OXIDATIVE IMPAIRMENTS IN MACROMOLECULES IN IMMATURE MAMMALIAN TESTIS UNDER EXPERIMENTALLY INDUCED DIABETES: BIOCHEMICAL AND FUNCTIONAL CONSEQUENCES

A Thesis

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by

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under the supervision of

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DECLARATION

I hereby declare that the thesis entitled '**OXIDATIVE IMPAIRMENTS IN IMMATURE MAMMALIAN TESTIS UNDER EXPERIMENTALLY INDUCED DIABETES: BIOCHEMICAL AND FUNCTIONAL CONSEQUENCES**' submitted to the University of Mysore for the award of the degree of **Doctor of Philosophy** in **Biochemistry** is the result of research work carried out by me under the guidance and supervision of Dr. Muralidhara, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore during the period October 2005 – January 2009.

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

CHANDRASHEKAR K.N.

Place: Mysore
Date: May 2009


To my parents


To my parents


To my parents

Dr. Muralidhara
Scientist 'F' (Deputy Director)
Department of Biochemistry and Nutrition

CERTIFICATE

I hereby certify that the thesis entitled **" OXIDATIVE IMPAIRMENTS IN MACROMOLECULES IN IMMATURE MAMMALIAN TESTIS UNDER EXPERIMENTALLY INDUCED DIABETES: BIOCHEMICAL AND FUNCTIONAL CONSEQUENCES"** submitted by **Mr. Chandrashekar K.N** for the degree of **Doctor of Philosophy** in **Biochemistry**, University of Mysore is the result of research work carried out by him at the Department of Biochemistry and Nutrition, CFTRI, Mysore under my guidance and supervision during the period of October 2005 - January 2009.

Dr. MURALIDHARA
Guide

Place: Mysore

Date: May 2009

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Chandrashekar K.N

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LIST OF SYMBOLS AND ABBREVIATIONS

AA	Ascorbic acid
ADP	Adenosine di phosphate
ATP	Adenosine tri phosphate
b.w.	Body weight
CDNB	1-chloro-2,4 di nitrobenzene
Conc.	Concentration
CTR	Control
Cyto	Cytosol
DCF	Dichlorofluorescein
dL	Decilitre
EDTA	Ethylene diamine tetraacetic acid
g	Gram
G6PDH	Glucose 6-phosphate dehydrogenase
GSH	Glutathione
GPx	Glutathione peroxidase
GST	Glutathione –S- transferase
h	Hours
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
i.m	Intra muscular
Kg	Kilo gram
KRBS	Kreb's Ringer Bicarbonate Solution
LDH-X	Lactate dehydrogenase
LPO	Lipid peroxidation
M	Molar
MEC	Molar Extinction Coefficient
MDA	Malondialdehyde
MDH	Malate dehydrogenase
mg	Milli gram
min	Minutes
Mito	Mitochondria
mL	Milli liter
MPT	Membrane permeability transition
N	Normality
n	Number
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide reduced
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate reduced
nm	Nanometer
nmol	Nanomolar
NPSH	Non-protein thiols
O^{2-}	Superoxide
pmol	Picomolar
ROS	Reactive oxygen species
SE	Standard error
SOD	Superoxide dismutase
STZ	Streptozotocin
T1DM	Type1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
tbHP	tertiary butyl hydroperoxide
TSH	Total thiols
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
µ	Micro
p	Pico
˚C	Degree Celsius

ABSTRACT

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and protein with an increased risk of vascular complications. The incidences of diabetes in young men/children are increasing alarmingly over the past decade exposing them to a greater risk of developing the macrovascular and microvascular complications during their prime of their life. Sexual dysfunction is frequently associated with diabetes in men and infertility is a common outcome in diabetic men. Although oxidative stress has been implicated as the primary causative factor, the underlying biochemical mechanism/s that leads to testicular dysfunctions is poorly understood. Further, most of what we know about diabetes associated reproductive dysfunctions in animal models is based on experimentally-induced diabetes in adult rodents (rats/ mice) by either Streptozotocin (STZ) or Alloxan. As a result of which there is a big lacuna in our understanding of the vulnerability of prepubertal testis to diabetes. Given the scenario that diabetes is becoming prevalent in children and adolescents and is likely to increase in phenomenal proportions, it is pertinent to obtain comprehensive insight into the biochemical /.functional impairments and their subsequent implications on steroidogenesis /spermatogenesis during the reproductive period.

Hence, in the present study, attempts have been made to obtain evidence in favor of our central hypothesis that "Immature and developing testis is likely to be more susceptible to oxidative stress under diabetic conditions and the biochemical and functional alterations may have far reaching consequences (transient or permanent) resulting in sub-fertility or infertility". The basic objectives envisaged were (i) To understand the nature, pattern and progression of diabetes induced oxidative damage in immature and developing testis (ii)To elucidate the biochemical and physiological implications in testis and its impact on functional development of testis (iii) To understand the spectrum of oxidative impairments in spermatozoa and its correlations with reproductive outcome and infertility and iv) to ascertain

whether these oxidative impairments are amenable for amelioration by phytochemicals/nutrients.

Streptozotocin administration (STZ, 90mg/kg bw) to prepubertal rats caused marked hyperglycemia together with marked increase in oxidative stress response. The oxidative induction was robust in testis compared to other somatic organs (viz., liver and brain) as evidenced by elevated levels of ROS and MDA, perturbations in the activities of antioxidant enzymes / non-enzymic antioxidants. Further, studies carried out to ascertain vulnerability pattern among two age group PP rats to diabetes induced oxidative stress revealed that the susceptibility increased with downward shift in age (4wk-old > 6wk-old) as evidence by enhanced levels of ROS and MDA, perturbations in antioxidant defenses and functional enzyme activities in both cytosol and mitochondria. Testis mitochondria of diabetic rats also exhibited altered activities of TCA cycle and oxidative phosphorylation enzymes together with loss in membrane potential and mitochondrial membrane leakage. Collectively, these findings clearly demonstrate increased susceptibility of 4wk- old prepubertal testis to diabetes induced oxidative stress.

In prepubertal diabetic rats, the oxidative impairments were discernable in all the major testicular subcellular organelles viz., cytosol, mitochondria and microsomes as evidenced by enhanced levels of ROS and MDA, perturbations in the activities of antioxidant enzymes and non-enzymic antioxidants, elevation in protein carbonyl content and in vital functional enzymes. In testis cytosol, the oxidative response induction was evident during both acute (<5d) and progressive phase (7/14d) while both mitochondria and microsomes exhibited marked oxidative alterations only during progressive phase. Among diabetic PP rats, the activities of functional enzymes such as SorDH and LDH were consistently reduced throughout. However, the activities of ADH and AR were reduced during progressive phase. Diabetic testis further showed robust increase in ascorbate levels, non-protein thiols were significantly elevated at all sampling days in contrast to the decreased tocopherol levels evident only during progressive phase. Further, a progressive decrease in the activities of mitochondrial TCA cycle enzymes such as citrate synthase, succinate dehydrogenase and malate

dehydrogenase were also evident in testis of diabetic PP rats. Further, diabetic testis mitochondria showed a preferential increase in the activity of NADH-cyt C reductase compared to succinate-Cyt C reductase accompanied by loss of mitochondrial potential/integrity. These changes were accompanied by altered activities of cytochrome-p450 enzymes in testis microsomes both during acute and progressive phase. These findings clearly suggest that testis of prepubertal rats is indeed subjected to significant oxidative stress during conditions of diabetes, and the testicular impairments observed during diabetes is a cumulative effect of the oxidative responses elicited by different organelles to varying degrees. Further, data obtained from flow cytometric analysis of diabetic prepubertal testis over one spermatogenic cycle (56days) revealed complete loss of germinal cell population and other cell types. These changes were accompanied by elevated activities of Caspase-3 and increased incidence of DNA fragmentation. Collectively, these observations clearly demonstrates involvement of apoptosis mediated cell death as the underlying mechanism for the higher incidence of testicular degeneration observed during diabetes that leads to decreased fertility.

Results of ameliorative studies with supplementation of D-Aspartic acid (D-Asp, i.p), testosterone (TP, i.p) and *Withania somnifera* (*WS*, oral) showed varying degree of protection in testis of diabetic PP rats. Supplementation with TP or *WS* significantly offset the STZ-induced oxidative impairments as evidenced by reduction in blood glucose levels, lowered levels of ROS/ MDA, restoration of protective thiols and antioxidants defenses in testis (cytosol/mitochondria) of PP rats. In the interactive model, D-Asp administered diabetic rats showed significantly reduced levels of oxidative stress markers together with upregulation in antioxidant defenses and the major steroidogenic enzyme, 3β-hydroxy steroid dehydrogenase (3β-HSD) suggesting the potential of D-Asp to attenuate testicular oxidative stress during diabetic conditions.

CONTENTS

PREFACE TO THE GENERAL INTRODUCTION

In the first part of the thesis titled ‘general introduction’, an attempt has been made to review the relevant literature under six separate sections.

The **first section** gives an overview of oxidative stress and its major complications on human health have been presented comprehensively. The emphasis has been made on the cellular sources of ROS, chemistry and biochemistry of ROS, oxidative damage to biomolecules, antioxidant defense mechanisms and the role of oxidative mechanisms in various diseases including male infertility.

The **second section** focuses on the structure, physiology and functional aspects of mammalian male reproductive system and enumerates various aspects related to male infertility in humans.

Further, in the **third section** a brief account on the role of oxidative stress as an etiological factor in testicular dysfunctions leading to male infertility is also provided.

The **fourth section**, provides a current and general understanding on Diabetes mellitus, types, prevalence of diabetes among children/adolescents, pathophysiology and mechanism/s underlying well known long term complications.

In the **fifth section**, diabetes associated testicular dysfunctions has been discussed briefly.

In the **sixth section,** ameliorative role of phytochemicals/nutrients under conditions of diabetes induced testicular oxidative stress has been presented. In the end, current literature on testosterone, D-Aspartic acid and *Withania somnifera* which were employed in the modulatory studies is presented.

1.0 OXIDATIVE STRESS: A GENERAL OVERVIEW

Free radicals are produced continuously by cells as part of normal cellular functions. About 95% of oxygen consumed by tissues is utilized in metabolic processes, but approximately 5% of the oxygen consumed is transformed into reactive oxygen species (ROS) (Valko et al., 2006). Our body is equipped with efficient enzymatic/non-enzymatic antioxidant molecules that effectively detoxify the free radicals generated during various metabolism. However, under pathological conditions, an imbalance between production and detoxification of free radicals occur either due to increased production of ROS/ their inefficient removal or both resulting in development of 'Oxidative stress ' (Klaunig et al., 1998, Halliwell and Gutteridge, 1999).

Free radicals

Free radicals are defined as molecules or molecular fragments that contain one or more unpaired electrons in atomic or molecular orbitals. The occurrence of unpaired electron results in high reactivity and affinity to donate/obtain another electron to attain stability (Kohen and Nyska, 2002). Accordingly, oxidants are being classified based on their *chemical nature (radical or non radical), production site and reactivity towards biological targets.* The radical group includes oxygen /nitrogen derived free radicals such as superoxide, hydroxyl and nitric oxide, while the non-radical category includes metabolites of oxygen free radicals such as hydrogen peroxide, hypochlorous acid and aldehydes. These free radicals being very reactive can potentially damage the biological architecture either alone or as a source for the generation of more reactive/damaging species (Halliwell and Gutteridge, 2006).

Reactive Oxygen Species (ROS)

The cellular toxicity of oxygen has been attributed to its affinity in accepting electrons from other molecules. Reactive oxygen species category includes both oxygen radicals/oxygen derived non-radicals that possess high energy oxidizing potential eg., superoxide (O_2^{2-}) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO*) and the very reactive hydroxyl (OH*) radicals. Most of

these free radicals including NO and $ONOO^-$ anion have been considered to play a significant role in the pathogenesis of variety of diseases.

Singlet oxygen: Is the more reactive form of oxygen, generated by rearrangement of electrons in presence of high energy input that increases its oxidizing ability (Halliwell, 2006).

Superoxide anion (O_2^{2-}): is a negatively charged free radical produced by a one electron reduction of molecular oxygen either by autooxidation or by the action of various oxidases. Superoxide exists as O_2^{2-} at physiological pH and as hydroperoxyl ion (HO_2^-) at lower pH (Schaffer and Buettner, 2001). Being very reactive, the hydroperoxyl radical, easily penetrates the biological membrane than the charged form (O_2^{2-}) and reduces ferric iron.

The most important reaction of superoxide radical is dismutation, where two superoxide molecules undergoes redox reaction, where one gets oxidized to oxygen and the other being reduced to hydrogen peroxide. Although the rate constant for this spontaneous reaction is low, the reaction becomes faster at acidic pH.

$$O_2 + e^- \xrightarrow{\text{Oxidase}} O_2^{2-}$$

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

Superoxide anion, arising either through metabolic processes or following oxygen 'activation' by physical irradiation, is considered as the 'primary ROS' and can further interact with other molecules to generate 'secondary ROS' either directly or through enzyme/ metal- catalyzed processes (Valko et al., 2005).

Hydrogen peroxide (H_2O_2)*:* is a non-radical form of ROS formed as a result of dismutation of superoxide radicals. H_2O_2 is also synthesized in the reactions catalyzed by various oxidases including amino acid oxidase and ascorbate oxidase. Although H_2O_2 is not a radical by definition, it causes cellular damage relatively at a lower concentration (10μM) and brings about release of iron from heme proteins, inactivation of enzymes, oxidation of DNA, lipids and -SH

groups. Further, it also serves as a source for the generation of OH* and HClO either by reacting with superoxide anion (Haber – Weiss reaction) or with free iron (Fenton reaction) (Halliwell et al., 2000).

$H_2O_2 + O_2^{2-} \text{---------} \rightarrow O_2 + HO + HO^*$ (Haber – Weiss reaction)

$Fe^{2+} + H_2O_2 \text{----------} \rightarrow Fe^{3+} + HO + HO^*$ (Fenton)

Hydroxyl radicals (HO):* are extremely energetic, short lived (10^{-9} sec) and neutral form of the hydroxide ion. Being a powerful oxidizing agent, it can react at a faster rate with most organic/inorganic molecules in the cell including DNA, protein, lipids, amino acids and metals. The toxicity of H_2O_2 and superoxide is often attributed to their conversion into hydroxyl radical. Hydroxyl radicals are formed *In vivo* when metal ions such as iron, copper, chromium and cobalt reacts with hydrogen peroxide.

Nitric oxide radical (NO)*: is a small molecule that contains one unpaired electron on the antibonding 2π* orbital of oxygen. Nitric oxide (NO) is produced by the oxidation of one of the terminal guanido nitrogen atoms of L-arginine by Nitric Oxide Synthase (NOS) that requires NADPH, flavin nucleotides (FMN/FAD), tetrahydrobiopterin, calmodulin, and calcium as cofactors for its activity.

L-Arginine + NADPH --------→ L-citrulline + NO

NOS isoenzymes (nNOS, eNOS and iNOS) responsible for NO biosynthesis are localized in many tissues and share 60% amino acid homology amongst each other. The ultimate effects of NO (prooxidant/antioxidant) depends largely on its concentration/ interaction with the type of free radical/substances that coexists. Overproduction of reactive nitrogen species results in 'nitrosative stress' (Klatt and Lamas, 2000; Ridnour et al., 2004).

Sources of ROS

Exogenous sources: Cellular exposure to ionizing/non-ionizing irradiations constitutes a major source of ROS resulting in the production of a range of

radical/non-radical species due to ionization of intracellular water (Shadyro et al., 2002). Air pollutants such as vehicle exhaust, cigarette smoke, industrial effluents and variety of xenobiotics (eg., toxins, pesticides /herbicides and drugs) produce ROS as a by-product during their metabolism *in vivo.* Another chief source of oxidants is food, as for the large portion of the food we consume gets oxidized generating a variety of peroxides, aldehydes, oxidized fatty acids and transition metals which have proven prooxidant potential (Lijinsky, 1999).

Endogenous sources: Although the exogenous sources play a major role for the increased oxidative damage, the relative impact of endogenous sources of ROS on cellular biochemistry is also high. The major source of free radicals inside the cell originates during ATP production in mitochondria, in the steps that reduce cellular oxygen to water. White blood cells, including neutrophils, eosinophils, basophils, monocytes and lymphocytes, with their mechanisms to combat bacteria and other invaders turn out to be the major sources of ROS (Forman and Torres, 2001; Halliwell and Gutteridge, 2006).

Mitochondria and Microsomes: principal sources of ROS

Among the various organelles in the cell, mitochondria are the major site of ROS generation during normal metabolic processes. During cellular oxidative phosphorylation, the univalent reduction of oxygen results in the formation of water through the mitochondrial cytochrome oxidase system with the generation of ROS. In addition, several other enzymic reactions viz., Xanthine oxidase, aldehyde oxidase, monamine oxidase etc., in mitochondria may also lead to the univalent reduction of oxygen to produce O_2^{2-} and H_2O_2. Sequential reduction of favoproteins, ubiquinone and cytochromes by one electron transfer reactions also results in the generation of ROS (Cadenas and Davies, 2000; Starkov and Fiskum, 2003). The autooxidation of reduced favoprotein by dehydrogenase is another possible source of O_2^{2-} in mitochondria. Superoxide are also formed from both complex I and III of the electron transport chain, which in its anionic form readily crosses mitochondrial membrane (Rolo and Palmeira, 2006) and gets either

dismutated to H_2O_2 or amplifies the oxidative damage by release of cytochrome c into cytosol (Petrusillo et al., 2003; Lambert and Brand, 2004).

Microsomes are the second major source of endogenous ROS generation in the cell. During reactions catalyzed by cytochrome P450 and NADPH dependent dehydrogenases microsomes produce $O_2^{2\cdot-}$ and H_2O_2. Further, Endoplasmic reticulum contains several mixed-function oxidases targeted to oxidize xenobiotics before it gains entry inside the cells. Activation of nucleophiles through their reduction by the favin monooxygenase system is another potential source of ROS in microsomes. A well-balanced SOD system in microsomes converts $O_2^{2\cdot-}$ to H_2O_2 and prevents potential damage caused by ROS. The rate of H_2O_2 production is also influenced by the presence of glycolate oxidase, D-amino acid oxidase and urate oxidase in microsomes (Vallyathan and Shi, 1997).

Peroxisomes consume major portion of cellular oxygen and participate in several metabolic functions that results in the formation of H_2O_2. The organelle is particularly being rich in catalase, decomposes H_2O_2 immediately into water (Valko et al., 2004).

Free radicals induced mitochondrial damage

Although hydrogen peroxide freely diffuses through mitochondria, other radicals such as superoxide and hydroxy radicals are largely restricted inside and are likely to inflict damage directly to inner membrane, the site of their production. Further, the inner membrane lodges many iron/copper complexes, which further amplifies the damage cascade. Owing to their close proximity to the site of ROS production, mitochondrial DNA is particularly more vulnerable to free radical attack. In addition, mitochondrial proteins are easily modified by nitration, s-nitrosylation and S-glutathionylation resulting in altered function (Cadenas and Davies, 2000).

Oxidative stress and damage

Irrespective of the source (endogenous or exogenous), the free radicals generated bring about potential damage to cellular structure, nucleic acids and proteins (Halliwell and Whiteman, 2004).

Oxidative damage to membrane lipids

Owing to the higher degree of unsaturation, cellular membranes are exceptionally susceptible to oxidation. The damage to lipids, usually called as 'lipid peroxidation' occurs in three stages; the first *initiation stage* - involves the abstraction of hydrogen atom from a methylene group by the reactive oxygen metabolite resulting in weakening of the double bond and its subsequent breakage. Subsequently, the remaining fatty acid rearranges to form a conjugated diene, which in presence of sufficient oxygen, forms lipid peroxy radical (LOO*), the *propagation step*. These radicals further abstract hydrogen atom and form lipid hydroperoxides that decompose forming wide range of aldehydes, cyclic endoperoxide/isoprostanes. The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxy2-nonenal (4-HNE) and are shown to be mutagenic in both bacteria and mammalian cells. In the *chain termination* step, interaction of one LOO* with another occurs. However, the lipid radicals formed can diffuse through membranes, modify the structure/ functional integrity.

