamin E in testis were accompanied with significant, varying degree of reduction in the activities of testicular antioxidant enzymes (viz., CAT, SOD, GPx and GR) suggesting oxidative impairment occurring *in vivo*

5. Consistent enhanced activities of testicular GST suggests the vital role played by this enzyme in detoxification of free radicals generated under STZ-induced diabetes

6. Marginal to moderate increase in protein carbonyls in testis are indicative of significant protein oxidation occurring in the organ

7. Significant pathological lesions in testis beyond 3 weeks and marked reduction in the activities of testicular dehydrogenases (viz., G6PDH

and SDH) beyond 4 weeks suggest the gradual development of pathophysiology and function.

8. Significant increase in cholesterol and phospholipid levels in testis and fatty acid profile are indicative of altered lipid metabolism associated with oxidative damage in diabetic situations

9. Enhanced lipid peroxidation in epididymal sperms (ES) sampled at one week and beyond which was accompanied with marked perturbations in the activities of various antioxidant enzyme indicates the vulnerability of sperms to oxidative damage in the epididymal milieu

10. Our experimental data in growing rats showed that significant hyperglycemia could be obtained only at a higher dose of STZ (90mg/kg bw) compared to adult rats

11. Testis of growing rats rendered diabetic appeared to be subjected to relatively higher degree of oxidative impact as evidenced by marked increase in MDA levels, and enhanced generation of ROS

12. Marked alterations in GSH (reduced) levels in the testis and enhanced activity of GST observed in testis (and liver) suggested the vital role played by this pathway in response to oxidative stress in the developing testis

13. Marginal to moderate increase in protein carbonyl levels were also evident in growing testis suggesting protein oxidation

14. Elevated levels of biochemical constituents viz., cholesterol and triglycerides in the developing testis are indicative of the lipid dysmetabolism which is likely to affect the testicular physiology and function

CHAPTER 3:

EARLY OXIDATIVE DAMAGE IN TESTIS, RESPONSE TO ANTIOXIDANTS AND GENOTOXIC IMPLICATIONS: MICE MODEL

INTRODUCTION

The diabetogenic agent Streptozotocin (STZ) is a monofunctional nitrosourea derivative, having a broad-spectrum antibiotic activity. The genotoxic effects of STZ have been extensively studied in a variety of *in vitro* systems, and is a potent alkylating agent known to directly methylate DNA (*Randerath et al., 1981),* induce DNA strand breaks *(Erickson et al., 1978),* chromosomal aberrations, sister chromatid exchanges and DNA damage in mammalian as well as insect cells (*Capucci et al., 1995; Bolzan and Bianchi, 2002)*. STZ is also demonstrated to be carcinogenic in rats, mice and hamsters (*as reviewed by Bolzan and Bianchi, 2002)*.

STZ has drawn attention as a potential inducer of OS and several lines of evidences indicate that the ROS generated by STZ is responsible for its wide spectrum of genotoxic effects in mammalian cells. While the genotoxic effects of STZ are fairly well established in various *in vitro* systems, data on its potential to induce mutagenic alterations *in vivo* are limited. More importantly, despite the knowledge on the clastogenic action of STZ *in vivo* (e.g., induction of micronuclei in mouse bone marrow), there are no data with regards to the mutagenic effects of STZ in germ cells (*Liegibel et al., 1992; Chinnaswami et al., 1998)*.

Currently, it is well established that damage to testicular male germ cells induced by various xenobiotics, products of abnormal metabolism, or ROS can result in testicular dysfunction leading to infertility (*Sikka, 2001; Agarwal et al., 2004; Agarwal and Said, 2005)*. In addition numerous epidemiological and experimental evidences have emphasized a potential relationship between oxidative damage in testis and sperms and testicular dysfunction (*Fraga et al., 1996; Rajeshkumar and Muralidhara, 1999; Rajeshkumar et al., 2002*).

Reproductive disturbances in diabetic males are well established. In animal models of diabetes (induced either by STZ or Alloxan) earlier workers have demonstrated various male reproductive dysfunctions both structurally and functionally (*Paz and Homannai 1979; Orth et al., 1979; Cai et al., 2000)*. Similar findings were documented in spontaneously diabetic rats such as Goto-kakizaki rats (*Palmeira et al., 2001*). Earlier (Chapter 2) we demonstrated that both testis and epididymal sperms of adult rats are subjected to significant OS as early as two weeks under STZ-induced diabetic situation. However, data on the degree of oxidative damage during early phase of diabetes and its correlation with DNA damage, are totally lacking. More importantly, data on the progression of oxidative damage and its impact on sperm morphology, development and their possible genotoxic implications are nonexistent.

Hence, our further investigations focused on early oxidative damage (EOD) in testicular and epididymal milieu, its persistence and correlation with DNA damage in testis and ES, progression of oxidative damage and genotoxic consequences. Data obtained have been presented under two sections A and B. **Section A** describes investigations on quantification of EOD in both testis and ES of mice following STZ administration, its attenuation by oral antioxidant supplements. **Section B** describes studies pertaining to progression of oxidative damage over four weeks, its correlation with testis pathology, DNA damage in testis and epididymal sperm counts. Data on the genotoxic effects of STZ-induced diabetes as ascertained by sperm abnormality assay and induction of male-mediated dominant lethal (DL) mutations are also presented.

SECTION A

EARLY OXIDATIVE DAMAGE IN TESTIS OF ADULT MICE AND RESPONSE TO ANTIOXIDANTS

EXPERIMENTAL DESIGN

Preliminary studies

Adult mice (8-10 wk old) were used for all the investigations. Preliminary dose-determinative studies comprised of administration (i.p.) of graded acute doses of STZ (dissolved in freshly prepared citrate buffer, pH 4.5; conc. 75mg/ml) in the range of 50-200mg/kg bw to groups (n=4) of male mice. Hyperglycemic response and mortality were determined.

Determinative studies

For all the determinative experiments, adult mice were administered STZ at the dosage of 150mg/kg bw.

Hyperglycemic response with optimum dose

Pattern of hyperglycemia induction was obtained by measuring the plasma glucose levels of STZ-treated mice at 3 and 5 days following administration.

Evidence of early oxidative damage in testis and epididymal sperms

Induction of early oxidative damage

Mice rendered diabetic were sampled at various time points (3 and 5 days) to measure the degree of oxidative damage in testis and epididymal sperms. To determine the degree of oxidative damage, a set of established biomarkers such as lipid peroxidation, ROS generation, activity of antioxidant enzymes and protein carbonyls were assessed in both testis and epididymal sperms.

Attenuation of early oxidative damage by antioxidants

In order to examine whether, STZ-induced oxidative damage in testis during the early phase is amenable for attenuation, the modulatory effect of two antioxidants viz., ascorbic acid (AA) and taurine (TAU) were investigated.

Pre-treatment regimen with ascorbic acid

Adult mice were administered (oral) with ascorbic acid at a dosage of 10mg/kg bw/d for five days and were subsequently (on the sixth day) administered an acute dose of STZ (150mg/kg bw). Animals with blood glucose levels above 200mg% were selected and randomly distributed into four groups as follows:

Group I - control; Group II – AA; Group III – STZ; Group IV - AA + STZ.

Pre-treatment regimen of Taurine

Adult mice were administered (oral) Taurine at a dosage of 1g/kg bw/d for five days and were subsequently (on the sixth day) administered an acute dose of STZ (150mg/kg bw). Animals with blood glucose levels above 200mg% were selected and randomly distributed into four groups as follows:

Group I – control; Group II – TAU; Group III – STZ; Group IV - TAU + STZ

The mice were sacrificed five days after STZ administration under mild diethyl ether anesthesia. Samples of blood were collected by cardiac puncture for obtaining plasma. Liver, testis and epididymis were excised and weighed. The effect of antioxidant treatment on diabetes induced oxidative damage in testis was investigated by assaying the following parameters: blood glucose levels, LPO status, generation of ROS and selected enzymic and non-enzymic antioxidants.

SECTION B

PROGRESSION OF OXIDATIVE DAMAGE AND GENOTOXIC IMPLICATIONS

EXPERIMENTAL DESIGN

Blood glucose and testosterone levels

Progression of Hyperglycemia in mice

In order to obtain the pattern of hyperglycemia induction, plasma glucose levels of STZ-treated mice were determined at 1, 2, 3 and 4 weeks following administration.

Measurement of serum testosterone level

Testosterone levels were quantified in fresh serum samples of mice 1, 2 and 4weeks after STZ administration.

Morphological and histopathological investigations

Body weights of both control and treated animals were recorded at weekly intervals. At each sampling point, mice of both groups were sacrificed and the testis and cauda epididymis were excised, freed from the tissue mass, rinsed in ice-cold physiological saline, blotted and weighed. Further, one testis and cauda epididymis from both control and treated animals were fixed in Bouin's fluid, embedded in paraffin, processed by standard histological techniques, stained and examined by light microscopy.

Progression of oxidative damage

Oxidative damage in testis

In a separate study, adult mice rendered diabetic (STZ: 150mg/kg bw) were sacrificed at weekly intervals to assess the progression of oxidative damage in testis (cytosolic and mitochondrial fractions). The

biochemical assays included assessment of LPO status and generation of ROS levels, measurement of selected enzymic and non-enzymic antioxidants.

Pattern of progression of oxidative damage in ES

The progression of oxidative damage was also determined in epididymal sperms. Oxidative damage indices such as LPO, ROS, antioxidant enzymatic levels, glutathione content and protein carbonyl content were analyzed at all sampling weeks.

Sperm number and morphology

Effect on sperm number

In a series of separate studies, following STZ treatment, cauda epididymis from both control and diabetic mice were excised at one, two, three and five weeks, weighed and processed for preparation of sperm suspensions. Suitable aliquots were used to determine the sperm counts at all sampling weeks.

Incidence of sperm head abnormalities

Aliquots of sperm suspensions were stained in eosin-Y and the smears were examined for sperm head abnormalities. The sperm suspensions were also processed and subjected to scanning electron microscopy to visualize the structural alterations.

Genotoxic consequences

DNA damage in testis

STZ-induced oxidative DNA damage in testis and epididymal sperms were quantified at 2 and 4 weeks of sampling. The extent of strand breaks was measured by FADU assay.

Induction of dominant lethal type mutations

In this investigation, adult proven males were administered (i.p.) a single dose of STZ (150mg/kg bw) and these males were individually caged with adult virgin females (1:2 ratio) each week for 5 consecutive weeks.

The males remained caged with each pair of females for 7 days and were subsequently transferred to the next virgin pair. Successful mating was ascertained by the presence of vaginal plugs and all the pregnant females were humanely sacrificed on 14-16th day of post-detection of plugs. Both the uterine contents were analyzed for total implantation sites, live embryos, resorptions and fetal deaths. Males administered a single dose of cyclophosphamide (CP, 100mg/kg bw) and mated with virgin females for five consecutive weeks served as the positive control.

RESULTS

SECTION A

EARLY OXIDATIVE DAMAGE IN TESTIS AND RESPONSE TO ANTIOXIDANTS

Mortality response to STZ treatment

STZ at the lowest dose (50mg/kg bw) failed to induce any significant increase in plasma glucose levels measured at three days of post-treatment and also did not induce any clinical signs of toxicity or mortality (data not shown). A dosage of 100mg/kg bw induced 20% mortality and induced only marginal hyperglycemia. However, a dosage of 150mg/kg bw induced a significant (2-3 fold) increase in the plasma glucose levels and a 30% mortality was recorded during four weeks of post administration. Furthermore, at the highest dose (200mg/kg bw), STZ induced nearly 70% mortality within five days of administration.

Hyperglycemic response with optimum dose

An acute dose of STZ (150mg/kg bw) was considered as the optimum dose since it induced a significant increase in the plasma glucose levels at 72h and beyond. While, the plasma glucose levels in controls were in

the range of 145-160mg%, that of the STZ-treated mice ranged between 499-578mg%.

In general, there was a 2-3-fold increase in the plasma glucose levels in STZ-treated mice measured at 3-5 days of post administration. The hyperglycemic responses of individual mice illustrated in Fig 3.1 clearly indicate that a majority of the animals responded to the STZ treatment.

Early oxidative damage in testis and ES

Incidence and magnitude during early phase

An acute dose of STZ induced a significant increase in the TBARS levels in both testis and epididymal sperms (Table 3.1) measured at three and five days of post treatment. In testis, STZ induced nearly 34% increase in LPO over the endogenous levels (controls) measured after three days, while the increase was 67% after five days. Similar enhanced MDA levels (95 and 68% increase over the controls) were evident in epididymal sperms of mice at both sampling times. A 301% increase in the LPO was also evident in hepatic homogenates at five days of post administration (Fig. 3.2).

A marked increase (64%) in the endogenous TBARS levels was evident in mitochondrial fractions of testis at five days after STZ treatment. However, there was a 108% increase in the LPO in hepatic mitochondrial fractions at five days of post administration (Fig. 3.2). Interestingly, STZ-treatment also induced a relatively higher degree of ROS (Fig. 3.3) in the testicular homogenates (35%), mitochondrial fractions (48%) and epididymal sperms (81%) after five days.

Interestingly, during the early phase, STZ induced no significant alterations in the testicular GSH levels, but induced a marginal increase (13%) in epididymal sperms and marginal decrease (10%) in liver GSH levels (data not shown). Data on the protein carbonyl content in both

testis and liver of mice is illustrated in Fig. 3.4. Marginal increase in protein oxidation was observed in testis (24 and 26%) and liver (10 and 24%) at three and five days of post administration of STZ.

Significant alterations in antioxidant enzyme activities (CAT, GPx and GST) in testis were also demonstrable suggesting STZ-induced oxidative stress (Fig. 3.5). The activities of both GPx (23 and 63%) and GST (26 and 21%) were significantly elevated at three and five days of post administration. However, there was a marginal decrease in the activity of CAT (19 and 17%) at both sampling times.

The effect of STZ treatment on antioxidant enzymes in epididymal sperms measured at three and five days is presented in Fig. 3.6. Significant decreases in the activities of CAT (34 and 31%), GST (32 and 20%) and GPx (13 and 17%) were evident in treated mice at both sampling times.

Pretreatment with antioxidants: attenuation of early oxidative damage

Effect on plasma glucose levels

Pretreatment of mice with antioxidants viz., ascorbic acid (AA) and Taurine (TAU) yielded only a marginal to moderate protection against STZ-induced elevation in the plasma glucose levels (Table 3.2). While AA treatment reduced the STZ-induced hyperglycemia by nearly 40%, TAU treatment reduced the plasma glucose levels by 10%.

Modulatory effect on lipid peroxidation and ROS levels

Data on the lipid peroxidation status in testis, liver and ES of mice with or without ascorbic acid pretreatment is presented in Table 3.3. While AA pretreatment *per se* had no effect on the LPO in testis, liver and ES, pretreatment with AA appeared to completely offset the STZ-induced oxidative damage. AA provided a significant protection against STZ-induced LPO in testis (100%), liver (98%) and epididymal sperms (57%). Likewise, varying degree of protection was also offered by AA against

STZ-induced LPO in mitochondrial fractions of testis (46%) and liver (43%) (Fig. 3.7).

The influence of taurine pretreatment on STZ-induced LPO is illustrated in Table 3.4. As evident from the table, TAU offered a marked protection against LPO in testis (61%) and liver (100%).

Data on the effect of AA and TAU on ROS generation levels of STZ treated animals in both testis and liver is presented in Fig 3.8. Administration of AA to diabetic rats provided a significant protection against ROS generation in testis (63%), ES (40%) and hepatic (30%) homogenates. Likewise, pretreatment of TAU to STZ treated mice led to a significant protection against ROS generation in both testis (82%) and ES (39%).

Modulation of antioxidant enzymes in testis and ES

Data on the protective effects of AA and TAU against perturbations in antioxidant enzyme activities (GST and GPx) are presented in Fig. 3.9. Pretreatment with AA induced a marked inhibition (86%) in the STZ-induced elevated activity of GST. While, GPx activity was unaltered in AA pretreated mice, complete restoration was observed in the activity of GST enzyme (100%) in TAU pretreated mice (Fig 3.9).

Effect of AA and TAU pretreatment on protein oxidative damage

The effect of pretreatment of AA and TAU against STZ-induced protein oxidative damage in homogenates of testis and liver is presented in Table 3.5. Pretreatment with AA yielded a significant protection against protein oxidative damage in testis (45%) and liver (100%). Although, there was a significant protection (89%) against the protein oxidative damage in liver of TAU pretreated mice, there was no protective effect evident in the testicular homogenates of the same group.

RESULTS

SECTION B

PROGRESSION OF OXIDATIVE DAMAGE AND GENOTOXIC IMPLICATIONS

Hyperglycemic response: Progression

Blood glucose levels measured at various sampling intervals (wk 1-5) in STZ administered mice revealed a consistent, progressive elevated levels (2.7 - 3.8 fold) compared to control mice (Table 3.6).

Serum testosterone level

The effects of STZ on the serum testosterone levels of diabetic mice were determined at week two and four of post administration. While there was only a marginal decrease at week 1 (data not shown), the serum testosterone levels were significantly (45%) lower than the control group at week two, and were markedly reduced (75%) at week four of sampling (Fig. 3.10).

Morphological and histopathological investigations

Effects on body and testicular weights

Body weights of diabetic mice at week two were significantly decreased (41%) compared to controls (data not shown). Likewise, there was a significant reduction in the testicular (11%) and epididymal weights (18%) in STZ-induced diabetic mice when compared to the controls at week two and beyond (data not shown).

Assessment of pathological lesions in testis

Histopathological lesions were studied in the testis at weeks 1-4. There were no obvious pathological alterations during the first three weeks (data not shown). However, significant lesions were evident in testis of diabetic mice sampled at 4 and 5 weeks. Some of the typical lesions in the testis of are resented in Plate 5 (a-d). The salient lesions were:

typical reduction in the size of the seminiferous tubules with reduced tubule diameter, reduction in the number of leydig cells and impaired spermatogenesis. The incidence of tubules showing various lesions gradually increased from fourth week onwards. Most of the tubules from the testis sampled at fifth week, showed marked atrophy with varying degree of spermatogenetic arrest with total absence of sperms in the lumen (Plate 5).

Progression of oxidative damage: weekly sampling

Oxidative damage progression in testis

Data on the LPO levels in testis and ES sampled at weeks two and four after administration of STZ is presented in Table 3.7. While the testicular homogenates showed an increase in LPO by 35 and 110% over the controls, there was an 80 and 116% increase in LPO in mitochondrial fractions during two and four weeks of post administration. The sperm suspensions of STZ treated mice showed nearly 62-79% increase in LPO at both sampling times. Measurement of ROS levels in both testis and ES among STZ treated mice revealed a similar pattern of response as illustrated in Fig 3.11. Marginal to moderate increases (23-69%) in ROS levels was evident in testis, at week 2 and 4 of sampling.

There was a significant reduction (23 and 30%) in testicular GSH levels (Fig. 3.12) sampled at wk 2 and 4. In testis, alterations in the activity of vital AOE viz., CAT (18 and 37% decrease), GPx (14% increase and 22% decrease), GST (26 and 33% decrease) and GR (7% increase and 28% decrease) were evident during 2 and 4 weeks of sampling (Fig. 3.13, upper panel). The activities of selected dehydrogenases in testis were also significantly altered in diabetic mice (Fig. 3.14). While the activity of G6PDH enzyme was decreased by 27 and 37% among diabetic mice sampled at week two and four, decreased activity of SDH (45%) was also evident at various weeks of sampling.

Oxidative stress progression in ES

The endogenous TBARS levels in the epididymal sperms at week two and four of post administration are presented in Table 3.7. The degree of LPO induction was significant and ranged between 62-79% at various sampling times. Further, the endogenous ROS levels (Fig. 3.11) in epididymal sperms was significantly higher (32-110%) in diabetic mice compared to the controls at week two and four of sampling. In addition, GSH levels in ES were also found to be significantly reduced (30-36%) at both sampling times (Fig. 3.12).

Data on activities of antioxidant enzymes in epididymal sperms are presented in Fig. 3.13 (lower panel). In general, there was a decreased activity of GPx (4 and 29%), GST (21 and 37%) and GR (20 and 24%) in diabetic mice sampled at week two and four of post administration.

Oxidative damage and genotoxic consequences

Effect on epididymal sperm number

The effect of STZ-induced diabetes on epididymal sperm counts is depicted in Table 3.8. The epididymal sperm counts were significantly decreased in the diabetic group (51 and 76%) when compared to that of control group at the first (7.32 ± 0.53 vs. 3.6 ±0.58; 51%) and second week (8.12 ± 0.71 vs. 3.01 ± 0.4; 76%) of post administration. The pattern of the decline in the sperm count was progressive and time-dependent, ranging between 50-89% at weeks one, two, three and five of post administration.

Sperm morphology characteristics: incidence of head abnormalities

The frequency of sperms with head abnormalities determined during various weeks of post treatment of STZ is presented in Table 3.8. The incidence of spontaneous abnormal sperms among controls ranged from 1.2 to 1.4%, which were within the background incidence reported earlier for this strain from our laboratory. However, STZ treatment

induced a significant elevation in the incidence and frequency of abnormal sperms at all sampled weeks. Significant increase in the percent (2-12 fold higher) of abnormal sperms was observed at all sampling weeks. The major abnormalities consisted of amorphous heads, coiled tails, balloon heads, hook less and hammer head (Plate 6) as originally described by Wyrobeck and Bruce (1978).

DNA damage in testis and epididymal sperms

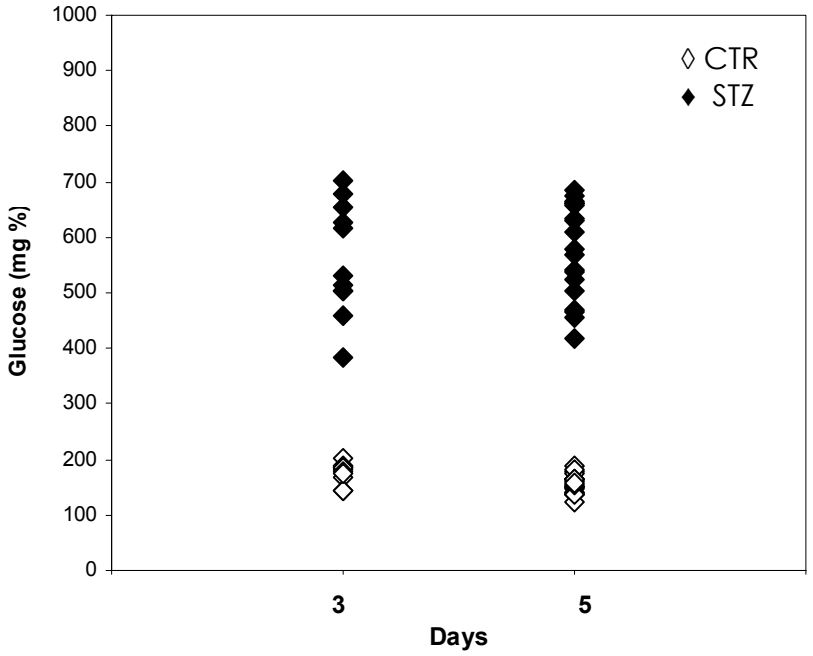
The effect of STZ-induced diabetes on DNA damage quantified in testis and epididymal sperms at two and four weeks of post administration is presented in Fig. 3.15. The formation of strand breaks, leading to a decreased percent of double stranded DNA was evident at week two. While the background DNA damage in the testis was 26%, the STZ-induced diabetic rats showed an increase (32-37%) in DNA damage at week two and four.

Induction of dominant lethal type mutations

In general, STZ-induced diabetic males showed a significant reduction in mating efficiency at all weeks and the efficiency decreased further during weeks four and five. The percentage of pregnancy induced by diabetic males ranged from 42 at week one to 16 at week five (Table 3.9). There was a significant decrease in the number of live implantations during all weeks except week five. The percent of dead implantations ranged from 15 (week one, three and four) and 35 (week two). This study suggested that STZ also induces a stage specific effect on the post meiotic germ cells.

In order to compare the DL-mutation response of STZ with a known mutagen, data was also obtained on the incidence of DI following treatment of males with an acute dose of cyclophosphamide (CP, a positive mutagen). Among the CP treated males, a 7-fold increase in the DI per litter was evident during the first two weeks and a 3- fold increase during the third week (Table 3.10).

Fig.3.1: Plasma glucose levels in adult mice rendered diabetic by an acute dose of STZ (150mg/kg bw)



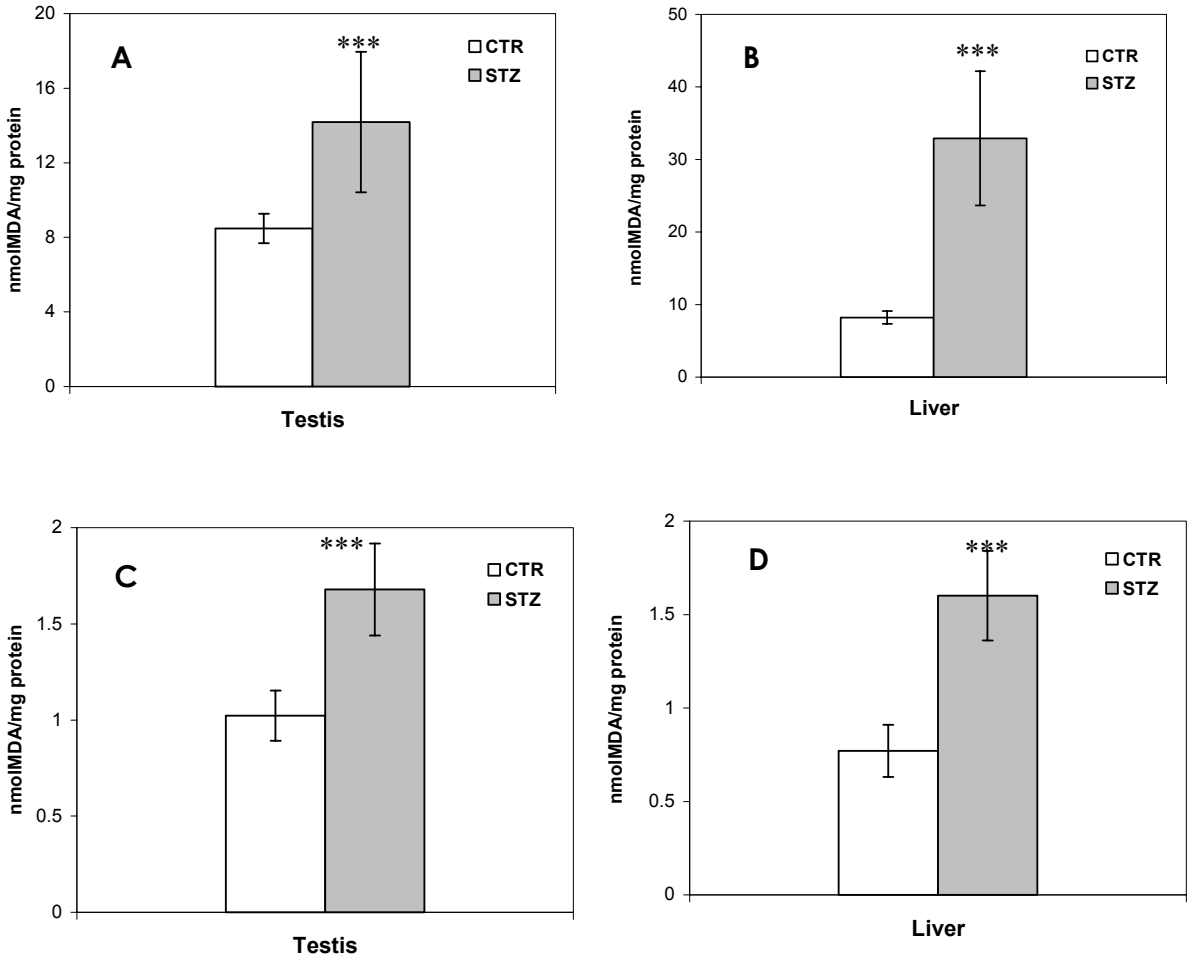
Data pooled from three independent experiments (n=12)

Table 3.1: TBARS levels in testicular homogenates (TH) and epididymal sperms (ES) of adult mice following STZ administration

Group	Malondialdehyde (ηmol/mg protein)			
	Days			
	3		5	
	TH	ES	TH	ES
CTR	9.49 ± 1.32	15.76 ± 2.53	8.49 ± 1.79	15.11 ± 1.23
STZ	12.70** ± 1.77	30.73*** ± 2.61	14.18*** ± 1.77	25.41*** ± 1.27

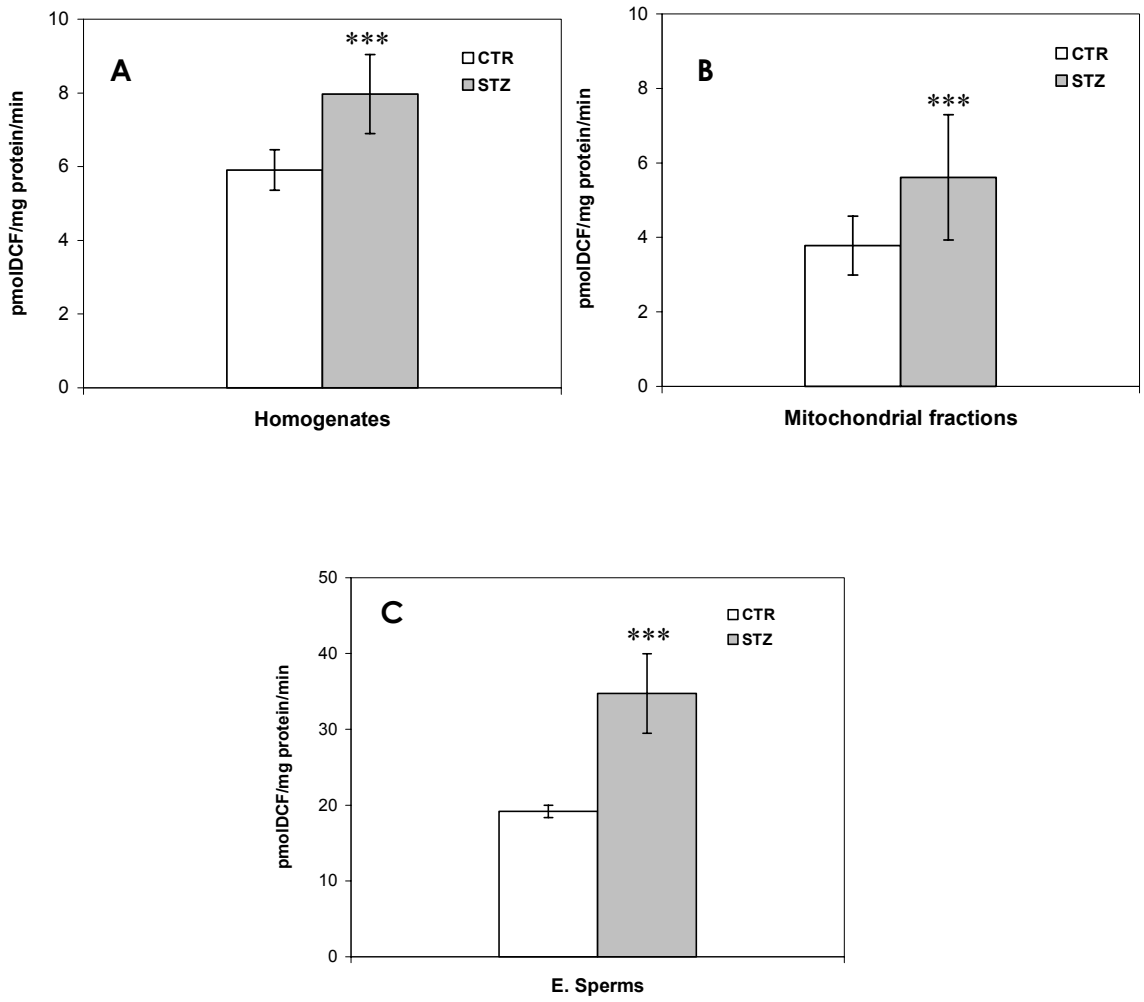
Values are mean ± SD (n=4)
Data analyzed by student's 't' test; **P<0.01; ***P< 0.001

Fig. 3.2: TBARS levels in testicular/hepatic homogenates (A & B) and mitochondrial fractions (C & D) of adult mice determined on day 5 following STZ administration (150mg/kg bw)



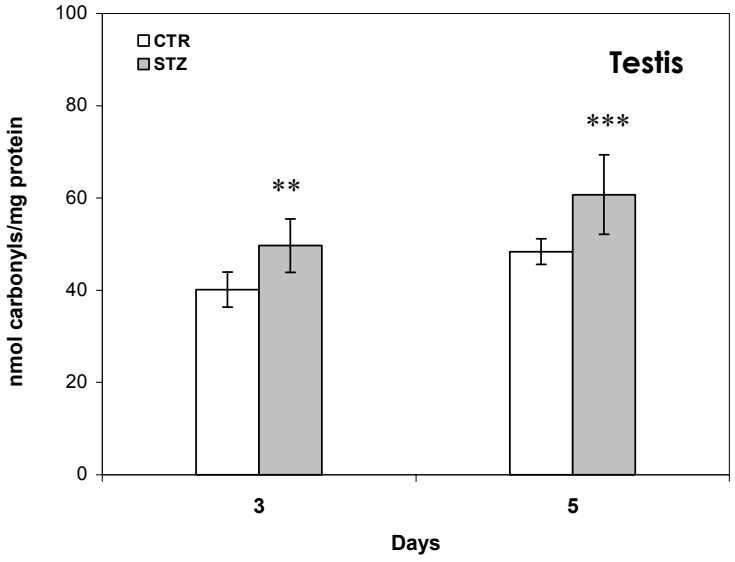
Values are mean ± SD (n=4)
Data analyzed by student's 't' test ***$P<0.001$*

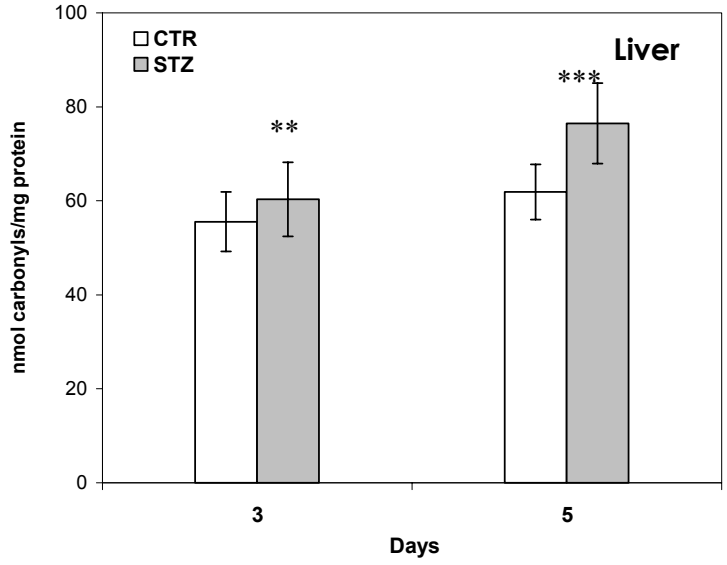
Fig. 3.3: ROS levels in testicular homogenates (A), testicular mitochondrial fractions (B) and epididymal sperms (C) in adult mice determined on day 5 following STZ administration



Values are mean ± SD (n=4)
Data analyzed by student's 't' test ***$P<0.001$

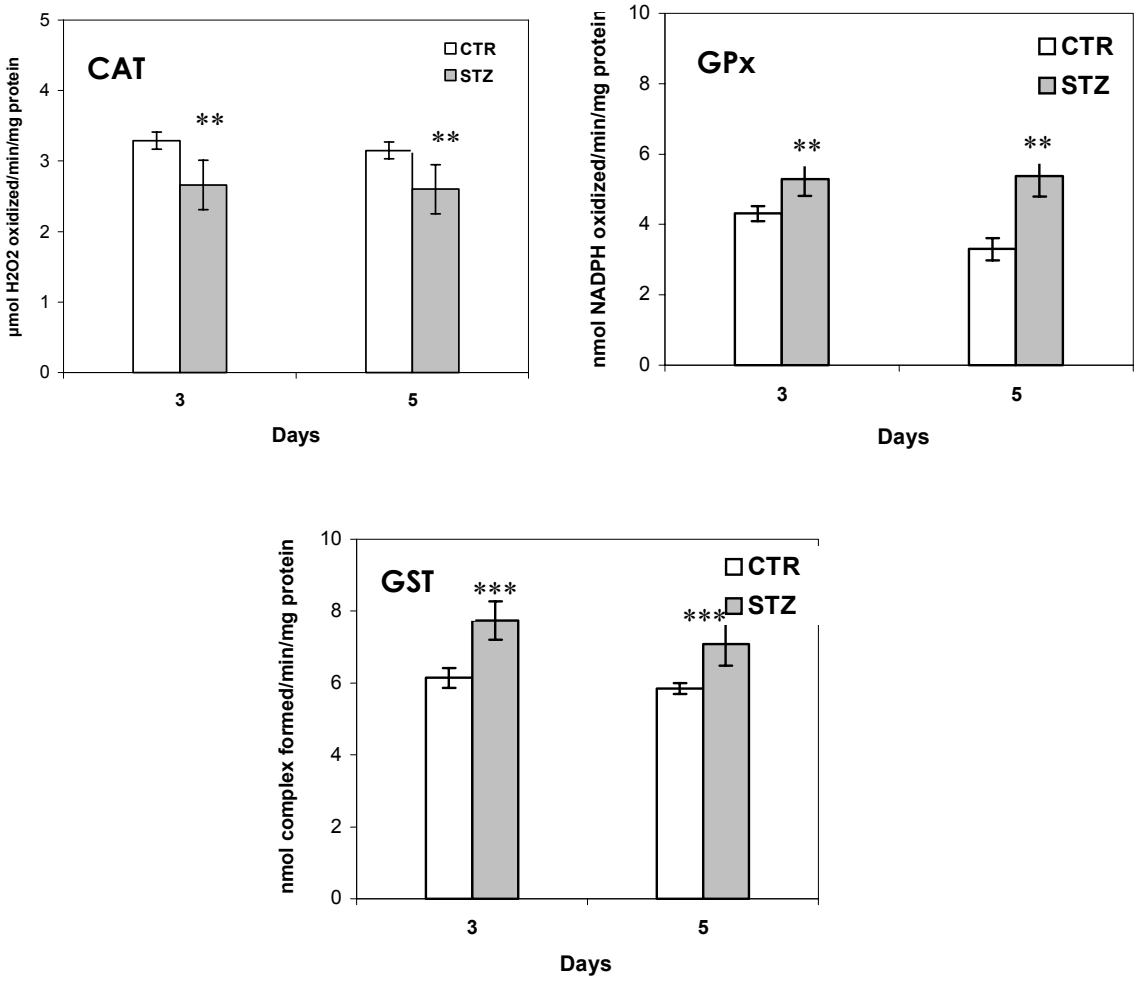
Fig. 3.4: Protein carbonyl content of testis and liver of mice administered (i.p.) an acute dose of STZ (150mg/kg bw)





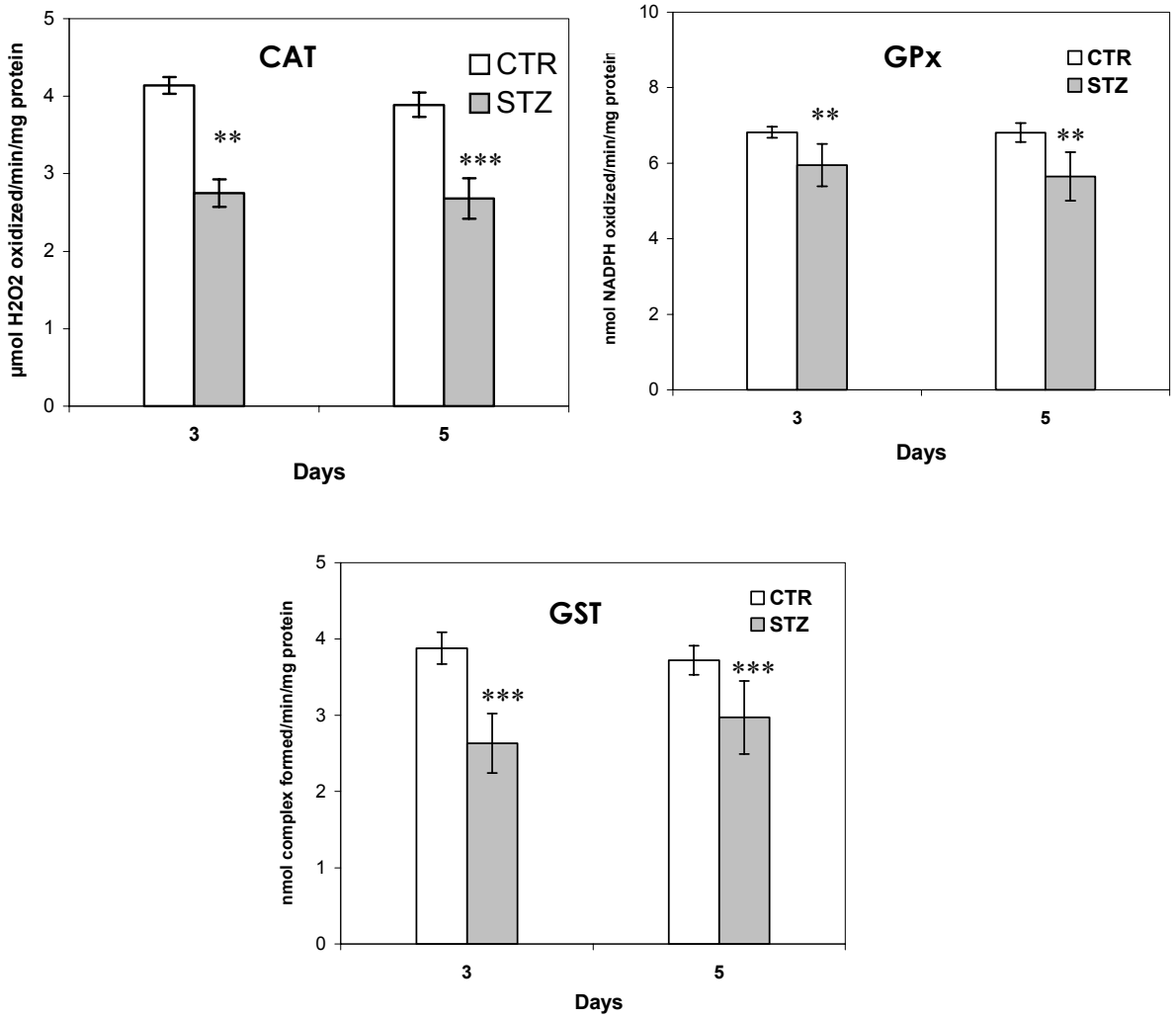
Values are mean ± SD (n=4)
Data analyzed by student's 't' test; ***P<0.01; ***P<0.001*

Fig. 3.5: Early perturbations in antioxidant enzymes in testis of mice administered (i.p.) an acute dose of STZ (150mg/kg bw)



CAT: µmol H_2O_2 oxidized/min/mg protein
GST: µmol product formed/min/mg protein
GPx: nmol NADPH oxidized /min/mg protein
Values are mean ± SD (n=4)
Data analyzed by student's 't' test ***P<0.01; ***P<0.001*

Fig. 3.6: Early perturbations in antioxidant enzymes of epididymal sperms in mice administered (i.p.) STZ (150mg/kg bw)



CAT: µmol H_2O_2 oxidized/min/mg protein
GST: µmol product formed/min/mg protein
GPx: nmol NADPH oxidized /min/mg protein
Values are mean ± SD (n=4)
Data analyzed by student's 't' test ***P<0.01; ***P<0.001*

Table 3.2: Plasma glucose levels of adult mice pretreated with ascorbic acid (AA)/Taurine (TAU) and administered an acute dose of STZ (150mg/kg bw; i.p.)

Group	Plasma glucose* (mg %)
CTR	177 .66[a] ± 25.36
AA	178.23[a] ± 19.54
TAU	187.75[a] ± 21.62
STZ	570.99[b] ± 107.89
AA + STZ	302.33[a] ± 111.52
TAU + STZ	500.33[b] ± 21.35

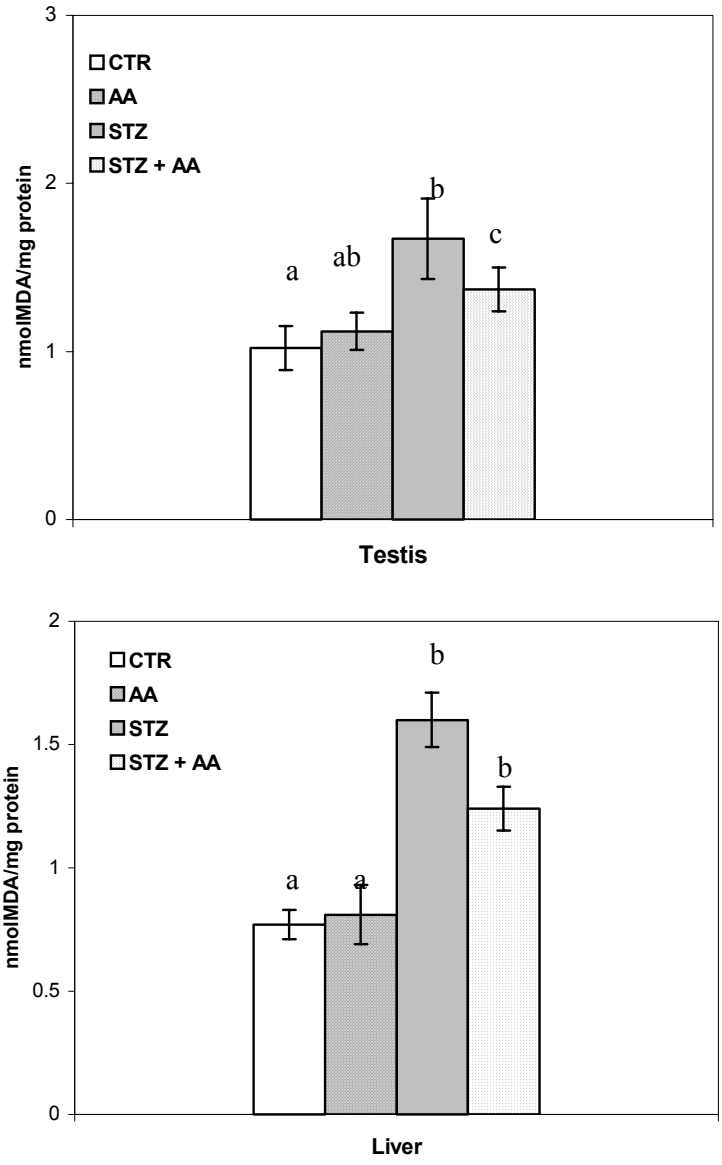
* Pooled data of two separate experiments
Values are mean ± SD (n=8)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 3.3: Influence of ascorbic acid (AA) pretreatment on STZ-induced lipid peroxidation in testis, liver and epididymal sperms (ES)of adult mice

Group	Malondialdehyde (ηmol/mg protein)		
	Testis	**ES**	**Liver**
CTR	8.27^{b} ± 0.61	15.76^{a} ± 2.53	12.38^{a} ± 2.56
AA	8.06^{b} ± 0.44	14.82^{a} ± 2.19	13.01^{a} ± 1.99
STZ	10.31^{c} ± 0.53	30.73^{c} ± 4.61	23.26^{b} ± 4.32
STZ + AA	6.82^{a} ± 0.52	22.20^{b} ± 3.76	12.63^{a} ± 3.72

Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Fig. 3.7: Modulatory effect of ascorbic acid (AA) pretreatment on STZ-induced early LPO in Testis and liver mitochondrial fractions in adult mice



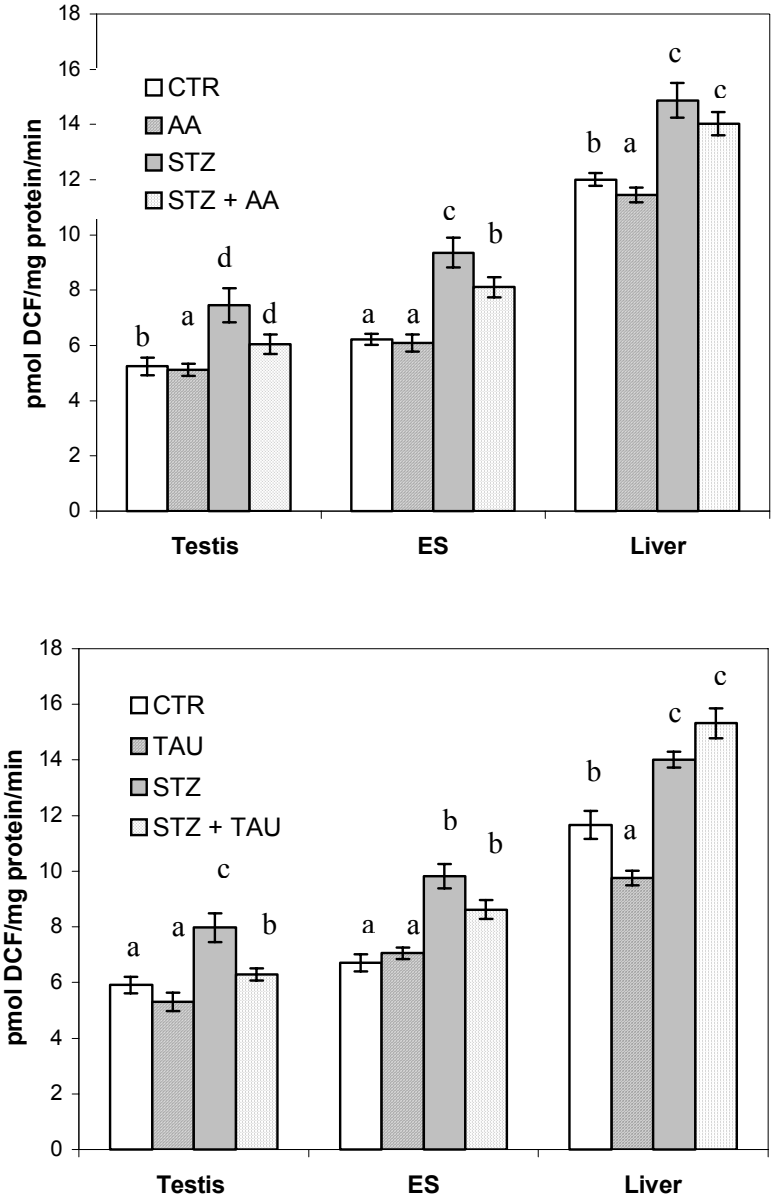
Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 3.4: Influence of Taurine (TAU) pretreatment on STZ-induced lipid peroxidation in testis and liver of adult mice

Group	Malondialdehyde (ηmol/mg protein)	
	Testis	**Liver**
CTR	8.95^{a} ± 1.94	8.30^{a} ± 0.63
TAU	10.70^{ab} ± 0.50	7.82^{a} ± 0.90
STZ	11.94^{b} ± 2.03	17.79^{b} ± 1.12
STZ +TAU	10.12^{ab} ± 1.97	8.33^{a} ± 1.54

Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

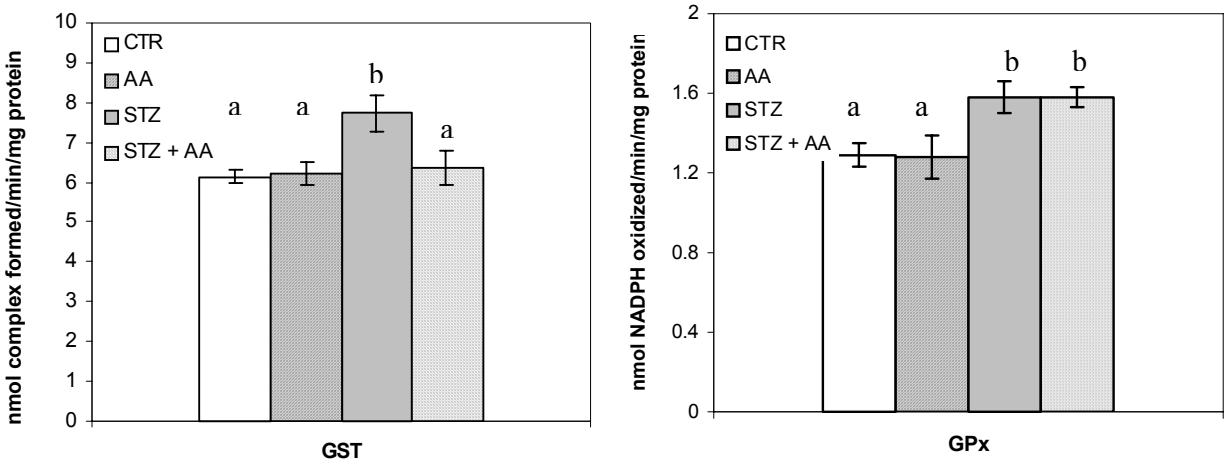
Fig 3. 8: Pretreatment with ascorbic acid (AA)/taurine (TAU) and its attenuation of STZ-induced ROS levels in Testis, liver homogenates and epididymal sperms (ES)of adult mice