Oxidative damage to membrane proteins

Protein oxidative modifications induced by ROS or by the byproducts of oxidative stress (Stadtman, 2006) results in either fragmentation of the polypeptide chain, oxidation of amino acid side chains, and/or formation of protein-protein cross linkages (Stadtman and Levine, 2000). Amino acids that are highly susceptible to oxidation include Arginine, Cysteine, Glutamate, Histidine, Leucine, Lysine, Methionine, Phenylalanine and Tyrosine (Cakatay, 2005). ROS mediated oxidation of protein/lipids and glycation reactions result in a) Schiff-base cross linkages, between carbonyl group of one protein with the $N^{€}$- amino group of a lysine of another b) Oxidation of two cysteine residues forming intra-/ inter disulfide cross-linkages c) Michael addition of histidine, lysine or cysteine with HNE d) Interaction of lysine residues of two different proteins with the aldehyde groups of MDA and e) formation of dityrosine cross linkages (Stadtman, 2006). The maintenance of protein redox status is of fundamental requisite for normal cell physiology/ function, and any

structural changes in proteins are considered to be one among several mechanisms underlying the progression of disease complications.

Oxidative damage to DNA

The hydroxyl radical is known to interact with all the components of DNA molecule, damaging both purine/pyrimidine bases and deoxyribose backbone (Halliwell and Gutteridge, 1999). The most extensively studied DNA lesions include formation of 8-OH-G, modification of DNA bases, single / double-strand breaks, loss of purine bases, DNA-protein cross linkage, and damage to DNA repair systems (Helbock et al., 1999).

Table 1

Reactive oxygen and nitrogen species of biological interest

Reactive species	Symbol	Half life	Reactivity
Reactive oxygen species			
Superoxide	O^{2-}	10^{-6} sec	Generated in mitochondria, less reactive
Hydroxyl radical	*OH	10^{-9} sec	Highly reactive, generated during iron overload
Hydrogen peroxide	H_2O_2	Stable	Formed during various oxidase mediated reactions, yields potent *OH species
Peroxyl radical	ROO*	Sec	Very reactive, damages membrane lipids, proteins and sugars
Organic hydroperoxide	ROOH	Stable	Reacts with transitional metal ions to yield reactive species
Singlet oxygen	$^1O_2$	10^{-4} sec	Highly reactive, formed during photosensitization
Reactive nitrogen species			
Nitric oxide	NO*	Sec	Neurotransmitter, blood pressure regulator, prooxidant during pathological states
Peroxynitrite	$ONOO^-$	10^{-3} sec	Formed from NO* and O^{2-}, highly reactive.
Peroxynitrous acid	ONOOH	Fairly stable	Protonated form of ONOO-
Nitrogen dioxide	NO_2	Sec	Formed during atmospheric pollution

Source : Devasagayam et al., 2004

Cellular oxidative stress response

Cellular response towards oxidative stress depends on the cell type and level of stress applied. Generally during stress conditions, it has been demonstrated that the intracellular free Ca^{2+} level rises and so do the levels of catalytic iron for free radical reactions. However, tissue/cells respond to mild oxidative stress by proliferating or by up-regulating the defense and/or repair systems. It is likely that upregulation may partially/ completely offer protection against the damage or even overprotect the cells by rendering them resistant to higher levels of oxidative stress imposed subsequently. The adaptation response need not always involve increases in antioxidants, but instead can lead to decreases in ROS-producing machinery or increases in other protective mechanisms (such as chaperones, heat shock proteins etc.). A moderate oxidative stress usually halts/ drives the cell cycle into senescence; thus the cell survives but no longer divides as a stress shock response. Severe oxidative damage, especially to DNA, may trigger death by apoptosis/ necrosis or mechanisms that involve features of both (Cave et al., 2005).

Antioxidant defenses

Aerobic organisms have adapted for existence under continuous exposure to ROS. Among the various adaptive mechanisms, the cellular antioxidant defense system plays a major role in removal of pro-oxidants and ensuing maximum protection. The cellular defense system can be classified into the *enzymes group* and the *low molecular weight antioxidant* group (Granot and Kohen, 2004, Sies et al., 2005).

Antioxidant enzymes

Superoxide dismutase: catalyzes the spontaneous dismutation of superoxide into hydrogen peroxide. The enzyme exists in two forms, a manganese containing SOD (Mn-SOD, mitochondria) and a copper-zinc dependant (Cu-Zn SOD, cytoplasm). Other types of SOD, such as extracellular SOD and Fe-SOD are seen only in plants. These enzymes are the first line of defense against oxidative stress (Landis and Tower, 2005).

Catalase: an unique enzyme that catalyzes the hydrolysis of hydrogen peroxide into water and oxygen. With a high K_m value for its substrate it decomposes hydrogen peroxide when present in high concentrations while glutathione peroxidase hydrolyzes relatively at low concentration with subsequent utilization of reduced glutathione (Sies, 1997).

Glutathione peroxidase: catalyzes the detoxification of both inorganic/ organic peroxides into water/alcohol via the oxidation of reduced glutathione. However, the insoluble form of glutathione peroxidase (phospholipid hydroperoxide glutathione peroxidase) acts on lipid hydroperoxide resulting in alcohols. Glutathione peroxidase competes with catalase for H_2O_2 and is the major source of protection against low levels of oxidative stress (Andreyev et al., 2005).

The activities of antioxidant enzymes are supported by glucose-6-phosphate dehydrogenase and glutathione reductase that supplies cofactors/coenzymes viz., NADPH and GSH for normal function.

Antioxidant molecules

In addition to the primary defense against ROS by antioxidant enzymes, significant protection is also offered by small molecules that efficiently scavenge the free radicals either directly or indirectly preventing them from participating in the metal mediated Haber-Wiess reaction.

Glutathione (L-γ-glutamyl-L-cysteinylglycine; GSH): is the most abundant non-protein thiol found in large quantities in organs exposed to toxins viz., kidney, liver, lungs etc., The antioxidant property of GSH is mainly attributed to its sulfhydryl (SH) group in its structure, that confer protection against damage by variety of oxidants, electrophiles and free radicals. Cellular GSH plays a key role in detoxification processes by acting as a cofactor for peroxidases, and efficiently decomposing H_2O_2 and acting as a substrate for Glutathione-S-transferase. Besides detoxification, GSH is also involved in recycling of ascorbic acid and in signal transduction (Masella et al, 2005).

Ascorbic acid: is a water soluble antioxidant synthesized from glucose by plants and few animals. Humans, primates and guinea pigs cannot synthesize ascorbic acid due to the lack of *ketogulonate synthetase*, and are completely dependent on the dietary sources. The antioxidant property of ascorbate is attributable to its inherent capability to donate electrons ($2e^-$) and form ascorbyl radical, which is stable and a powerful antioxidant. The radical may lose its electron and gets transformed to dehydroascorbic acid or regenerated to the reduced form either by GSH or NADH (Halliwell, 2001). Besides being an antioxidant, ascorbic acid is also a cofactor for various enzymes eg., proline hydroxylase, dopamine hydroxylase etc (Rigoni and De Tullio, 2002).

Vitamin E (α- tocopherol): Is a lipophilic antioxidant that scavenge LOO* and inhibits the lipid peroxidation process in biological membranes. Following interaction, tocopherol is converted to tocopherol quinine/ quinone. As with other scavengers, the tocopheryl radical is recycled to its active form by ascorbate and/or GSH (Kojo, 2004).

2.0 MALE REPRODUCTIVE SYSTEM

Male reproductive system comprises of a penis, a pair of gonads (testes), epididymis and accessory sex glands. Each testis is surrounded by two membranes –the outer *tunica vaginalis*, derived from the peritoneum and the inner *tunica albuginea.* Septal extensions of the tunica albuginea divide testis into many wedge-shaped compartments or *lobules*. Each lobule contains one to four tightly coiled *seminiferous tubules*, the actual 'sperm factories'. Further, the seminiferous tubules of each lobule converge to form a *tubulus rectus* that conveys sperm to *epididymis* through *rete testis.*

Surrounding the seminiferous tubules are the interstitial cells, or Leydig cells that are functionally differentiated to produce androgens (testosterone) which gets secreted into the surrounding interstitial fluid. The Sertoli cells function from the blood testis barrier that partitions the seminiferous epithelium into a basal compartment containing spermatogonia, early spermatocytes and an adluminal compartment containing fully developed spermatogenic cells. Nutrients, hormones and other chemicals pass either

between or through Sertoli cells in order to diffuse from one compartment to another. Germinal cells are found either between adjacent pairs of Sertoli cells or inside their luminal margin. Sertoli cells secrete various hormones/proteins that include plasminogen activator, Androgen binding protein (ABP), inhibin and transferrin that regulate spermatogenesis/testicular function. Thus, the sperm-producing and hormone-producing functions of the testis are carried out by completely different cell populations. Testis is richly supplied with blood from the arteries that branch from abdominal aorta and drained by highly innervated veins.

Spermatogenesis

Spermatogenesis is a chronological process spanning about 42 days in rodents and 72 days in man. During this period, relatively undifferentiated spermatogonia, the immature germ cells undergo several mitotic divisions to generate a large population of cells called primary spermatocytes, which produce haploid germ cells (spermatids) by two meiotic cell divisions. *Spermiogenesis* is the transformation of spermatids into the elongated flagellar germ cells capable of motility. The release of mature germ cells is known as *spermiation*.

Phase I: Mitosis: formation of spermatocytes - Spermatogonial stem cells are the outermost, least differentiated cells which are in direct contact with the basal lamina undergoes repeated mitotic divisions until puberty forming spermatoginal. Following puberty, each mitotic division of a spermatogonium results in two distinctive daughter cells- *types A and B*. Type A daughter cell remains at the basement membrane to maintain the germ cell line. The type B cell gets pushed toward the lumen, where it becomes a *primary spermatocyte* destined to produce four sperm.

Phase II: Meiosis: Spermatocytes to spermatids - Each *primary spermatocyte* generated during the first phase undergoes repeated meiotic divisions (meiosis I and II), forming spermatids and small round cells with large spherical nuclei.

Phase III: Spermiogenesis: Spermatids to sperm- Although each spermatid has the correct chromosomal number for fertilization (n), it is non motile. It

undergoes a streamlining process called *spermiogenesis*, during which it sheds its superfluous cytoplasmic baggage and fashions a tail. The resulting sperm or spermatozoon has three major regions: a head, a mid piece and a tail, which correspond roughly a genetic, metabolic and locomotor region.

Throughout the spermatogenic process, descendants of the same spermatogonium remain closely attached to one another by cytoplasmic bridges. Additionally, they are surrounded by and connected to supporting cells of a special type, called *sustencular cells* or *Sertoli cells*, which extend from the basal lamina to the lumen of the tubule. The tight junctions between the sustencular cells form the blood-testis barrier. This barrier prevents the membrane antigens of differentiating sperm from escaping through the basal lamina into blood stream. Since sperm are not formed until puberty, they are absent when the immune system is being programmed to recognize one's own tissues early in life (Sikka, 2001).

Hormonal regulation of male reproductive function

Spermatogenesis and testicular androgen production is under strict control by the hormones released by hypothalamus. Further, a potential interaction has been shown to exist between hypothalamus, anterior pituitary gland, and testes, also called *brain-testicular axis.* Hypothalamus secretes gonadotropin releasing hormone (GnRH), which controls the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates Sertoli cells to release androgen-binding protein (ABP) that concentrates testosterone, which in turn stimulates spermatogenesis. LH binds to the interstitial cells and stimulates them to secrete testosterone (and a small amount of estrogen). Both the hypothalamus and anterior pituitary are subjected to feed back inhibition by blood borne hormones. Testosterone inhibits hypothalamic release of GnRH and on the anterior pituitary to inhibit gonadotropin release while Inhibin, a protein hormone produced by the Sertoli cells, inhibits pituitary release of FSH and GnRH and thereby regulates spermatogenesis.

3.0 OXIDATIVE STRESS AND TESTICULAR DYSFUNCTIONS

In the recent past, greater emphasis has been placed on understanding the role of oxidative stress in the development of various testicular dysfunctions in experimental animals and male infertility in humans (Aitken, 1994; 1995). Peroxidative damage to sperm plasma membrane by ROS is one of the major causes of defective sperm function (Alvarez et al., 1987; Alvarez and Storey, 1989; Aitken et al., 1993; Chen et al., 1997). At the level of the isolated spermatozoan, ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of these cells and their ability to support normal embryonic development. In testes, oxidative stress disrupts the steroidogenic capacity of Leydig cells as well as the capacity of the germinal epithelium to differentiate into normal spermatozoa (Vaithianathan et al., 2008). A large number of independent clinical /experimental studies have demonstrated a potential relationship that exists between male infertility and testicular oxidative stress (Mahfouz et al., 2008). Although oxidative stress is clearly a dominant feature in the aetiology of male infertility, the underlying causative mechanisms remain unresolved.

While testis is endowed with antioxidant defense molecules, their concentrations are known to be relatively lower compared to hepatic levels (Bauche, 1994). An elevated production of ROS in testis has the propensity to cause significant alterations in tissue physiology or induce oxidative damage to DNA, which is of potential risk to the offspring (Ames, 1995). This has been demonstrated under exposures to various xenobiotics (Latchoumycandane et al., 2003; Kaur et al., 2006; Saradha et al., 2008,2009) and during metal toxicity (Stohs and Bagchi, 1995; Samanta and Chainy, 1997, Batra et al., 2001; Rajesh Kumar and Muralidhara, 2002; Doreswamy et al., 2004; Kaur and Bansal, 2004). Further, prooxidant induced genotoxic and fertility consequences viz., organic hydroperoxides, ‘acute iron overload’ are well documented employing rodent models (Lucesoli and Fraga, 1999; Rajesh Kumar et al., 2002, Doreswamy and Muralidhara, 2005, Rajesh kumar and Muralidhara, 2007; Kaur et al., 2008). Besides, certain situations of oxidative stress in humans such as ascorbic acid deficiency (Oteiza et al., 1995) and

varicocele (Agarwal et al., 2009) are shown to cause an increase in sperm DNA oxidation and poor antioxidant levels in semen, clearly emphasizing a potential relationship between oxidative damage to testis/sperms and mammalian male reproductive dysfunction/s.

Vulnerability of prepubertal testis to oxidative stress

Despite of large number of reports available on effect of xenobiotics, metals and disease conditions in adult testis, our understanding on the susceptibility and biochemical consequences following such exposures in developing testis are rather limited. This assumes greater relevance since testicular dysfunction/s during developmental stage can have profound implications on adult testicular physiology/function.

Developing testis is far more different both biochemically and functionally from that of the adult in terms of cell types, expression of receptors, testosterone levels and protective antioxidant machinery. Further, the cell types in testis are shown to undergo extensive morphological /biochemical changes during development and are particularly susceptible to oxidative stress. Although classification of experimental animals based on age is practically difficult, the age range from 28-42 days postnatal has been conservatively classified as adolescence (Spear, 2007). This period of variable duration has been classically considered as a quiescent phase of testicular development but is actually characterized by intense, yet inapparent activity. Prepubertal period is characterized by increase in testicular volume due to growth in length of seminiferous cords marked with active proliferation of infantile Sertoli cells while Leydig cells show recrudescence after birth, possibly determined by an active gonadotrophic-testicular axis results in increased testosterone secretion of uncertain functional role. This postnatal activation slowly subsides during late infancy and spurts again following activation of the hypothalamo-pituitary–testicular axis during puberty. The marked testicular growth in this stage is due to progressive increase in seminiferous tubule diameter, differentiation of Sertoli cells, lumen formation, and acquiring cyclic morphological and metabolic variations characteristic of the mature stage. All of these modifications indicate that, far from being

quiescent, testis undergoes numerous changes during infancy, and that the potential for pubertal development and normal adult fertility depends on successful completion of these changes (Chellakooty et al., 2003).

Antioxidant defenses of testis

Despite low oxygen tensions that characterize the testicular micro-environment, the tissue remains highly vulnerable to oxidative stress due to the abundance of unsaturated fatty acids (particularly 20:4 and 22:6) and the presence of potential ROS-generating systems. The richest source of ROS are mitochondria and variety of oxidases that includes xanthine- / NADPH-oxidases (Banfi et al., 2001; Kumagai et al., 2002) and cytochrome P450 enzymes (Zanger et al., 2004). In order to protect against the free radical attack, testis is endowed with a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents. The induction of oxidative stress in testes precipitates a response characterized by NF-kB mediated upregulation of SOD, GPx and GST activities (Kaur et al., 2006) that rapidly convert superoxide/hydrogen peroxide to water (Peltola et al., 1992).

Testis contains not only the conventional cytosolic (Cu/Zn) and mitochondria (Fe/Mn) forms of SOD, it also features an unusual extracellular SOD (SOD-Ex) produced by both Sertoli and germinal cells. There are evidences which suggest that the germ cells may stimulate the secretion of SOD-Ex by Sertoli cells through the actions of cytokines such as interleukin-1α. The importance of the cytosolic form of SOD (SOD1) was recently been investigated employing SOD1-knockout mice subjected to testicular heat stress where significant enhancement of DNA strand breakage and cytochrome c leakage from mitochondria has been observed (Ishii et al., 2005). Similarly, the importance of the mitochondrial form of SOD (SOD2) in controlling superoxide leakage from testicular mitochondria has been emphasized by the finding that the mRNA for this enzyme is markedly higher in the testes than the liver, unlike GPx and catalase. Although catalase is of limited importance in the testes, there are several isoforms of GPx in this tissue that use glutathione (GSH) as a source of electrons to reduce hydrogen peroxide to water which are mainly concentrated in mitochondria, nucleus and

acrosomal domain of differentiating spermatozoa (Vaisberg et al., 2005). The phospholipid- hydroperoxide-GPx (PhGPx) is the most important GPx isoform expressed in both spermatogenic and Leydig cells (Baek et al., 2007).

Small molecular antioxidants: In addition to the major antioxidant enzymes, testes rely heavily on small molecular weight antioxidant factors for protection against oxidative damage

*Zinc***:** is an acknowledged antioxidant factor for various free radical scavenging enzymes viz., SOD, CAT and a recognized protector of sulhydryl groups. Zinc administration has been shown to counteract the lead/ cadmium induced testicular oxidative stress and decline in testosterone/ sperm production (Ozkan et al., 2004; Amaral et al., 2008).

α-Tocopherol (Vitamin E): a powerful lipophilic antioxidant vital for the maintenance of spermatogenesis is particularly rich in Sertoli cells/ pachytene spermatocytes. Ascorbic acid and GSH also contributes to much of its activity by reducing α-tocopherol and maintain in an active state (Paolicchi et al., 1996; Sonmez et al., 2005). Vitamin E has been shown to offer protection against prooxidant induced lipid peroxidation in various organs (Lucesoli and Fraga, 1999; Gavazza and Catala, 2006).

Melatonin: a pineal hormone plays a major role in protecting testes from oxidative stress (Mogulkoc et al., 2006). Melatonin has two major attributes that set it apart from most other antioxidants, it undergoes $2e^-$ oxidation when acting as an antioxidant; being soluble in both lipid/aqueous environments, it cross blood-testes barrier and confer germinal cell protection. Melatonin administration has proved to beneficial in infertile patients exhibiting varicocele/ non-obstructive azoospermia (Semercioz et al., 2003; Awad et al., 2006).

Cytochrome C: a low molecular weight free radical scavenger, recently been shown to play a major role in reducing hydrogen peroxide. The testis specific isoforms are powerful activators of apoptosis thus provide additional protection by virtue of their ability to facilitate the depletion of damaged germ cells.