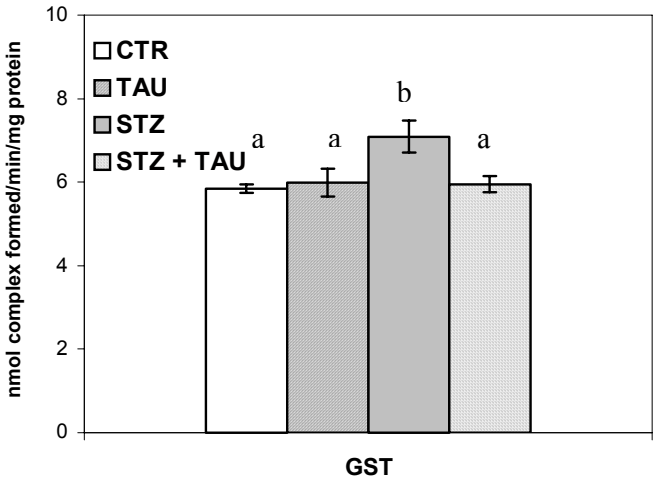
Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Fig. 3.9: Modulatory effect of Ascorbic acid and Taurine pretreatment on antioxidant enzyme activities in Testis of STZ treated mice

Ascorbic acid



Taurine



Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 3.5: Attenuation of STZ -induced protein oxidative damage in testis and liver homogenates by ascorbic acid (AA) and Taurine (TAU)

Group	ηmol carbonyls/mg protein	
	Testis	**Liver**
CTR	36.81^{a} ± 1.46	54.99^{b} ± 0.19
AA	35.66^{a} ± 2.31	52.03^{ab} ± 0.26
TAU	32.66^{a} ± 2.31	49.77^{a} ± 5.31
STZ	49.28^{bc} ± 2.75	76.82^{c} ± 0.26
STZ +AA	43.62^{b} ± 2.01	47.40^{a} ± 5.20
STZ +TAU	49.79^{c} ± 2.18	57.47^{b} ± 4.24

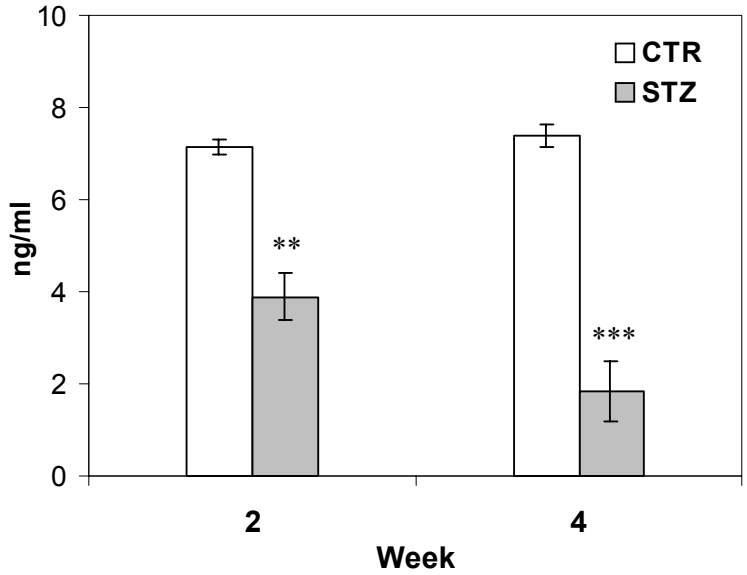
Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 3.6: Plasma glucose levels in adult mice rendered diabetic by an acute dose of STZ (150mg/kg bw)

Group	Plasma glucose (mg %)				
	Week				
	1	2	3	4	5
CTR	143.66 ± 11.81	152.00 ± 12.20	140.66 ± 6.59	136.33 ± 11.08	137.43 ± 10.10
STZ	570.50*** ± 88.5	665.25*** ± 66.48	528.33*** ± 15.32	663.50*** ± 10.5	623.50*** ± 11.25

Values are mean ± SD (n=4)
Data analyzed by student's 't' test; ****P*< 0.001

Fig. 3.10: Testosterone levels in serum of mice administered an acute (i.p) dose of STZ (150mg/kg bw)



Values are mean ± SD (n=4)
Data analyzed by student's 't' test; ***P<0.01; ***P< 0.001*

Table 3.7: MDA levels in testis (homogenates / mitochondria) and epididymal sperms of mice rendered diabetic by an acute dose of STZ (150mg/kg bw)

	MDA (nmol/mg protein)	
	Week	
Group	2	4
Testis homogenates		
CTR	8.98 ± 0.28	8.91 ± 0.60
STZ	12.13** ± 1.05	18.71*** ± 0.78
Testis mitochondria		
CTR	1.28 ± 0.28	1.46 ± 0.24
STZ	2.30** ± 1.17	3.16*** ± 1.65
Epididymal sperms		
CTR	15.33 ± 0.82	15.87 ±0.42
STZ	24.85*** ± 1.51	28.40*** ± 1.73

Values are mean ± SD (n=4)
Data analyzed by student's 't' test; ***P<0.01;* ****P*< 0.001

Fig. 3.11: ROS levels of testis and epididymal sperms (ES) of mice rendered diabetic by an acute dose of STZ

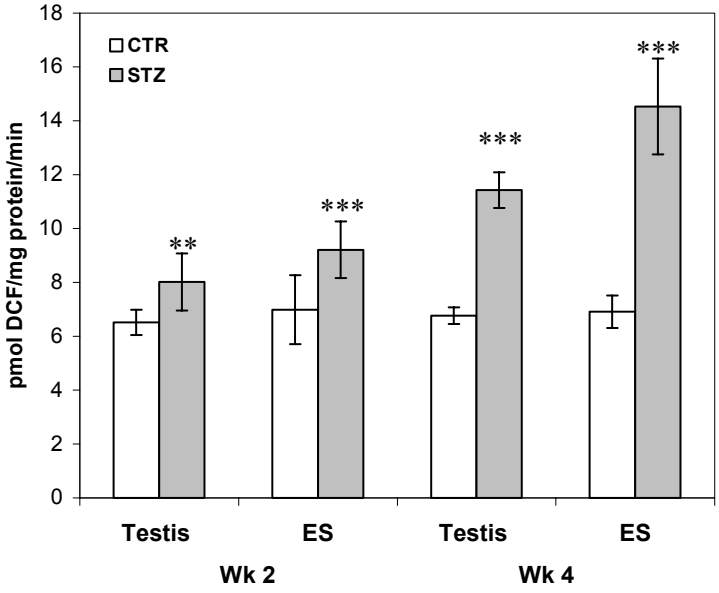
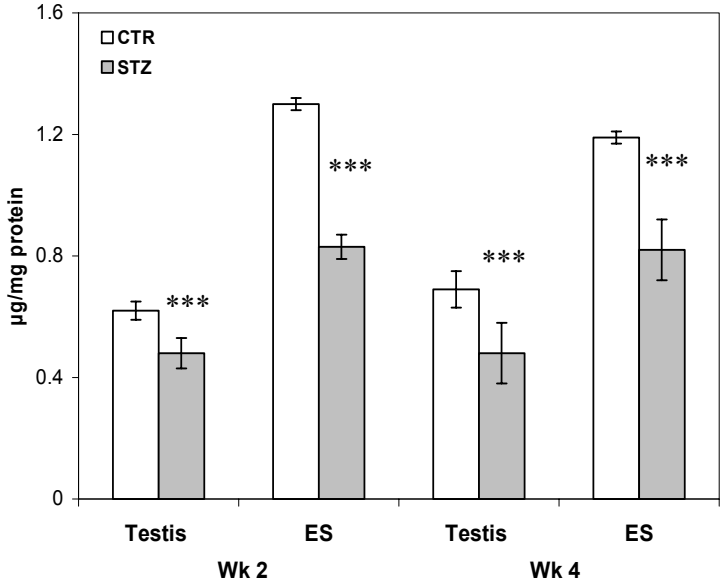
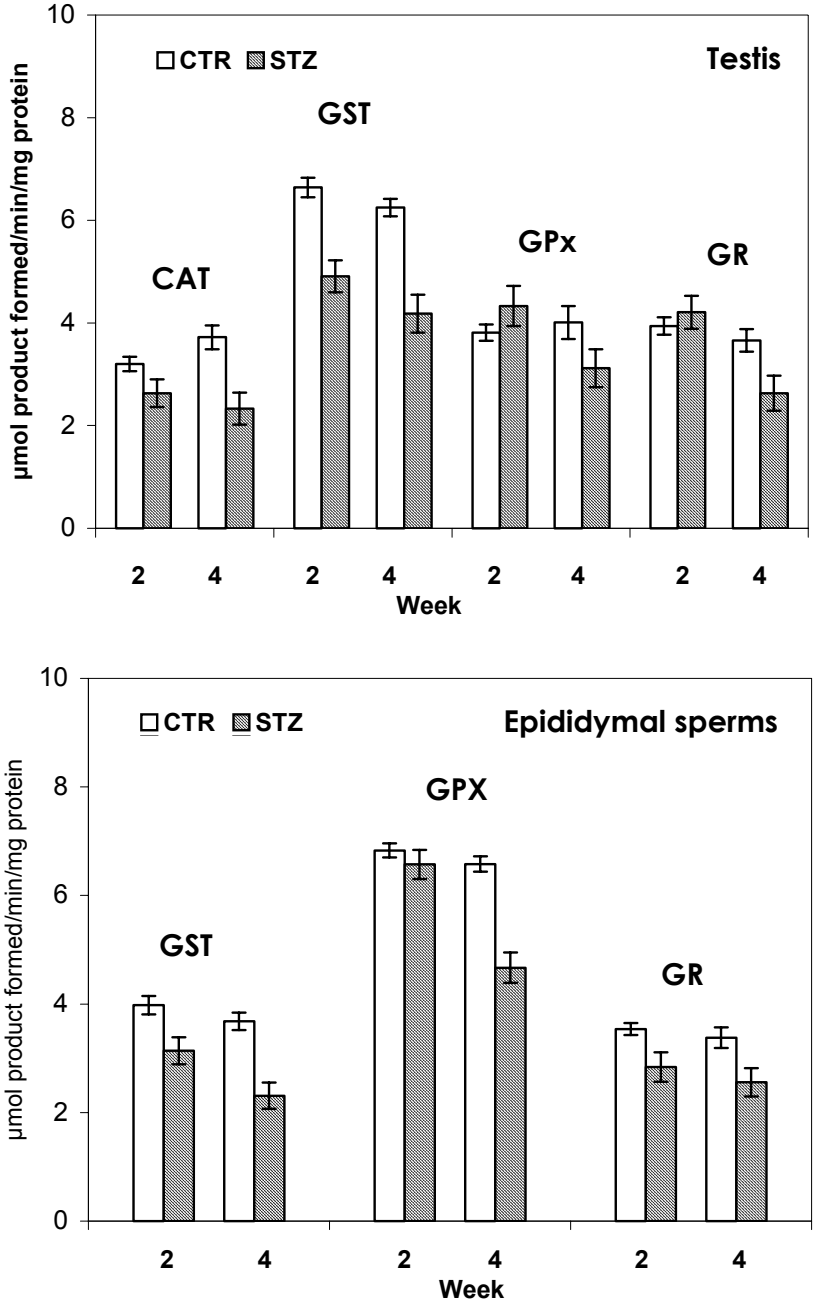


Fig. 3.12: Glutathione levels in testis and epididymal sperms (ES) of mice rendered diabetic by an acute dose of STZ



Values are mean ± SD (n=4)
Data analyzed by student's 't' test; *$P<0.01$; ***$P<0.001$

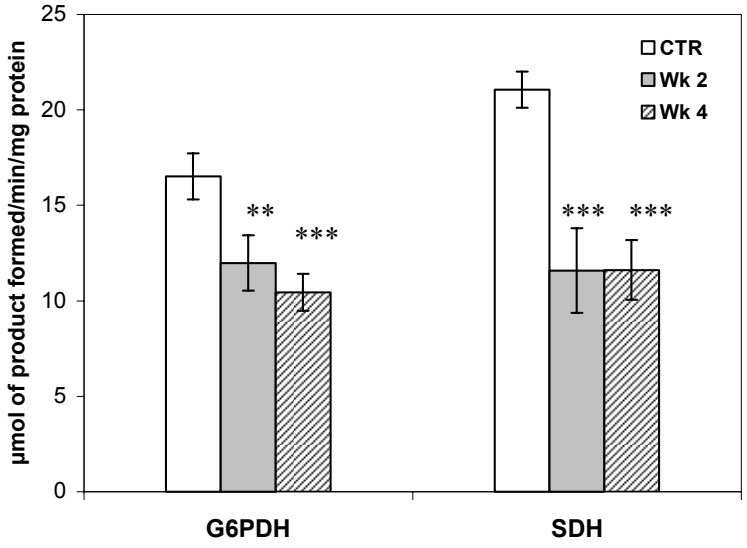
Fig: 3.13: Antioxidant enzyme activities in testis and epididymal sperms of mice rendered diabetic by an acute dose of STZ



CAT: µmol H_2O_2 oxidized/min/mg protein; GST: µmol product formed/min/mg protein; GPx: nmol NADPH oxidized /min/mg protein

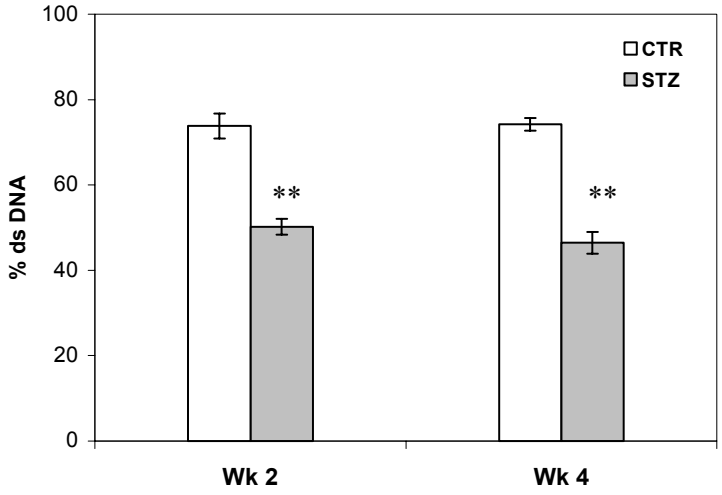
Values are mean ± SD (n=4); Data analyzed by student's 't' test

Fig 3.14: Activities of dehydrogenases in testis of mice rendered diabetic by an acute dose of STZ (150mg/kg bw)



Values are mean ± SD (n=4)
Data analyzed by student's 't' test; *$P<0.01$; ***$P<0.001$

Fig. 3.15: DNA damage in testis of mice rendered diabetic by an acute dose of STZ (150mg/kg bw) at various sampling intervals



Values are mean ± SD (n=4)
Data analyzed by student's 't' test; ***P<0.01*

Table 3.8: Sperm counts and incidence of abnormal sperms in mice rendered diabetic by an acute dose of STZ (150mg/kg bw)

Group	Week							
	1		2		3		5	
	Count‡	ABS	Count	ABS	Count	ABS	Count	ABS
CTR	7.38 ± 0.53	1.46 ± 0.20	8.12 ± 0.71	1.35 ± 0.54	7.83 ± 0.51	1.32 ± 0.22	7.01 ± 0.21	1.23 ± 0.36
STZ	3.6 ± 0.58	2.55* ±1.50	3.01 ± 0.24	3.04* ± 0.22	0.91 ± 0.69	17.43* ± 2.91	0.80 ± 0.39	12.10* ± 2.92

‡ expressed as $X10^6$/cauda epididymis
ABS: percent abnormal sperms
Values are mean ± SD (n=4)
Data analyzed by Mann-Whitney *U*-test; **P<0.001*

Table 3.9: Incidence of total implantations, live embryos and dead implantations resulting from male mice rendered diabetic by an acute dose of STZ (150mg/kg bw)

Treatment	Index	Weeks				
		1	2	3	4	5
CTR	TI/Litter	11.7	11.6	11.5	11.7	11.7
STZ		11.0	7.70*	11.5	10.2	10.0
CP		8.7*	11.3	10.1*	11.3	10.8
CTR	LI/litter	10.7	10.6	10.2	10.7	10.7
STZ		9.4*	5.0***	9.0	8.6	9.0
CP		3.4***	5.0***	6.5*	10.3	9.8
CTR	DI/litter	1.0	1.0	1.3	1.0	1.0
STZ		1.6*	2.7**	2.5**	1.4	1.0
CP		5.3***	6.3***	3.6**	1.4	1.0

TI: total implantations; LI: live embryos; DI: Dead implantations
CP: cyclophosphamide
Data analyzed by Mann-Whitney *U*-test
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.002$

Table 3.10: Dominant lethal mutation induction in male mice rendered diabetic by an acute dose of STZ (150mg/kg bw)

Treatment	DI per litter (%)				
	Weeks				
	1	2	3	4	5
CTR	8.69	8.06	11.30	8.56	8.65
STZ	14.50*	34.78***	20.00**	15.70	10.00
CP	60.90***	55.8***	35.6***	12.40	9.26

DI: Dead implantations
CP: cyclophosphamide (100mg/kg bw)
Data analyzed by Mann-Whitney *U*-test
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.002$

DISCUSSION

Early oxidative damage (EOD) in testis and epididymal sperms

The primary aim of this series of investigations in mice was to ascertain whether significant oxidative stress can be detected in the testis during early time points following administration of an acute diabetogenic dose of STZ. For this, an optimum dose of STZ (150 mg/Kg bw), which induced significant hyperglycemia and low mortality, was selected and our investigations focused on early oxidative damage (EOD) by measurement of various biomarkers of OS in both testis and ES. Since liver is also one of the primary targets of STZ, we have measured oxidative damage in liver so as to obtain a comparative data. Adult mice were employed as experimental models for these studies for two reasons viz., (i) that mice required a lower dose of diabetogenic agent (STZ/mouse) and (ii) the ease of multiple sampling and processing of the testicular tissue. Further, earlier workers (*Mauer et al., 1978; Yaksumoto et al., 1997*) have also reported the advantages of mice model since they develop nephropathy earlier than rats suggesting there could be an early onset of oxidative damage in mice tissues in response to STZ.

Data on the oxidative damage in male reproductive system in mice under diabetic conditions is rather limited. More importantly, there have been absolutely no data which describe the occurrence of oxidative disturbances at *early time points* in testis and epididymal sperms (ES). Hence we designed experiments to ascertain the induction of EOD by following approaches: (a) determination of LPO status (b) measurement of ROS levels (c) response of antioxidant enzymes (AOE) and (d) formation of protein carbonyls.

Our preliminary investigations showed no significant increases in testicular MDA levels at 24 and 48 hrs of STZ administration (data not

shown). However, enhanced MDA levels in both testis and ES were evident during sampling times of 3 and 5 days of post administration, the magnitude being higher at the later time point (Table 3.1). Further, the TBARS levels measured on day 5 were significantly enhanced not only in testicular and hepatic homogenates, but also in mitochondria, liver homogenates and mitochondria. Concomitantly, higher degree of ROS generation was evidenced in testicular homogenates (>50%), mitochondria (>75%) and ES (>75%) of STZ treated mice clearly suggesting the early oxidative perturbations. These observations appear to be the first report, since there is paucity of data on the induction of oxidative damage *in vivo* at early time points. Additionally, we found decreased activity of CAT, increased activities of GPx and GST enzymes in the testis during both sampling times. Research database shows no total agreement about the effects of diabetes on the activities of some of these AOE in somatic organs (*Maritim et al., 2002*). Interestingly, both elevations and reduced activity of CAT, GPx are documented in liver, kidney, RBC and retina. These variations are primarily because of different time points at which measurements are made.

To the best of our knowledge, there are no reports describing the early induction of oxidative stress *in vivo* in testicular tissue. Our principle findings in testis viz., enhanced LPO and ROS levels, perturbations in AOE and increased protein carbonyls clearly suggest that administration of STZ induces significant early oxidative damage. This raises a logical question whether this effect could be termed a primary effect of STZ in testis *in vivo*. The ability of STZ to act as a prooxidant *in vitro* is well demonstrated and numerous *in vitro* studies have documented the potential of STZ to induce generation of free radicals ($OH^{\cdot}$, $O_2^{\cdot-}$, NO), DNA damage, apoptosis and necrosis (*Maritim et al., 2002*).

The consequences of increased ROS generation can have profound effect on the testicular physiology. Although testis is endowed with sufficient antioxidative defenses, earlier workers have demonstrated altered steriodogenesis as a consequence of increased OS with several xenobiotics and alcohol (*Peltola et al., 1996*). During metabolism of xenobiotics by cytochrome P450 enzymes, radical metabolites or ROS are known to be produced and altered steroidogenesis by itself can generate free radicals. Hence even slight alterations in ROS levels and their detoxification can substantially affect the spermatogenetic process since germ cells are more susceptible to peroxdiaitve damage than sertoli cells (*Bauche et al., 1994*).

Interestingly, these observations were confined not only to the testicular milieu as we observed similar oxidative disturbances in the epididymal sperms sampled simultaneously. Markedly enhanced MDA levels, increased generation of ROS levels and reduced activities of AOE were a hallmark among the ES during both time points. Again a logical question arises whether this is a primary effect of STZ on the sperms, which were already present in the epididymis at the time of STZ administration. Our observations during sampling days 3 and 5 can be interpreted to mean that STZ has a prooxidant action *in vivo* during early time points. However, our further observations on the persistence of oxidative damage during week 1 and 2 indicate that it may be a combination effect of both primary and secondary (diabetes associated hyperglycemia) consequence. Since EOD was quite obvious in the mice model, it is possible that OS may be significantly contributing to the development of pathophysiological complications in the testis.

Another approach adopted by us during EOD studies was to examine the ameliorating effects of ascorbic acid (AA) and taurine (TAU) on STZ-induced EOD. The hypothesis was that if oxidative

damage does occur in testis during early time points following STZ administration, exogenous supplementation of model antioxidants should significantly reduce the extent of EOD. Interestingly, pretreatment with AA or TAU markedly offset the STZ induced EOD in testis, ES and liver. While TAU had no effect on hyperglycemic response, AA pretreatment partially decreased the extent of hyperglycemia. While the exact mechanism by which this protection occurs is not known, it may be related to the impaired ascorbic acid metabolism in STZ-induced diabetic animals.

AA, a naturally occurring major antioxidant is essential for scavenging free radicals in both plasma and tissues (*Frie et al., 1988)*. While AA levels in plasma and tissues of diabetic patients and animals have been demonstrated to be low, supplementation of AA is shown to decrease sorbitol levels (*Cunningham et al., 1994; Wang et al., 1995*) thereby preventing the development of various diabetic complications (*Brownlee et al., 1988)*. Interestingly, impaired urinary excretion of AA in diabetic patients and impaired AA metabolism in STZ-induced diabetic rats have also been demonstrated (*Kashiba et al., 2002*).

In our EOD studies, AA supplementation was very effective since it totally abolished STZ-induced peroxidation in testis and liver homogenates and protein oxidative damage (100% protection). Additionally, markedly reduced ROS levels in cytosol, moderate protection (50%) in mitochondria, and modulation of AOE (GPx, GST) are indicative of the ameliorating influence of AA in testis. These data corroborate the earlier findings in various organs of STZ-induced diabetic model (*Hassan et al., 2002; Naziroglu, 2003; Kutlu et al., 2005)*. While the protective effects of oral supplementation of AA in testis of diabetic rats are limited, few workers have shown enhanced testicular

antioxidants in STZ (*Naziroglu, 2003)* and alloxan -induced diabetic rats (*El-Missiry, 1999*).

Taurine (2-aminoethane sulfonic acid), one of the most abundant free amino acids in many tissues, is well known to have several beneficial physiological actions such as antioxidation, detoxification, osmo-regulation, cell membrane stabilization and neuromodulation (*Wright et al., 1986*). The protective role of TAU in diabetes and its role in reducing the development of diabetic complications have been recently investigated (*Hansen, 2001; Odetti et al., 2003*). Studies in mice have shown that TAU supplementation (*5%* w/v, *7* d, drinking water) significantly enhanced GSH related enzymes and reduced the formation of lipid peroxides in hepatic tissue in alloxan induced T1 DM. In the current study, oral doses of TAU (1g/Kg bw) for 10 days completely mitigated STZ-induced LPO, normalized the ROS levels in both testis and liver. Consistent with our findings TAU has been shown to protect against hepatotoxicity induced by several free radical generators (*Hagar, 2004*) and drugs (*Waters et al., 2001*).

Although the precise mechanism of action of TAU in diabetic testis cannot be defined from the present study, it is attributable to its antioxidant properties. This data is consistent with the idea that TAU can not only function as a direct antioxidant that scavenges free radicals thus inhibiting LPO, but also function as an indirect antioxidant which increase the membrane permeability in many tissues such as liver as speculated earlier (*Chen, 1993*). Alternatively, TAU may also enhance the activity of endogenous antioxidants such as hepatic GSH, GPx and CAT (*Hagar, 2004*). Taken together, the results of our studies with TAU demonstrate that exogenous supplementation of TAU has a protective role in mitigating STZ-induced EOD possibly through its cytoprotective activities such as antioxidant activity.

Progression of oxidative damage and genotoxic implications

Having established that EOD occurs in testis, we were further interested in investigating its progression over four weeks. We aimed to obtain data on the relationship between LPO and DNA damage in testis *in vivo*, the consequences of oxidative damage on sperm morphology, and their possible implications on fertility.

At the selected optimum dosage (150mg/Kg bw), STZ induced a consistent hyperglycemia (3-4-fold) measured at all sampling weeks. The serum testosterone levels were significantly lower in the STZ administered mice (55% of that of control at week two and 25% of that of control at week four), which are consistent with earlier reports *(Tanaka et a., 2001; Sudha et al., 2000)*. While there was no significant alteration in serum testosterone levels at five days, only a marginal reduction (15%) was observed during the tenth day of STZ post administration.

In the second set of studies, testis sampled at week two and four showed consistent elevation in MDA levels (35 and 110%)and higher ROS levels indicating a clear progression of oxidative damage. Interestingly, testicular mitochondrial fractions appeared to be more susceptible since, we observed higher MDA levels at both sampling times. In general, the susceptibility of mitochondria to the damaging effects of ROS leading to both apoptosis and necrosis under various degenerative disorders is well known (*Halliwell and Gutteridge, 1999*). Both elevated OS and ROS productions have been reported in the mitochondrial membranes of various somatic tissues in diabetic rats (*Kowluru and Abbas 2003; Raza et al., 2004*). Mitochondria are an important source of ROS production and are the primary targets of ROS induced toxicity and cell death. Recent studies (*Raza et al., 2004*) have shown a marked increase in mitochondrial CYP 2E1and GST A44 and

lowered mitochondrial GSH pool in somatic tissues of STZ-induced diabetic rats and opined that altered mitochondrial biosynthetic and metabolic activities may be the contributing factors in T1DM. Enhanced ROS levels in mitochondria, MDA levels, reduced cytosolic GSH levels and significantly reduced activities of AOE (CAT, GPx, GST) observed in testis at week two and four in the present study clearly indicate impairment of mitochondrial ROS metabolism.

In the present study, we also examined the relationship between LPO and DNA damage in testis. For this we chose to quantify DNA damage at week two and four in the testis. The increased LPO in testis was concurrent with significant oxidative damage in testicular DNA at both the sampling times. We also found evidence of significant varying degree of pathological lesions in the testis. Although STZ is a well-known genotoxic agent in *in vitro* cell models, its potency to induce genotoxic effects in germ cells is less studied (*Bolzan and Bianchi, 2002a*). It is shown to be an alkylating agent, inducing DNA strand breaks, alkali labile sites, unscheduled DNA synthesis, DNA adducts, and chromosomal abbreations in various *in vitro* systems. In rodent models, STZ is shown to be a good inducer of micronuclei in mouse bone marrow cells (*Liegibel et al., 1992; Chinnaswamy et al., 1998; Bolzan and Bianchi, 2002b*). However, to the best of our knowledge there are no reports indicating the mutagenic effects of STZ in testis in well-established *in vivo* mutagenicity assays.

With regard to the DNA damaging ability of STZ *in vivo*, in a recent time course study in mice, Imaeda *et al.,* (*2000*) reported induction of DNA damage in liver and kidney as early as one hour after STZ administration and its persistence up to four weeks as measured by comet assay. Our data on testicular DNA damage is consistent with early and late DNA damage reported in the above study. It is quite

logical to speculate that the damaged DNA under such situations can get incorporated into germ cells and it is likely that physiologically normal germ cells could carry a modified DNA that could affect the progenies. Similar hypothesis have been extended earlier in humans (*Fraga et al., 1996*). In view of this possibility, in the current study, we assessed the genotoxic implications measured in terms of incidence of sperms with abnormal heads over an entire spermatogenic cycle (35 days) and frequency of male mediated dominant lethal mutation induction response in such males (5 weeks).

We have earlier shown the susceptibility of ES to diabetes associated OS (*Discussion of chapter 2*). Oxidative damage to DNA has been postulated to have biological implications in mammals (*Ames, 1995*). Under *in vivo* situation, it is difficult to characterize different DNA modifications by oxidative reaction because of rapid metabolism of nuclei acid oxidation products, high repair systems and delayed expression of such modifications. In the present study, we have demonstrated diabetes induced DNA damage *in vivo* in testis by FADU assay, which basically quantifies the strand breaks. These results correlate well with the higher peroxidation state of the testis and suggest the involvement of lipid hydroperoxide derived radicals in inducing DNA damage. The DNA damage may be triggered by two major mechanisms. In the first one, LPO products generated under diabetic condition may play significant role in inducing the DNA damage. Further, iron released due to peroxidation of membranes may play a key role in mediating and adding to the production of free radicals under diabetic situations and propagate the radical mediated pathological process (*Latour et al., 1995*).

In general, LPO products besides inducing breaks in the genome have also been implicated in mutagenicity. Lipid peroxides are known

to decompose into a wide range of non-radical such as aldehydes, keto, hydroxy, epoxy, carboxy and peroxy as well as alkanes and alkenes and many are highly reactive and have been shown to be metabolized to epoxides, compounds known to be DNA alkylating agents with high mutagenic activity. While malondialdehyde, major end product of LPO has been shown to be a potent mutagen in bacterial systems (*Marnett et al., 1985*), both MDA and other aldehydes are shown to be mutagenically active in various cell models *in vitro*.

In spite of strong evidences, which show a good correlation between LPO and genotoxicity under *in vitro* condition, data on the mutagenic implications of OS *in vivo* is lacking. Increases in the number of abnormally shaped sperms or decrease in sperm count or motility are associated with reduced fertility in humans and animals. Sperm morphology assay (SMA) provides an effective method for identifying potential mutagens by assessment of morphological changes in treated animals (*Topham, 1983*). A large number of autosomal as well as sex-linked genes appear to be involved in the coding for sperm head shape. Any alterations in these genes can manifest in development of a sperm with abnormal head. Several compounds have been labeled as germ cell mutagens by employing SMA (*Topham, 1983*). However, in some cases the induced changes in sperm head morphology of treated F_0 male may not be only due to mutation, but may arise from interference with the complex differentiation of the spermatozoa.

In the present study, STZ treatment produced a significant increase in the frequency of abnormal sperms during weeks 1, 2, 3 and 5, which was consistent and progressive as revealed by both light and scanning electron microscopy. These findings need to be interpreted very cautiously since they are likely to be due to different mechanisms. In general, the most plausible explanations for these observations are:

(a) STZ may have a direct effect on the epididymal sperms in the first week (b) Induction of diabetes has a significant effect on the sperm development in the epididymal compartment at least during the first two weeks, (during storage and transit time of 10-12 days) and (c) That post-meiotic stages are more susceptible to diabetes induced oxidative stress. However, further detailed investigations are required which would allow us to delineate the primary effect of STZ on the ES at least during the initial stages of diabetes induction. Further, the observed increase in abnormal sperms during the third week may be a combination effect since by that time leydig cell functions appear to be significantly altered as evidenced by reduced serum testosterone levels and diminished testicular testosterone levels (*Sudha et al., 2000; Tanaka et al., 2001*).

Interestingly, oxidative damage to sperm DNA is not always associated with decreased viability or motility. As a result, sperms containing mutations that arise during spermatogenesis can reach ova and affect the fertility outcome. Therefore, in order to investigate any lethal mutations in sperms, which can lead to embryonic failure due to STZ treatments, DLT assay was conducted. Dominant lethal (DL) mutation assay is a genotoxic assay, which provides a unique opportunity to assess mutations in germ cells (*Ehling, 1977; Dean, 1981*). The term dominant lethal describes a genetic change in a gamete that kills the conceptus early during development. Any induced change that affect the viability of the germ cells themselves or render the gametes incapable of participating in fertilization are excluded. The induction of dominant lethal is determined by the increase in pre-implantations and post implantation loss of embryos in experimental group over the loss in the control group. However, several authors believe that preimplantation loss cannot be taken as good indicator of

DL assay as mother can also contribute to the loss (*WHO, 1985; Muralidhara and Narasimhamurthy, 1996*). Therefore, in the present study we have considered only the 'incidence of post implantation losses as the index of DL type mutations.

The suitability of CFT-swiss mice for investigating the induction of dominant lethal type mutations has been established earlier (*Muralidhara and Narasimhamurthy, 1996; Rajeshkumar and Muralidhara, 1999*). Administration of an acute dose of STZ markedly affected the mating efficiency of males as the percent pregnancies were drastically reduced (control: 83.3 – 91.6% vs. STZ: wk 1: 41.6%; wk 2: 50%; wk 3/4: 66.6%; wk 5: 16.6%). Among the STZ males, the implantation rate was also decreased significantly at all weeks, the degree being highest in wk two. A significant decrease in the LI with a concomitant increase of DI was noticeable during the first three weeks among the STZ-treated males. Higher incidence of DI (DI/litter control 1.0-1.3 vs. STZ: 1.6-2.7) clearly suggested that STZ treatment had a stage specific effect on the post meiotic germ cells. While a 2-fold increase of DI during week one and two is indicative on the effect of sperms, the response during the third week can be interpreted as a direct effect on the spermatids. In general, the various germ cell stages have shown to have different sensitivity to the induction of DL mutations by chemical mutagens (*WHO, 1985*), and the germ cell stage assay depends on the interval between treatment and mating.

In the present study, significant decreases in the pregnancy rate were also observed during the fourth and fifth week. Interestingly, varying degree of pathological lesions was manifested beyond four weeks suggesting altered androgenesis and impaired spermatogenesis. These data corroborate with the earlier reports on structural and functional alterations in leydig cells in STZ-induced diabetic rats (*Orth et*

al., 1979; Paz and Homonnai, 1979). In animal models, various reproductive disturbances such as suppression of mating behavior (*Foglia et al., 1969*), oligospermia (*Paz et al., 1978)* and involution of secondary sex organs have been reported which may at least in part result in depressed levels of testosterone levels in diabetic rats. Earlier authors (*Orth et al., 1979*) have demonstrated various ultra structural changes in leydig cells such as accumulation of lipid droplets, reduced smooth endoplasmic reticulum, myelin like structures in mitochondria and opined that leydig cell dysfunction leads to altered synthesis of androgen. In the present study, we limited ourselves to measurement of oxidative biomarkers up to fourth week since significant testicular atrophy was evident at fourth and fifth weeks.

In conclusion, our data from EOD investigations in the mice model, may be interpreted to mean that the male reproductive milieu (testis and ES) is subjected to early oxidative stress, and is likely to significantly contribute to the development of testicular dysfunction leading to altered steriodogenesis and impaired spermatogenesis. Data on the decreased serum testosterone levels only beyond two weeks, progressive and consistent oxidative damage associated with pathological alterations in the testis, alterations in sperm number/ development and reduced fertility in diabetic males clearly indicate the importance of understanding the potential contribution of oxidative stress mechanisms in diabetes associated male reproductive dysfunction.

SUMMARY

1. Our findings (in the first set of investigations) clearly demonstrate that STZ administration causes early oxidative damage in both testicular and epididymal milieu in adult mice

2. Enhanced testicular MDA and ROS levels, accompanied with marked perturbations in activities of antioxidant enzymes noticed at both 3 and 5 days of post administration suggest induction of marked induction of oxidative stress

3. While AA showed no hypoglycemic effect in diabetic mice in the short term treatment, TAU exhibited marginal hypoglycemic action

4. Oral administration of antioxidants viz., ascorbic acid and taurine (pre and post treatment) in adult mice rendered diabetic significantly reduced lipid peroxidation in testicular homogenates, mitochondria and completely normalized the GST levels and protein oxidation

5. Oxidative impairments in the epididymal sperms were also protected to varying degrees with short-term treatment with AA

6. Marked attenuation of oxidative damage in both testis as well as epididymal sperms were evident following antioxidant supplementation of ascorbic acid (10mg/kg bw/d) and taurine (1g/Kg bw/d) suggesting that STZ-induced EOD is amenability to modulation

7. Our findings during the progressive phase (beyond two weeks), indicate that diabetes induced oxidative damage in male reproductive milieu is consistent and progressive in adult mice

8. Marked reduction of serum testosterone levels were evident only beyond two weeks of STZ administration

9. Significant oxidative damage in testis was accompanied by varying degree of pathological lesions at four and five weeks

10. In mice administered STZ, significant DNA damage in testis and ES was associated with high incidence of abnormal sperms during first week and further (1, 2, 3, 4 and 5 wks) suggesting that the ES are also the targets of diabetes associated oxidative stress

11. In the DL assay, STZ induced significantly higher incidence of dead implantation (DI) in the first week of mating indicating that sperms present in the epididymis were susceptible to STZ induced oxidative stress, which may be interpreted as the primary effect of STZ

12. However, the decreased DI at weeks three and five are a combination effect of reduced testosterone (impaired spermatogenesis) and decreased sperm counts

CHAPTER 4:

AMELIORATION OF DIABETES INDUCED TESTICULAR OXIDATIVE DAMAGE

INTRODUCTION

Involvement of OS and its role in the development of various diabetic complications is well known (*Baynes and Thorpe, 1999)*. Enhanced cellular OS and altered AO pool have been implicated under both clinical and experimental T1DM (*Martin-Gallan et al., 2003; Varvaroska et al., 2004; Martin-Gallan et al., 2005*). There is evidence for multiple pathways of increased generation of ROS in diabetes, which may alter several redox sensitive genes and or cellular signaling pathways (*Brownlee, 2001; Evans et al., 2002)*. Consistent with a role for oxidative mechanisms in the pathogenesis of diabetic complications *in vivo*, numerous antioxidant dietary supplements such as vitamin E, C, NAC, oxerutin, taurine, α-lipoic acid have all been found to attenuate OS in experimental models of diabetes (*Odetti et al., 2003*).

Currently, it is also well known that OS mechanisms are involved in xenobiotic-induced testicular dysfunctions leading to male infertility (*Stohs and Bagchi 1995; Rajeshkumar et al., 2002; Doreswamy et al., 2004; Doreswamy and Muralidhara, 2005*). Further, the free radical theory of male infertility (*Aitken, 1994*) emphasizes the significant role played by OS mechanisms in the development of male infertility. Earlier, few studies have recognized the potential advantages of antioxidant therapy on human reproductive functions (*Luck et al., 1996; Irvine, 1996*; *Lenzi et al., 1998*). Recently the role of antioxidants in the treatment of male infertility in humans has been reviewed (*Agarwal et al., 2004*). The results of clinical trials using antioxidants in the treatment of infertile men remain an issue of controversy (*Ford and Whittington, 1998; Lenzi et al., 1998; Tarin et al., 1998*). Nevertheless, the beneficial effects of antioxidants such as vitamin C, E and β-carotene in male infertility have been demonstrated both *in vitro* and *in vivo*. Some of the salient findings reported were: improvement in sperm quality in smokers

following oral doses of vitamin C (*Dawson et al., 1992*); improvements in sperm motility in asthenozoospermic; by oral doses of vitamin E patients (*Suleiman et al., 1996)*. Interestingly, GSH treatment protected against LPO and dyspermia in human spermatozoa (*Lenzi et al., 1993; Lewis et al., 1997*).

Currently, there has been a growing interest to identify natural antioxidants from various plant sources since they are known to provide valuable degree of protection against several oxidant related diseases including diabetes (*Yeh et al., 2003*). Numerous pharmaceutical interventions based on biological antioxidants have been carried out (*Oberley, 1988; Maritim et al.,* 2003). While many of these antioxidants have been demonstrated to offer protection against OS in somatic organs, data on the ability to protect testis, the male reproductive organ are very limited.

It is logical to hypothesize that, if OS constitutes an important etiological factor in the development of male reproductive dysfunctions under diabetic situations, antioxidants may play a significant role in the amelioration of oxidative damage in the reproductive milieu. Further, as we have gathered evidences (chapter 2 and 3), which show that testis and ES are indeed subjected to significant OS during early and progressive phases, the possibilities of modulating this phenomenon employing oral supplementation of a polyphenol (viz., ferulic acid) and β-carotene, a caroteniod whose role in spermatogenesis is well known) were chosen for our investigation. Another approach attempted was to identify the potential of *Ficus bengalensis* seed powder to ameliorate diabetes associated oxidative damage in testis in growing rats.

ATTENUATION OF DIABETES INDUCED OXIDATIVE DAMAGE IN RAT TESTIS

EXPERIMENTAL DESIGN

In vitro screening of polyphenols and aqueous extracts

Preliminary investigations

Initially, the antioxidant activity of aqueous extracts of plant parts viz., *Ficus bengalensis* (FB), *Brassica alba* (seed powders), *Withania sominifera* (root powder) was assessed in rat liver homogenates. In a preliminary study, of the three, FB showed higher radical scavenging ability and hence was selected for further studies. In addition, the antioxidant activities of selected standard polyphenols viz., ferulic acid (FA), protocatechuic acid (PCA), quercetin (Q) and caffeic acid (CA) were also evaluated deploying rat liver homogenate system. Of the four, FA was selected for *in vivo* studies owing to its higher antioxidant activity.

Induction of LPO in testicular homogenates and explants

Initially, using ferrous ascorbate system (Fe-As), pattern of LPO induction was assessed in both testicular homogenates and explants. To obtain the concentration response, both whole homogenates (WH) and testicular explants (TE) (50mg of seminiferous tubules) were exposed to Fe-As for 30min at three concentrations (25, 50 and 100µM of ferrous sulphate; 0.1mM of ascorbic acid) and the LPO assay was performed in duplicate after 30 min of incubation.

Antioxidant activity of polyphenolic acids in vitro

Employing whole homogenates of testis, the potency of four polyphenols viz., FA, Q, PCA, CA to inhibit Fe-As induced lipid peroxidation was studied. For this study, aliquots of testicular

homogenates were co-incubated with aqueous extracts of polyphenols at concentrations ranging from 25-100μM and Fe-As radical generating system. Following 30min incubation at 37°C, the degree of inhibition of LPO was determined by quantification of TBARS levels. Further, the degree of protection offered by the compounds was quantified by measuring the percent decrease in LPO over prooxidant treatment.

Antioxidant activity of *Ficus bengalensis* (FB) *in vitro*

Total phenolic content of FB extracts

Total phenolic content of the aqueous extracts (cold and hot water) of FB powder was determined using gallic acid as the standard and the polyphenol content in the extracts was expressed as mg gallic acid equivalents /g powder.

Scavenging of DPPH radicals

Aliquots of aqueous extracts (cold and hot water) of FB (100mg/ml) were tested for the free radical scavenging activity employing the DPPH system. DPPH solution was incubated with aqueous extracts (0.5mg-2mg) and ethyl alcohol for 20min at room temperature. The absorbance of this reaction mixture was read at 517nm. Radical scavenging activity was measured as a decrease in the absorbance of DPPH and scavenging effect (%) was calculated.

Inhibition of Ferrous-ascorbate induced lipid peroxidation in testis

Testicular homogenates were pre-incubated for 3-5min with freshly prepared aqueous extracts of FB at 1, 2 and 4mg equivalents and then exposed to Fe-As (50μM) radical generating system for 30min at 37°C. The degree of inhibition of induced LPO was determined by quantification of TBARS levels, and the protection offered by the compounds was quantified by measuring the percent decrease in LPO over prooxidant induced levels.

Attenuation of Diabetes associated oxidative damage *in vivo*

Preliminary studies

Initially, growing rats (5 wks) were administered an acute dosage of STZ (60mg/kg bw), and the hyperglycemic response was measured at the end of 72h. Owing to a poor hyperglycemic response at this dosage, a higher dosage of 90mg/kg bw was employed for rendering the rats diabetic.

Determinative studies

Two sets of experiments were conducted in growing rats to assess the attenuation of diabetes induced testicular oxidative damage. For comparison, hepatic tissue was also subjected to analysis.

Modulation of testicular oxidative damage by oral supplements of ferulic acid (FA) and β-carotene (BC)

Following administration of STZ (90mg/kg bw), blood was drawn from retro-orbital plexus and fasting blood glucose levels were monitored. Rats with glucose levels >200mg% were selected for the study. They were randomly distributed into 6 groups as follows:

Group I: control; Group II: FA; Group III: BC; Group IV: STZ;

Group V: STZ + FA; Group VI: STZ + BC.

Rats were given oral supplements of FA and BC (20mg/kg bw) on alternate days for a period of four weeks (a total of 15 supplements) and were maintained on a commercial pellet diet (M/s Lipton India Ltd., India). Terminally, all the rats were sacrificed under light diethyl ether anesthesia. Blood was drawn by cardiac puncture for separation of serum. Organs (Liver and Testis) were excised, rinsed in physiological saline, blotted, weighed and processed. The following biochemical indices of oxidative damage were determined in testis and liver in order to ascertain the modulatory potency of oral supplements: TBARS and

ROS levels, Reduced GSH content, protein carbonyls, selected enzymic antioxidants and lipid/fatty acid profiles.

Modulation of testicular oxidative damage by dietary *Ficus bengalensis* (FB)

In a separate study, following administration of STZ (90mg/kg bw), fasting blood glucose levels were monitored in rats and those showing blood glucose levels > 200mg% were selected and randomly distributed into four groups as follows:

Group I: control; Group II: FB (2%); Group III: STZ; Group IV: STZ + FB (2%).

Weighed amounts of diet (control and FB supplemented) were fed to the rats for a period of four weeks. The food intake of rats was monitored by weighing the residual diet each day. At weekly intervals, body weights were recorded and terminally, the rats were sacrificed under light diethyl ether anesthesia. Blood was drawn by cardiac puncture for separation of serum. Organs (Liver and Testis) were excised, rinsed in physiological saline, blotted, weighed and processed. The modulatory effects of FB seed powder on the diabetes-induced oxidative damage were assessed employing the following selected markers: TBARS and ROS levels, reduced GSH content, protein oxidation, enzymic antioxidants and lipid/fatty acid profiles.

RESULTS

Screening of polyphenols and FB extracts *in vitro* study

Ferrous-ascorbate induced lipid peroxidation in rat testis: in vitro

Our preliminary studies indicated that ferrous-ascorbate at a concentration of 50µM induced a significant increase in LPO during a 30min incubation period in both testicular whole homogenate (TH) and testicular explant (TE) models. Further, a time-course study showed an optimum LPO response following a 30 min exposure (data not shown).

For the concentration-response study, a total of three concentrations viz., 25, 50 and 100µM of ferrous-ascorbate were tested in both the models. In the TH model, the lower concentration (25µM) induced only a moderate increase (75%) in LPO, while higher concentrations (50 and 100µM) induced nearly 4-6-fold increase in LPO response over the basal levels (Fig. 4.1). Further, in the TE model, ferrous-ascorbate (25µM) induced a 65% increase over control levels. However, nearly 3-4.5-fold increase in LPO response was observed at 50 and 100µM respectively.

Modulation of Fe-As induced LPO by polyphenols

Co-incubation of whole homogenates with polyphenols viz., FA, CA, Q and PCA resulted in a marked inhibition of Fe-As induced LPO (Fig. 4.2). In general, all phenolics induced a concentration-dependent inhibition of LPO. With FA, the degree of protection ranged between 40-80%. At the highest concentration, both FA and quercetin offered 80% protection. The order of protection was FA>Q>CA>PCA.

Total phenolic content of Ficus bengalensis extracts

The total phenolic content of FB determined employing gallic acid as the standard ranged from 8.2-8.9mg of GA equivalents/g powder.

There was only a marginal difference in the activity among the cold and hot water extracts

DPPH scavenging activity of Ficus bengalensis

The pattern of scavenging of DPPH radical by both cold and hot water extracts of FB powder is shown in Fig.4.3. A concentration dependent scavenging of DPPH radical was evident. The DPPH radical scavenging activity ranged between 40-80% at the concentrations of 0.5-2mg equivalent of FB powder.

Modulation of LPO by FB in testicular fractions

Data on the pattern of protection offered by FB in various testicular fractions are presented in Fig 4.4. In general FB offered significant protection against induced LPO in all the three fractions. In the mitochondrial fraction, all the concentrations showed 40-45% protection, while in WH and microsomal fractions, a concentration-dependent protection against LPO was evident. The degree of protection by FB in these two fractions ranged from 15-45% (WH) and 12-22% (Mic).

Attenuation of oxidative damage by Ferulic acid and β-carotene

Effects on growth, plasma glucose and testis weights

Data on the plasma glucose levels, absolute body weights and testicular weights of control and various treatment groups are presented in Table 4.1. Terminally, the average body weight gain among STZ administered rats was significantly lower (30g) compared to that of control rats (100g). The plasma glucose levels in STZ rats were significantly elevated (1.5 - 2 fold) compared with controls. Oral supplementation with FA or BC *per se* had no obvious effect on the blood glucose levels and also did not alter the blood glucose levels in STZ rats. Further, both FA and BC did not significantly affect the body

weight gain of diabetic rats and also had no appreciable effect on the testicular weights.

Effects on testicular TBARS and ROS levels

In general, oral supplements of FA or BC to non-diabetic rats had no appreciable effect on various oxidative indices since the values were highly comparable to those of non-diabetic controls. Induction of diabetes caused a significant increase in TBARS as well as ROS levels in both testis and liver (Table 4.2). In testis, STZ caused a marked (98%) elevation in MDA levels. Interestingly, with FA supplementation, there was a dramatic decrease in the MDA levels in testis (100% protection), while in liver a 56% protection was evident (Fig 4.5). Further, BC supplements in diabetic rats also markedly reduced (81%) the testicular MDA levels, while the degree of protection was marginal (13%) in liver.

ROS levels in testis of diabetic rats were significantly enhanced (28%) compared with non-diabetic rats (Table 4.2). With FA supplements, the ROS levels were completely normalized in testis (100% protection) and significantly reduced in liver (46% protection) (Fig.4.5). Correspondingly, BC supplements also resulted in complete normalization of testicular ROS levels (100% protection), while only a marginal decrease was evident in hepatic ROS levels.

In mitochondria of testis, the ROS levels were markedly enhanced (63%) in STZ administered rats compared to controls (Table 4.2). Interestingly, FA supplements resulted in a marked (79%) protection in testis and marginal protection (18%) in liver against STZ-induced increase in the ROS levels (Fig. 4.5). On the other hand, BC supplements offered only a marginal protection in testis (17%) and liver (28%) against the diabetes-induced increase in ROS levels.

Effects on testicular GSH and protein carbonyl content

In general, FA or BC supplements *per se* had no measurable effect on the testicular or hepatic GSH levels in non-diabetic rats. In diabetic rats, testicular GSH levels were significantly decreased when compared with non-diabetic rats (Fig.4.6 Upper panel). FA supplementation in diabetic rats significantly offset the decrease in both the testis and liver GSH levels. A similar pattern of protection was also evident with BC supplementation in diabetic rats. In general, both the antioxidants completely normalized the GSH levels in testis and liver of diabetic rats.