Table 1.2

Oxidative stress mediated testicular dysfunctions

	Nature of oxidative dysfunctions	Reference
Testicular torsion	Common; testicular ischaemia, High levels of H_2O_2, NO, LPO; antioxidant enzyme depletion, mitochondria-mediated apoptosis	Chaki et al., 2003; Anim et al., 2005; Lysiak et al., 2007
Varicocele	Impaired venous drainage, disruption in spermatogenesis, elevated ROS, high rates of DNA damage, depletion in antioxidant levels in seminal plasma	Agarwal et al., 2006; Shiraishi and Naito, 2006
Hyperthyroidism	Elevated oxidative stress, elevated GSH/antioxidant enzyme activities	Sahoo et al., 2007
Infection	Elevated oxidative stress, generation of proinflammatory mediators viz., IL-1β, reduction in StAR/3β-HSD activities	Allen et al., 2004; Reddy et al., 2006
Physical exertion	Elevated oxidative stress, high levels of LPO, upregulation in antioxidant enzyme activities	Somani and Husain, 1996; Manna et al., 2003
Reproductive hormone imbalance	Diminished testosterone levels, anti oxidant defences, increased germ cell apoptosis	Ghosh et al., 2002; zini and Schlegel, 2003; Chaki et al., 2006
Xenobiotics	Elevated oxidative stress, reduced activities of antioxidant/ and steroidogenic enzymes	Fraga et al., 1996 Maneesh et al., 2008

4.0 DIABETES MELLITUS: AN OVERVIEW

Diabetes mellitus (DM), is a group of syndromes characterized by hyperglycemia with altered metabolism of lipids, carbohydrates and protein (Pickup and Williams, 2003). Long considered a disease 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 (King et al., 1998; Wild et al., 2004). Pronounced changes in the human environment and in behaviour/lifestyle have attributed to a greater extent for the escalating rates of people diagnosed under diabetes.

Diabetes mellitus may present as a relatively sudden, potentially lethal metabolic catastrophe or it can be associated with few if any, symptoms or signs and may escape detection for many years. These extremes of clinical manifestations constitute the basis for subdividing the disease into the insulin dependent (IDDM or type1DM) and the non-insulin dependent (NIDDM or type 2DM) types.

Type I diabetes (Insulin dependent): is primarily due to autoimmune-mediated destruction of pancreatic β-cell islets that culminates in absolute insulin deficiency. People with type I diabetes require exogenous insulin for survival to prevent the development of keto-acidosis. The prevalence of Type1DM is relatively less and accounts 5-10% of all diagnosed cases.

Type II diabetes (non-insulin dependent) : Encompasses the most prevalent form of the disease characterized by two metabolic defects 1) derangement of insulin secretion that is either delayed or insufficient relative to glucose load and is characterized by insulin resistance and/or abnormal insulin secretion 2) Inability of peripheral tissues to respond to insulin- called *insulin resistance*. People with T2DM are not dependent on exogenous insulin, but may require it for control of blood glucose levels if this is not achieved with diet alone or with oral hypoglycemic agents. T2DM accounts for over 90% of cases globally and is strongly associated with sedentary lifestyle/obesity. Although T2DM is numerically more common in general population, T1DM is the most common chronic disorder among children.

Global prevalence

According to WHO reports, the prevalence of type 2DM has projected an upsurge in the prevalence from 170 million in 2002 to 366 million by 2030 and the major burden will occur in the developing countries (Wild et al., 2004). Further, a recent study by American Diabetes Association, estimates that approximately 8% of the population in the United States is diabetic with over 6 million people yet to be diagnosed (American Diabetic Association, 2008).

Diabetes in India: The prevalence of T2DM is fastly becoming recognized in all non-industrialised populations. It has been estimated that by 2025, three-

quarters of the world's 300 million adults with diabetes will be in non-industrialised countries and almost a third in India and China alone. A national survey of diabetes conducted in six major cities in India showed an upsurge in the prevalence in urban adults compared to that in rural (Raman Kutty et al., 2000; Mohan et al., 2001). In another study, the onset of diabetes was reported to occur before the age of 50 years in 54.1% of cases, implying that these subjects developed diabetes in the most productive years of their life and had a greater chance of developing the chronic complications of diabetes (Ramachandran et al., 2001).

Diabetes in children and adolescents: Global Scenario

T2DM hitherto was considered as a disease of adults and rare in the pediatric population. Over the last decade, however, there has been a disturbing trend of increasing cases of T2DM in children, particularly adolescents, with a greater proportion of minority children being affected. Reports from different parts of the world, especially from the United Kingdom and the United states, show an increasing occurrence of T2DM in children (Barrett et al., 2001, Grinstein et al., 2003). The American Diabetes Association has highlighted a high prevalence of T2DM in children in ethnic minority populations such as the American and Canadian Indians, Hispanics, African Americans, Japanese, Pacific Islanders and Asian and Middle Eastern populations (American Diabetes Association, 2000). In Japan, 80% of all new cases of diabetes in children and adolescents are diagnosed as T2DM (Cockram, 2000; Rosenbloom, 2002; Vikram et al., 2005). According to another report, in Japan the incidence of T2DM among school children increased 10-folds (from 0.2per 100,000 in 1976 to 2 per 100,000 in 1995) in contrast to the incidence of T1DM which did not change appreciably over 20 years (Lee, 2000). Similar increasing trend was also reported from Singapore, Hong Kong and Taiwan accounting for ~10% of all new cases of childhood diabetes (Lee, 2000; Huen et al., 2000; Wei et al., 2003). Studies conducted in United Kingdom, showed a higher prevalence of T2DM in children <16 years of age (Barret et al., 2002).

Prevalence of childhood/adolescent diabetes: Indian Scenario

India has the highest number of adult diabetes cases (20 million) and this number is expected to rise to 57 million by 2020 (King et al., 1998). Although, there are few reports of T2DM in children and adolescents in India, there are several reasons to believe that this type of diabetes will be fastly recognized in greater numbers in near future (Narayana et al., 2001). The rising prevalence of obesity in children, globalization and industrialization, sedentary lifestyle and socio-economic status, genetic predisposition are important risk factors for the development of disease (Ehtisham et al., 2000; Larzon, 2002). Further, pre-diabetic conditions such as impaired glucose tolerance/fasting glucose are also on the rise, indicating the possibility of further rise in the prevalence.

In concurrence with the reports from developed countries, obesity, female sex, parental history of type2 diabetes, and pubertal age appears to be strongly associated with the disease in the Asian children (Kapil et al., 2002; Ramachandran et al., 2003). Unlike in children with T1DM who have acute onset of the disease with severe symptoms and ketonuria, lean body weight and lack of familial aggregation, the type2 children share the features similar to classic adult-onset T2DM.

Glucose intolerance: a pre-disposing factor

The rise in prevalence of T2DM is viewed largely as an urban phenomenon, showing four-fold higher rates than rural populations (Ramachandran et al., 2001). In contrast, the prevalence of impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG), considered as predisposing factors for the onset of diabetes, appearing in both urban and rural communities implicating a higher burden of incipient disease in rural populations in the near future. IGT in developing countries has been observed phenomenally in young adults (Wild et al., 2004). A recent survey by Raghupathy et al., (2007) showed a remarkably high prevalence of T2DM and IGT at a young age in rural and urban southern Indian communities implicating a rapid increase in disease settings in both the groups. While this situation is alarming, it provides an opportunity for intervention programme to

help young adults to lead healthier lives. However, the prevention and treatment of T2DM in children/youth is a daunting challenge because of the enormous behavioral influence and difficulty in reversing obesity in this age group. Thus, T2DM is fast being recognized as health problem of international scope, following much the same pattern as in adults.

Involvement of oxidative stress in diabetes associated complications

Oxidative stress is widely accepted to play a key role in the development and progression of various pathological diseases by depleting antioxidant defenses and generating free radicals (Ha and Lee, 2000; Bonnefont-Rousselot, 2002; Evans et al., 2003, Maritim et al., 2003; Agarwal, et al., 2008). A growing body of evidence implicates the underlying mechanism associated with diabetes mellitus to be the glucose induced generation of free radicals, decrease in antioxidant potential and subsequent development of insulin resistance (Brownlee, 2001). The pathogenetic effect of hyperglycemia, possibly in concert with free fatty acid release, is mediated to a large extent via increased production of ROS. Free radicals generated activate a number of cellular-stress-sensitive pathways leading to tissue damage. Oxidative stress may even decrease insulin sensitivity and injure the insulin-producing cells within the pancreas (Lepore et al., 2004; Chen et al., 2005). In addition, free fatty acids induced ROS production, have been shown to contribute significantly to mitochondrial DNA damage and impaired pancreatic β-cell function. As a result, it is the activation of oxidative stress pathways that plays a key role in the development of not only the late complications of diabetes, but also insulin resistance (Rachek et al., 2006) (Scheme 1).

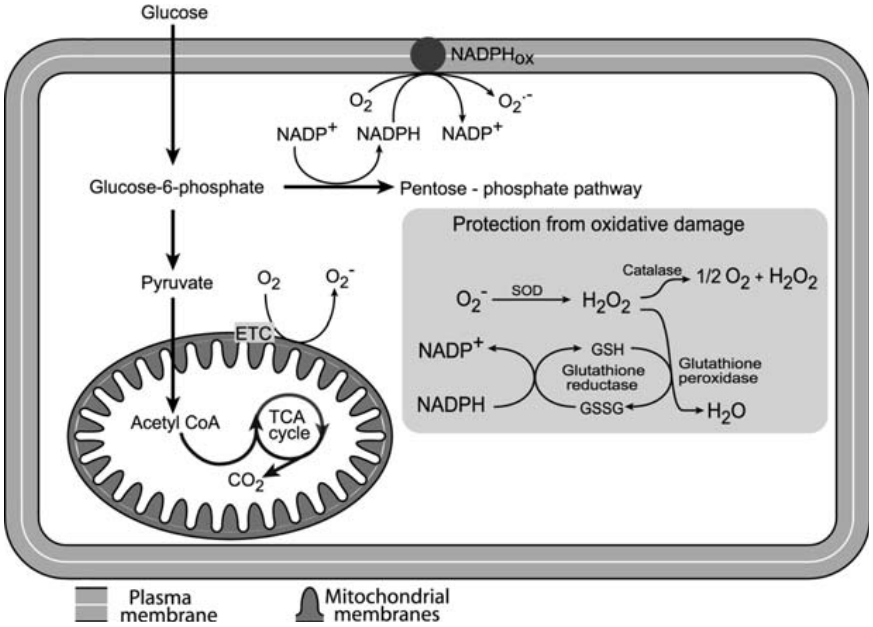
Oxidative stress exacerbates type1 and 2 diabetic complications

It has been shown recently that, there is a correlation between iron stores and susceptibility to T2DM, suggesting raised levels of redox-active metals leads to enhanced generation of ROS and increased probability of diabetes. However, there are no reports regarding the involvement of oxidative stress in the development of T1DM as it is an autoimmune disease.

T2DM and oxidative stress: There is still a debate whether the primary defect in T2DM is that of response to insulin (insulin resistance) or a β-cell defect as hyperglycemia arises only when insulin production/ release is inadequate. A growing body of evidence suggest that the early targets of oxidative stress are membrane proteins in general and Na^+/H^+, Na^+/Ca^{2+} antiporters, GLUT2 transporters in particular. The other key biochemical changes includes, increased production of cholesterol due to activation of HMG CoA reductase, insufficient acetyl CoA clearance, higher flow of Ca^{2+} ions into cells, inactivation of membrane ATPases, increased platelet aggregation, increased free radical production/ lipid peroxidation and exhaustive consumption of antioxidants (West, 2000).

Scheme 1.1

Sites of production of reactive oxygen species (ROS) and antioxidant systems in a generic cell type (Newsholme et al., 2007)



The toxicity due to hyperglycaemia depends on the shift of glucose metabolism from the major glycolytic pathway to minor forms viz., sorbitol, hexosamine and advanced glycation end-products. These oxidative stress

pathways are found to be responsible for the block in insulin signal transduction/insulin resistance (Baynes and Thorpe, 1999) (Scheme 2).

Increased polyol pathway flux: In the polyol pathway, aldehydes generated by ROS are reduced to inactive alcohols and glucose to sorbitol by the cytosolic aldose reductase utilizing NADPH as cofactor. Under normoglycemic state, aldose reductase has a low affinity (high K_m) for glucose, and metabolism by this pathway is very limited. However, during hyperglycemic state, increase in intracellular glucose results in increased enzymatic conversion to sorbitol, with concomitant decrease in NADPH. Further, sorbitol is oxidized to fructose by sorbitol dehydrogenase with NAD being reduced to NADH.

A number of mechanisms have been proposed to explain the potential detrimental effects of hyperglycemia-induced increased in polyol pathway flux. As sorbitol formed does not diffuse across cell membrane, it has been suggested that increased accumulation is likely to cause osmotic damage to the microvascular cells. Further, the oxidation of sorbitol increases cytosolic NADH: NAD+ ratio, inhibiting the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which subsequently increases the formation of both methyl glyoxal and diacylglycerol (DAG) thus activating AGEs/PKC pathways. In addition reduction of glucose to sorbitol consumes NADPH which is reduces intracellular GSH pool thereby elevating oxidative stress.

Advanced glycation end-products (AGE): are from non-enzymatic reactions between extracellular proteins and glucose/glucose-derived dicarbonyl precursors generated intracellularly viz., glyoxal, methylglyoxal, glucose-derived 1-amino-1-deoxyfructose lysine adducts (Amadori product) etc. Thus, intracellular hyperglycemia is the primary initiating event in the formation of both intra- and extracellular AGE (Degenhardt et al., 1998). Production of intracellular AGE precursors damage target cells by intracellular modifications in extracellular matrix components, plasma proteins and in cell membrane receptors thereby generating ROS, subsequently activating several transcription factors that alters gene expression (Brownlee, 2001).

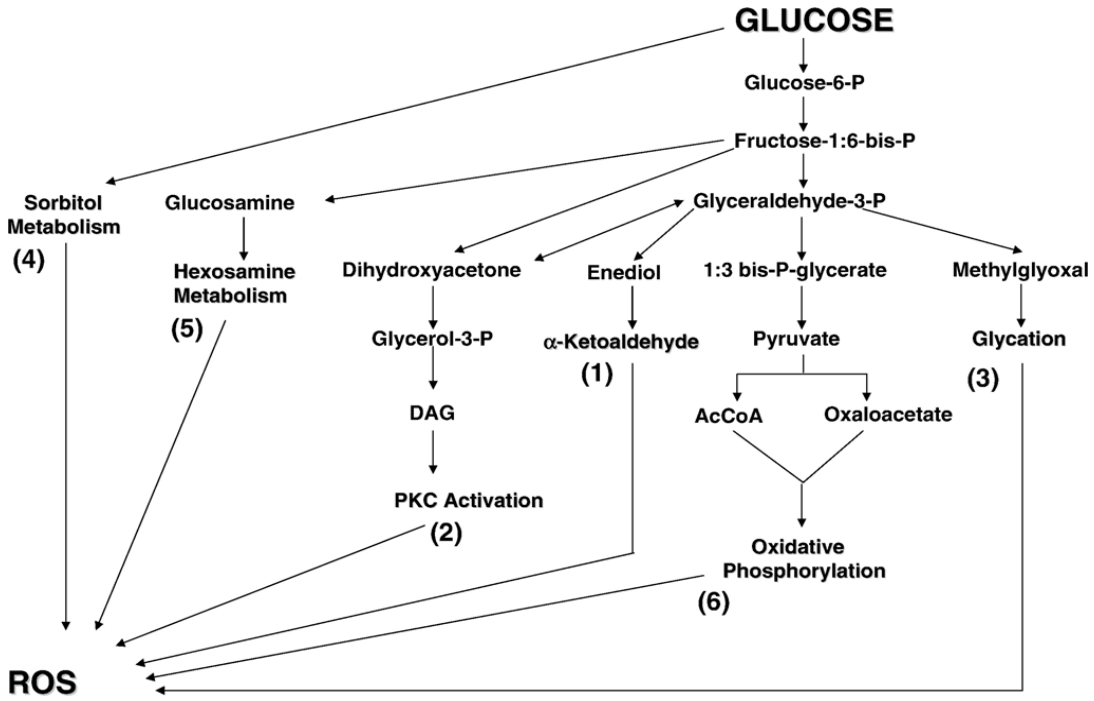
Activation of protein kinase C: PKC family is a group of structurally related enzymes that require phosphotidylserine /diacylglycerol/ free fatty acids

and/or Ca^{2+}/ Mg^{2+} for their activation. PKC are activated by the Diacyl glycerol formed form stepwise acylation of glyceraldehyde 3-phosphate (Koya and King, 1998). In experimental diabetes, activation of PKCβ isoforms has been shown to mediate insulin-stimulated eNOS activity, VEGF and TGF-β activation (Kuboki et al., 2000).

*Hexosamine pathways: S*hunting of excess intracellular glucose into the hexosamine pathway also cause several manifestations of diabetic complications. During normal physiology, approximately 3% of the glucose is channeled into the hexosamine pathway. The enzyme *glutamine: fructose-6-phosphate amidotransferase* catalyzes the conversion of fructose-6-phosphate to glucosamine 6-phosphate. The later compound rapidly metabolizes to UDP-N-acetyl-glucosamine and other hexosamines, which are the substrates for the synthesis of glycoproteins, proteoglycans, gangliosides and glycolipids. During hyperglycaemia, increased glucose flux follows hexosamine pathway and results in raised glucosamines that causes insulin resistance in skeletal muscle and adipocytes (Balasubramanyam et al., 2002).

Scheme 1.2

Pathways along which glucose metabolism can form ROS (Robertson, 2004)



Diabetes associated long term complications

Cardiovascular disease: The incidence of this disease in people with DM is three to four times that of non-diabetic individuals who are prone to dyslipidemia, hypertension and smoking. Oxidative stress has been implicated to be the underlying mechanism for the development of atherosclerosis. Increased concentrations of oxidized-LDL, auto-antibodies to both oxidized/glycated LDL during diabetes suggest enhanced oxidative stress *in vivo* (Jilal et al., 2002).

Nephropathy: is another important microvascular complication of DM. It occurs in about one-third of patients with insulin dependent diabetes and is the single largest cause of end-stage renal disease requiring chronic dialysis/ transplantation. Although the pathophysiology is not well defined, recent studies have implicated that persistent hyperglycemia activates various kinases viz., PkC, MPK etc., which aggravates diabetic complications (Anjaneyulu et al., 2004).

Neuropathy: Chronic hyperglycemia plays a critical role in the development /progression and causes rapid changes in glial cells and upregulates glial fibrillary acidic proteins that eventually end up in neurodegeneration (Obrosova, 2008).

Retinopathy: is caused due to early selective loss of pericytes and retinal endothelial cells mediated by the expression of NF-kB before any diabetic pathological symptoms are detected (Kowluru et al., 2003).

5.0 DIABETES, TESTICULAR DYSFUNCTIONS AND MALE INFERTILITY

Male reproductive disturbances have been widely reported in individuals with diabetes. The effect of diabetes in men may be directly linked to the effects of the disease process on erection, ejaculation, spermatogenesis and even embryo development. According to a recent survey, about 90% of diabetic patients have disturbances in sexual function, including a decrease in libido, impotence and infertility in the later case due to testicular dysfunction associated with sustained hyperglycemia (Amaral et al.,

2006). The prevalence of patients with erectile dysfunction (ED) has been reported to be 30 million only in United States (Levine, 2000) and the prevalence has been shown to vary in different communities from 7% to 57% (Lewis, 2002). The main causative factors contributing to the development of ED during diabetes are insulin deficiency, generation of free radicals, imbalance in hypothalamo-pituitary- testicular axis, lowered testosterone and decreased NO levels. Insulin besides maintaining blood glucose levels, by yet unexplained mechanism/s has been shown to regulate the Leydig/ Sertoli cell function (Glenn et al., 2003). Further, induction of diabetes brings about changes in seminiferous tubules that includes increased tubular wall thickness, severe germ cell depletion and sertoli-cell vacuolization resulting in reduced testicular volumes, semen volume, and motile sperm output. The increased vulnerability of testis to free radical damage can partly be attributed to higher levels of unsaturated fatty acids particularly n-6 and n-3 and also on both steroidogenic and spermatogenic processes (Unlucerci et al., 2000).

Testicular function is primarily controlled by pituitary hormones viz., follicle stimulating hormone (FSH) regulating spermatogenesis, while luteinizing hormone (LH) controlling Leydig cell function (Ward et al., 1991). The onset of Diabetes diminishes the stimuli induced secretion of FSH and LH, indicating a direct relationship that exists between insulin/glucose and LH/FSH levels in serum. However, the mechanism/s of hormonal regulations by insulin/glucose is far from clear understanding.

The involvement of oxidative stress in male infertility is becoming increasingly apparent based on experimental as well as epidemiological evidences (Saleh and Agarwal, 2002). Our recent findings in STZ-diabetic adult rodent models have shown the occurrence of oxidative stress in testis during acute phase and its progression (Shrilatha and Muralidhara, 2007a,b). Recent studies have demonstrated decreased testicular weight, sperm count, sperm motility, testosterone levels and increased frequency to abnormal spermatogenesis following experimental induction of diabetes. Furthermore, induction of diabetes has been shown to increase apoptosis in testicular germ cells in mice (Koh, 2007).

Animal models of Diabetes

Various animal models have been employed to obtain basic insights on the potential adverse effects of diabetes upon human male reproductive system. Experimental T2DM can be induced by any of the following methods (1) Chemical destruction or surgical removal of part of the β-cell mass, (2) Lesioning the ventromedial hypothalamus (3) feeding with high-fat/high sugar diets (4) malnutrition *in utero* (5) High doses of glucocorticoids and (6) prolonged cell exposure to hyperinsulinemia (Pickup and Williams, 2003). Of all the methods available, chemical destruction of pancreatic β-cells using diabetogenic agents viz., Alloxan and Streptozotocin is the most commonly employed to induce experimental diabetes in rodents. Both the chemicals have been shown to preferentially accumulate in pancreatic β-cells *via* the GLUT2 glucose transporter and selectively destroy pancreatic β-cells.

Streptozotocin induced diabetic model

Streptozotocin (STZ) is a nitrosourea analogue in which the N-methyl-N-nitrosourea moiety is linked to the carbon-2 of a hexose. Nitrosoureas are usually lipophilic and are rapidly taken up by the cells; however, hexose substitution as in STZ makes the compound less lipophilic. STZ is selectively accumulated in pancreatic beta cells *via* the low-affinity GLUT-2 glucose transporter in the plasma membrane. Thus, cells which do not express the transporter are resistant to streptozotocin action. The importance of the GLUT2 glucose transporter in this process is also shown by the observation that streptozotocin damages other organs expressing this transporter, particularly kidney and liver. The toxicity of streptozotocin is attributed to the DNA alkylating activity of its methylnitrosourea moiety at O^6 position of guanine. The transfer of the methyl group from STZ to the DNA molecule causes damage, which along a defined chain of events, results in the fragmentation. Further, protein glycosylation may be an additional damaging factor. In the attempt to repair DNA, poly (ADP-ribose) polymerase (PARP) is overstimulated. This diminishes cellular NAD+, and subsequently ATP stores. The depletion of the cellular energy stores ultimately results in beta cell necrosis.

Thus, STZ is the preferred diabetogen in animal models as it has more specificity towards b-cell cytotoxicity. The sensitivity varies with species, strain, sex and nutritional state. Further, there is a huge controversy that still exists in the type of diabetes obtained upon administration of streptozotocin. The more traditional procedure involves a single toxic dose which causes β-cell death in 2-4 days which has been related closely to type2 diabetes of human beings. A multiple low dose administration of STZ results in a more immunologically based disease with insulitis and the activation of C-type retroviruses, resembling type1 diabetes in humans (Lenzen, 2008).

6.0 MODULATION OF DIABETES INDUCED TESTICULAR OXIDATIVE STRESS

Over the past decade, there is a growing body of evidence on employing biologically active compounds to treat various diseases for their known health benefits. These potentially active biomolecules called 'phytonutrients' have been used as therapeutics or dietary supplements in treating or alleviating various disease conditions such as cancer, cardiovascular diseases, diabetes etc (Yeh et al., 2003) owing to their potent antioxidant, anticarcinogenic properties. These plant-based chemicals have been extensively used to ameliorate diabetes associated complications as they are intended to improve glucose metabolism and enhance the overall health of diabetic patients by improving lipid metabolism, increasing antioxidant status, improving capillary function, and lowering blood pressure and cholesterol endocrinal discrepancies (Broadhurst et al., 2000).

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. 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 and fail to significantly alter the course of diabetic complications. As the

knowledge of heterogeneity of this disorder increases, there is a constant need for more efficacious agents with lesser side effects.

D-Aspartic acid, its role in testicular functions

D-Aspartic acid, one of the most common D-amino acid is widely found in nature eg., vegetables, coffee, eggs, marine animals and alcoholic beverages etc., (Friedman, 1999) (Table 1.3). D-Asp is an endogenous amino acid, which is also found in nervous and endocrine tissues of both invertebrates and vertebrates. In organs such as pituitary and pineal glands, D-Asp concentrations have found to reach values from 4-10 times higher than other tissues/organs. The natural storage in these tissues suggests a possible role of D-Asp in mechanisms related to the activities of these glands, which may be likely related to reproduction (D'Aniello 1998).

D-Asp is a novel type of messenger in the mammalian body that is synthesized by specific tissues and cells, including the anterior lobe of the pituitary gland and the seminiferous tubules of the testis. D-Asp is released from the cell after its synthesis and may act in an autocrine and paracrine fashion on the synthesizing cell itself and/or neighboring cells. An example of such autocrine activity is that in the anterior lobe of the pituitary gland, prolactin producting cells synthesize D-Asp that then stimulates these cells to secret more prolactin. An example of paracrine activity is that D-Asp secreted by the seminiferous tubules may stimulate Leydig cells in the interstitial space to increase their testosterone production by stimulating StAR gene expression. D-Asp secreted by tissues that synthesize it may also act in an endocrine fashion on other tissues by entering the vascular system and being taken up by the cells bearing the L-Glu transporter.

D-asparate is found to occur in specific cells at distinct periods during the development of rat brain, adrenal, pineal, pituitary glands and testis. D-aspartate appears to be synthesized by the pituitary gland and testis and then secreted into the vascular system which transports it to tissues such as the adrenal and pineal glands, which takes up via L-glu transporter.

Table 1.3

Foods containing D-amino acids

Food Categories	Items	Source
Alcoholic beverages	Beer, Wines, Vinegar	D-Pro
Baked products	Bread, Dough, Wheat flour	D-Ala, D-Glu
Coffee	Green, Instant, Roasted	D-Asp, D-Glu, D-Phe
Dairy products	Cheese, Milk, Cream, Buttermilk	D-Ala, D-Asp, D-Glu, D-Lys, D-Ser
Food colorants, Flavor enhancers		D-Glu
Fruits and vegetables	Apples, Carrot, Cabbage, Garlic, Grapes, Lemon	D-Ala, D- Arg, D-Asp, D-Glu
Honey		D-Leu, D- Phe, D-Pro
Meat and meat products	Fish meal, Bacon, Beef, Chicken soup	D-Asp

Source : Friedman, 1999.

D-Aspartic acid and Testis

Besides brain, testis is the second organ where D-Asp is synthesized and secreted. In adult rats, D-Asp has been shown to get accumulated in the cytoplasm of germ cells, particularly in the elongate spermatids. D-Asp is apparently synthesized inside the seminiferous tubules and secreted out of the tubules into the interstitial space where it stimulates leydig cells to increase testosterone production by stimulating Steroid acute Regulatory protein (StAR) gene expression. However, exogenously administered D-Asp accumulates in the interstitial spaces of the testis rather than being incorporated into the inside of the tubules. It appears that when the elongate spermatids are released as free spermatozoa into the epididymis to continue their development, they leave their D-Asp behind in the seminiferous tubule, because D-Asp levels in the rat epididymis and epididymal fluid are very low compared with those of the testis, suggesting that most of the D-Asp in the elongate spermatids is left behind in the cytoplasmic fragment remaining in the seminiferous tubules when the cells expel most of their cytoplasm in the tubules and are released into the epididymis. Thus, D-Asp appears to play a significant role(s) in the testis rather than in the spermatozoa. The secreted

testicular D-Asp acts to modulate testosterone synthesis by Leydig cells. Earlier studies with purified rat Leydig cells have demonstrated, D-Asp induced increase in testosterone synthesis (Nagata et al., 1999). However, it appears that rat Leydig cells express a L-glu transporter subtype denoted as GLAST that may specifically mediate their D-Asp uptake.

Mechanism of testosterone release by D-Aspartic acid

Under normal physiological conditions in Leydig cells, cholesterol is transported across the outer mitochondrial membrane to the inner mitochondrial membrane where it is converted to pregnenolone by P-450cc. Pregnenolone is then converted to testosterone via the steroidogenic pathway in endoplasmic reticulum. The process of translocation of cholesterol to the inner mitochondrial membrane, the rate-limiting step for testosterone biosynthesis is activated in Leydig cells. Stimulation of Luteinizing receptor (LH) by human chorionic gonatotropin (hCG) results in increased cAMP levels, which subsequently stimulates StAR gene expression. Although, the exact mechanism of D-Asp induced testosterone biosynthesis is not clear, it has been proposed that it is taken up by Leydig cells via L-glutatamate transporters, and induce the expression of StAR protein, thus facilitating testosterone biosynthesis.

Testosterone

Testosterone is the primary male sex hormone secreted by the testes in response to luteinizing hormone (LH) produced by the pituitary gland. Testosterone circulates in plasma either in bound form ie. sex hormone-binding globulin (SHBG) or with albumin, or in a small fraction unbound or free (more bio- available). Androgens acting through the androgen receptor have a function in prenatal sexual differentiation, pubertal change, development of male secondary sexual characteristics and subsequently body composition (Gobbi et al., 2003). Testosterone is converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase, which has greater binding affinity for the androgen receptor, or by the enzyme aromatase into estradiol. The mode of androgen action has been demonstrated to involve both classical and

nonclassical pathways that might involve nuclear androgen receptor-mediated transcriptional activation of target genes. In men, circulating levels of testosterone increase at the time of birth and at puberty (Bhasin, 2008).

Testosterone Deficiency

Male hypogonadism is defined as a clinical syndrome which results from failure of the testes to produce physiological concentrations of testosterone and the normal number of spermatozoa. Traditionally hypogonadism is classified as either primary testicular failure with elevated luteinizing hormone (LH), or secondary, hypothalamic-pituitary failure together with decreased LH. However, hypogonadism can still occur with gonadotropin levels within the normal range (American Society of Andrology, 2006). Symptoms of hypogonadism includes loss of libido, erectile dysfunction, difficulty achieving orgasm, diminished sexual penile sensation as well as other symptoms such as fatigue, lack of physical strength, impaired cognitive function, and depressed mood.

Testosterone and the Metabolic Syndrome

Prevalence of the metabolic syndrome is significantly higher in men with erectile dysfunction than in healthy controls (26.7% vs 13%). Furthermore, metabolic syndrome is associated with a more severe erectile dysfunction, which is further exacerbated by the coexistence of hypogonadism. Both clinical observations and experimental data suggest that the metabolic syndrome and its components are associated with low serum concentrations of testosterone in men. The metabolic syndrome defined as a cluster of comorbidities, which is associated with an increased cardiovascular risk, is often found together in viscerally obese patients and has insulin resistance as the common denominator. Indeed, insulin resistance plays a key role in the pathogenesis of the metabolic syndrome. According to the International Diabetes Federation (IDF, Scheen et al., 2006), for an individual to be defined as having the metabolic syndrome, they must have central obesity (defined as a waist circumference of 94 cm or more for Europid men and 80 cm or more for Europid women) and any two of the following factors:

hypertriglyceridemia, low high-density lipoprotein cholesterol, hypertension, and raised fasting blood glucose or previously diagnosed type 2 diabetes mellitus. It is well established that erectile dysfunction is very common in diabetic men, with prevalence reports of between 30% and 90% (Cho et al., 2005). A recent study has shown that levels of both bioavailable and free testosterone (but not total testosterone) were significantly lower in diabetic men with erectile dysfunction as opposed to those without. However, the severity of erectile dysfunction as assessed by International Index of Erectile Function (IIEF) scores did correlate with total testosterone as well as the bioavailable and free fraction of testosterone (Kapoor et al., 2007).

Evidence suggests that insulin sensitivity, obesity, and testosterone are interlinked, with testosterone having beneficial effects on obesity and insulin resistance. Indeed, low levels of total and bioavailable testosterone in men have been associated with type 2 diabetes, visceral obesity, insulin resistance, hyperinsulinemia, and dyslipidemia in cross-sectional studies. In the study of Stellato et al., (2000), total and free testosterone levels were significantly lower among men, who later developed diabetes,

In addition, testosterone replacement therapy in hypogonadal men with diabetes has been reported to improve insulin sensitivity (Kapoor et al., 2006). Low testosterone levels have also been demonstrated to predict insulin resistance and future development of T2DM diabetes in men (Stellato et al., 2000), and epidemiological studies have showed an increasing prevalence of hypogonadism in diabetic men compared with nondiabetic men (Dhindsa et al., 2004). A potential correlation has been proposed to exist between Low circulating levels of total/ bioavailable testosterone and obesity in men diagnosed as diabetic (Laaksonen et al., 2003). Recent studies have demonstrated that increased deposition of abdominal adipose tissue during hypogonadism upregulates aromatase activity that eventually leads to greater formation of 17-β-estradiol from testosterone. As a consequence, there will be further reduction in serum/ tissue concentrations of testosterone that results in male infertility or subfertility (Kapoor et al., 2005).

Testosterone therapy has been shown to restore erectile function in the majority of hypogonadal patients and could be considered a first line of

therapy in patients with hypogonadal ED /associated comorbidities such as diabetes mellitus and metabolic syndrome.

Phytotherapeutic approach to treat diabetes

In recent years, there has been an upsurge in the clinical use of indigenous drugs. Indian medicinal plants and their derivatives have been an invaluable source of therapeutic agents to treat various disorders including diabetes. Although, several therapies are in use for treatment, there are certain limitations viz., high cost, side effects such as development of hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity etc., (Dey, et al., 2002). Based on recent advances and involvement of oxidative stress in complicating diabetes mellitus, efforts are on to find suitable antidiabetic and antioxidant therapy. It is assumed that herbal medicines can only be effective as an alternative to oral hypoglycemic agents in the treatment of T2DM, where pancreatic islets are not totally destroyed.

Withania somnifera

Is popularly known as Ashwagandha or Winter Cherry (Andallu and Radhika, 2000) is a green shrub (family Solanaceae) whose various parts have been used for centuries to treat variety of ailments as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia and senile dementia. Clinical trials and animal research support the use of Ashwagandha for anxiety, cognitive and neurological disorders, inflammation and Parkinson's disease. (Kulkarni et al., 1998; Bhattcharya et al., 2001) (Table 1.4)

The major biochemical constituents of Ashwagandha root are steroidal alkaloids and steroidal lactones in a class of constituents called *Withanolides* (steroidal lactones with erogstane skeleton). The Withanolides have the structural resemblance with the active constituents present in the plant *Panax gingseng* known as ginsenosides. The main withanolides characterized includes withanine, somniferine, somnine, withananine, pseudo-withanine, tropine, psuedotropine, 3α-glyoxytropane, anaferine, glycowithanoloids, and acyl steryl glucosides. Much of ashwagandha's pharmacological activity has

been attributed to two main withanolides, withaferin A and withanolide D (Grandhi et al., 1994). The protective effects of *Withania* has been attributed to its anti-inflammatory, antitumor, antistress, immunomodulatory and rejuvenating properties besides its positive influence on the endocrine and central nervous system.

Mechanism of action

The Withanolides serve as important hormone precursors that can convert into human physiologic hormones as needed. Ashwagandha is thought to be amphoteric ie., it can help to regulate important physiologic processes. The theory is that when there is an excess of a certain hormone, the plant based hormone precursor occupies cell membrane receptor sites so the actual hormone cannot attach and exert its effect. If the hormone level is low, the plant-based hormone exerts a small effect. Ashwagandha is also considered to be an adaptogen, facilitating the ability to withstand stressors, and has antioxidant properties as well.

Table 1. 4

Therapeutic use of *Withania somnifera*

Properties	References	Properties	References
Analgesic	Dhuley, 2001; Davis and Kuttan, 1998	Anticancer	Kaur et al., 2004; Mathur et al., 2006
Adaptogenic	Bhattacharya and Murugandam, 2003	Antiperoxidative	Dhuley, 1998
Antiinflammation	Dhuley, 2000	Antioxidant	Bhattacharya et al., 1997, 2001
Antipyretic	Dhuley, 2000	Anticonvulsant	Kulkarni and George, 1996
Cardioprotective	Dhuley, 2000; Mohanty et al., 2004; Hamza et al., 2008	Immunomodulator	Ziauddin et al., 1996; Rassol and Varalakshmi, 2006
Hemopoetic	Davis and Kuttan, 1998	Thyroid hormone regulating effect	Panda and Kar, 1998

6.0 SCOPE OF THE PRESENT INVESTIGATION

Diabetes mellitus is becoming a significant health care concern worldwide. This metabolic disorder is characterized by insulin resistance and physiological/biochemical malfunctioning of different metabolic pathways associated with carbohydrate metabolism resulting in increased endogenous output. An individual with insulin resistance is strongly predisposed to an increased risk for life threatening clinical conditions including Type2 diabetes (T2DM), and the metabolic syndrome (also known as the insulin resistance syndrome). Long considered a disease of minor significance to world health, is now taking its place as one of the major threats to human health in the 21st century. The global figure of people with T2DM has reached epidemic proportions in both developed and developing countries with a current estimate of 150 million to 220 million in 2010 and 300 million in 2025. According to a recent estimate, there are nearly 32 million diabetics in India and the number is expected to rise phenomenally in the near future. Eventhough, diabetes is viewed as an urban phenomenon, the prevalence of prediabetic risk factors viz., impaired glucose tolerance/fasting glycemia are fastly appearing in rural communities suggesting a high burden of incipient disease on population health.

Until recently, T2DM had been considered as an adult disease associated with obesity, sedentary life style and a positive family history. Over the last decade, there has been a disturbing trend of increasing cases of children/adolescents presenting with clinical features of T2DM. Increasing number of cases with T2DM in children/adolescents has been reported from developed countries such as UK, USA with highest prevalence in Japan. A growing concern of this epidemic is that a lowering in age of onset of type2 diabetes will be a major influence on the future burden of the disease. Onset in childhood heralds many years of disease and an accumulation of the full range of micro- and macro vascular complications. As in adults, sedentary life style, obesity, arterial hypertension, hyperlipidemia, non-alcoholic-steatohepatitis, psychological and social problems are the possible risk

factors that predispose for the development of T2DM in children and adolescents.

Epidemiological evidences emphasize the potential contribution of increased oxidative stress to the development of various diabetic complications. Both clinical and experimental evidences equally suggests increased production of ROS, decreased antioxidant defenses, alterations in membrane biochemistry leading to cell death are common features in diabetes. Male infertility is an unavoidable consequence of overt diabetes and the nature of pathobiochemical changes associated during chronic stages is currently receiving wide attention. Animal models with spontaneous and chemically induced diabetes have been employed to obtain basic insights into the adverse effects of diabetes on human male reproductive dysfunctions both structurally and functionally. Earlier studies have shown various testicular pathologies such as seminiferous tubular thickening, germ cell depletion, diminished testosterone levels etc., among STZ-diabetic rats. Given the current understanding on the reproductive pathologies associated with diabetes is largely known in adults, a basic insight into disease pathogenesis during developmental stage is lacking. Developing testis is biochemically and functionally different from the adult ones in terms of cell types, expression of receptors, membrane proteins, protective antioxidant defenses (molecules/ enzyme levels) and high levels of circulating growth hormone which renders them more susceptible to oxidative stress. To the best of our knowledge, studies pertaining to the impact of diabetes during childhood /adolescents on testicular biochemistry /physiology and its associated fertility consequences have not been conducted. Further, the involvement of oxidative stress mechanism/s in the development of pathologies in immature/prepubertal testis under diabetic situations has not been comprehensively investigated.

Given the scenario that diabetes is becoming prevalent in children/ adolescents and is likely to increase in phenomenal proportions, it is pertinent to obtain newer and comprehensive insight into the biochemical and functional impairments that occur under such conditions. It is anticipated from the above studies that, a newer insights into the disease manifestation/progression, genetic mechanisms could be obtained in greater

details. Moreover, implications of diabetes in children offer some unique opportunities to understand the causes of the disease/insulin resistance and to plan primary intervention.