FA and BC supplements marginally decreased the protein carbonyl (PC) content in testis of non-diabetic rats. The PC content of both testis and liver were significantly elevated in diabetic rats (Fig. 4.6, lower panel). Interestingly, in diabetic rats both the antioxidants significantly reduced the testicular PC levels (FA: 43%; BC: 68%). Similarly, the PC levels in liver were markedly reduced (FA: 51%; BC: 42%) in diabetic rats.

Effect of antioxidant enzyme activities

In non-diabetic rats, FA or BC supplements did not appreciably alter the testicular GST activity since the activities were *on par* with those of controls (Fig 4.7, upper panel). Further, the supplements had no effect on the STZ- induced increase (27%) in GST activity in testis. However, in liver, both the supplements significantly normalized the STZ-induced changes in GST activity. In testis, supplementation of non-diabetic rats with FA or BC marginally (11%) enhanced testicular GPX activity (Fig 4.7, Lower panel). While STZ–induced a significant decrease in testicular GPX activity, both FA and BC supplementation significantly normalized the diabetes-induced decrease in the GPx activity.

Effect on testis lipid profile

Serum lipids: The serum lipid profile of rats given oral supplements of FA or BC to both non-diabetic and diabetic groups is presented in Table 4.3. In general, both FA and BC had a significant hypocholeserolemic effect (30%) in non-diabetic rats as well as diabetic rats. However, the serum of diabetic rats showed significantly higher (19%) cholesterol levels compared to non-diabetic group. In general, supplementation of FA or BC dramatically lowered the elevated cholesterol levels in diabetic rats. Interestingly, the cholesterol levels were brought down by both treatments to those of FA/BC treated non-diabetic rats.

Supplementation of FA or BC *per se* reduced the TG levels (25 and 28% respectively) compared to the non-diabetic rats. However, a significant increase (33%) was observed in the phospholipid (PL) levels among the diabetic rats (Table 4.3). The elevated PL levels were markedly reduced by oral supplementation of FA (86%) and BC (88%) among the diabetic rats.

Testis lipids: The testicular lipid profile of rats given oral supplementation of FA or BC is presented in Table 4.3. In general, there was a marginal increase (21%) in the total cholesterol levels in diabetic rats compared to the non-diabetic rats. However, supplementation of FA or BC to diabetic rats significantly reduced (69% and 47%) the cholesterol levels compared to non-diabetic rats. Correspondingly, a significant increase (66%) in the TG levels was evident among the diabetic rats. Supplementation of FA or BC significantly lowered (82% and 41%) the enhanced levels in diabetic rats. However, no significant alterations were evident in the testicular PL levels among diabetic and non-diabetic animals with or without FA or BC supplementation.

Effects on fatty acid composition

Serum: The serum fatty acid profile of growing rats supplemented with FA or BC is given in Table 4.4. Among diabetic rats, the level of 18:2 and 20:4 were found to be decreased by 31% and 38% respectively. However, this reduction in the level of 18:2 and 20:4 was not observed in diabetic rats either fed with FA or BC. The P/S ratio in STZ-treated group was significantly lowered compared to all other groups.

Testis: The fatty acid profile of testis in rats supplemented with FA or BC in STZ induced diabetes is presented in Table 4.5. FA and BC supplementation to non-diabetic rats had no measurable effect on the testicular fatty acid profile. However, significant decrease in the levels of 18:2, 20:4 and 22:5 were observed in diabetic rats. In contrast, FA or BC supplementation to diabetic animals resulted in a significant increase in the level of 18:2, 20:4 and 22:6 fatty acids.

Attenuation of testicular oxidative damage by *Ficus bengalensis*

Effect on plasma glucose, body and testis weights

In general, supplementation of FB to both non-diabetic and diabetic rats had no effect on the plasma glucose levels (Table 4.6). There was a significant increase (2 fold) in the plasma glucose levels of diabetic rats. Likewise, supplementation of FB *per se* had no effect on the body weight gain pattern in non-diabetic rats. There was a significant reduction (32%) in the body weight gain among the diabetic rats compared to non-diabetic rats. Dietary supplementation of FB marginally improved (20%) the weight gain pattern in diabetic rats. Conversely, the testicular weights of diabetic rats were significantly reduced (18%) compared to the non-diabetic rats. Supplementation of FB to diabetic rats did not have any appreciable effect on the testis weights.

Effect of FB on testicular and hepatic MDA and ROS levels

In general, FB supplementation *per se* had no effect on the MDA levels in testicular homogenates of non-diabetic rats. FB supplementation offered varying degree of protection against STZ-induced oxidative damage (Fig. 4.8). Significant elevation (90%) in the testicular MDA levels was evident in diabetic rats while supplementation with FB significantly lowered (44%) the enhanced testicular MDA levels in diabetic rats. However, the elevated hepatic MDA levels were normalized (100% protection) by FB supplementation to the diabetic animals. Dietary FB significantly lowered the diabetes-induced elevated ROS levels in both testicular and hepatic sub-cellular fractions. Diabetes induced a significant increase in testicular (39%) and hepatic (48%) ROS levels. FB supplementation to diabetic rats offered a significant reduction in the testicular (38% protection) and hepatic (83% protection) ROS levels. Likewise, mitochondrial ROS levels were reduced (testis: 36% protection; liver: 100% protection) in diabetic rats supplemented with FB. A similar pattern of protection was observed in microsomal ROS levels of both testis (61%) and liver (82%) among diabetic rats supplemented with FB.

Effect on testicular GSH and Protein carbonyl content

Testicular and hepatic GSH levels were marginally decreased (13% and 18%) in diabetic rats compared to that of non-diabetic rats (Fig. 4.9). Supplementing diabetic rats with FB normalized both the testicular and hepatic GSH levels.

Likewise, FB marginally reduced the PC content in both testis (18%) and liver (9%) in non-diabetic rats. FB supplementation to diabetic rats significantly reduced the testicular (85%) and hepatic (16%) protein carbonyl levels (Fig. 4.9).

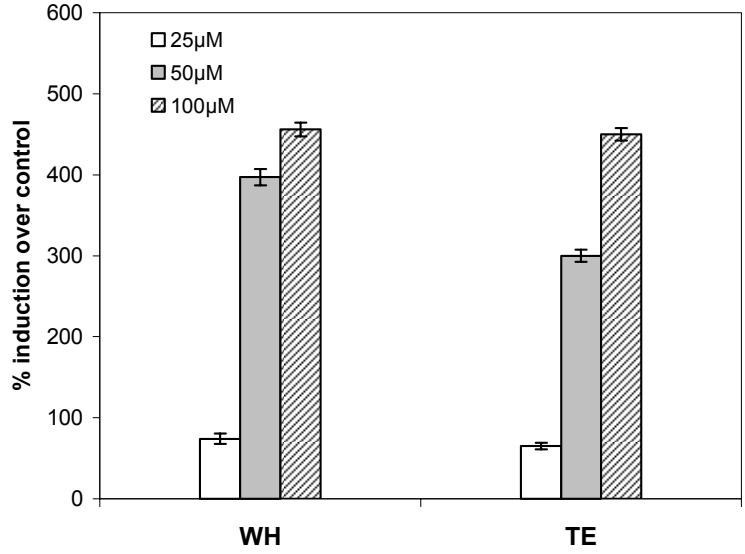
Effect on testicular antioxidant activities

The effect of dietary FB on the activity of enzymic antioxidants is depicted in Fig 4.10. In general, dietary FB *per se* improved the activities of testicular antioxidant enzymes viz., CAT, GST, GPx and GR in non-diabetic rats. Significant decreases in GPx (32%), GR (25%), CAT (48%) and increase in GST (30%) activities were evident in diabetic rats. FB supplementation to diabetic rats significantly improved GST (43% protection), GPx (65% protection), GR (72% protection) and CAT (19% protection) activities.

Effect on Testis lipid profile

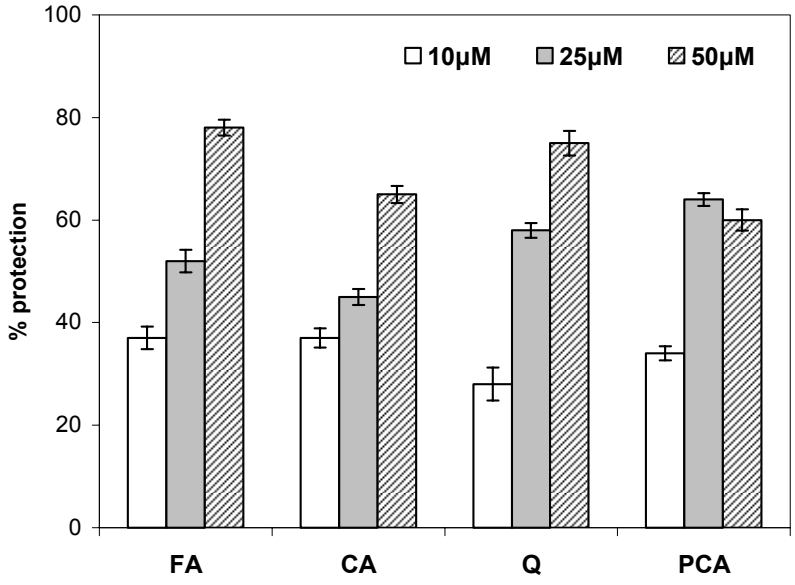
In general, dietary intake of FB *per se* reduced (28%) the cholesterol levels compared to the controls. The total cholesterol levels in diabetic rats were found to be elevated by 26% compared to non-diabetic rats (Table 4.7). Supplementation of FB to diabetic rats normalized the elevated cholesterol levels. Likewise, the triglyceride and phospholipid levels in diabetic rats were significantly increased (22 and 11%) compared to non-diabetic rats. Supplementation of FB to diabetic rats normalized the elevated levels of TG and phospholipids compared to non-diabetic rats.

Fig. 4.1: Pattern of ferrous-ascorbate induced lipid peroxidation in rat testicular homogenates and explants *in vitro*



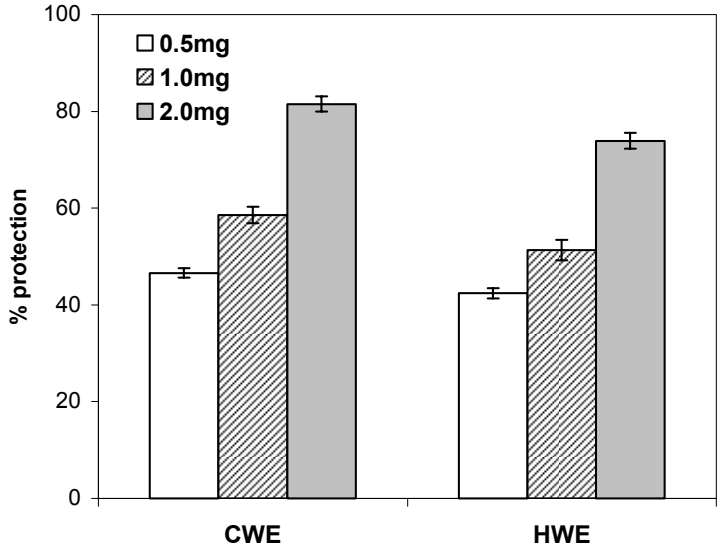
WH: whole homogenate; TE: testicular explants

Fig 4.2: Modulation of Fe-As (50µM)-induced LPO in rat testicular homogenates by polyphenols *in vitro*



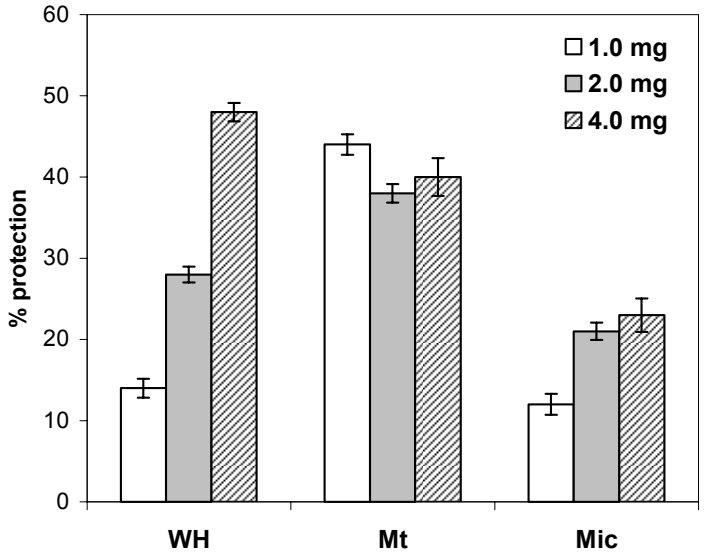
FA: ferrulic acid; CA: caffiec acid; Q: quercetin; PCA: protocathechuic acid

Fig. 4.3: DPPH radical scavenging activity of aqueous extracts of FB powder *in vitro*



CWE: cold water extract; HWE: hot water extract

Fig. 4.4: Modulation of Fe-As (50µM)-induced LPO in rat testis *in vitro* by *Ficus bengalensis*



WH: whole homogenates; Mt: mitochondria; Mic: microsomes

Table 4.1: Effect of oral supplementation of ferrulic acid (FA) and β-carotene (BC)on plasma glucose, body weights and testicular weights of growing rats rendered diabetic by STZ

Parameters	CTR	FA	BC	STZ	STZ + FA	STZ + BC
Glucose (mg%)	157.5^{a} ± 7.66	150.5^{a} ± 5.40	159.5^{a} ± 5.50	502.2^{c} ± 12.62	421.4^{bc} ± 26.14	454.5^{bc} ± 28.43
Body weight (g)						
Initial	118.5 ± 4.50	105.5 ± 3.50	105.2 ± 2.80	106.5 ± 2.90	106.0 ± 2.90	105.7 ± 2.50
Final	208.25 ± 12.75	228.25 ± 12.75	187.5 ± 5.50	135.80 ± 27.9	131.40 ± 27.72	138.75 ± 32.33
Testis weight (g)						
Absolute	2.16 ± 0.11	2.35 ± 0.11	2.46 ± 0.04	1.63 ± 0.63	1.50 ± 0.79	1.48 ± 0.71
Relative	1.03 ± 0.86	1.02 ± 0.68	1.31 ± 0.07	1.20 ± 0.25	1.14 ± 0.84	1.06 ± 0.19

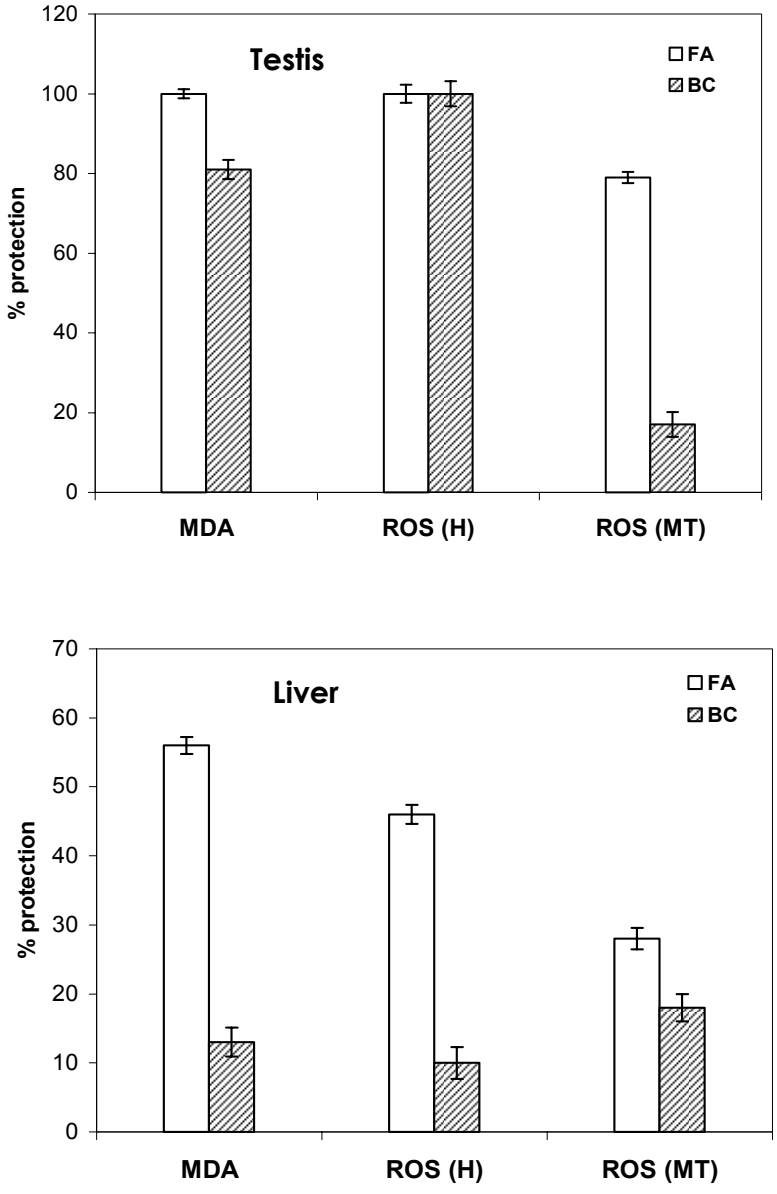
Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 4.2: Effect of oral supplementation of ferrulic acid (FA) and β-carotene (BC) on testicular and hepatic TBARS and ROS levels in growing rats rendered diabetic by STZ

Parameter	CTR	FA	BC	STZ	STZ + FA	STZ + BC
MDA (homogenates)						
Testis	3.75^{a} ± 0.75	4.04^{a} ± 0.50	3.59^{a} ± 0.31	7.45^{b} ± 0.67	3.29^{a} ± 0.71	4.44^{a} ± 0.28
Liver	4.61^{a} ± 0.57	4.07^{ab} ± 0.63	4.72^{a} ± 0.59	7.89^{c} ± 1.26	6.06^{abc} ± 1.80	7.45^{bc} ± 1.07
ROS (homogenates)						
Testis	3.09^{ab} ± 0.39	3.14^{ab} ± 0.57	2.79^{ab} ± 0.22	4.03^{b} ± 0.45	2.72^{a} ± 0.65	3.02^{ab} ± 0.87
Liver	8.01^{a} ± 1.78	8.17^{a} ± 0.59	8.13^{a} ± 0.90	9.57^{b} ± 3.13	8.85^{a} ± 1.32	10.43^{a} ± 3.01
ROS (Mitochondria)						
Testis	3.21^{a} ± 0.18	3.32^{a} ± 0.51	3.26^{ab} ± 0.23	5.23^{c} ± 1.55	3.63^{a} ± 0.20	4.89^{bc} ± 0.69
Liver	8.77^{a} ± 1.04	8.23^{ab} ± 1.58	8.34^{b} ± 1.90	11.66^{ab} ± 0.88	11.13^{ab} ± 3.33	10.86^{ab} ± 1.20

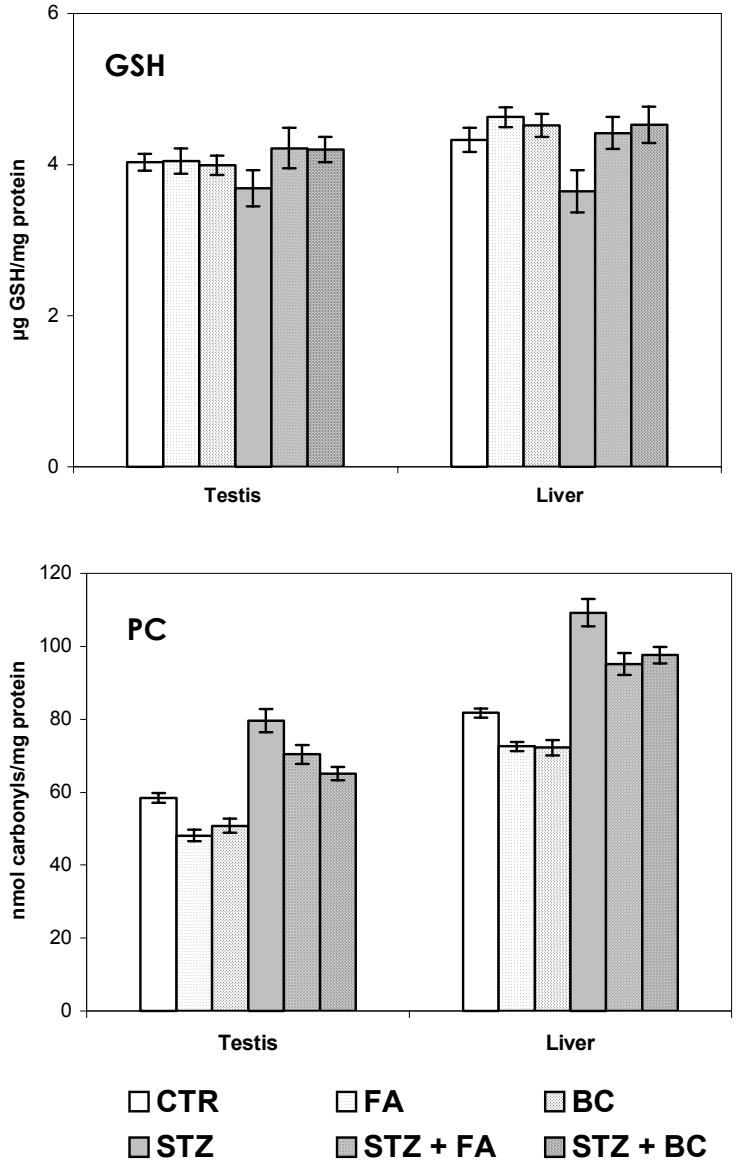
MDA: nmol /mg protein; ROS: pmol DCF/mg protein/min
Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Fig. 4.5: Percent protection offered by ferrulic acid (FA) and β-carotene (BC) against testicular and hepatic oxidative stress in growing rats rendered diabetic by STZ



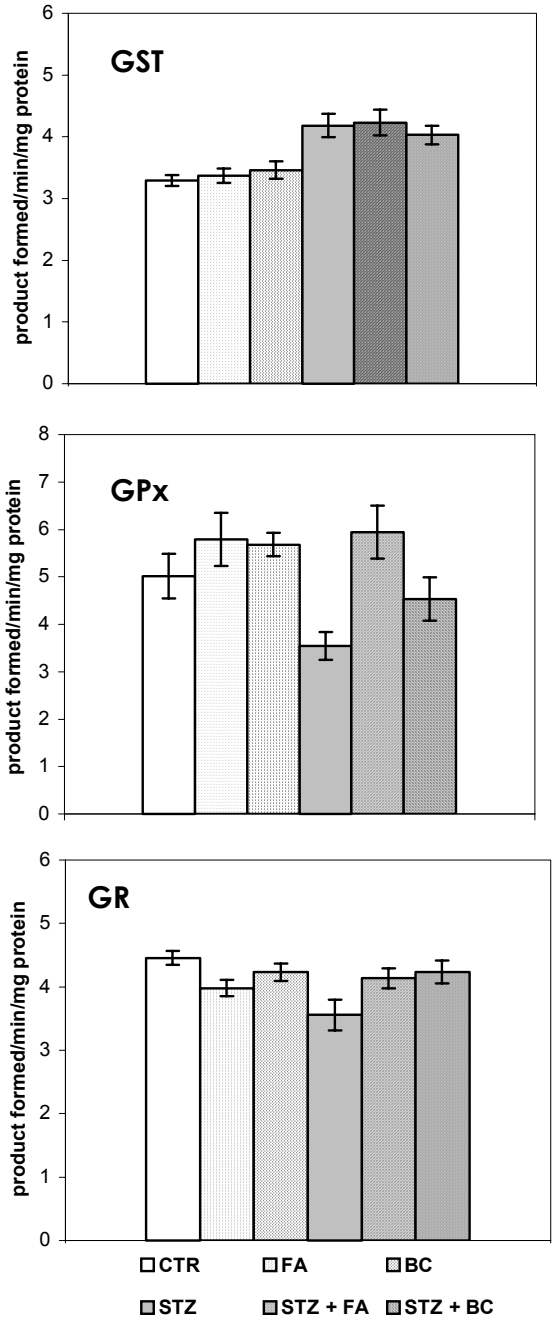
Values are mean ± SD (n=4)

Fig. 4.6: Effect of oral administration of ferrulic acid (FA) and β-carotene (BC) on GSH and protein carbonyl content in testis and liver of growing rats rendered diabetic by STZ



Values are mean ± SD (n=4); Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Fig. 4.7: Effect of oral administration of ferrulic acid (FA) and β-carotene (BC on the activities of GST, GPx and GR in testis of growing rats rendered diabetic by STZ



Values are mean ± SD (n=4); Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 4.3: Effect of oral administration of ferrulic acid (FA) and β-carotene (BC) on serum and testis lipid profile in STZ-treated rats

Parameter	CTR	FA	BC	STZ	STZ + FA	STZ + BC
Serum Cholesterol	68.85^{b} ± 3.46	47.97^{a} ± 3.64	45.67^{a} ± 6.45	81.97^{c} ± 3.80	53.80^{a} ± 6.91	47.11^{a} ± 2.87
Triglycerides	79.44^{c} ± 3.78	59.53^{a} ± 2.83	57.08^{a} ± 4.56	94.29^{d} ± 3.33	64.88^{b} ± 6.19	58.37^{a} ± 2.43
Phospholipids	86.87^{a} ± 5.90	91.37^{bc} ± 2.39	84.75^{bc} ± 6.71	115.9^{d} ± 10.37	91.00^{cd} ± 15.39	90.37^{ab} ± 8.54
Testis Cholesterol	3.58^{a} ± 0.38	3.14^{a} ± 0.30	3.35^{a} ± 0.31	4.32^{b} ± 0.44	3.81^{a} ± 0.41	3.97^{a} ± 0.20
Triglycerides	4.16^{a} ± 0.36	4.67^{ab} ± 0.46	4.64^{ab} ± 0.45	8.25^{c} ± 0.53	5.27^{b} ± 0.30	7.77^{c} ± 0.43
Phospholipids	4.90^{a} ± 0.35	4.47^{a} ± 0.45	4.44^{a} ± 0.46	5.39^{ab} ± 0.38	5.94^{bc} ± 0.48	5.54^{c} ± 0.31

Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 4.4: Effect of oral administration of FA and BC on serum fatty acid composition (mg %) of growing rats rendered diabetic by STZ

Fatty acid	CTR	FA	BC	STZ	STZ + FA	STZ + BC
16:0	28.43 ± 1.81	28.18 ± 1.00	28.25 ± 0.39	29.80 ± 1.34	29.14 ± 1.36	30.38 ± 1.02
16:1	1.61 ± 0.18	1.34 ± 0.18	1.44 ± 0.04	1.16 ± 0.39	1.91 ± 0.60	1.12 ± 0.21
18:0	9.80 ± 1.15	7.12 ± 3.39	9.63 ± 0.77	11.54 ± 0.85	10.36 ± 1.41	11.59 ± 1.51
18:1	21.72 ± 3.06	27.06 ± 0.63	27.31 ± 1.25	32.38 ± 3.18	29.25 ± 1.37	26.04 ± 0.96
18:2	18.75 ± 3.06	15.57 ± 0.43	15.57 ± 1.09	12.96 ± 1.58	12.16 ± 0.91	14.61 ±1.53
20:4	19.02 ± 3.06	17.08 ± 1.57	15.89 ± 0.09	11.76 ± 2.66	16.82 ± 1.29	16.60 ± 0.25
SFA (S)	38.23	35.30	37.88	41.34	39.50	41.97
PUFA (P)	37.77	32.65	31.46	24.76	28.98	31.21
P/S	0.98	0.92	0.83	0.59	0.73	0.74

Values are mean ± SD (n=4)

Table 4.5: Effect of ferrulic acid and β-carotene on testis fatty acid composition (mg %) of growing rats rendered diabetic by STZ

Fatty acid	CTR	FA	BC	STZ	STZ + FA	STZ + BC
16:0	28.84 ± 1.73	29.29 ± 0.71	28.64 ± 1.45	30.79 ± 2.85	32.98 ± 3.00	33.42 ± 0.92
16:1	2.90 ± 0.08	2.62 ± 0.51	2.15 ± 0.29	1.92 ± 0.24	1.56 ± 0.34	1.73 ± 0.36
18:0	8.21 ± 0.61	8.37 ± 0.14	8.80 ± 0.76	8.11 ± 0.41	8.22 ± 0.11	8.41 ± 0.23
18:1	14.24 ± 0.36	11.49 ± 6.50	13.49 ± 0.81	13.84 ± 0.80	13.36 ± 0.59	13.53 ± 0.42
18:2	13.04 ± 1.06	12.72 ± 0.48	14.03 ± 0.19	10.26 ± 1.65	12.99 ± 1.23	11.31 ± 1.04
20:4	18.92 ± 1.85	19.8 ± 1.0	18.8 ± 0.93	15.81 ± 1.69	17.02 ± 0.39	17.21 ± 0.21
22:5	13.0 ± 0.45	14.67 ± 0.40	13.64 ± 0.54	8.94 ± 0.05	9.83 ± 1.33	10.91 ± 0.55
SFA (S)	37.05	37.66	37.44	38.90	41.20	41.83
PUFA (P)	44.96	47.19	45.47	40.98	39.84	36.46
P/S	1.21	1.25	1.21	1.05	0.96	0.87

Values are mean ± SD (n=4)

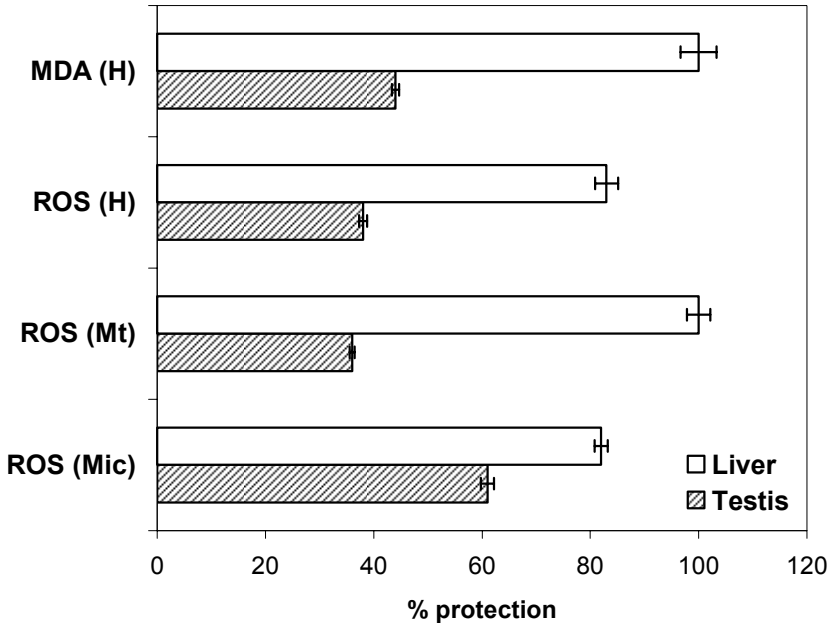
Marginal decrease observed in 20:4 in the STZ administered with or without FA/BC

Table 4.6: Effect of dietary *Ficus bengalensis* seed powder on plasma glucose, body and testicular weights in STZ–treated rats

Parameter	CTR	FB	STZ	STZ + FB
Glucose (mg%)	155.33^{a} ± 14.73	155.33^{a} ± 33.07	360.33^{b} ± 30.76	342.50^{a} ± 82.43
Body weight (g)				
Initial	145.6 ± 5.38	148.0 ± 5.71	145.8 ± 7.88	150.75 ± 8.40
Final	231.33^{c} ± 30.80	238.66^{c} ± 23.47	158.33^{a} ± 36.57	173.25^{b} ± 45.43
Testis weight (g)				
Absolute	2.51^{b} ± 0.23	2.78^{b} ± 0.06	2.05^{a} ± 0.36	1.95^{a} ± 0.49
Relative	1.08 ± 0.43	1.16 ± 0.12	1.29 ± 0.15	1.12 ± 0.23

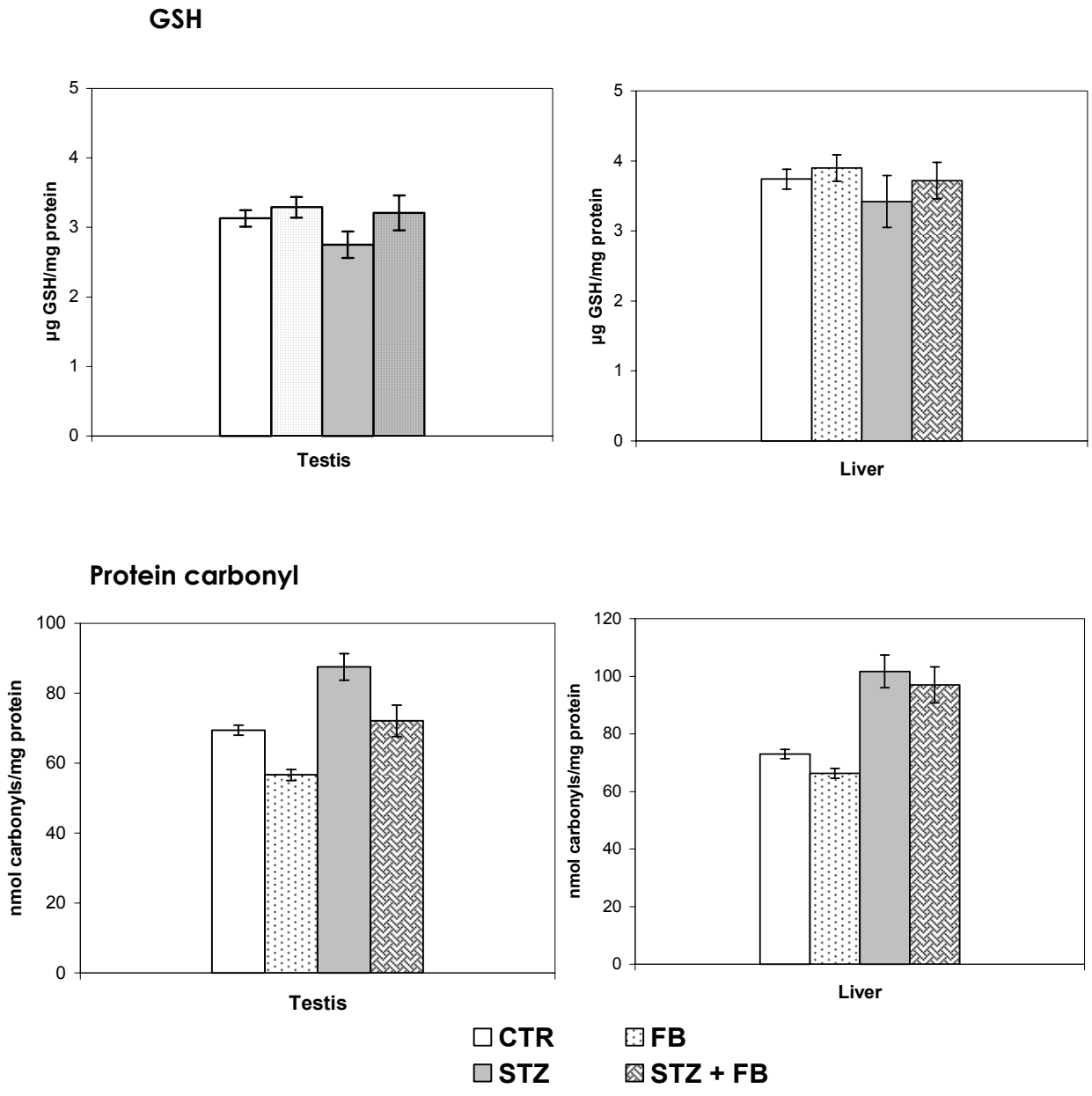
Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Fig. 4.8: Protective effect of dietary of *Ficus bengalensis* (FB) on testicular/hepatic MDA and ROS levels in growing rats rendered diabetic by STZ



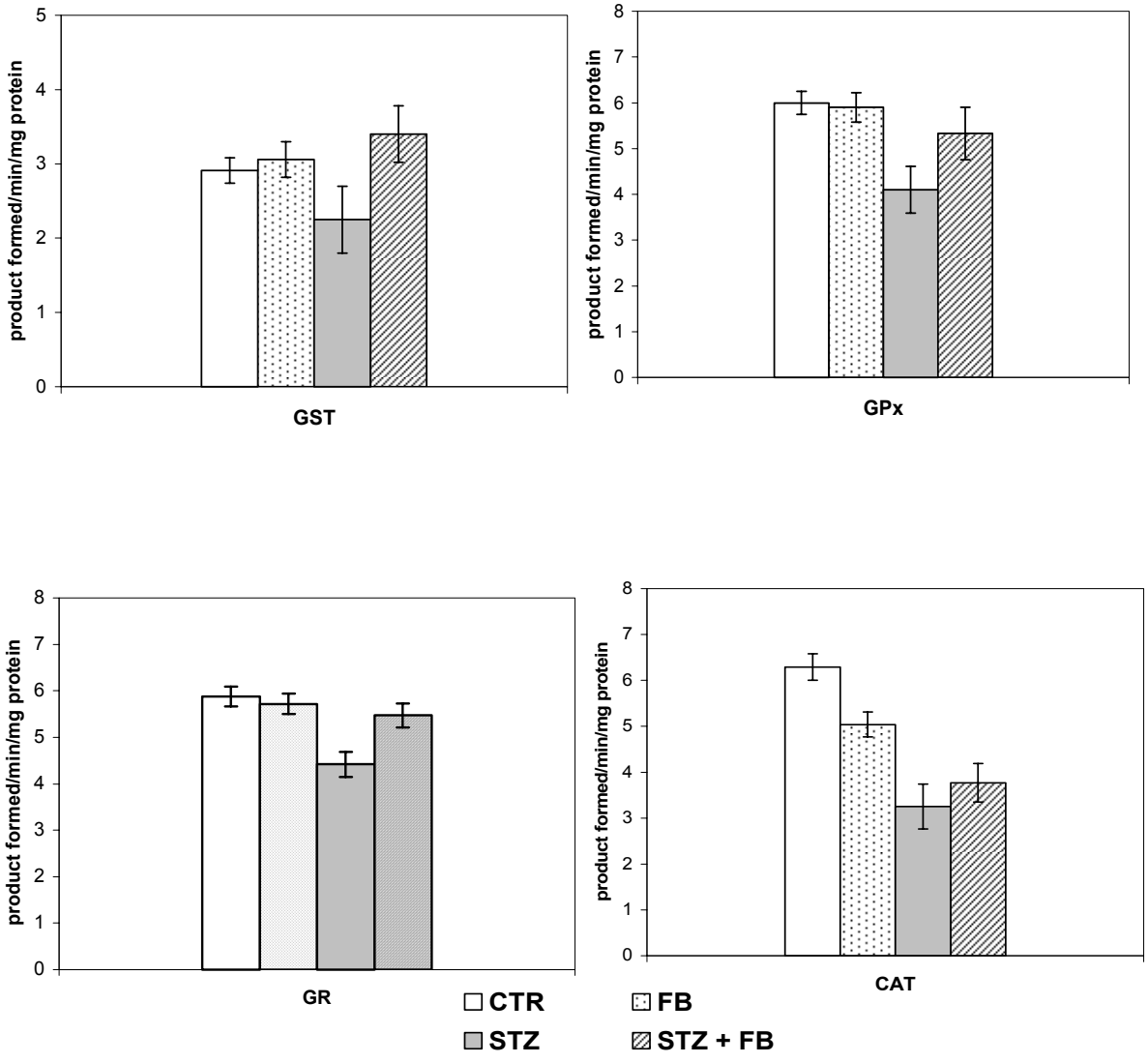
Values are mean ± SD (n=4).
H: homogenates; Mt: mitochondria; Mic: microsomes

Fig. 4.9: Effect of *Ficus bengalensis* on GSH and protein carbonyl content in testis and liver of growing rats rendered diabetic by STZ



Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Fig. 4.10: Effect of dietary *Ficus bengalensis* on the activity of testicular antioxidant enzymes of growing rats rendered diabetic by STZ



Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

Table 4.7: Effect of dietary *Ficus bengalensis* on testis lipid profile in STZ-treated growing rats rendered diabetic by STZ

Group	Cholesterol	Triglycerides (mg/g tissue)	Phospholipid
CTR	3.57 ± 0.23^{b}	4.97 ± 0.37^{a}	4.14 ± 0.18^{b}
FB	2.58 ± 0.29^{a}	4.68 ± 0.19^{a}	5.05 ± 0.18^{d}
STZ	4.50 ± 0.18^{c}	6.06 ± 0.22^{b}	4.60 ± 0.34^{c}
STZ + FB	2.82 ± 0.34^{a}	4.74 ± 0.09^{a}	3.88 ± 0.40^{a}

Values are mean ± SD (n=4)
Data analyzed by ANOVA: mean with different superscripts are significantly different from each other (α=0.05)

DISCUSSION

The primary aim of this study in growing rats was to examine the possibilities of ameliorating diabetes induced testicular oxidative damage employing ferulic acid (FA), a well-known phenolic acid commonly found in fruits and vegetables, and demonstrated to have antioxidant property both *in vitro* and *in vivo*. In addition, to obtain a comparative data, β-carotene, a well-known lipid soluble antioxidant was also included in the study.

Recently, much attention is focused on the protective function of naturally occurring antioxidants in biological systems (*Grover et al., 2002; Scott and King, 2004*). Phenolic compounds, widely distributed in plants, have been considered to play an important role as dietary antioxidants for the prevention of oxidative stress associated with diabetes (*Gomez et al., 1995; Kobayashi et al., 2001*). FA, a ubiquitous phenolic acid shows good antioxidant property against protein oxidation (*Graf, 2000*) reduces free radical damage in neuronal cells (*Kanski et al., 2002*), possess antihypertensive (*Suzuki et al., 2002)* and hypolipidemic (*Balasubashini et al., 2004*) properties. The beneficial effects of FA on sperm viability, motility and oxidative damage to sperm membranes in both fertile and infertile individuals have been demonstrated (*Zheng and Zhang, 1997*). Recently, few investigators have also reported hypoglycemic activity of FA in STZ-induced diabetic conditions in both rats (*Balasubashini et al.,* 2004) and mice (*Ohnishi et al., 2004)*.

In the present study, oral supplements of FA over a month (15 administrations, on alternate days) induced only a marginal decrease in blood glucose levels in STZ-administered rats. In contrast, earlier workers showed significant hypoglycemic action of FA following long-term

administration or dietary feeding (*Balasubashini et al., 2004*; *Ohnishi et al 2004*). FA supplements *per se* had no significant effect on any oxidative stress biomarkers such as endogenous MDA, ROS and GSH levels in non-diabetic rats excepting for a significant hypocholesterolemic effect in testes (30% decrease). Interestingly, FA marginally decreased the basal levels of testis protein carbonyls in both diabetic and non-diabetic rats.

In diabetic rats, FA offered varying degrees of protection against STZ-induced testicular oxidative damage. While it completely decreased the MDA and ROS levels in testicular homogenates to a normal range, the mitochondrial ROS levels were markedly diminished. However, FA appeared to protect hepatic lipid peroxidation to a lesser degree, since only moderate degree of protection (46-56%) was evident in terms of both MDA and ROS levels. Our findings in hepatic tissue are consistent with recent reports, which show that FA significantly alleviated STZ-induced lipid peroxidation in rat liver (*Balasubashini et al., 2004)* and brown adipose tissue of mice *(Ohinishi, et al., 2004)*. Our major finding that FA alleviates testicular oxidative damage under diabetic situations is a new finding. However, the underlying mechanism/s by which FA protects oxidative damage is not clear.

Several mechanism/s can be speculated to explain the mode of action by which FA supplements could ameliorate STZ-induced oxidative damage *in vivo*. FA and its esters are shown to be powerful free radical scavengers (e.g., DPPH radicals, peroxyl and hydroxyl radicals) in chemical systems (*Kikuzaki et al., 2002*) and cell models (*Kanski et al., 2002*). Although the data on the potency of FA to modulate oxidative stress *in vivo* are limited, few recent reports showed enhanced activity of antioxidant enzymes (CAT, GPx, SOD) in liver following oral administration of FA at dosages of 10 and 40 mg/kg bw for 45 days.

GSH and its oxidized counterpart GSSG constitute a major redox buffer system. GSH can act as a non-enzymic antioxidant by direct interaction of –SH group with ROS or it can be involved in the detoxification reaction for ROS, as a cofactor or coenzyme. In the present study, testis GSH levels in diabetic rats were significantly depleted indicating the importance of this non-enzymic antioxidant. Interestingly, FA supplements however, restored the GSH levels to normal endogenous levels. A similar trend was also evident in the hepatic tissue of diabetic rats suggesting that the protective ability of FA could be partly ascribed to this effect. Furthermore, in this study, the levels of testicular enzymes such as GR and GPX were also significantly reduced in diabetic rats. FA supplements however, markedly restored the GPX and the GR levels in testis of diabetic rats to normal levels suggesting the important role played by these redox enzymes under diabetes induced oxidative stress condition. GR catalyzes the conversion of GSSG to GSH and the enzyme is known to contain one or more sulfhydryl group residues, which are essential for the catalytic activity and are vulnerable to free radical mediated inactivation. It is quite apparent that the decreased activity of testicular GR may be an important factor for the depletion of GSH content in diabetic testis.

On a comparative basis, the degree of amelioration of oxidative damage by FA appeared to be relatively higher when compared to BC supplements with respect to various biomarkers measured in the study. BC is shown to be an effective antioxidant not only against singlet oxygen (*Burton and Ingold, 1984*) but also against lipid peroxidation and the highly destructive hydroxyl radical, which is implicated in many diseases such as cancer and heart disease (*O'Neill and Thurnham, 1998*). Since BC is a lipophilic substance, it exerts its action in hydrophobic environment such as the lipid core of membranes.

In the present study, BC *per se* neither showed any hypoglycemic effect nor had any significant effect on the oxidative stress biomarkers in non-diabetic rats excepting for significant hypolipidemic activity. Interestingly, BC marginally reduced the PC levels in both testis and liver of diabetic as well as non-diabetic rats. In diabetic rats, BC supplements offered varying degrees of protection against STZ-induced testicular oxidative stress. While BC completely normalized (100% protection) the ROS levels in diabetic testis, it offered only 80% protection against testicular lipid peroxidation. However, only marginal protection was evident in the mitochondria in terms of restoration of ROS levels. These findings are consistent with the basic concept that BC can significantly mitigate varying situations of oxidative stress conditions *in vivo* (*Burton and Ingold, 1984; Cooper et al., 1999*). BC has been demonstrated to markedly ameliorate OS induced by xenobiotics (*el-Demerdash et al., 2002, 2004*), radiation (*Manda and Bhatia, 2003*) and diabetes (*Maritim et al., 2002*).

Under experimentally–induced diabetic situations, numerous workers have reported varied protective effects of BC in somatic organs such as liver, kidney, heart and retina (*Mekinova et al., 1995; Aruna et al., 1999; Maritim et al., 2002; 2003; Berryman et al., 2004*). Multiple mechanisms have been suggested to explain the protective action of BC in diabetic situations, which include: (i) the reversal of lipid peroxidation indicating lower levels of damage caused by free radicals, which is attributable to physical quenching of FR, (ii) interaction with BC with GSH pathway and (iii) possible enhancement of antioxidant defenses. In the present study, ameliorative effects observed with BC on testicular oxidative damage could be speculated to involve any of the above three mechanisms.

In summary, our findings have reinforced the conclusions drawn by earlier workers who opined that BC influences the ability of various organs to handle OS (*Maritim et al., 2002, Berryman et al., 2004*). The reversal of elevated LPO in testis (and liver) of diabetic rats support our working hypothesis that antioxidants such as FA and BC can modulate diabetes induced oxidative impairments in testis. However, further experiments are required to identify the extent of benefits provided with these antioxidants to different cell types of testis. Furthermore, since BC is shown to confer greater protection when administered along with other antioxidants such as vitamins C and E, it may be worthwhile to investigate the protective effects of combination of phenolic acids with BC and/or other antioxidant vitamins.

In the present study, we also investigated the potency of dietary FB seed powder to protect against diabetes-induced testicular oxidative damage. One of the major reasons why we chose FB seed for the modulation studies was based on the personal discussion we had with a local practicing ayurvedic doctor who has been prescribing the FB seed powder to human patients with proven oligospermia. His personal observations included improvement in the sperm number among patients following 1-2 months therapy. A variety of dietary antioxidants have been shown to protect different somatic tissues against experimentally induced oxidative damage (*Grover et al., 2002*,) while data on their ability to protect germ cells are limited. Epidemiological studies correlating the intake of various phenolic compound sources have been generally suggestive that green tea, red wine and more generally flavonoid intake protects against various diseases (*Scott and King, 2004*) Significant levels of biologically active compounds present in fruits, seeds and vegetables are believed to

impart health benefits by way of antioxidant, anti-mutagenic and anti-carcinogenic properties (*Singh et al., 2004)*.

FB is an indigenous tree grown all over India to which many medicinal properties have been attributed (*Grover et al., 2002*). Various parts of the plant specially the bark have been shown to possess antioxidant, anti-inflammatory, hypoglycemic and several other therapeutic effects (*Geetha et al., 1994; Daniel et al., 2003; Augusti et al., 1994*). Several flavonoid compounds isolated from the bark of FB have been shown to possess significant antioxidant and hypoglycemic properties *in vitro* and *in vivo*. Oral administration of FB bark extracts is shown to enhance serum insulin levels in both normo-glycemic and diabetic rats (*Achrekar et al., 1991*). Ficus flavonoids have also been shown to have significant hypocholesterolemic effects in both rats and dogs.

In the present study, dietary FB *per se* failed to induce any significant hypoglycemic effect. Further, one month feeding had no significant effect on the oxidative stress biomarkers either in testis or liver of non-diabetic rats. However, in diabetic rats, FB offered varying degree of protection against STZ-induced testicular oxidative damage. While MDA levels in liver were completely normalized (100% protection), the testis showed marked restoration (50% protection). Our other findings in testis viz., moderate modulation in cytosolic ROS levels (38% protection), mitochondria (36% protection) and microsomes (61%) were accompanied by marked alleviation in liver biomarkers such as cytosol ROS levels (83% protection), mitochondrial ROS levels (100% protection) and microsomes (82% protection). More importantly, GSH levels, which were depleted in the testis of diabetic rats, were also restored to normal levels upon FB feeding. Furthermore, the activities of several testicular AOE such as GPX, GST and GR, which were found to be significantly

lowered in diabetic rats, were completely normalized in FB fed rats clearly suggesting that FB may be enhancing the total redox state of the testis. Other major protective effects of FB were related to the hypolipdemic properties since testis lipid constituents viz., cholesterol, triglycerides and phospholipids which were significantly enhanced in diabetic rats were also restored to normal levels upon FB feeding. However, the mechanism by which altered lipid constituents in diabetic rats are restored by FB seed is not clear. Further, the implications of this protection in terms of preventing or restoration of diabetic associated complications in testis needs to be further investigated.

Taken together, our data on and FB seed powder in growing diabetic rats clearly modulation of testicular oxidative damage by ferulic acid, β-carotene emphasize that diabetes–induced oxidative stress in testis is amenable for amelioration by dietary approaches. However, further focused investigations are needed to understand the underlying mechanism/s, which may be operative under *in vivo* situation and also the degree to which diabetes associated testicular dysfunctions can be attenuated.