It is becoming increasingly clear that oxidative stress mechanisms are involved in xenobiotic- induced testicular dysfunctions leading to male infertility. Consistent with the role for oxidative mechanisms in the pathogenesis of diabetic complications mediated mainly by hyperglycemia *in vivo*, various pharmacologically active compounds have been employed to study their attenuating influence during such situations and is likely that these bioactives either delay/hinder the pathological events associated with development of diabetic complications. While large part of the protective effects of these bioactives is available on somatic organs, data on their ability to protect testis are very limited. Hence, it is hypothesized that, if oxidative stress constitutes single most etiological factor in the development of male reproductive dysfunctions under diabetes, these bioactives may play a significant role in the amelioration of oxidative damage in the reproductive milieu.

Accordingly, in the present study, attempts have been made to obtain evidence in favor of our central hypothesis tested that Immature/ developing testis is likely to be more susceptible to oxidative stress under diabetic conditions and the biochemical and functional alterations may have far reaching consequences (transient or permanent) resulting in sub-fertility or infertility. The objectives employed at the beginning of the investigation to assess the implications of diabetes on immature/developing testis are as follows:

a) To understand the nature, pattern and progression of diabetes induced oxidative damage in immature and developing testis
b) To elucidate the biochemical and physiological implications in testis and its impact on functional development of testis
c) To understand the spectrum of oxidative impairments in spermatozoa and its correlations with reproductive outcome and infertility
d) Dietary modulation of testicular oxidative damage and associated biochemical/ physiological alterations and impact on fertility outcome.

SECTION-A

MATERIALS

Chemicals

2,7 dichlorofluorescein diacetate, 1,1,3,3- tetra methoxy propane, 2-thiobarbituric acid, reduced glutathione, oxidized glutathione, 5,5-dithio-bis(2nitrobenzoic acid), ascorbic acid, hydrogen peroxide (3% w/v), quercetin, tetramethylethylene diamide, glutathione reductase, tert-butyl hydroperoxide, nicotinamide adenine di- nucleotide phosphate (reduced), nicotinamide adenine dinucleotide (reduced), nicotinamide adenine dinucleotide (oxidized), bovine serum albumin, cytochrome C, rotenone, acetyl coenzyme A, p-iodo-nitrotetrazolium violet, α-ketovaleric acid, ouabain, glucose 6-phosphate, adenosine di-, tri-phosphate, rhodamine 123, 5-pregnen-3β-ol-20-one, fura 2-acetoxy methyl ester, L-nitro-arginine methyl ester, trypsin, collagenase, proteinase K, mercaptoethanol were purchased from M/s Sigma Chemicals, St.Louis, MO, USA. All other chemicals used were of analytical grade purchased from M/s Sisco Research Laboratories, Mumbai, India.

Diabetogen

Streptozotocin (CAS No. 18883-66-4) was purchased from M/s Sigma Chemicals Co., (St.Louis, MO,USA).

D-Aspartic acid

D-Aspartic acid (CAS No.1783-96-6) was purchased from M/s Sigma-Aldrich Chemicals Co., (St. Louis, MO, USA).

Testosterone propionate

Testosterone propionate (CAS No 57-85-2) was purchased from M/s Sigma-Fluka Chemicals (St. Louis, MO, USA).

Withania somnifera

Standardized root extract of *Withania Somnifera powder* (C81015, Withanolides, 2.57%; Withaferin A, 2.38%) was purchased from M/s Sami Labs Ltd., Bengaluru, India

Animals and care

Prepubertal male wistar rats (4wk old, 55 ± 5g) were used throughout the investigation. Animals were randomly drawn from the 'Institute animal house facility' and housed in rectangular polypropylene cages (n=3) kept on racks built of slotted angles and the cages were provided with dust free paddy husk as the bedding material. The animals were acclimatized for a week prior to the start of the experiment in a controlled atmosphere with a 12h light :dark cycle and maintained on a commercial pellet diet (supplied from M/s Saidurga Feeds and Foods Pvt. Ltd, Bangalore, India) with free access to tap water *ad libitum* throughout the studies.

Ethical considerations

All experiments inclusive of animal handling and sacrifice were conducted strictly as per the guidelines of 'Institutional Ethics Committee' regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, India.

Preliminary study

Initially, a dosage of 60mg/kg bw was chosen to induce hyperglycemia, based on our previous findings in adult rats (Shrilatha and Muralidhara, 2007). However, at this dosage STZ failed to induce significant hyperglycemia. Hence a higher dosage of 90mg/kg bw was chosen.

Dose optimization study (Chapter 2, Section A) comprised of administration of STZ at dosages of 30, 60, 90 and 120mg/kg bw. For all other investigations (Chapter2, Section B, Chapter 3 and Chapter 4) the optimum dosage, 90mg/kg bw were used.

Preparation of STZ and induction of hyperglycemia

Streptozotocin was dissolved in freshly prepared 0.1M citrate buffer (pH 4.5, conc. 75mg/mL) and administered to prepubertal rats intraperitoneally. The treated rats were given 5% dextrose solution for a period of 48h to prevent hypoglycemic mortality. After 72h, blood glucose levels were monitored to ascertain the induction of hyperglycemia.

Autopsy of experimental animals

Terminally, animals were sacrificed under light ether anesthesia, blood was collected by cardiac puncture in tubes containing anticoagulant and used for estimation of plasma glucose and lipid profile. Testis was excised, washed free of blood in ice cold saline and kept at -20 º C until use.

Isolation of testis cytosol, mitochondria and microsomes

Testis mitochondria were prepared by differential centrifugation technique according to the method described by Trounce et al., (1996) with minor modifications. Testis was decapsulated, seminiferous tubules were gently expressed and homogenized in Mannitol-Sucrose-HEPES (MSH) buffer, pH 7.4 using a glass teflon grinder. The homogenate was then centrifuged at 1,000Xg for 10min to remove nuclear and tissue debris. Mitochondria were isolated by centrifuging the post nuclear supernatant at 10,000 X g for 10min. The pellet was washed twice with Tris- sucrose buffer (pH 7.4) and centrifuged at 7,000Xg for 10min and finally suspended in the same buffer, stored at -20˚C until use.

Microsomes were isolated from the post mitochondrial supernatant by centrifuging at 1,00,000 Xg for 60min. The microsomal pellet obtained was finally suspended in Tris-buffer.

Preparation of testicular homogenates

Rats were sacrificed under light ether anesthesia, testes were excised and decapsulated. A 10% homogenate was prepared using Krebs-Ringer Bicarbonate solution (KRBS, 110mM NaCl, 4.6mM KCl, 1.9mM $CaCl_2$, 6.6mM $MgCl_2$, 25mM $NaHCO_3$, 1.4mM NaH_2PO_4 and 15mM glucose/L, pH 7.2) and centrifuged at 800Xg for 10min to remove tissue debris. The supernatant served as testicular homogenate.

Preparation of testicular explants

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 were dispensed into each well of a sterile tissue culture plate (12 wells of 24mm diameter). Tubules were suspended in small volume of media, teased apart, and were cut into 3-5mm fragments. Finally, testicular explants were suspended in a required volume of KRBS and used for *in vitro* studies.

Preparation of testicular cell suspensions

Testicular cell suspension was prepared following the method of Romrell et al., (1976) with minor modifications. Rats were sacrificed under light ether anesthesia, testis was excised, decapsulated and the seminiferous tubules were gently expressed. The aggregate of tubules were incubated with Krebs-Ringer bicarbonate solution containing collagenase (0.5mg/mL) followed by trypsin (0.5mg/mL) treatment at 32˚C for 15min on a shaking water bath. The cell suspension was gently pipetted, filtered through nylon mesh (5uM pore size) and washed. Finally, cell numbers were adjusted to 5 million/mL and used for *in vitro* incubations.

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 Span diagnostics, Mumbai, India). The intensity of the colored solution was measured at 505nm and expressed as mg glucose/dL.

Serum triglycerides and cholesterol estimation

The serum cholesterol level were estimated as per the specification, using commercially available kit (M/s Aspen diagnostics, Mumbai, India) which involves cholesterol oxidation in presence of cholesterol oxidase (COD) and phenol aminoantipyrine (PAP). Serum triglycerides were estimated enzymatically in presence of glycerol phosphate oxidase (GPO) and

peroxidase (POD). The intensity of the colored solution was measured at 505nm and expressed as mg cholesterol or triglycerides /dL.

Markers of oxidative stress

Measurement of Reactive Oxygen Species *(Driver et al., 2000)*

Generation of reactive oxygen species (ROS) in testis subcellular organelles were measured using dihydrodichlorofluorescein diacetate (DCF-DA), a non-polar compound that after conversion to its polar derivative by intracellular esterases, rapidly reacts with ROS to form a highly fluorescent dichlorofluorescein (DCF). An aliquot (equivalent to 0.2mg protein) of cytosol /mitochondria/ microsomes was dispensed into tubes containing Locke's solution (154mM NaCl, 5.6mM KCl, 3.6mM $NaHCO_3$, 5.0mM HEPES, 2.0mM $CaCl_2$ and 10mM glucose/L, pH 7.4) to which 10µL of DCF-DA was added to a final concentration of 5µM/mL and incubated for 30 min at room temperature. The fluorescence was measured with excitation and emission wavelengths at 480 and 530nm. Background fluorescence was corrected by including parallel blanks. ROS generation was quantified from a DCF standard curve and expressed as pmol DCF formed/min/mg protein.

Lipid peroxidation (LPO) assay *(Ohkawa et al., 1979)*

The extent of lipid peroxidation was quantified by measuring thiobarbituric acid reactive substances (TBARS) in testis subcellular organelles. The reaction mixture contained an aliquot of (equivalent of 1mg protein) cytosol/mitochondria/ microsomes, 1.5mL of acetic acid (pH 3.5, 20% v/v), 0.2mL SDS (8%, w/v) and 1.5mL of thiobarbituric acid (0.8%, w/v). The mixture was heated in a boiling water bath for 45 min, adducts formed were extracted into 3mL of 1-butanol, and the color intensity was measured at 532nm. Lipid peroxidation was quantified as malondialdehyde (MDA) equivalents using 1,1,3,3- tetramethoxy propane as the standard.

Measurement of hydroperoxide levels *(Wolff, 1994)*

Water soluble hydroperoxide levels were determined according to the method of ferrous iron oxidation with xylenol orange (FOX1). An aliquot of the

test sample was added to 950 µL of FOX1 reagent and incubated at room temperature for 30min, at which the color development was virtually complete. The mixture was centrifuged (to remove flocculent material) and the absorbance was measured at 560nm (€-1.5X10^4 mol /cm).

Protein carbonyl levels *(Levine et al., 1990)*

Protein carbonyl content in testicular subcellular fractions was evaluated according to the method of Levine et al., (1990). Testis cytosol / mitochondria (equivalent to 0.5mg protein) was incubated with 2,4-dinitrophenyl hydrazine (DNPH) for 60min at room temperature. Following, protein precipitation by adding 20% trichloroacetic acid, the pellet was washed three times with acetone to remove excess reagent and dissolved in 1mL of tris buffer containing sodium dodecyl sulphate (8% w/v, pH 7.4). The absorbance was measured at 370nm and expressed as nmol carbonyl/mg protein (€ -22000 nmol/cm).

Determination of Nitric Oxide levels

Nitric oxide (NO) levels were measured in testis cytosol/ mitochondria employing commercially available Griess reagent (1.5% Sulphanilamide and 0.15% N-1-naphthyl-ethylene diamine in 1N HCl) procured from M/s Sigma Chemicals (St. Louis, MO, USA). Nitric Oxide levels were quantified from sodium nitrite standard curve measured at 560nm.

Quantification of Fluorescent products (Perez-Severiano et al., 2004)

Testis was homogenized in ice-cold saline (10% w/v) and extracted with chloroform and methanol (2:1).The fluorescence of the clear chloroform layer was measured at excitation, emission wavelengths of 370 and 430nm respectively and expressed as relative fluorescence units.

Antioxidant molecules

Measurement of reduced glutathione levels *(Hissin and Hilf, 1976)*

Testis cytosol/mitochondria or microsomes (equivalent to 0.1mg protein) was added to 1mL sodium phosphate buffer (0.1M, pH 8.0) containing 0.005M EDTA and 25% HPO_3. The reaction mixture was

centrifuged at 10,000 X g for 30min. An aliquot of the supernatant was diluted in 4.5mL of phosphate-EDTA buffer (pH 8.0). The assay mixture (2mL) contained 0.1mL of diluted supernatant, 1.8mL of phosphate-EDTA buffer and 0.1mL of O-phthaldehyde dissolved in methanol (1mg/mL). After thorough mixing and incubation at room temperature for 15 min, the fluorescence was determined with excitation at 350nm and emission at 420nm. Glutathione levels were quantified from the standard curve and expressed as µg GSH/mg protein.

Measurement of reduced glutathione levels *(Mokrasch and Teschke, 1984)*

Testis cytosol/mitochondria or microsomes (equivalent to 0.1mg protein) was added to 1mL formic acid (0.1M) and centrifuged at 10,000 Xg for 10min. An aliquot of the supernatant was mixed with 0.1M buffered formaldehyde (1:4, formaldehyde: 0.1M Na_2HPO_4) and added to sodium phosphate buffer (0.1M, pH 8.0, 5mM EDTA) and O-phthaldehyde (1mg/mL). Following incubation for 45 min at room temperature, the fluorescence was measured at excitation and emission wavelengths of 345 and 425nm respectively. Glutathione levels were quantified from the standard curve and expressed as µg GSH/mg protein.

Estimation of total thiols *(Ellman, 1959)*

Total thiol content in testicular subcellular compartments was estimated according to the method of Ellman (1959). An aliquot of cytosol/mitochondria /microsome (equivalent to 0.5mg protein) was added to Tris buffer (0.2M, pH 8.2) containing 25µL of dithio-nitrobenzoic acid (DTNB,10mM in methanol) and 1.975mL of methanol. Following incubation for 30min at room temperature, the tubes were centrifuged at 3,000Xg for 10min. The clear supernatant was read at 412nm against distilled water blank and expressed as nmol DTNB oxidized /mg protein (€-13.6/mM/cm).

Non-protein thiols *(Ellman, 1959)*

Testis cytosol/ mitochondria/microsomal protein (0.1mg) was added to 5% trichloroacetic acid and centrifuged at 10,000Xg for 30min. An aliquot of

protein free supernatant was added to 1mL Tris buffer (0.4M, pH 8.9) containing 25µL DTNB (10mM in methanol) and kept in dark for 15min at room temperature. The absorbance was read at 412nm and expressed as nmol DTNB oxidized/mg protein (€-13.6/mM/cm).

Tissue ascorbic acid levels *(Omaye et al., 1979)*

Testis ascorbic acid levels were measured using 2,4-dinitrophenyl hydrazine. Testis was homogenized in 5% TCA and centrifuged at 10,000Xg for 10min. An aliquot of the supernatant was added to 0.25mL of Dinitrophenylhydrazine-Thiourea-Copper sulphate solution and incubated for 3h at 37˚C.. Following addition of ice-cold sulphuric acid (65% v/v, 0.2mL) the tubes were incubated further at room temperature for 30min. The absorbance was measured at 520nm and expressed as µg/mg tissue.

Quantification of vitamin E levels *(Zaspel and Csallany, 1983)*

Testis was homogenized in 1.15% KCl and tocopherols were extracted into 1mL of hexane containing 0.002% butylated hydroxyl toluene (BHT). Samples were centrifuged at 1,500Xg for 5min at 4˚C. The hexane layer was pooled and dried under a stream of nitrogen, and reconstituted in 0.1mL of spectroscopic grade methanol. Tocopherols were separated by injecting 10µL of the sample to 3µm C18 reverse phase HPLC column (Merck), and eluated using acetonitrile-methanol (1:1) with a flow rate of 1mL/min at a wavelength of 340nm.

Antioxidant enzyme assays

Superoxide dismutase *(Vladimir et al., 1989)*

Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of quercetin autoxidation. To 1mL reaction mixture containing 890µL of phosphate buffer (0.016M, pH 7.8) and 50µL of TEMED – EDTA (8mM /0.08mM), 50µL of quercetin (0.15% in dimethyl formamide) was added. The rate of quercetin autoxidation was monitored for 3min at 406nm. Following addition of sample (5-10µg cytosol/mitochondrial protein), the decrease in

absorbance was monitored. The amount of protein that inhibits quercetin oxidation by 50% was defined as one Unit.

Catalase *(Aebi, 1984)*

Catalase (CAT) activity was measured by the method of Aebi (1984). An aliquot of testis cytosol (equivalent to 0.1mg protein) was added to reaction mixture containing 850µL of phosphate buffer (0.1M, pH 7.4) and 50µL of H_2O_2 (8.8mM). The decrease in absorbance was monitored at 240nm and the activity was expressed as µmol H_2O_2 decomposed/min/mg protein (€- 43.6/mM/cm).

Glutathione peroxidase *(Flohe and Gunzler, 1984)*

The activity of glutathione peroxidase (GPx) was determined according to Flohe and Gunzler (1984) using t-butyl hydroperoxide (tbHP) as the substrate. The reaction mixture containing 50µg of cytosol/mitochondrial protein, Phosphate buffer (0.1M, pH 7.0, 2mM EDTA), 100µL glutathione reductase (0.24U), 100µL GSH (1mM) and 100µL NADPH (0.15mM) was incubated for 3min at 37˚C. The reaction was initiated by addition of 100µL tbHP (0.12mM) and the change in absorbance was monitored for 3min at 340nm and the activity was expressed as nmol NADPH oxidized/min/mg protein (€- 6.22/mM/cm).

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

Glutathione-S- transferase (GST) activity was measured by monitoring the enzyme catalyzed conjugation of glutathione with 1-chloro,2,4 dinitro benzene. An aliquot of testis cytosol/mitochondria was added to reaction mixture containing phosphate buffer (0.1M, pH 6.5, 0.5mM EDTA), 50 µL CDNB (1.5mM) and 50 µL GSH (1mM). The increase in absorbance was monitored for 3min at 340nm. The activity was expressed as µmol conjugate formed/min/mg protein (€- 9.6/mM/cm).

Glutathione reductase *(Carlberg and Mannervick, 1985)*

Glutathione reductase (GR) activity was measured in a one mL reaction mixture that contains 900µL of phosphate buffer (0.2M, pH 7.0, EDTA

2mM), 0.2mg cytosol / mitochondrial protein and 50 µL of oxidized glutathione (20mM). The reaction was started by addition of 50 µL NADPH (2mM) and the decrease in absorbance was monitored at 340nm for 3 min. The activity was expressed as µmol NADPH oxidized/min/mg protein (€- 6.22/mM/mg protein).

Thioredoxin reductase *(Luthman and Holmgren, 1982)*

Thioredoxin reductase (TRR) activity was measured by the addition of cytosol/mitochondria (50µg protein equivalent) to potassium phosphate buffer (0.1M, pH 7.0, 10mM EDTA) containing 0.2mM NADPH, 0.2mg BSA, 5mM DTNB. The absorbance was measured at 412nm for 3min, the activity was calculated using the formula $dA_{412}X0.5$ /13.6X2, since one mol of NADPH yields 2mol of thionitrobenzoate and expressed as µmol NADPH oxidized/min/mg protein.

Functional enzyme activities

Sorbitol dehydrogenase *(Gerlach, 1974)*

The activity of sorbitol dehydrogenase (SorDH) was determined as the amount of fructose reacted per unit time, measured by the decrease in the absorbance due to oxidation of NADH at 340 nm. An aliquot (0.2mg protein) cytosol was added to tris buffer (111mM, pH 7.5) and NADH (1.8mM) in a final volume of 1mL. The reaction was started by the addition of fructose (66.6mM) and decrease in absorbance was monitored for 3 min. The activity was expressed as µmol NADH oxidized/min/mg protein (€-6.22 mM/cm).

Glucose 6-phosphate dehydrogenase (*Deutsch et al., 1974*)

Glucose 6-phosphate dehydrogenase (G6PDH) was measured in the testis cytosol following the rate of reduction of NADP at 340nm. An aliquot of cytosol (equivalent to 0.2mg protein) was added to sodium phosphate buffer (0.1M, pH7.8) containing $MgCl_2$ (63mM) and glucose-6-phosphate (33mM). The reaction was started by the addition of NADP (3.8mM) and increase in absorbance was monitored for 3min at 340nm. The activity was expressed as µmol NADPH formed/min/mg protein (€-6.22mM/cm).

Aldehyde dehydrogenase *(Canuto et al., 1983)*

Aldehyde dehydrogenase (ALDH) activity was determined in 1mL reaction mixture containing Sodium phosphate buffer (60mM-1mM EDTA, pH7.8), NAD^+ (1mM) and propionaldehyde (1mM). The reaction was initiated by addition of cytosol/mitochondria protein (0.1mg) and the increase in absorbance was monitored at 340 nm (€-6.22mM/cm).

Aldose reductase *(Nishikawa et al., 2001)*

Aldose reductase (AR) activity was determined in 1mL reaction mixture containing sodium phosphate buffer (0.1M, pH 7.0), NADPH (1mM) and methylglyoxal (1mM). The reaction was initiated by addition of testis cytosol (0.1mg protein) and the decrease in absorbance was monitored at 340 nm (€-6.22mM/cm).

Lactate dehydrogenase *(Goldberg and Hawtrey, 1967)*

The activity of lactate dehydrogenase (LDH-X) was measured in a reaction mixture containing Tris-HCl buffer (0.1M, pH 7.0), α-ketovaleric acid (5mM), NAD^+ (10mM). The reaction was initiated by addition of cytosol protein (0.1mg) and change in absorbance was monitored at 340nm for 3min and expressed as µmol NADH oxidized/min/mg protein (€- 6.22 /mM /cm).

3 β- hydroxysteroid dehydrogenase *(Qujeq, 2002)*

3β-hydroxysteroid dehydrogenase (3β-HSD) activity in testis cytosol was determined by measuring rate of conversion of pregnenolone into progesterone. The enzyme was assayed in a reaction mixture containing Tris-HCl buffer (0.15M, pH 7.25), NAD^+ (400µM), Pregenolone (200µM), nitroblue tetrazolium (953µM) in a total volume of 3.2mL. The reaction was started by the addition of the 0.25mg cytosol protein and incubated at 37˚C for 60min. Incubation of testis homogenate and NAD^+, without addition of pregnenolone was simultaneously carried out as a control. Terminally, the reaction was stopped by the addition of 2.0mL of phthalate buffer (pH 4.25), centrifuged at 5,000Xg for 30min, and the supernatant was read at 570nm. The enzyme activity was calculated from the NADH standard curve and expressed as nmol NADH formed/h/mg protein.

Determination of ATPase activity *(Desaiah and Ho, 1979)*

ATPase activity was measured by determining the inorganic phosphate liberated from ATP according to the method of Desaiah and Ho (1979).Total ATPase was assayed in a medium containing Tris-HCl buffer (50mM, pH 7.4), NaCl (100mM), KCl (20mM), $MgCl_2$(5mM) final concentration. Mg^{2+} ATPase activity was measured in a medium containing Tris-HCl buffer (50mM, pH 7.4), ATP (5mM), KCl (20mM), $MgCl_2$ (5mM) and 0.1mM Ouabain with the omission of NaCl and KCl. The reaction was started by the addition of 50µg cytosol/mitochondrial protein and incubated for 15min at 37ºC. The reaction was terminated by addition of 20% TCA. Following centrifugation at 1,500 Xg for 10min the inorganic phosphate in the protein free supernatant was determined according to Fiske and Subbarow (1921). Blanks without enzyme were carried throughout the entire procedure. Enzyme activity was expressed as µg inorganic phosphate liberated/mg protein for 15min. Na^+ K^+ ATPase activity was calculated as the difference of the activity between total ATPase and Mg^{2+} ATPase.

Intracellular Ca^{2+} levels (*Hitoshi et al., 1997*)

Intracellular Ca^{2+} levels were measured in testicular cytosolic preparation using Fura-2AM. Testis cytosol equivalent to 0.25mg protein was incubated with Locke's buffer (pH 7.4, free from Ca^{2+}) containing Fura-2AM (5µM/mL) for 45min at 37ºC. The fluorescence was measured at excitation and emission wavelengths of 488 and 525nm.

Tissue iron levels *(Peters et al., 1956)*

Tissue iron levels were quantified using bathophenanthroline, a ferrous iron sensitive dye. A 10% homogenate of testis was prepared using protein precipitating agent (TCA 0.1% v/v, 0.3mL thioglycollic acid, 0.02 mL HCl). The homogenate was centrifuged at 3,000Xg for 10min and the supernatant was used for the estimation. A known volume of the supernatant was added to 1mL of bathophenanthroline color reagent (bathophenanthroline- thioglycollic acid: sodium acetate: water; 1:20:20) and incubated at room temperature for

10min. The absorbance was measured at 535nm and quantified using a reference standard (0-100µg).

Mitochondrial enzyme activities

Citrate synthase *(Srere, 1969)*

Citrate synthase (CS) activity was determined by monitoring the oxidation of DTNB at 412nm.Testis mitochondria (0.1mg protein) was added to a reaction mixture containing Tris-HCl buffer (0.1M, pH 8.1 containing 0.1% Triton X-100), 0.2mM DTNB and 0.1mM acetyl CoA. The reaction was initiated by addition of oxaloacetate (10mM). The enzyme activity was expressed as nmol thiol group oxidized/min/mg protein (€- 13.6/mM /cm).

Succinate dehydrogenase *(Penington, 1961)*

Succinate dehydrogenase (SDH) activity was determined by incubating mitochondrial protein (0.05mg) with 50mM potassium phosphate (pH 7.4) containing sodium succinate (0.01mol/L) and *p*-iodonitrotetrazolium violet (2.5µg/mL) for 10 min. The reaction was stopped by addition of 10% TCA. The color obtained was extracted with ethyl acetate: ethanol: trichloroacetic acid (5:5:1, v:v:w) and measured at 490 nm. The activity was expressed as mmol substrate oxidized /mg protein (€- 2000 /mM /cm).

Malate dehydrogenase *(Kitto, 1969)*

Malate dehydrogenase (MDH) activity was measured by addition of testis mitochondria (0.1mg protein) to potassium phosphate buffer (0.1M, pH 7.5) containing 14.3mM NADH and 20mM Oxaloacetate and the decrease in absorbance was monitored at 340nm for 3min. The enzyme activity was expressed as µmol NADH oxidized/min/mg protein (€-6.22mmol/cm).

Mitochondrial complex enzyme activities

NADH:Cytochrome c reductase (Complex I-III) (*Navarro et al., 2004*)

Testis mitochondria (0.1mg protein) was added to phosphate buffer (0.1M, pH 7.4) containing NADH (0.2mM) and KCN (1mM). The reaction was initiated by addition of 0.1mM cytochrome C and decrease in absorbance was

monitored for 3min at 550nm. The activity was expressed as µmol cytochrome c reduced/min/mg protein (€-19.6 mM/cm).

Succinate: Cytochrome C reductase (Complex II-III) (*Navarro et al., 2002*)

Testis mitochondrial protein (0.1mg) was added to phosphate buffer (0.01M, pH 7.4, 2mM EDTA) containing succinate (20mM) and KCN (1mM). The reaction was initiated by addition of 0.1mM cytochrome C and decrease in absorbance was monitored for 3min at 550nm. The activity was expressed as µmol cytochrome c reduced/min/mg protein (€-19.6 mM/cm).

Mitochondrial integrity assays

Mitochondrial permeability transition pore opening (MPT) *(Masaki et al., 1989)*

Testis mitochondria (1mg protein) was suspended in swelling medium consisting of mannitol (215mM), sucrose (71mM), HEPES (3mM) and sodium succinate buffer (5mM, pH 7.4). Mitochondrial swelling was monitored following the addition of $CaCl_2$ (100µM) at 540nm.

Mitochondrial membrane potential *(Shimizu et al., 1999)*

Testis mitochondria (0.05mg protein) was added to 0.1M phosphate buffered saline (PBS, pH 7.4) containing Rh 123 (1.5µM /mL) and incubated at 37˚C for 30 min. Following centrifugation at 10,000X g for 10min at 4˚C, the fluorescence intensity of the supernatant was measured at excitation and emission wavelengths of 490nm and 520nm respectively.

MTT reduction assay **(***Berridge and Tans, 1993*)

Testis mitochondria were incubated in a Mannitol-Sucrose-HEPES buffer (pH 7.4) containing sodium succinate (20mM) and 15µL MTT (5mg/mL) at 37˚C. for 60min. The formazan crystals formed were dissolved in aqueous 10% SDS- 45% DMF buffer (v/v, pH 7.4). The absorbance of the clear solution was measured at 570nm and expressed as OD/mg protein.

Caspase 3 Assay

Caspase 3 activity was estimated employing commercially available kit procured from M/s Sigma Chemicals (St. Louis, MO, USA). The assay is based on the hydrolysis of the substrate acetyl-Asp-glu-Val-Asp-7-amido-4-methylcoumarin by Caspase 3, resulting in the release of the fluorescent 7-amino-4-methyl coumarin (AMC) moiety. The fluorescence was measured at the excitation and emission wavelengths of AMC at 360nm and 460nm respectively. The concentration of the AMC released was calculated from AMC calibration curve and expressed as nmol AMC/min/mL.

Microsomal NADPH-cyt P450 reductase **(***Dignam and Strobel, 1975)*

The method is based on the reduction of cytochrome c in a medium containing NADPH at 550nm. Cytochrome c (75μM in 0.3M phosphate buffer, pH 7.8), 0.1mM NADPH and 0.1mg microsomal protein was added to the reaction mixture. The decrease in absorbance was measured for 3min and the activity was expressed as μmol cytochrome c reduced/min/mg protein (€-21.0 mM/cm).

Ethoxy Resorufin –O- Dealkylase (EROD)

The reaction mixture contained Tris–HCl (0.05M, pH 7.4), 2.6μM ethoxy-resorufin, NADPH-regenerative system (1mM NADP+, 1U/ml glucose-6-phosphate dehydrogenase, 4.4mM glucose-6-phosphate, 5mM $MgCl_2$, 1mg BSA) and testicular microsomal protein (0.2mg) in a total volume of 1mL. The samples were incubated for 20min at 37˚C. The reaction was initiated by adding 0.5 ml of NADPH-regenerative system and terminated by adding 2mL of methanol. Following incubation for 5 min at room temperature tubes were centrifuged at 10,000×g and the supernatant was measured fluorimetrically (Ex 544; Em 590nm) and quantified using resorufin standard.

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

An aliquot of cytosol/mitochondria/ microsomal suspension was made upto 1mL with Lowry's reagent (2% Na_2CO_3 in 0.1N sodium hydroxide containing 1% copper sulphate and 2% sodium potassium tartarate) and incubated for 10min at room temperature. Following addition of 0.1mL of

Folin-Ciocalteu reagent (1N) the reaction mixture was allowed to stand for 20min at room temperature. The absorbance was measured at 750nm and the concentration of protein was determined using BSA as the standard.

COMET Assay *(Singh et al., 1988)*

Alkali labile fragmented DNA was ascertained by quantifying the incidence of comets as described by Singh et al., (1988). Testis was decapsulated and seminiferous tubules were gently expressed and incubated with collagenase (0.5mg/mL) followed by trypsin at 37˚C for 15 min on a shaker. The cell suspension was gently aspirated, filtered through 100micron sieve, washed twice with KRBS and the cell number was adjusted to $5X10^{6}$ cells/mL.

A pre-cleaned fully frosted microslide was covered with a thin layer of normal melting agarose (0.75%). Testicular cells suspended in low melting agarose (0.5%) were layered carefully followed by a third layer of low melting agarose (0.5%). The slides were allowed to solidify and then immersed in cold lysing solution (2.5M NaCl, 100mM EDTA and 10mM Tris, pH 10) overnight at 4˚C. The slides were subjected to electrophoresis by immersing in alkaline buffer (1mM Na_2EDTA, 300mM NaOH, pH 10) for 10min at 50V under dim light.

Following electrophoresis, the slides were neutralized with Tris buffer (0.4M, pH 7.5) and stained with ethidium bromide (0.002%). The slides were examined under fluorescent microscope at a magnification of 40X. Cells with damaged DNA appeared as comets and the incidence was measured in a field of 100 cells, taken at random.

Flow cytometric analysis of testicular cells (*Krishnamurthy et al., 1998*)

Testicular cell suspension was prepared as described earlier were fixed in 70% ice-cold alcohol. For flow cytometric analysis, the alcohol fixed cells were washed with PBS and subjected to pepsin (1% w/v) digestion. For measurement of DNA content, $2X10^{6}$ cells were stained with hypotonic propidium iodide (PI, 50µg/mL) containing 0.1% sodium citrate and 0.1% triton X-100. The analysis was performed using a Becton Dickinson FACScan flow

cytometer. A total of $2X10^4$ events were accumulated for each measurement. After gating of the cells by light scatter and DNA width versus area to eliminate doublets, DNA content was analyzed from fluorescent intensity distribution histograms in which peaks were, represented haploid, diploid, tetraploid and S-phase cells. The haploid region corresponds to the spermatids which include both immature/mature spermatid populations. The diploid region represents populations of G1-phase spermatogonia, preleptotene spermatocytes/somatic cells while tetraploid region represent spermatogonia in G2/M phase. The S-phase cell region (between diploid and tetraploid cells) represent cells actively synthesizing DNA.

Protein profile by SDS-PAGE *(Laemmli, 1970)*

Testis cytosol/ mitochondrial protein were prepared as described in earlier. The sample for electrophoresis was prepared by derivatizing cytosol/ mitochondrial protein (25µg) with 0.1% SDS in presence of 5% β-mercaptoethanol at 90ºC for 5min. After heating, SDS-PAGE was performed using 12% acrylamide gels. A constant current of 50mA was employed. After the run, the proteins were stained with 0.25% Coomassie Brilliant Blue for 5hr.

Histopathological examination

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

Statistical analysis

Experimental data obtained were expressed as mean ± Standard error mean (SEM) and analyzed by Student's 't' test. Data obtained in modulation of oxidative damage in prepubertal rats are expressed as mean ± SEM and analyzed by one way ANOVA using Statplus professional statistical software 2007 version 4.9. Post hoc multiple comparisons were performed between the groups using Tukey's or Duncan's Multiple Range Test (DMRT). P value was set at 0.05 as the minimum level of significance.

1.0 INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from defective insulin secretion, resistance to insulin action or both**.** The past two decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide. The global figure of people with diabetes is set to rise from the current estimate of 150million to 220million in 2010 and 300million in 2025 (Fagot-Campagna, 2001). A national study projected that a major proportion of world's diabetic population will be in non-industrialised countries and India will have the major burden (Ramachandran et al., 2003). According to a recent estimate, there are nearly 32million diabetics in India and the number is expected to raise phenomenally in the near future (Wild et al., 2004). Even though, diabetes is viewed as an urban phenomenon, the prevalence of prediabetic risk factors viz., impaired glucose tolerance and impaired fasting glycemia are fastly appearing in rural communities (Ramachandran et al., 2001) suggesting a high burden of incipient disease on population health. Over the last decade, there has been a disturbing trend of increasing cases of children/adolescents presenting with clinical features of type2 diabetes (T2DM). In developed countries, a ~10 fold increase in the incidence of T2DM among young individuals (Rosenbloom, 2002) and the increasing proportion up to one third- in children and adolescents with diabetes is reported (Pinhas-Hamiel et al., 2005). Similar trends have also been reported from other countries (Kitagawa et al., 1998; Likitmaskul et al., 2003).

Being a systemic disorder, diabetes has been shown to affect almost all the organs of the body and the major long term complications are retinopathy, nephropathy, neuropathy and testicular dysfunction. It has been suggested that enhanced production of free radicals and oxidative stress is a central event to the development of diabetes/associated complications (Vincent et al., 2002). The increased blood glucose level during early stages of diabetes becomes a chief source of free radicals in the chronic stage damaging almost all organs. Epidemiological evidences and clinical data emphasize that oxidative stress is increased in diabetes due to the

overproduction of reactive oxygen species (ROS) and decreased efficiency of antioxidant defenses (Baynes 1991; Baynes and Thorpe, 1999; Zimmet, 2001; Obrosova, 2008). High reactivity of ROS determines chemical changes in virtually all cellular components, leading to lipid peroxidation. Further, elevated glucose interacts with specific amino acids on proteins forming AGE that can lead to modification in cell signaling and upregulation of cell death events (Rahimi et al., 2005).

Several epidemiological evidences clearly emphasize the role of oxidative stress in the etiology of male infertility (Aitken, 1995; Sikka, 2001; Saleh and Agarwal, 2002). Diabetes associated male reproductive dysfunctions are well known and sustained hyperglycemia is associated with development of testicular impairments observed during diabetes (Sexton and Jarrow, 1997). About 90% of diabetic patients have disturbances in sexual function, including a decrease in libido, impotence and infertility. Animal models with spontaneous and induced diabetes have attempted to provide basic insight into the potential adverse effects of diabetes on human male reproductive dysfunction. In animal models of diabetes, earlier workers have demonstrated various structural and functional reproductive anomalies (Paz and Homonnai, 1979) viz., seminiferous tubular wall thickening, germ-cell/Leydig cell depletion, Sertoli cell vacuolization, hypo gonadism, decreased sperm motility along with diminished testosterone levels. While the participation and contribution of oxidative stress mechanism/s in development of various diabetic complications is well known, the possible involvement of such mechanism/s in testicular dysfunctions is only beginning to be understood (El-missiry, 1999; Cai et al., 2000; Unlucerci et al., 2000). In this regard, recent studies from our laboratory have demonstrated the occurrence of oxidative stress in adult testis and epididymal sperm under experimentally-induced diabetes both during acute and progressive phase in experimental animals (Shrilatha and Muralidhara, 2007a,b).

Mammalian testis is anatomically and physiologically more prone to free radical mediated oxidative attack which in part is attributed to its unique lipid composition (Cummins et al., 1994; Bauche et al., 1994) that becomes main substrate during peroxidative attack and adversely effecting testicular

physiology/ function (Aitken et al., 1989). Although testis is bestowed with various antioxidant defenses, their efficiency is restricted due to their limited concentration and varied distribution. Hence it is likely that oxidative stress mechanism/s may play a predominant role in testis under diabetic situations. Given the current understanding on the reproductive pathologies associated with diabetes is largely known in adults, a basic insight into disease pathogenesis during developmental stage is lacking. However, to the best of our knowledge, no data exists on the vulnerability of immature (prepubertal) testis to oxidative stress under experimentally-induced diabetes. Owing to the phenomenal increase in the incidence of diabetes among children/adolescent males, comprehensive studies are warranted to understand the nature/extent of oxidative impairments and associated biochemical dysfunctions in immature testis. This rising prevalence of T2DM in children will expose them to the risk of developing the macrovascular and microvascular complications during the prime of their life (their productive period), which will have an adverse impact male reproductive health (Sahay and Sahay, 2003).

Accordingly in the present study, the major emphasis was to understand whether testes of prepubertal rats are subjected to marked oxidative stress during the acute phase of diabetes. The results have been presented under two sections A and B. *Section A* describes studies related to the determination of an optimum dosage of STZ that causes significant, consistent hyperglycemia and low mortality among prepubertal rats. Further, the relative susceptibility of testis to oxidative stress was ascertained along with other vital organs such as liver and brain. *Section B* describes investigations carried out to understand the susceptibility pattern of testis of two age group rats (4 and 6-wk old) in the Streptozotocin diabetic PP model.

2.0 OBJECTIVE

In this chapter, we have primarily addressed several issues related to the question of vulnerability of prepubertal rat testis to diabetes induced oxidative stress employing STZ-model. The study comprised of preliminary dose selection study, dose response study and a detailed investigation among two age group prepubertal rats (4- and 6- wk old).

SECTION - A

STZ INDUCED OXIDATIVE DYSFUNCTIONS IN TESTIS OF PREPUBERTAL (PP) RATS: DOSE RESPONSE

3.0 EXPERIMENTAL DESIGN

Preliminary study

In order to determine the optimum dosage of Streptozotocin (STZ) that would induce low mortality, consistent hyperglycemia accompanied by testicular impairments, a preliminary study was conducted. Prepubertal male rats (4wk old, 50±5g) procured from 'Institute animal house facility', were used for the investigation. STZ was dissolved (50mg/ml) in freshly prepared citrate buffer (0.1M, pH 4.5). Groups of male rats (n=6) were administered (intraperitoneally) an acute dose of STZ ranging from 30 - 120mg /kg bw. STZ treated rats were provided with glucose water (5% w/v) for 24h to prevent mortality by hypoglycemic shock. Mortality was recorded as and when it occurred.