SUMMARY

1. Our *in vitro* investigations showed that, of all the polyphenols screened, FA showed maximum inhibition of ferrous induced lipid peroxidation in rat testicular homogenates

2. Aqueous extracts of FB seed powder exhibited marked DPPH radical scavenging activity and also significantly inhibited Fe/As induced lipid peroxidation in testicular homogenates

3. Oral administration of both FA and BC failed to show any significant hypoglycemic effect in diabetic rats

4. Oral supplementation of FA to growing diabetic rats markedly ameliorated testicular oxidative damage measured in terms of MDA levels and ROS generation

5. While FA in non-diabetic rats did not affect testicular GSH levels and activities of AOE, FA supplements in diabetic rats completely restored GSH levels (in testis and liver) and normalized the activities of GPx and GR clearly suggesting the involvement of GSH pathway in regulating the diabetes induced testicular OS

6. BC supplements also significantly mitigated the testicular oxidative damage in growing diabetic rats, although the degree of protection was relatively lower compared to that provided by FA supplements

7. FA and BC supplementation reduced the protein oxidation in testis of diabetic rats measured in terms of protein carbonyl content

8. Both FA and BC showed significant hypocholesterolemic effect in testis and serum among both non-diabetic and diabetic rats

9. Dietary FB seed powder (2%) failed to show any hypoglycemic effect in diabetic rats, while it improved the weight gain among diabetic rats

10. FB markedly protected testicular oxidative damage measured in terms of both MDA levels and ROS generation pattern (cytosol and mitochondria)

11. FB also offered varying degrees of protection in terms of restoring GSH levels, normalization of AOE activities and reduction of protein carbonyl formation in testis of diabetic rats

12. FB exhibited marked hypocholesterolemic effect in testis since it completely normalized STZ-induced elevation in cholesterol, phoshpolipids and triglycerides level suggesting its protective action in diabetes induced lipid dysmetabolism

CONCLUSIONS

CONCLUSIONS

1. Our principle findings viz., enhanced lipid peroxidation, higher ROS levels, alterations in key non-enzymic antioxidants (vit E and GSH) and activities of enzymic antioxidants (CAT, SOD, GST, GPx and GR) in testis of adult diabetic rats (rendered diabetic by STZ) clearly demonstrate the occurrence of significant oxidative stress (OS) in the testis during early (week 1) and progressive phase (week 2 onwards).

2. The degree of oxidative damage in testis although marginal during the induction phase, was progressive and consistently higher at week 4 and beyond and was associated with varying degree of pathological lesions (evident beyond 3-4 weeks).

3. Elevation in the protein carbonyl content, marked reduction in the testicular dehydrogenases accompanied with altered lipid metabolism (in terms of increased cholesterol and phospholipids) are indicative of diabetes associated disturbances in testicular physiology.

4. Sperms sampled at various weeks from the epididymis exhibited varying degree of oxidative impairments indicating their vulnerability to diabetes induced oxidative stress. Although speculative, the excessive and repeated OS in the epididymal milieu is likely to affect the post-testicular maturation process of sperms and their subsequent functional competence.

5. Taken together, we interpret our data to mean that excessive oxidative insult in the testis and epididymal milieu in adult rats may participate as well as contribute significantly to the development of various diabetes associated male reproductive dysfunctions and

thereby emphasizes the need and scope for further investigations in this area.

6. Our data in growing diabetic rats are suggestive of the increased susceptibility of the developing testis to oxidative damage as evidenced by consistently enhanced MDA and ROS levels, elevated mitochondrial ROS production and increased formation of protein carbonyls.

7. Depletion of reduced GSH levels and dramatic elevation in testicular GST activity in testis suggested the involvement of GSH pathway in regulation of diabetes associated oxidative stress in developing testis.

8. Hyperglycemia in growing testis was associated with significant perturbations in testicular lipid dysmetabolism and is likely to have direct impact on the testicular steriodogenesis, which merits further studies.

9. Induction of oxidative damage in developing testis was nearly 2-4 fold higher at early sampling times (week 1 and 2) when compared to the moderate damage in adult testis evident at similar sampling times. The dramatic elevation (4.5 fold) of testicular GST suggests the magnitude of oxidative impact occurring *in vivo* and merits further investigation.

10. Our studies clearly demonstrate the induction of early (3/ 5 days) oxidative damage in the testis of diabetic mice model which was detectable in terms of selected biochemical markers such as lipid peroxidation, increased generation of ROS levels, perturbations in GSH levels, alterations (reduction) in antioxidant enzymes and enhanced protein carbonyl levels.

11. The early oxidative damage was not confined only to the testicular milieu, since major oxidative dysfunctions were also discernible in the epididymal sperms during early time points emphasizing the dual targets of diabetes induced OS in the male reproductive system.

12. Interestingly, STZ-induced early oxidative damage in mice was amenable for amelioration by short-term oral treatment with antioxidants viz., AA and TAU. Both antioxidants provided total protection by inhibiting lipid peroxidation and generation of ROS in testicular homogenates/ mitochondria and restoration of antioxidant enzymes to varying degree.

13. During the progressive phase, increased LPO in mice testis was accompanied by decreased serum testosterone levels, reduced sperm numbers and varying degree of pathological alterations which are attributable to a combined consequence of impaired steriodogenesis and spermatogenesis.

14. High levels of ROS in ES correlated with an increased incidence/ magnitude of abnormal sperms and DNA damage, which are indicative of derangements in the regulation of spermiogenesis.

15. Induction of stage–specific DL type mutations during the first three weeks of mating in diabetic male mice suggested that the primary targets are the post-meiotic germ cells (sperms and spermatids). While it is difficult to delineate the primary genotoxic effects of STZ and OS mediated effects, nevertheless, this data is indicative of the wide range of genotoxic consequences likely to occur under diabetic situations.

16. Oral supplementation of ferulic acid to diabetic rats over a 4-week period significantly offset diabetes-induced oxidative damage in testis measured in terms of MDA/ ROS levels, activities of AOE (GR/GPX), GSH levels and formation of protein carbonyls.

17. Supplementation of β-carotene in diabetic rats also markedly mitigated the diabetes-induced oxidative damage in testis as indicated by various biomarkers of oxidative damage measured.

18. Dietary supplementation of FB seed powder to diabetic rats showed no hypoglycemic effect, while it offered significant degree of protection against oxidative stress in both testis and liver, and ameliorated the diabetes associated lipid dysmetabolism in testis.

BIBLIOGRAPHY

REFERENCES

Achrekar S, Kaklij GS, Pote MS and Kelkar SM, (1991), *In vivo*, **5**(2): 143-147

Aebi H, (1984), *Methods Enzymol* **105**: 121-125

Agarwal A and Said TM, (2003), *Hum Reprod Update*, **9**: 331-345

Agarwal A and Said TM, (2005), *B J U Intl*, **95**: 503-507

Agarwal A and Saleh RA, (2002), *Urol Clinc N Am*, **29**: 817-827

Agarwal A, Nallella KP, Allamaneni SR and Said TM, (2004), *Reprod Biomed Online*, **8**(6): 616-627

Aitken RJ, Buckingham DW and West KM, (1992), *J Cell Physiol,* **151**(3): 466-77

Aitken RJ, (1995), *Reprod Fertil Dev*, **7**: 659-68

Aitken RJ, (1999), *J Reprod Fertil*, **115**: 1-7

Aitken RJ, Buckingham DW and Harkiss D, (1993), *J Reprod Feril,* **97**: 441-450

Aitken RJ, Buckingham DW, Harkiss D, Paterson M, Fisher H and Irvine S, (1996), *Mol Cell Endocrinol*, **117**: 83-93

Aitken RJ, Clarkson JS and Fishel S, (1989), *Biol Reprod*, **40**: 183-187

Aitken RJ, (1994), *Reprod Fertil Dev*, **6**: 19-24

Alarcon-Aguilara FJ, Roman-Ramos R, Perez-Gutierrez S, Aguilar-Contreras A, Contreras-Weber CC and Flores-Saenz JL, (1998), *J Ethnopharmacology,* **61**(2): 101-110

Alvarez JG and Storey BT, (1995), *Mol Reprod Dev***, 42**: 334-346

American Diabetes Association, (2000), *Diabetes Care*, **23**: 381-389

Ames BN, (1995), *Proc Natl Acad Sci USA,* **92**: 5258-5265

Anderson JE and Thliveris JA, (1987), *Am J Anat*, **180**(1): 41-48

Aravinda S, Gopalakrishna B, Dey CS, Totey SM, Pawshe CH, Salunke D, Kaur K and Shaha C, (1995), *J Biol Chem*, **270**: 15675-685

Aruna RV, Ramesh B and Kartha VN, (1999), *Indian J Exp Biol*, **37**: 399-401

Aruoma OI, Halliwell B, Hoey BM and Butler J, (1988), *Biochem J,* **256**:251-255

Asada K and Kiso K, (1973), *Eur J Biochem,* **33** (2): 253-257

Augusti KT, Regi Susan D, Cherian S, Sheela CG and Sudhakaran CR, (1994), *Indian J Med Res*, **99**: 82

Babior BM, (1984), *J Clin Invest*, **73**(3): 599-601

Balasubashini M, Rukkumani R, Vishwanathan P and Menon VP, (2004), *Phytotherapy Res*, **18**: 310-314

Balazy M, Iesaki T, Tiang H, Park JL, Kaminski PM and Wolin MS, (1999), *Circulation*, **100**: 1-14

Ball BA, Vo AT and Baumber J, (2001), *Am J Vet Res*, **62**: 508-515

Ballard DJ, Humphrey LL, Melton LJ III, *et al*., (1988), *Diabetes*, **37**:405-412

Bar-On H, Roheim PS and Eder HA, (1976), *J Clin Invest*, **57**: 714-721

Bauche F, Fouchard MH and Jegou B, (1994), *FEBS Lett*, **349**: 392-396

Baynes J, (1991), *Diabetes*, **40**: 405-412

Baynes JW and Thorpe SR, (1996), *Curr Opin Endocrinol*, **3**: 277-284

Baynes JW and Thorpe SR, (1999), *Diabetes,* **48**: 1-9

Benedetti MS, Russo A, Marrari P and Dostert P, (1991), *J Neural Transm Gen Sect,* **86**(3): 191-203

Berlett BS and Stadtman ER, (1997), *J Biol Chem,* **272**(33): 20313-16

Berryman AM, Maritim AC, Sanders RA and Watkins JB III, (2004), *J Biochem Mol Toxicol,* **18** (6): 345-52

Birnboim HC and Jevcak J, (1981), *Cancer Res*, **41**: 1889-92

Blum J and Fridovich I, (1985), *Arch Biochem Biophys*, **240**: 500-508

Bolle P, Evandri MG and Saso L, (2002), *Contraception*, **65**: 313- 315

Bolzan AD and Bianchi MS, (2002a), *Mutat Res*, **7690**, 1-14

Bolzan AD and Bianchi MS, (2002b), *Mutat Res*, **503**: 63-68

Borenshtein D, Ofri R, Werman M, Stark A, Tritschler HI, Moeller W, and Madar Z, (2001), *Diab Metab Res Rev,* **1**7: 44-50

Bradley DE and Minn DB, (1992), *Crit Rev Food Sci Nutri,* **31**: 211-236

Brash AR, (1999), *J Biol Chem*, **274**: 23679-82

Brownlee M, Cerami A and Vlassara H, (1988), *Diabetes Metab Rev*, **4**(5): 437-51

Brownlee M, (2001), *Nature,* **414**: 813-820

Brenner RR, Peluffo RO, Mercuri O and Restelli MA, (1968), *Am J Physiol*, **215**: 63-70

Burton GW and Ingold KU, (1984), *Science*, **224**: 569-573

Cai L, Chen S, Evans T and Deng DX, (2000), *Urol Res*, **28**: 342-347

Cameron DF, Murray FT and Drylie DD, (1985), *Anat Rec*, **213**: 53-62

Capucci MS, Hoffmann ME and Natarajan AT, (1995), *Mutat Res*, **347**: 79-85

Caragay AB, (1992), *Food Technol*, 46: 65-70.

Carlberg I and Mannervick B, (1985), *Methods Enzymol*, **113**: 507-510

Carlsen A, Giwercman N, Keiding and Skakeback NE, (1992), *Br Med J*, **305**: 609-613

Cates W, Farley TMM and Rowe PJ, (1985), *Lancet II*, 596-598

Cerutti PA and Trump BF, (1991), *Cancer Cells*, **3**(1): 1-7

Cerutti P, Ghosh R, Oya Y and Amstad P, (1994), *Environ Health Perspect,* **102**: 123-9

Chance B, Sies H and Boveris A, (1979), *Physiol Rev*, **59**: 527-605

Chen YX, (1993), *Chin Med. J. Engl*, **73**: 276-279

Chinnaswamy N, Fairbairn LJ, Laher J, Willington MA and Rafferty JA, (1998), *Mutat Res,* **416**: 1-10

Comhaire FH, Christophe AB, Zalata AA, *et al*., (2000), *Prostaglandins, Leukotriens and Essential Fatty acids,* **63**: 159-165

Cooper DA, Eldridge AL and Peters JC, (1999), *Nutr Rev*, **57**: 201-214

Costa M, Canale D, Filicori M *et al*., (1994), *Andrologia*, **26**: 355-359

Craig WJ, (1997), *J Am Diet Assoc*, **97**S: 199-204

Creasy DM and Foster PMD, (1991), In: Hand book of toxicologic Pathology, Haschek WM and Rousseaux CG (eds.), Academic press, San Diego, CA, pp. 829-889

Cross CE, Halliwell B, Borish EI, *et al*, (1987), *Ann Intern Med*, **107**: 526-45

Cummins JM, Jequier AM and Kan R, (1994), *Mol Reprod Develop*, **37**: 345-362

Cunningham JJ, Mearkle PL and Brown RG, (1994), *J Am Coll Nutr*, **13**: 344-350

Daar A and Merali Z. Infertility and social suffering: the case of ART in developing countries. In: Vayena E, Rowe P, Griffin D, (eds). Report of a meeting on "Medical, Ethical, and Social Aspects of Assisted Reproduction; 2001 17-21 Sept; Geneva, Switzerland: WHO: 20021,p16- 21

Daniel RS, Devi KS, Augusti KT and Sudhakaran Nair CR, (2003), *Indian J Exp Biol*, **41**(4): 296-303

Dawson EB, Harris WA, Teter MC *et al*., (1992), *Fertil Steril*, **58**: 1034-1039

de Catalfo GE and Gomez Dumm IN, (1998), *Int J Biochme Cell Biol*, **30**: 1001-1010

de Lamirande E and Gagnon C, (1995), *Hum Reprod,* **10**:15-21

Dean BJ, Anderson D and Sram RJ, (1981), In: Comparative Chemical Mutagensis, de Serres FJ, Shelby MD (eds.) New York, pp 487-538

Degenhardt TP, Thorpe SR and Baynes JW, (1998), *Cell Mol. Biol*. **44**, 1139-1145

Desco MC, Asensi M, Marquez E *et al*., (2002), *Diabetes*, **51**: 1118-24

Deutsch J, (1974), *Methods in Enzymatic Analysis*, III edition, vol-III, 190-197

Dinulovic D and Radonjic G, (1990), *Arch Androl*, **25**: 277-293

Dominguez C, Gussinye M and Carrascosa A, (1999), *Diabetes Care*, **22**: 871-73

Doreswamy and Muralidhara (2005), *Toxicology*, **206**: 169-178

Doreswamy K, Shrilatha B, Rajeshkumar T and Muralidhara, (2004), *J Androl*, **25**(6): 996-1003

Dounelly ET, McClure N, Lewis SEM, (1999), *Fertil Steril*, **72**(3): 484-495

Dounelly ET, McClure N, Lewis SEM, (2000), *Mutagenesis*, **15**: 61-68

Driver AS, Rao PSK, William R and Mundy, (2000), *Neurotox Teratol,* 175-181

Du XL, Edelstein D, rossetti L, Fantus JG, *et al.,* (2000), *Proc Natl Acad Sci, USA*, **97**: 12222-12226

Duru NK, Morshedi M and Ohininger S, (2000), *Fertil Steril*, **74**: 1200-1207

Ehling UH, (1977), *Arch Toxicol*, **38**: 1-11

El-Demerdash E, el-Denshary Eel-D, el-didi M, Al-Gharabli N and Osman AM, (2002), *Anticancer Res,* **22**(2A): 977-84

El-Demerdash FM, Yousef MI, Kedwany FS and Baghdadi HH, (2004), *Fd Chem Toxicol*, **42**: 1563-1571

El-Khatib AS, Moustafa AM, Abdel Aziz AA, Al Shababah OA, El-Kashef HA, (2001), *Pharmacol Res,* **43** (3): 233-240

El-Missiry MA, (1999), *Comp Biochem* Physiol, **124**: 233-237

Erickson LC, Bradley MO and Kohn KW, (1978), *Cancer Res*, **38**: 3379-84

Evans JL, Goldfine ID, Maddux BA and Grodsky GM (2002), *Endocr Rev*, **23**: 599-622

Fagot-Campagna A *et al*., (2000), *J Pediatr*, **136**: 664-672

Fisher M, Zuckerman M, Fishkin RE, Goldman A, Neeb M, Fink PJ, Cohen SN, Jacobs JA and Weisberg M, (1984), *J Androl*, **5**: 8-16

Fletcher MJ, (1968), *Clin Chim Acta*, **22**: 303 – 307

Flohe L and Gunzler WA, (1984), *Methods Enzymol*, **105**: 114-121

Flohe L and Otting A, (1984), *Methods Enzymol*, **105**: 93-96

Foglia VG, Rosner JM, Lema BE and Cattaneo PR (1969), *Hormone Metab Res*, **1**: 71-77

Folch J, Lee M and Sloane-Stanley GH, (1957), *J Biol Chem,* **226**: 497 – 509

Ford WCL and Whittington K, (1998), *Hum Reprod*, **13**: 1416-1419

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM and Ames BN, (1996), *Mutat Res*, **351**: 199-203

Fridovich I, *Annu Rev Biochem,* (1995), **64**: 97-112

Frie B, Stocker R and Ames BN, (1988), *Proc Natl Acad Sci*, **85**: 9748-9752

Gagnon C, Iwasaki A, de Lamirande E and Kovalski N, (1991), *Ann NY Acad Aci*, **637**: 436-44

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F and Lenzi A, *Hum Reprod,* (2000), **15**(4): 830-9

Geetha BS, Biju CM and Augusti KY, (1994), *Indian J Physiol Pharmacol*, **38**: 220-223

Georgellis A, Montelius J and Rydstrom J, (1987), *Toxicol Appl Pharmacol*, **87**: 141-154

Gerlach U, (1974), *Methods in Enzymatic Analysis*, III edition, vol-**III**, 112-117

Gil-Guzman E, Ollero M, Lopez MC *et al*, (2001), *Hum Reprod*, **16**: 1922-30

Giugliano D, Paolisso G and Giugliano D, (1996), *Diabetes care*, **19**: 257-67

Glogoswski EA, Tsiani E, Zhou X, Fantus IG and Whiteside C, (1999), *Kidney Intl*, **55**: 486-499

Goldberg HJ, Scholey J and Fantus IG, (2000), *Diabetes,* **49**: 211-219

Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS and Aitken RJ, (1995), *J Androl*, **17**: 276-287

Graf E, (2000), *Free Radic Biol Med*, **28**: 1249-1256

Gressier B, Lebegue S, Brunet C, Luyckx M, Dine T, Cazin M and Cazin JC, *Pharmazie,* (1994), **49**(9): 679-81

Grover JK, Yadav S and Vats V, (2002), *J Ethnopharmacology*, **81**: 81-100

Guthenberg C, Alin P and Mannervik B, (1985), *Methods Enzymol*, **113**: 507- 510

Hagar HH, (2004), *Tox Lett*, **151**: 335-343

Halliwell B, (1995), *Biochem Pharmacol,* **49**(10): 1341-48

Halliwell B and Aruoma OI, (1991), *FEBS Lett,* **281**(1-2): 9-19

Halliwell B and Gutteridge JMC, (1989), *Free Radicals in Biology and Medicine*, 2nd ed. Oxford, Clarendon Press, pp. 126-131

Halliwell B and Gutteridge JMC, (1990), *Methods Enzymol*, **186**: 1-85

Halliwell B and Gutteridge JMC, (1999), *Free Radicals in Biology and Medicine*, 3rd edn, Oxford University Press: Oxford, 31-230

Halliwell B, (1994), *J Roy Soc Med*, **82**: 747-52

Halliwell B, Aeschbach R, Loliger J and Aruoma OI, (1995), *Fd Chem Toxicol,* **33**(7): 601-17

Hanna AN, Sharma HM, Kauffman EM and Newwan HA, (1994), *Phaarmacol. Biochem Behav,* **39**: 505-510

Hansen SH, (2001), *Diab Metab Res Rev,* **17**(5): 330-346

Hassan MY, Alshuaib WB and Fahim MA, (2002), *Endo Res*, **28**(1-2): 49-59

Hayes JD and Pulford DJ, (1995), *Crit Rev Biochem Mol Biol*, **30**: 445-600

Hemachand T and Shaha C, (2003), *FEBS Lett*, **538**: 14-18

Hendin B, Kolettis P, Sharma RK, Thomas AJ Jr and Agarwal A, (1999), *J Urol*, **161**:1831-4

Howland BE and Zebrowski EJ, (1980), *Experientia,* **36**(5): 610-1

Huang YS, Horrobin DF, Manku MS, Mitchell J and Ryan MA, (1984), *Lipids*, **19**: 367-370

Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, *et al*., (1985), *Br Med J*, **291**: 1693-1697

Imaeda A, Kaneko T, Aoki T, Kondo Y and Nagase H, (2000), *Fd Chem Toxicol*, **40**: 979-987

Imlay JA and Linn S, (1986), *Science,* **240**:1302-1309

Irvine DS, (1996), *Rev Reprod*, **1**: 6-12

Isabella DD, Rossi R, Giustarini D, Milzani A and Colombo R, (2003), *Clinica Chem Acta*, **329**: 23-38

Isogawa A, Noda M, Takahashi Y, Kadowaki T and Tsugane S, (2003), *Lancet*, **361**: 703-704

Iwanier K and Zachara BA, (1995), *J Androl,* **16**(5): 441-47

Iwasaki A and Gagnon C, (1992), *Fertil Steril*, *57*: 409-416

Jackobson CF and Miller MG, (1997), *Toxicology*, **123**: 15-26

Jamieson D, Chance B, Cadenas E and Boveris A, (1986), *Annu Rev Physiol,* **48**: 703-19

Jiang Zy, Woollard AC and wolff SP, (1990), *FEBS Lett*, **268**(1): 69-71

Jones R, Mann T and Sherins RJ, (1978), *Fertil Steril*, **31**: 531-537

Kanski J, Aksenova M, Stoyanova A and Butterfield DA, (2002), *J Nutr Biochem*, **13**: 273-281

Karakaya S and Kavas A, (1999), *J Agric Food Chem*, **79**: 237-242

Kashiba M, Oka J, Ichikawa R, Kasahara E, Inayama T, Kageyama A *et al*., (2002), *Free Radic Biol Med*, **33**(9): 1221-30

Kaul N, Siveski-Iliskovic N, Hill M, *et al*., (1996), *Mol Cell Biochem*, **160/161**: 283-288

Kedziova-Kornatowska KZ, Luciak M, Blaszczyk J and Pawlak W, (1998), *Clin Chem Lab Med*, **36**: 771-775

Keenoy B, Vertommen J and Leeuw I, (1999), *Diab Nutr Metab,* **12**: 256-263

Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, *et al*., (2003), *Arch Androl*, **49**(2): 83-94

Kikuzaki H, hisamoto M, Hirose K, Akiyama K and Taniguchi H, (2002), *J Agric Food Chem*, **50**: 2161-2168

Kitagawa T, Owada M, Urakami T and Yamanchi K, (1998), *Clin Pediatr*, **37**: 111-116

Klein R and Duwall ES, (1994), *Endocrinologist* **4**:252–269

Kobayashi H, Larson K, Rakesh K, Sharma, Nelson DR, Evenson DP, Toma H, *et al*., (2001), *Fertil Steril*, **75**: 469-75

Kobayashi Y, Motohashi Y, Miyazaki Y, Yatagai M and Takano T, (1991), *Int J Biometeorol,* **35**(4): 234-8

Kocak G, Aktan F, Canbolat O, Ozogul C, Elbeg S, Yildizoglu-Ari N and Karasu C, (2000), *Diab Nutr Metab*, **13**: 308-318

Kodama H, Yamaguchi R, Fukuda J, Kasai H and Tanaka T, (1997), *Fertil Steril*, **65**: 519-524

Kowluru RA and Abbas SN, (2003), *Inves Opthalmol Vis Sci*, **284**: E1089-E1097

Krolewski AS, Warram JH, Rand LI, Christlieb AR, Busick EJ and Kahn CR (1986), *Diabetes Care*, **9**:443-452

Kuboki K, Jiang ZY, Takahara N, Ha SW, Igarashi M, Yamauchi T, Feener EP, *et al*., (2000), *Circulation*, **101**(6): 676-81

Kutlu M, Nazioglu M, Simsek H, Yilmaz T and Sahap Kukner A, (2005), *Int J Vitam Nutr Res*, **75**(1): 71-80

Lammarrone E, Balet R, Lower AM, Gillot C and Grundzinskas JG, (2003), *Best Prac Res Clin Obst Gynaec*, **17**(2): 211-229

Langenstroer P and Pieper GM, (1992), *Am J Physiol,* **63**:H 257–65

Latour I, Demoulin JB and Buc-Calderon P, (1995), *FEBS lett*, 373: 299-302

Lean ME, Noroozi M, Kelly I, Burns J, Talwar D, Sattar N and Crozier A, (1999), *Diabetes,* **48**(1): 176-81

Lee AY and Chung SS, (1999), *FASEB J*, **13**, 23-30

Lee GL, (1960), Manual of histopathologic staining methods of the Armed forces Institute of Pathology, III edition, American Registry of Pathology, Mcgraw-Hill Book Company, pp. 258.