Determinative study

Prepubertal male rats were randomly assigned to three groups: Group I designated as 'non-diabetic controls' received citrate buffer and served as 'the normal controls'. To determine the minimum effective dose, STZ was administered at two dosage levels viz., 60 and 90mg/kg bw to group II and group III rats respectively.

Morphological investigations and autopsy

Growth pattern among control and STZ- treated rats were measured as gain in body weight on alternate days and on the day of sacrifice. Animals were sacrificed on day 7 of post STZ treatment under light ether anesthesia. Blood was collected by cardiac puncture and processed for glucose estimation. Testis, liver and brain were excised, rinsed with ice-cold saline, blotted and weighed.

STZ induced oxidative dysfunctions in prepubertal rats – Dose response

Although the main focus of this study was to assess the susceptibility pattern of prepubertal testis to STZ induced oxidative impairments, paucity of data on the relative susceptibility of other vital organs prompted us to determine the oxidative stress response induction in both liver and brain.

Induction of oxidative damage: Generation of ROS and formation of MDA

Following STZ administration, the generation of ROS was quantified in subcellular organelles (cytosol/mitochondria) of testis, liver and brain using dichlorofluorescein-diacetate (DCF-DA). Further, the status of lipid peroxidation was measured in terms of malondialdehyde (MDA) by thiobarbituric acid reaction.

Perturbations in antioxidant defenses

Alterations in the activity levels of both enzymic antioxidants viz., SOD, Catalase, GPx and GST together with non-enzymic antioxidant molecules viz., glutathione, total thiols and non-protein thiols were assayed in both cytosol and mitochondria of testis, liver and brain of prepubertal rats.

Alterations in the activities of functional enzymes

The activity levels of lactate dehydrogenase and sorbitol dehydrogenase were assayed in testis cytosol among both control and STZ-treated rats.

SECTION - B

DIFFERENTIAL INDUCTION OF OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTIONS IN TESTIS OF PRE-PUBERTAL (PP) RATS

3.0 EXPERIMENTAL DESIGN

Eventhough classification of experimental animals based on age is practically difficult, taking into consideration characteristic metabolic changes that occur during developmental stages (Spear, 2007) we have employed 4wk- old PP rats that represents early prepubertal stage and 6wk-old PP rats as the end stage of prepubertal stage or onset of puberty.

With an objective of determining the relative vulnerability of prepubertal testis to STZ induced oxidative stress, rats of two prepubertal age groups (4wk-old, 50±5g; 6wk-old, 110±5g) were randomly drawn from the stock colony. The animals were housed individually and fed with commercial pellet diet and tap water *ad libitum*.

Prepubertal male rats were randomly assigned to four groups; Group I and III animals received only citrate buffer and were designated as '4wk- and 6wk-old PP controls' respectively. Group II and IV animals were rendered 'diabetic by an acute dose of STZ (90mg/kg bw) and were designated as '4wk- and 6wk- old PP diabetic'. Age matched control rats received an equal volume of vehicle (citrate buffer, pH 4.5). STZ treated rats were provided with glucose water (5% w/v) to prevent hypoglycemic shock. Food intake was measured daily and growth pattern was measured as gain in body weight every alternate day and before sacrifice. Induction of hyperglycemia was ascertained by measuring plasma glucose levels among both control and STZ-treated rats.

Oxidative stress and mitochondrial dysfunctions in testis

Induction of oxidative damage: Generation of ROS and lipid peroxidation

Following STZ administration, the generation of ROS in cytosol/mitochondria of 4-and 6wk old PP testis were quantified using

dichlorofluorescein diacetate (DCF-DA), while lipid peroxidation was quantified using 2-thiobarbituric acid (TBA).

Perturbations in antioxidant defenses

The activities of antioxidant enzymes viz., SOD, Catalase, GPx and GST were assayed in testis cytosol and mitochondria. Further, the levels of non-enzymic antioxidants, viz., ascorbic acid, thiol status along with tissue iron and calcium levels were determined among both age group rats.

Alterations in functional enzyme activities

Following STZ administration, the activities of functional enzymes viz., LDH, SorDH, G6PDH and 3β-HSD were assayed in testis cytosol of both age groups.

Activity of TCA cycle enzymes

The activities of citrate synthase, malate dehydrogenase and succinate dehydrogenase were determined in freshly prepared mitochondria of testis among diabetic/ non-diabetic rats of both age groups.

Activity of NADH - cytochrome c reductase and succinate - cytochrome c reductase

The activity of mitochondrial NADH-cytochrome-C reductase and succinate-cytochrome C reductase were determined in fresh mitochondria of testis both age group rats.

4.0 RESULTS

SECTION - A

STZ INDUCED OXIDATIVE DYSFUNCTIONS IN TESTIS OF PREPUBERTAL (PP) RATS: DOSE RESPONSE

Salient findings of preliminary study

Administration of STZ at the lowest dose (30mg/kg bw) failed to induce any significant increase in blood glucose levels, while a mild hyperglycemia (2-folds) was apparent at a dosage of 60mg/kg b.w in PP rats. However, at 90mg/kg b.w, PP rats showed a significant increase in plasma glucose levels. Although, marked hyperglycemia (5-6 folds) was evident at the highest dosage (120mg/kg bw), there was a higher incidence of mortality within 72h of administration. Hence, in the determinative study, the effect of STZ at only two dosage levels (60 and 90mg/kg bw) was analysed.

Determinative study

Food intake, growth pattern and testis weight

Data on the growth pattern among different groups of rats measured as gain in body weight is presented in Table 2.1. PP rats treated with STZ initially showed a marginal decrease in food intake during the early days of administration (3days), which subsequently increased throughout the experiment period (25%) (data not shown). At both dosages, STZ treatment resulted in a significant reduction in body weight gain (lower dose, 25%; higher dose, 40%). Irrespective of the dosage, STZ-treated PP rats showed significant decrease in testis weights (72, 83%) while, the liver weight showed marginal to moderate reduction (23%, 36%).

Hyperglycemic response

Blood glucose levels measured terminally are presented in the Fig 2.1. STZ at the dosage of 60mg/kg bw induced 2 fold increase in the plasma glucose levels, while significant hyperglycemia (4.5 folds) was evident at the higher dose (90mg/kg bw).

Oxidative dysfunctions in testis

Generation of ROS and MDA levels

Status of generation of ROS and induction of lipid peroxidation in testis of both control and STZ treated rats is presented in Fig. 2.2A. STZ at both dosages, significantly enhanced the generation of ROS (40%, 60%), which were accompanied by robust elevation in MDA levels (4- 8 fold) (Fig. 2.2B).

Reduced glutathione and total thiol content

Data on levels of reduced glutathione and non-protein thiol content in testis cytosol is presented in Fig. 2.2C. There was no appreciable change in the glutathione levels at lower dose, while the levels were marginally elevated at higher dose (12%). On the other hand, irrespective of the dosage, non-protein thiol levels in testis were markedly elevated (50%) (Fig. 2.2D).

Perturbations in the activities of antioxidant enzymes

Data on the effect of STZ treatment on the activities of selected antioxidant enzymes is presented in Figures 2.3 A-F. Irrespective of the dosage, testicular cytosolic SOD activity was relatively less affected compared to other enzymes. The activity of catalase was significantly elevated (25%) at the lowest dose, while a marginal decrease (12%) was evident at the highest dose. Among diabetic rats, the activity of GPx was less altered at lower dose, while a significant decrease (33%) in the activity was evident at highest dose. However, the activity of GST was significantly elevated at both dosages (29-32%).

Alterations in the activities of functional enzymes

The effect of STZ induced diabetes on selected testicular functional enzyme activities is presented in Fig. 2.4. A dose dependant decrease in the activities of sorbitol dehydrogenase was evident in the diabetic testis cytosol (31, 66%), while the activity of LDH was elevated at both dosages (21 - 40%).

Oxidative dysfunctions in liver and brain

Generation of ROS and MDA levels

Table 2.2 shows dose related effect of STZ treatment on the generation of ROS and induction of lipid peroxidation in liver and brain of prepubertal rats. At both dosages, STZ failed to induce any significant increase in ROS levels in liver (cytosol and mitochondria) while the levels were markedly elevated in brain cytosol. However, brain mitochondria were relatively less affected. STZ also elicited a differential oxidative response in subcellular organelles of liver and brain. A robust increase in MDA levels was evident in hepatic mitochondria compared to cytosol, while a marginal elevation was evident in brain of diabetic PP rats.

Reduced glutathione and total thiol content

Alterations in the levels of reduced glutathione in brain and liver cytosol is represented in Table 2.3. A significant decrease in the reduced glutathione levels was evident in liver cytosol. On the other hand, levels were significantly increased in brain cytosol of diabetic rats. However, glutathione levels were less affected in both brain and liver mitochondria at both dosages.

Perturbations in the activities of antioxidant enzymes

Data on the effect of STZ treatment on the activities of selected antioxidant enzymes in brain and liver cytosol is also presented in Table 2.3. The activity of SOD was elevated marginally in both liver and brain cytosol while the activity of catalase decreased in both brain and liver cytosol only at the highest dose. However, the activity of GST was significantly elevated in brain cytosol.

SECTION- B

EVIDENCE OF DIFFERENTIAL INDUCTION OF OXIDATIVE STRESS AND MITOCHONDRIAL DYSFUNCTIONS IN PREPUBERTAL (PP) TESTIS

STZ treatment and growth pattern and testis weight

Administration of STZ to 4- and 6- week old PP rats at 90mg/kg bw did not induce any mortality during the experimental period. The food intake was comparable between both the age group (data not shown). A general decrease in body weight gain was evident as a result of diabetes induction among both age group rats. The weight gain was less among 4wk PP diabetic rats (9g *vs* control 49g) compared to 6wk PP diabetic rats (30g *vs* control 45g). An analysis of the body weight/testis ratio showed a robust reduction in the testis size among 4wk PP (39%) compared to 6wk PP rats (16%).

Hyperglycemic response

Plasma glucose levels measured terminally are presented in Table 2.4. More robust elevation in blood glucose levels was evident among 4wk PP diabetic rats (3.4 fold) compared to 6wk PP rats (2.4 fold).

Serum lipid profile

Analysis of serum lipids showed a significant reduction in triglycerides (4wk, 47%; 6wk, 71%) and cholesterol levels (4wk, 24%; 6wk, 50%) among diabetic rats (Table 2.4).

Oxidative dysfunctions in subcellular organelles of PP testis

Testicular oxidative stress markers

Effect of STZ administration on testicular oxidative stress parameters among both 4- and 6- week old PP rats is depicted in Fig. 2.5. A distinct differential oxidative induction response was discernable in diabetic groups and the response was more pronounced in cytoplasm compared to mitochondria. While the degree of induction was more robust in testis cytosol of 4wk PP rats (ROS, 114%; HP, 44% and MDA, 296%), the response was

less pronounced among 6wk PP rats (ROS, 89%; HP, 46% and MDA, 91%). However, the mitochondrial oxidative response was similar among both age group rats as evidenced by enhanced HP (20, 18%) and MDA levels (40, 60%). Induction of diabetes resulted in enhanced levels of cytosolic Nitric oxide (NO) among both age groups (4wk-108%; 6wk-193%), while the levels were less affected in mitochondria (data not shown).

Reduced glutathione and total thiol content

Significant alterations in the levels of both reduced glutathione and non-protein thiols were evident in testis (cytosol/mitochondria) (Table 2.5). While the levels of GSH were increased only in the mitochondrial compartment (52, 20%), the cytosolic form was less affected. In addition, irrespective of age group, the levels of non-protein thiols were elevated in testis. However, total thiols in testis were marginally decreased in both the diabetic groups. Further, induction of diabetes resulted in enhanced levels of protein carbonyls in both cytosol/mitochondria of 4wk- old PP diabetic rats (33%, 21%), while the levels were found to be less affected in 6wk- old diabetic rats.

Perturbations in the activities of antioxidant enzymes

Data on the effect of STZ treatment on the activities of selected antioxidant enzymes is presented in Table 2.6. Induction of diabetes caused significant perturbations in testicular antioxidant enzyme activities among both age groups. The activity of SOD was found to be elevated in 4wk PP diabetic rats (cytosol, 27%; mitochondria, 56%), while the activity was less affected in 6wk PP rats. The activity of GPx was relatively less affected in testis cytosol of both age groups, while a significant decrease (40%) was observed in mitochondria of 6wk old PP rats. Further, the activity of catalase was marginally elevated in the cytosol of 4 wk PP rats, while it decreased (31%) among 6wk PP rats. The activities of thioredoxin reductase (15-25%) and glutathione reductase (25-57%) were decreased among both age groups of diabetic rats. However, the activity of GST was elevated in both cytosol and mitochondria (15 - 45%) among both age groups.

Alterations in the activities of functional enzymes

The effect of STZ induced diabetes on the activities of selected testicular functional enzymes is presented in Table 2.7. Irrespective of age, a general decline in the activities of sorbitol dehydrogenase (54, 32%), lactate dehydrogenase (29, 22%) and glucose-6-phosphate dehydrogenase (28%) were evident in diabetic testis.

*Alterations in the activity of 3*β *–hydroxysteroid dehydrogenase*

Perturbations in the activity of 3β–hydroxysteroid dehydrogenase are presented in Fig. 2.6. STZ administration significantly reduced the enzyme activity in both age group rats (25%).

Alterations in the activities of mitochondrial TCA cycle enzymes

STZ induced alterations in the activities of mitochondrial TCA cycle enzyme is presented in Table 2.8. STZ markedly decreased the activities of aldehyde dehydrogenase (76%), citrate synthase (36%) and succinate dehydrogenase (47%) in mitochondria of 4wk old PP rats compared to 6wk old PP rats.

Alterations in the activities of electron transport enzyme complexes

Testis mitochondria of diabetic rats showed a differential response in the activities of ETC enzymes (Fig. 2.7). While the activities of NADH-cytochrome-C reductase and Succinate-cytochrome-C reductase were significantly elevated (69%) among 4wk PP rats, the activities were less robust among 6wk old PP rats.

Perturbations in the mitochondrial integrity

Diabetes induced mitochondrial membrane disturbances are presented in Fig. 2.8. Administration of STZ induced significant disturbances in mitochondrial membrane integrity in testis of diabetic rats as ascertained by enhanced swelling rate of mitochondria, loss in membrane potential and increase in intracellular calcium ions. Testis mitochondria of 6wk old PP rats were relatively more susceptible to MPT inducers (Ca^{2+}, 100μM) compared to 4wk PP rats.

Table 2.1

Gain in body weight, testis and liver weights of prepubertal (PP) rats rendered diabetic by an acute dose of Streptozotocin (STZ)

STZ (mg/kg bw)	Body weights (g)		Organ weights (g)	
	Initial	Final	Testis	Liver
0	52.3 ± 2.6	107.3 ± 4.4	0.710 ± 0.2	5.5 ± 0.4
60	50.6 ± 2.3	75.1 ± 6.5[b]	0.199 ± 0.15[c]	4.2 ± 0.4
90	51.3 ± 2.9	60.3 ± 1.8[c]	0.118 ± 0.02[c]	3.5 ± 0.3[b]

Values are mean ± SEM (n=6).

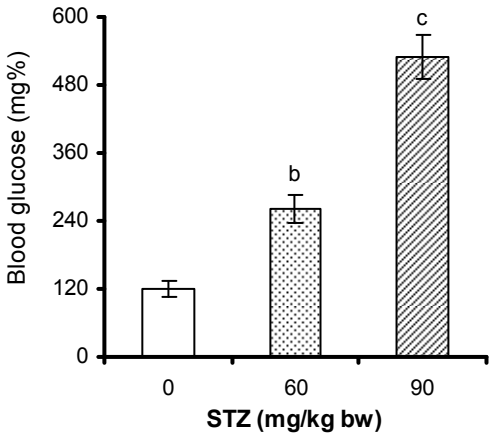
Data analyzed by student's 't' test. Values assigned with different alphabets differ significantly.

[b] $p < 0.01$, [c] $p < 0.05$.

Rats sampled 7d post STZ administration

Fig. 2.1

Effect of Streptozotocin administration on blood glucose levels in prepubertal rats



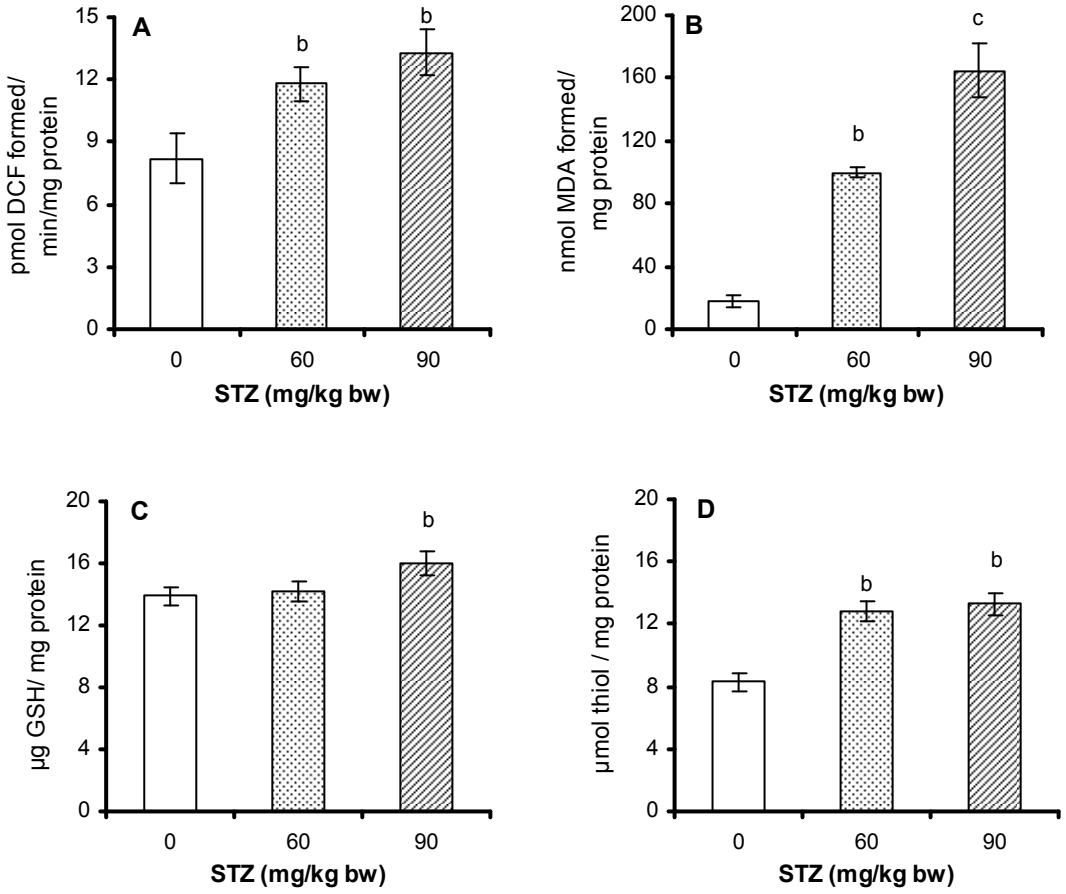
Values are mean ± SEM (n=6).

Data analyzed by student's 't' test. Values assigned with different alphabets differ significantly. [b]$p<0.01$,[c]$p<0.001$

Rats sampled 7d post STZ administration.