Lenzi A, Cualosso F, Gandini L, Lombardo F and Dondero F, (1993), *Hum Rep*, **9**: 2044-2050

Lenzi A, Gandini L, Maresca V, Rago R, Sgro P, Dondero F and Picardo M, (2000), *Mol Hum Reprod,* **6**(3): 226-31

Lenzi A, Gandini L and Picardo M, (1998), *Hum Reprod*, **13**: 1419-1421

Levin DE, Hollstein M, Christman MF, Schweirs EA and Ames BN, (1982), *Proc Nat Acad Sci,* **79**: 7445-7449

Levine RL, Garland D, Oliver C, Amici A, Climent I, Lenz A, Ahn B, Shaltiel S and Stadtman E, (1990), *Methods Enzymol,* **186**: 464-478

Lewin A and Laven H, (1997), *Mol Aspects Med*, **18**: S213-239

Lewis SE, Sterling ES, Young IS and Thompson W, (1997), *Fertil Steril*, **67**(1): 142-7

Liegibel U, Tinwell H, Callander RD, Schmezer P and Ashby J, (1992), *Mutagenesis,* **7**: 471-474

Lopez S, Jurisicova A, Sun J and Casper RF, (1998), *Hum Reprod*, **13**: 896- 900

Loven D, Schedl H, Wilson H, Daabees TT, Stegink LD, Diekus M and Oberley L, (1986), *Diabetes,* **35**(5): 503-7

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, (1951), *J Biol Chem*, **193**: 265-275

Luck RM, Jeyaseelan I and Scholes RA, (1996), *Biol Reprod*, **52**: 262-266

Malmezat T, Breuille D, Pouyet C, Mirand PP and Obled C, (1998), *J Nutr*, **128**(1): 97-105

Manda K and Bhatia AL, (2003), *J Envion Biol*, **24**(4): 369-72

Maritim A, Dene BA, Sanders RA and Watkin JB III, (2002), *J Biochem Mol Toxicol*, **16**: 203-208

Maritim AC, Moore BH, Sanders RA and Watkins JB, (1999), *Int J Toxicol*, **18**: 161-166

Maritim AC, Sanders RA and Watkins JB, (2003), *J Biochem Mol Toxicol,* **17**(1): 24-38

Maritim AC, Young AS, Lesan AC, Ndungu SG, Stagg DA and Ngumi PN, (1992), *Vet Parasitol,* **43**(1-2): 1-14

Marnett LJ, Hurd HK, Hollstein MC, Kevin DE, Esterbauer H and Ames BN, (1985), *Mutat Res*, **148**: 25-34

Marshell S, Bocote V, Traxinger RR, (1991), *J Biol Chem,* **266**: 4706 -4712

Martin-Gallan P, Carrascosa A, Gussinye M and Dominguez C, (2005), *Free Radic Res,* **39**(9): 933-42

Martin-Gallan P, Carrascosa A, Gussinye M, Dominguez C, (2003), *Free Radic Biol Med*, **34**(12): 1563-74

Marx JL, (1985), *Science,* **235**: 529-531

Mathis D, Vence L and Benoist C, (2001) Nature, **414**: 792-798

Matsugo S, Yan LJ, Han D, Trischler HJ and Packer L, (1995), *Biochem Biophys Res Commun,* **208**(1): 161-7

Mauer SM, Brown DM, Matas AJ and Steffes MW, (1978), *Diabetes*, **27**: 959-964

Mazilli F, Rossi T, Marchesini M, Ronconi C and Dondero, (1994), *Fertil Steril*, **62**: 862-868

Mc Lennan SV, Heffernan S, Wright L, *et al*., (1991), *Diabetes,* 40(3): 344-8

McGill HC, (1988), *Clin Chem*, **34**(8B): B33-9

Mekinova D, Chorvathova V, Volkovova K, Staruchova M, Grancicova E, *et al*., (1995), *Nahrung*, **39**(4): 257-61

Melo SS, Arantes MR, Meirelled MS, Jordao AA Jr and Vannucchi H, (2000), *Acta Diabetol*, **37**(1): 33-39

Meuleman EJ, (2002), *Int J Impot Res,* **14**:S22-8

Mokrasch LC and Teschike EJ, (1984), *Anal Biochem*, **140**: 506-508

Morrison MR and Smith M, (1963), *J. Lipid Res*, **5**: 600 – 608

Muralidhara and Narasimhamurthy K, (1996), *Lab Animals*, **30**: 138-142

Muralidhara and Narasimhamurthy K, (1996a), *J Fd Safety*, **11**:39-48

Murray FT, Cameron DF and Orth JM, (1983), *Metabolism*, **32**: 141-147

Nathan DM, Singer DE, Godine JE and Perlmuter LC, (1986), *Am J Med*, **81**:837-842

Naziroglu M, (2003), *Biol Trace Elem Res*, **94**(1): 61-72

Nerlich AG, Sauer U, Kolm-litly V, Wages E, Koch M and Schleicher ED, (1998), *Diabetes,* **47**, 170-78

Niki E, (1997), Free radicals in chemistry and biochemistry. In, *Food and Free Radicals,* p. 2. Ed. Hiramatsu *et al*., Plenum Press, New York

O'Neill ME and Thurnham DI, (1998), *Br, J Nutr*, **79**(2): 149-59

Oberley LW, (1988), *Free Radic Biol Med*, **5**: 113-124

Obrosova IG and Stevens MJ, (1999), *Invest Ophtalm Vis Sci*, **40**(3): 680-688

Odetti P, Pesce C, Traverso N, Menini S, Maineri EP, Cosso L, Valentini S, *et al*., (2003), *Diabetes*, **53**: 409-505

Oeda T, Henkel R, Ohmeri H and Schill WB, (1997), *Andrologia*, **29**: 125-131

Ohkawa H, Oshini H and Yagi K, (1979), *Anal Biochem,* **95**: 351-358

Ohnishi M, Matuo T, Tsuno T, Hosada A, Nomura E, Tanguchi H, Sasaki H and Morishita H, (2004), *Biofactors*, **21**(1-4): 315-9

Ohnishi M, Morishita H, Iwahashi H, Toda S, Shirataki Y, Kimura M and Kido R, (1994), *Phytochemistry*, **36**: 579-583

Oleinick, N.L., Chiu, S., Ramakrishman N. and Xue, L, (1986) *Brit J Cancer* **55**(8): 135-140

Ong CN, Shen HM and Chia SE, (2002), *Tox Lett*, **134**: 17-30

Oomah BD and Maaza G, (2000), In:The Wiley Encyclopedia of Science and Technology, 2nd edition, (ed. FJ Francis) New York: Wiley, 1176-1182

Orth JM, Murray FT and Bardin W, (1979), *Anat Rec*, **195**: 415-430

Oubre AY, Carlson TJ, King SR, Reaven GM, (1997), *Diabetologia*, **40**: 614-617

Packer AI, Besmer P and Bachvarova RF, (1995), *Mol Reprod Dev*, **42**(3): 303-10

Palmeira CM, Santos DL, Seiça R, Moreno AJ and Santos MS, (2001), *Am J Physiol Cell Physiol,* **281**:C1023–C1028

Pandey VN, Rajagopalan SS and Chowdhary DP, (1995), *J Res Ayurveda Siddha* **XVI**: 1-14

Pardon OF, Brackett NL, Sharma RK, Kohn S, Lyme CM, Thomas Jr AJ and Agarwal A, (1997), *Fertil Steril*, **67**: 1115-1120

Pardon RS, Dambay A, Saurez R and Mas J, (1984), *Acta Diabetol Lat* **21**: 115-121

Pasqualotto FF, Sharma RK, Kobayashi H, Nelson D, Thomas A Jr and Agarwal A, (2001), *J Androl*, **22**: 316-322

Pasqualotto FF, Sharma RK, Nelson DR, Thomas Jr AJ and Agarwal A, (2000), *Fertil Steril,* **73**: 459-464

Paz G and Homonnai ZT, (1979), *Experientia*, **35**: 1412-13

Paz G, Homonnai ZT, Drasnin N, Sofer A, Kaplan R and Kraicer PF, (1978), *Andrologia*, **10**: 127-136

Peltola V, Huntaniemi I and Ahotupa M, (1996), *Endocrinol*, **137**: 105-113

Peluffo RO, Ayala S and Brenner RR, (1970), *Am J Physiol*, **218**: 669-673

Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, *et al*., (1990), *Mech Ageing Dev*, **51**: 283-297

Pirart J, (1978), *Diabetes Care*, **1**:168-88, 252

Potts RJ, Jefferies TM and Notarianni LJ, (1999), *Hum Reprod*, **14**: 2513-2526

Prince PS and Menon VP, (1999), *J Ethnopharmacology*, **65**(3): 277-281

Purvis K and Christiansen E, (1992), *Annals Med,* **24**:259-72

Quinn M, Angelico MC, Cross A, Gearin G and Warram JH, (1992), *Diabetes* **41**(I 1): 121A-121A

Rajeshkumar T, and Muralidhara (1999), *Mutat Res*, **145**: 145-149

Rajeshkumar T, and Muralidhara (2002), *Toxicol in vitro*, **16**: 675-682

Rajeshkumar T, Doreswamy K, Shrilatha B and Muralidhara, (2002), *Mutat Res*, **513**: 103-111

Ramachandran A, Snehalatha C and Vishwanathan V, (2002), *Curr Sci*, **83** (12): 1471-76

Randerath K, reddy MV and Gupta RC, (1981), *Proc Natl Acad Sci USA,* **78**: 6126-6129

Rao AV and Saha H, (2000), *Free Radic Biol Med*, **10**: 1015-1027

Rauscher PM, Sanders RA, Watkins JB, (2000), *J Biochem Mol Tox,* **14**: 189-194

Raza H, Subbuswamy KP, Robin MA and Avadhani NG, (2004), *Diabetes*, **53**: 185-194

Reddy GP, Prasad M, Sailesh S, Kumar YV and Reddanna P, (1992), *Prostaglandins*, **44**: 497-507

Rhodes MJC, (1996), *Proc. Nutri. Soc*, **55**: 71-384

Riley JCM and Behrman HR, (1991), *Proc Soc Exp Biol Med*, **198**: 781-791

Rolf C, Cooper TG, Yeung CH and Nieschlag E, (1999), *Hum Reprod*, **14**: 1028-1033

Romrell LJ, Bellve AR and Fawcett DW, (1976), *Develop Biol*, 49: 119-131

Rosen M, Kahan E and Derazne E, (1987), *Lab Animals ,* **21**(4): 348-52

Rosen P, Nawroth PP, King G, Moller W, Tritschler HJ and Packer L, (2001), *Diabet Metab Res Rev* **17**: 189-212

Rosengren A, Dotevall A, Wilhelmsen L, Thelle D and Johansson S, (2004), *J Int Med*, **255**: 89-95

Rudel and Morris, (1976), *J Lipid Res*, **14**: 363-366

Said G, Goulon-Goeau C, Slama G and Tchobroutsky G, (1992), *N Engl J Med*, **326**:1257-1263

Sailaja Devi MM and Das SY, (2000), *J Pineal Res*, **29**(2): 108-115

Sakkas D, Mariethoz E, Manicardi GC, Bizarro D, Bianchi PG and Bianchi U, (1998), *Rev Reprod*, **4**: 31-7

Saleh R, Agarwal A, Sharma R, Nelson D and Thomas AJ, (2002), *Fertil Steril*, **78**: 491-499

Saleh RA and Agarwal A, (2002), *J Androl*, **23** (6): 737-752

Sanders RA, Rauscher FM and Watkins JB III, (2001), *J Biochem Mol Toxicol*, **1**5: 143-149

Sanguinetti RE, Ogawak K, Kurohmaru M and Hayashi Y, (1995), *Exp Animals*, **44** (1): 71-3

Santos MS, Santos DL, Palmeira CM, Seica R, Moreno AJ and Oliviera CR, (2001), *Diabet Metab Res Rev*, **17**: 223-230

Schubert SY, Lansky EP, Neeman I, (1999), *J Ethnopharmacology*, **66**(1): 11-17

Schuessler H and Schilling K, (1984), *Int J Radiat Biol,* **45**: 267-281

Scott JA and King GL, (2004), *Ann N Y Acad Sci*, **1031**: 204-213

Seethalakshmi l, Menon M and Diamond D, (1987), *J Urol*, **138**: 190-194

Sen CK, Roy S, Han D and Packer L, (1997), *Free Radic Biol Med,* **22**: 1241-1257

Sexton WJ and Jarrow JP, (1997), *Urology*, 508-513

Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, *et al*., (2002), *Fertil Steril*, **77**: 873-882

Sharma RK and Agarwal A, (1995), *J Androl*, **1**6: 464-8

Sharma RK and Agarwal A, (1996), *Urology*, **48** (6): 835-850

Shetty K, Curtis O, Levin RE, Witkowsky R and Ang W, (1995), *J Plant Physiol*, **147**:447-451

Sierens J, Hartley JA, Campbell MJ, Leathem AJ and Woodside JV, (2002), *Teratogenesis Carcinogenesis and Mutagenesis*, **22**: 227-234

Sikka SC, (2001), *Curr Med Chem*, **8**: 851-62

Singh N, Kamath V and Rajini PS, (2004), *Clinica Chem Acta*, **353**:165-175

Sokol RZ, Okuda H, Nagler HM, Berman N, (1994), *Toxicol Appl Pharmacol*, **124**: 310-316

Snedecor GW, and Cochran, WG. In: 'Statistical Methods', (1971) 6th ed: The Iowa state University Press, Ames, I. A., USA; p299-338

Stadtman ER, (1986). *Trends Biochem. Sci*. **11**:11-12

Stadtman ER, (1990), *Free Radic Biol Med,* **9**(4): 315-25

Stahlberg MR and Hietanen E, (1991), *Scand J Clin Lab Invest,* **51**: 125–30

Stefek M, Sotnikova R, Okruhlicova L, Volkovova K, Kucharska J, Gajdosik A, *et al*., *Acta Diabetol,* (2000), **37**(3): 111-7

Steffes MW, Osterby R, Chavers B and Mauer SM, (1989), *Diabetes,* **38**:1077-1081

Stewart JCM, (1980), *Anal. Biochem,* **104**: 10-14

Stohs SJ and Bagchi D, (1995), *Free Radic Biol Med*, **18**: 321-336

Storey BT, Alvarez JG and Thompson KA, (1998), *Mol Reprod Dev,* **49**(4): 400-7

Suarez G, Etlinger JD, Maturana J and Weitman D, (1995), *Arch Biochem Biophys,* **321**: 209-213

Sudha S, Valli G, Julie PM, Arunakaran J, Govindarajulu P and Balasubramanian K, (2000), *Exp Clin Endocrinol Diabetes*, **108** (1): 14-20

Sukalski KA, Pinto KA, Berntson JL. (1993), *Free Radic Biol Med,* **14**: 57–65

Suleiman SA, Ali ME, Zaki ZM, *et al*., (1996), *J Androl*, **17**: 530-537

Sun JG, Jurisicova A and Casper RF, (1997), *Biol Reprod*, **56**: 602-607

Suzuki A, kagawa D and Fujii A, (2002), *Am J Hypertension*, **15**: 351-357

Tadolini B, Pintus G, Pinna GG, Bennardini F and Franconi F, (1995), *Biochem Biophys Res Commun,* **213**(3):820-6

Tagami S, Kondo T, Yoshida K, Hirokawa J, Ohtsuka Y and Kawakami Y (1992), *Metabolism,* **41**:1053–8

Tanaka M, Nakaya S, Kumai T, Watanabe M, Matsumoto N and Kobayashi S, (2001), *Endocrine Res*, **27**(1&2): 109-117

Tarin JJ, Brines J and Cano A, (1998), *Hum Reprod*, **13**: 1415-1416

Thompson KH and Mc Neil JH, (1993), *Res Commun Chem Pathol Pharmacol*, **80** (2): 187-200

Tisch R and McDevitt H, (1996), *Cell*, **85**: 291-297

Topham JC, (1983), In: Chemical Mutagens, Serred FJD (ed) pp. 201-234

Traverso N, Menini S, Odetti P, Pranzato MA, Cattalosso D and Marinari UM, (2002), *Free Radic Biol Med*, **32**: 350-359

Trounce IA, Kim YL, Jun AS, Wallace DC, (1996), *Methods Enzymol*, **264**: 484-509

Twig J, Fulton N, Gomez E, Irvine DS and Aitken RJ, (1998), *Hum Reprod*, **13**: 1429-1236.

Uchida K and Stadman ER, (1993), *J Biol Chem,* **268**: 6388-6393

Uzel N, Sivas A, Uysal M and Oz H, (1987), *Horm Metab Res*, **19**: 89– 90

Varvarovska J, Racek J, Stetina R, Sykora J, Sykora J, Pomachacova R, *et al*., (2004), *Biomed Pharmacother,* **58**(10): 539-45

Varvarovska J, Racek J, Stozicky F, Soucek J, Trefil L and Pomahacova R, (2003), *J Diabetes Complications*, **17**: 7-10

Verma A and Kanwar KC, (1998), *Andrologia*, **30**: 325-329

Vezina D, Mauffette F, Roberts KD, *et al*., (1996), *Biol Trace Elem Res*, **53**: 65-83

Vincent Am, Brownlee M and Russell JW, (2002), *Ann N Y Acad Sci*, **959**: 368-383

Wang MY, Leihr JG *et al*., (1995), *Arch Biochem Biophys*, **316**: 38-46

Wang X, Sharma RK, Gupta A *et al*., (2003), *Fertil Steril*, **80**(2): 844-850

Waters E, Wang JH, Redmond *et al*., (2001), *Liver Physiol*, **280**: G1274-G1279

Wester KM, Ahuja MMS and Benner PH, (1983), *Diabetes Care*, **6**: 361-369

WHO, (1985), Environmental Health Criteria, No. 51 Guide to short term tests for detecting mutagenic and carcinogenic chemicals, Geneva: WHO, pp. 115-124

Willcox JK, Ash SL and Catignani GL, (2004), *Crit Rev Food Sci Nutr,* **44**: 275-295

Willett W, Manson JE and Liu S, (2002), *Am J Clin Nutr*, **76**: 274S-280S.

Wise JA, (2001), *In: vegetables, fruits and herbs in health promotion* ed. Watson RR, CRC Press, Bota Raton, 152

Wiseman H and Halliwell B, (1996), *Biochem J*, **313**: 17-29

Woolley DM, (2003), *Reproduction*, **126**: 259-270

World Health Organization, (1999), Definition, Diagnosis and classification of diabetes mellitus (Dept. of noncommunicable diseases, Surveillance, Geneva.

World Health Organization, (1999a), Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge, UK: university press.

Wright CE, Tallan HH, Lin YY and Gaull GE, (1986), *Ann Rev Biochem*, **55**: 427-453

Wyngaarden JB, Smith LH and Bennett JC (1992), (eds): *Cecil Textbook of Medicine*, 19th edition. WB Saunders, Philadelphia.

Wyrobeck AJ and Bruce WR, (1975), *Proc Nat Acad Sci*, **72**: 4425-4429

Yaniv Z, Dafni A Friedman J, Palevitch D, (1987), *J Ethnopharmacology,* **19**:145-151

Yatsumoto T, Naitoh T, Shikada K and Tanaka S, (1997), *Jpn J Pharmacol*, **75**: 59-64

Yeh GY, Kaptchuk TJ, Eisenberg DM and Phillips RS, (2003), *Diabetes Care*, **6**(2): 109-126

Yen GC and Chen HY, (1995), *J. Agric Fd Chem,* **43**: 27-32

Yen SSC and Jaffe, RB, (1991), (eds). *Reproductive Endocrinology, Third Edition*. Philadelphia: W.B. Saunders Co.

Yerneri KK, Bai W, Khan BV, Medford RM and Natarajan R (1999), *Diabetes,* **48**: 855-864

Yeung CH, Cooper TG and DeGeyter M, (1988), *Mol Hum Reprod*, **4**: 835-839

Zaspel BJ and Csallany AS, (1983), *Anal Biochem,* **130**: 146-150

Zheng RL and Zhang H, (1997), *Free Radic Biol Med*, 22(4): 581-586

Zimmet P, (2000), *J Intern Med,* **247**: 301-310

Zimmet P, Alberti KGMM and Shaw J, (2001), *Nature*, **414**: 782-787

Zimmet P, *Diabetologia*, (1999), **42**: 499-518

LIST OF PAPERS PRESENTED AT NATIONAL SYMPOSIA

1. **Shrilatha B,** Thyagaraju B M and Muralidhara, 2005
   Oral supplementation of Ferulic acid and beta-carotene mitigates diabetes induced oxidative damage in rat testis, *74th annual meeting of Society of Biological Chemists (SBC), November 7-10, 2005, Lucknow, India*

2. **Shrilatha B**, Thyagaraju B.M and Muralidhara, 2004
   Male-mediated dominant lethal mutations following administration of diabetogenic agent- Streptozotocin in mice. *International conference on role of Free Radicals and antioxidants in Human Health & disease and II Annual conference of Society for Free Radical Research, (SFRR) - India /, Jan 17-19, 2004, Chidambaram, TN, India*

3. **Shrilatha B** and Muralidhara, 2004
   Evidences of testicular oxidative damage in STZ-induced diabetes
   International conference on role of Free Radicals and antioxidants in Human Health & disease and ii Annual conference of Society for Free Radical Research, (SFRR) - India /, Jan 17-19, 2004, TN, India

4. **Shrilatha B**, Doreswamy K and Muralidhara, 2003
   Testicular oxidative stress in streptozotocin administered mice
   International conference on role of Free Radicals and antioxidants in Health & disease and ii Annual conference of society for Free Radical Research,Feb 10-12, Luckow, India.

5. Doreswamy. K, **Shrilatha. B** and Muralidhara 2002
   Susceptibility of testis to metal- induced oxidative stress and associated genotoxic consequences, *Proc. 71h Annual Meeting of Society of Biological chemistry, India (SBC), Nov 14-16, PAU Ludhiana, Punjab, India.*

6. **Shrilatha B**., Chandrashekar K N., Doreswamy. K and Muralidhara, 2002
   Modulation of oxidative stress response in rat hepatic and human erythrocyte membranes in vitro by aqueous extract of *Withania somnifera Proc. 71h Annual Meeting of Society of Biological chemistry, India (SBC), Nov 14-16, PAU Ludhiana, Punjab, India*

7. **Shrilatha. B**, Doreswamy K and Muralidhara, 2002
   Modulation of oxidative stress response in testicular tissue in vitro by aqueous extract of *Ficus bengalensis*, *Proc. 71h Annual Meeting of Society of Biological chemistry, India, Nov 14-16, PAU Ludhiana, Punjab, India*

8. Rajesh Kumar. T, Doreswamy. K, **Shrilatha. B**, and Muralidhara, 2001
   Induction of oxidative stress in male reproductive system of mice- effects of testicular DNA, frequency of abnormal sperms and fertility. *International conference on Natural Antioxidants and Free Radicals in Human Health & Radiation Biology (NFHR-2001) July, 22-25, 2001, organized by Society for Free Radical Research, (SFRR)-India/Asia, BARC, Mumbai, India.*

LIST OF PAPERS PUBLISHED/ TO BE PUBLISHED

1. K. Doreswamy, **Shrilatha. B**, Rajeshkumar T and Muralidhara, 2004
   Nickel induced oxidative stress in testis of mice: evidence of DNA damage and genotoxic effects, ***Journal of Andrology*, *25 (6) 996-1003***

2. Rajesh Kumar T, Doreswamy K, **Shrilatha B** and Muralidhara, 2002
   Oxidative stress associated DNA damage in testis: Induction of abnormal sperms and effects on fertility, ***Mutation Research (GTEM) 513, 103-111***

3. **Shrilatha B** and Muralidhara, 2005
   Evidences of early oxidative damage in testis and epididymal sperms of mice administered Streptozotocin, its response to model antioxidants and associated genotoxic implications
   Diabetes

4. **Shrilatha B** and Muralidhara, 2005
   Evidences for the involvement of oxidative stress mechanisms in diabetes induced testicular dysfunction in rat
   Fertility & Sterility

5. **Shrilatha B** and Muralidhara, 2005
   Oxidative stress response of developing testis under STZ-induced diabetes
   Toxicol Lett

6. **Shrilatha B** and Muralidhara, 2005
   Vulnerability of rat epididymal sperms to oxidative damage in vivo and associated alterations in the antioxidant enzymes under diabetes state
   Fertility & Sterility

7. **Shrilatha B** and Muralidhara, 2006
   Oral supplementation of ferulic acid and beta-carotene mitigates diabetes induced oxidative damage in rat testis
   J Biochem Mol Toxicol

8. **Shrilatha B** and Muralidhara, 2006
   Attenuation of testicular oxidative damage in diabetic rats by dietary supplementation of *Ficus bengalensis*
   Clinica Chemica acta

Plate 1

Photomicrographs of testis (transverse section) of adult rats (H & E):

Control group (Top panel): **1a** (X40); **1b** (X100)
Normal histoarchitecture: Typical appearance of seminiferous tubules showing different cellular associations

STZ group (bottom panel): **1c** (X40); **1d** (X100)
Testis sampled at week 4 of post administration;
Showing typical reduction in the size of seminiferous tubules; loss of interstitial cells and varying degree of spermatogenetic arrest.

Plate 2

Photomicrographs of testis (transverse section) of adult rats rendered diabetic, sampled at various weeks (H & E):

Testis sampled at week 4 and 5: 2a-d (X100)

To show various pathological alterations: shrinkage of seminiferous tubules, varying degree of germ cell depletion, necrosis of spermatogonia and spermatocytes; total germ cell loss; absence of spermatozoa in the lumen.

Plate 3

Photomicrographs of testis (transverse section) of adult rats rendered diabetic, sampled at various weeks (H & E):

Testis sampled at week 4 and 5: 3a-3d (X 400)

To show various pathological alterations at higher magnification
Typical appearance of a seminiferous tubule undergoing various degenerative changes; note thickening of seminiferous epithelium, irregular spermatocytes, multinucleate aggregation; necrotic appearance of germ cells; loss of cellular associations; lumen filled with cell debris and total absence of spermatozoa.

Plate 4

Photomicrographs of testis (transverse section) of growing rats of control and STZ group (H & E):

Control group: **4a** (X 100); **4c** (X 400)

Normal histoarchitecture: Typical appearance of seminiferous tubules showing the different cellular associations

STZ group: 4b (X 100); **4d** (X 400)
Testis sampled at week 3 and 4 showing various pathological alterations: shrinkage of seminiferous tubule; effect on spermatogonia; loss of cellular association; necrosis of round spermatids; absence of active spermatogenesis.

Plate 5

Photomicrographs of testis (transverse section) of adult mice from control and STZ groups (H & E):

Control group (Top panel): 5a (X40); **5b**(X100)
Normal histoarchitecture: Typical appearance of seminiferous tubules showing the different cellular associations

STZ group (bottom panel): **5c** (X40); **5d** (X100)
Testis sampled at week four of post administration;

Showing typical reduction in the size of seminiferous tubules; loss of interstitial cells and varying degree of spermatogenetic arrest.

Plate 6

Photomicrographs of sperms obtained from cauda epididymis of adult mice of the control group (stained with Eosin Y) (X1000):

Control group: 6a and 6b:

Showing normal shape of the head and normal attachment

STZ group (6c- h)

Showing various abnormal head shapes and attachments: note amorphous, hookless, hammerhead forms

Plate 7

Electron micrographs of sperms obtained from cauda epididymis of adult mice (X 8220):

Control group: 7a
Showing normal shape of the head and normal attachment

STZ group (7b - f)

Showing various abnormal head shapes and attachments:
Bent head, Hookless, amorphous, hammerhead, Coiled form, balloon form