Fig. 2.2

Generation of reactive oxygen species (A), induction of lipid peroxidation (B), glutathione (C) and non-protein thiol levels (D) in testis cytosol of diabetic prepubertal rats



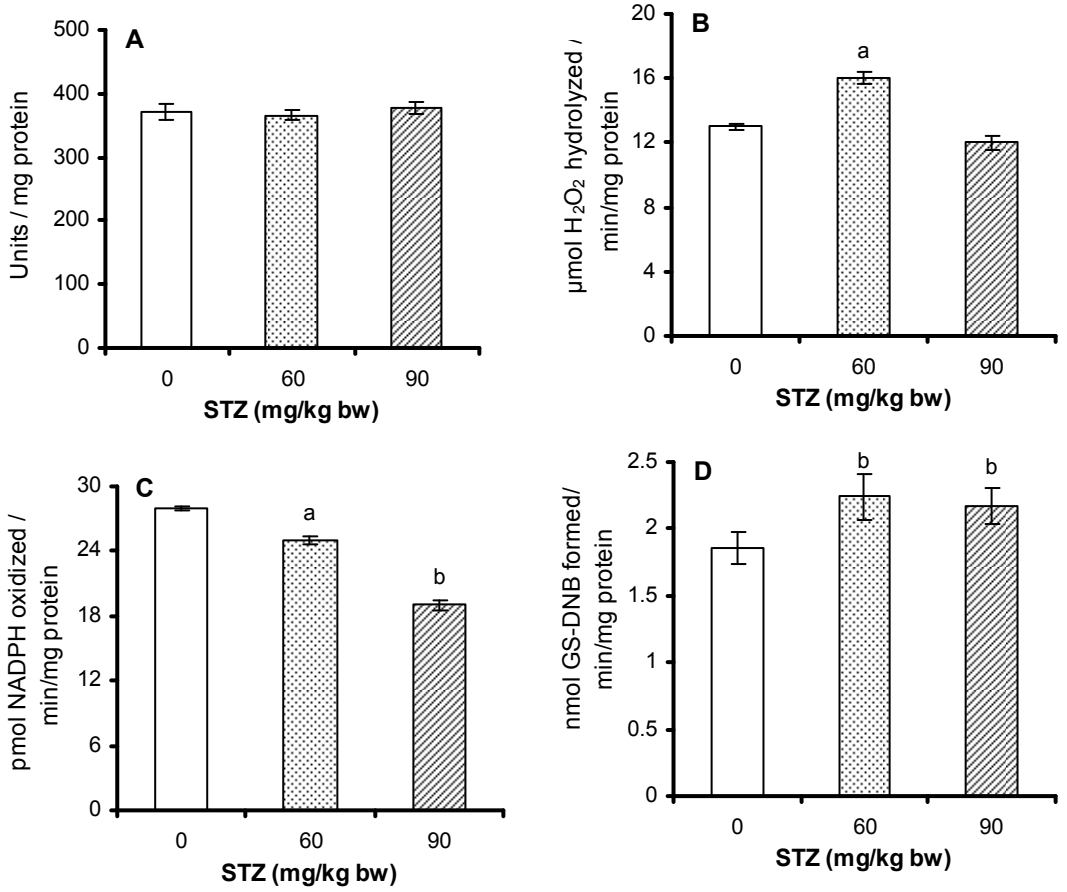
Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; ^{b} $p< 0.01$, ^{c} $p< 0.001$

PP rats sampled 7d post STZ administration

Fig. 2.3

Alterations in the activities of superoxide dismutase (A), catalase (B), glutathione peroxidase (C), glutathione–S-transferase (D) in testis cytosol of diabetic prepubertal rats



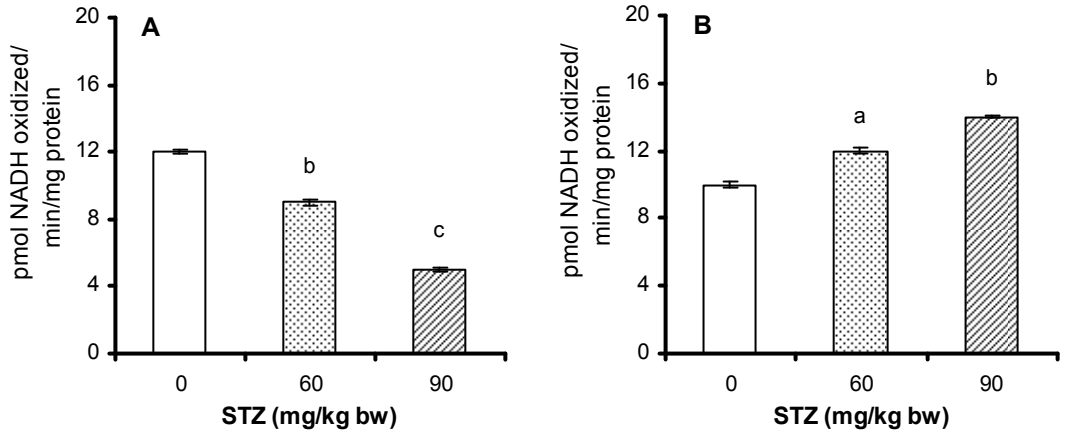
Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$

PP rats sampled 7d post STZ administration

Fig. 2.4

Alterations in the activities of sorbitol dehydrogenase (A) and lactate dehydrogenase (B) in testis cytosol of diabetic prepubertal rats



Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p<0.05$, [b] $p< 0.01$, [c] $p< 0.001$

PP rats sampled 7d post STZ administration

Table 2.2

Reactive oxygen species (ROS) and malondialdehyde (MDA) levels in cytosol and mitochondria of liver and brain of diabetic prepubertal rats

		STZ Dosage (mg/ kg bw)		
		0	60	90
Liver				
Cytosol	ROS	3.67 ± 0.04	3.53 ± 0.01	2.72 ± 0.09[a]
	MDA	36.7 ± 0.71	38.1 ± 0.33	32.8 ± 0.09
Mitochondria	ROS	14.3 ± 0.75	12.4 ± 0.53	12.1 ± 1.30
	MDA	54.2 ± 5.70	73.8 ± 5.30[b] (36)	94.8 ± 11.8[c] (75)
Brain				
Cytosol	ROS	1.08 ± 0.03	1.31 ± 0.05[a] (21)	1.78 ± 0.03[c] (64)
	MDA	44.9± 3.31	48.3 ± 2.11 (10)	54.7 ± 4.31[a] (20)
Mitochondria	ROS	0.59 ± 0.02	0.57 ± 0.07	0.65 ± 0.02
	MDA	213.2 ± 4.2	207.2 ± 7.10	197.0 ± 2.9

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$, [c] $p<0.001$

Values in parenthesis denote percent increase over control

PP rats sampled 7d post STZ administration

Table 2.3

Activities of enzymic and non-enzymic antioxidants in cytosol of liver and brain of diabetic prepubertal rats

STZ (mg/kg bw)	GSH[1]	Enzyme activities		
		SOD[2]	CAT[3]	GST[4]
Liver				
0	17.6 ± 0.9	346.9 ± 13.9	123.3 ± 7.5	1.05± 0.16
60	11.4 ± 0.2[b] (29)	411.2± 15.9 (18)	127.4 ± 11.3	0.98 ± 0.10
90	7.8 ± 0.3[c] (59)	425.8 ± 18.3[a] (22)	110.3 ± 6.2 (11)	0.94± 0.07
Brain				
0	8.3 ± 0.09	578.3 ± 22.0	6.1 ± 0.2	0.081± 0.009
60	9.5 ± 0.2 (27)	663.7± 5.9 (15)	6.8 ± 1.2	0.093± 0.011 (15)
90	13.8 ± 0.3[c] (50)	681.1 ± 19.9 (18)	5.0 ± 0.7 (18)	0.117 ± 0.02[b] (44)

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p< 0.05$, [b] $p< 0.01$, [c] $p< 0.001$

Values in parenthesis denote percent change over controls

[1] Reduced glutathione, µg / mg protein

[2] Superoxide dismutase, Units/mg protein

[3] Catalase, n mol H_2O_2 decomposed/min/mg protein

[4] Glutathione-S-transferase, nmol GS-DNB conjugate formed/min/mg protein

Table 2.4

Blood glucose, testis weights and serum lipids of two age group prepubertal rats rendered diabetic by an acute dose of Streptozotocin

Group	Blood glucose (mg/dl)	Testis wt (g)	Serum lipids	
			Triglycerides (mg/dl)	Cholesterol (mg/dl)
4 wk- old				
Control	86.8± 8.8	0.61 ± 0.03	117.2 ± 13.1	93.3 ± 6.7
STZ	384.4±12.5[b]	0.25± 0.06[b] (59)	61.5 ± 10.6[b] (47)	70.7 ± 4.3[a] (24)
6 wk- old				
Control	107.5 ± 9.1	2.03 ± 0.1	126.5 ± 5.5	78.6 ± 5.2
STZ	259.1±22.2[b]	0.87 ± 0.1[b] (57)	35.9 ± 2.1[c] (72)	39.2 ± 2.8[b] (50)

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test.

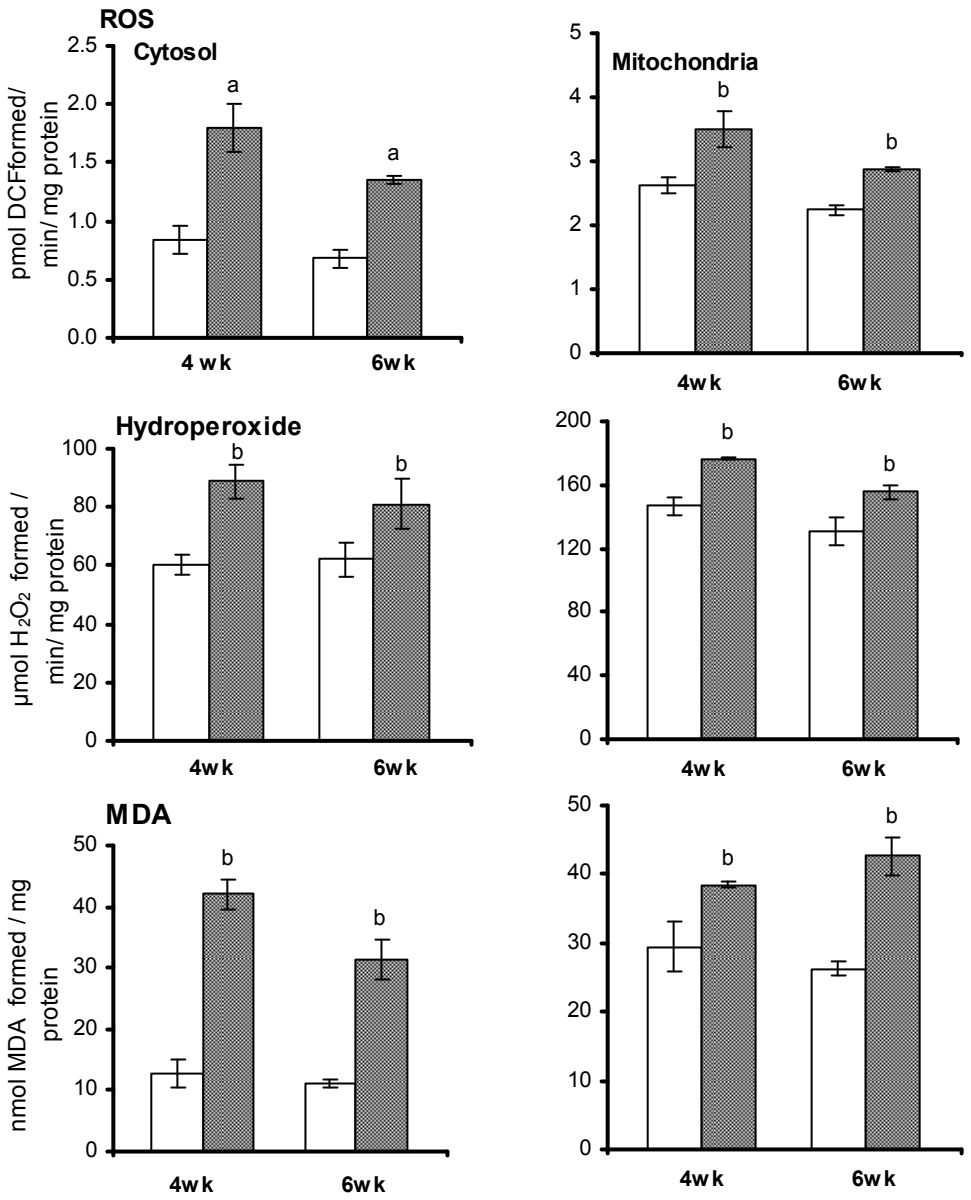
Values assigned with different alphabets differ significantly. [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$

Values in parenthesis denote the percent deviations from respective controls (to indicate degree of testicular atrophy)

PP rats sampled 15d post STZ administration

Fig. 2.5

Pattern of reactive oxygen species generation, hydroperoxide and malondialdehyde in cytosol and mitochondria of testis among diabetic prepubertal (4 wk- and 6wk- old) rats



Each value represents the mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$.

Table 2.5

Status of thiols and protein carbonyls in testis cytosol and mitochondria of diabetic prepubertal rats

	4wk old		6wk old	
	Cytosol	Mitochondria	Cytosol	Mitochondria
Glutathione[1]				
CTR	10.4 ± 0.4	8.6 ± 0.08	11.9 ± 0.7	8.4 ± 0.02
STZ	10.2 ± 1.1	13.1 ± 0.3[b]	10.8 ± 0.3	9.9 ± 0.07[a]
Total thiols [2]				
CTR	38.2 ±1.0	34.0 ± 2.0	33.3 ± 0.9	32.3 ± 0.9
STZ	25.3 ± 1.6[b]	26.7 ± 0.7[a]	27.4 ± 1.1[a]	22.4 ± 1.7[b]
Non protein thiols[3]				
CTR	8.3 ± 0.23	9.2 ± 0.9	8.5 ± 0.8	9.8 ± 0.3
STZ	13.3 ± 0.86[b]	11.8 ± 0.6[a]	10.6 ± 0.3[a]	11.3 ± 0.1
Protein carbonyls [4]				
CTR	1.8 ± 0.02	1.3 ± 0.04	7.2 ± 0.03	7.5 ± 0.03
STZ	2.4 ± 0.01[b]	1.6 ± 0.05[a]	8.1 ± 0.13	7.9 ± 0.01

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p<0.05$, [b] $p< 0.01$, [c] $p< 0.05$

[1] µg /mg protein,

[2,3] µmol thiol /min/mg protein,

[4] nmol carbonyls/mg protein

Table 2.6

Alterations in the activities of antioxidant enzymes in testis cytosol (cyto) and mitochondria (mito) of diabetic prepubertal rats

Enzymes		4 wk old		6 wk old	
		Control	STZ	Control	STZ
SOD[1]	Cyto	370.4 ±1.2	377.0 ± 8.3	332.8 ± 15.0	308.6 ± 12.2
	Mito	110.8 ± 3.1	175.8 ± 18.3[b]	137.2 ± 6.1	131.8 ± 19.9
GPx[2]	Cyto	0.028 ± 0.01	0.025 ± 0.01	0.025 ± 0.01	0.027 ± 0.01
	Mito	0.043 ± 0.01	0.040 ± 0.01	0.072 ± 0.01	0.043 ± 0.01[b]
GST[3]	Cyto	0.711 ± 0.04	1.03 ± 0.17[b]	0.83 ± 0.02	0.98 ± 0.03
	Mito	1.02 ± 0.09	1.44 ± 0.08[b]	1.52 ± 0.08	1.73 ± 1.2
GR[4]	Cyto	23.3 ± 0.05	25.7 ± 0.04	28.5 ± 0.05	31.3 ± 0.12
	Mito	69.3 ± 5.7	31.3 ± 4.4[b]	57.8 ± 4.2	29.7 ± 3.1[b]
TRR[5]	Cyto	0.064 ± 0.006	0.054± 0.008[a]	0.093 ± 0.02	0.074± 0.006[a]
	Mito	0.72 ± 0.008	0.54 ± 0.04[a]	0.76 ± 0.007	0.65 ± 0.16
CAT[6]	Cyto	53.3 ± 0.5	63.0 ± 1.5[a]	73.4 ± 0.5	50.1 ± 0.8[b]

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$

[1] Superoxide dismutase, Units/mg protein;

[2] Glutathione peroxidase, µmol NADPH oxidized /min/mg protein;

[3,] Glutathione S- transferase, µmol GS-DNB conjugate formed /min /mg protein;

[4] Glutathione reductase, µmol NADPH oxidized /min/mg protein;

[5] Thioredoxin reductase, µmol DTNB oxidized/min/mg protein;

[6] Catalase, µmol H_2O_2 hydrolyzed/min/mg protein.

Table 2.7

Alterations in the activities of functional enzymes in testis of diabetic prepubertal rats

	SorDH [1]	LDH[2]	G6PDH[3]
4 wk old			
Control	11.5 ± 2.6	12.3 ± 0.3	15.0 ± 1.0
STZ	5.3 ± 1.1[b]	8.7 ± 0.9[b]	10.7 ± 1.5[b]
6 wk old			
Control	14.3 ± 0.7	17.7 ± 0.3	14.7 ± 0.6
STZ	9.8 ± 0.6[b]	13.7 ± 1.4[b]	10.5 ± 1.1[b]

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test, [b] p< 0.01.

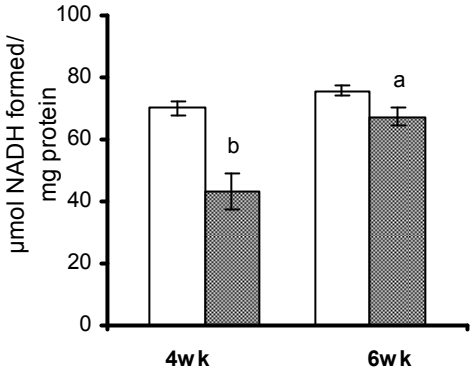
[1] Sorbitol dehydrogenase - µmol NADH oxidized /min/mg protein;

[2] Lactate dehydrogenase - µmol NADH oxidized /min/mg protein;

[3] Glucose 6phosphate dehydrogenase- µmol NADPH oxidized /min/mg protein

Fig. 2.6

Alteration in the activity of 3β-hydroxy steroid dehydrogenase in testis of diabetic prepubertal rats



Values are mean ± SEM (n=6).

Data analyzed by student 't'test; [b] P<0.05

Table 2.8

Alterations in the activities of mitochondrial enzymes in testis of diabetic prepubertal rats

	4 wk old		6 wk old	
	Control	STZ	Control	STZ
ALDH [1]	4.3 ± 0.8	1.0 ± 0.03[c]	4.8 ± 0.13	3.9 ± 0.5[a]
CS[2]	18.3 ± 0.3	11.7 ± 1.7[b]	16.7 ± 1.7	19.3 ± 0.9
SDH[3]	9.5 ± 0.8	5.0 ± 0.6[b]	12.3± 0.7	10.3 ± 0.2

Values are mean ± SEM (n=6).

Data analyzed by student's 't' test, [a] p < 0.05, [b] p< 0.01, [c] p< 0.001

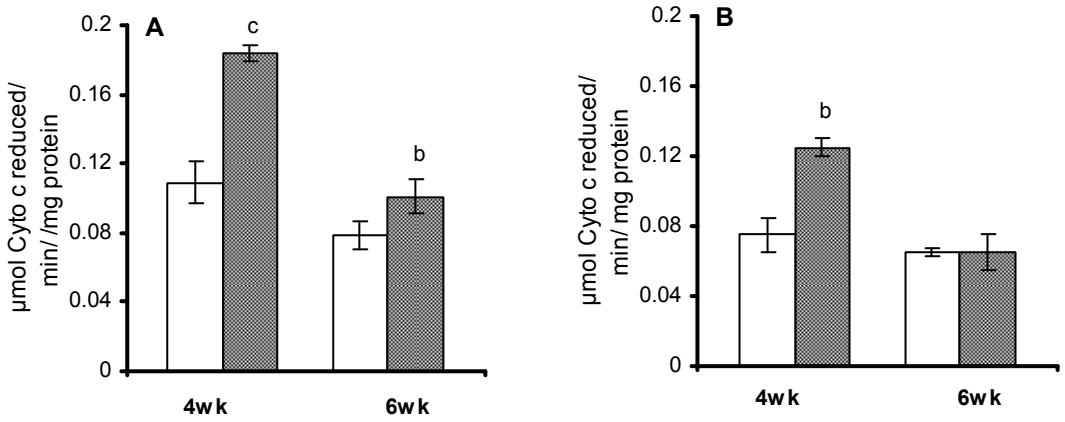
[1] Aldehyde dehydrogenase- µmol NAD reduced /min/mg protein ;

[2] Citrate Synthase- µmol thiol conjugated /min/mg protein;

[3] Succinate dehydrogenase - ηmol succinate oxidized /min/mg protein

Fig. 2.7

Alterations in the activities of mitochondrial enzymes in testis of diabetic prepubertal rats

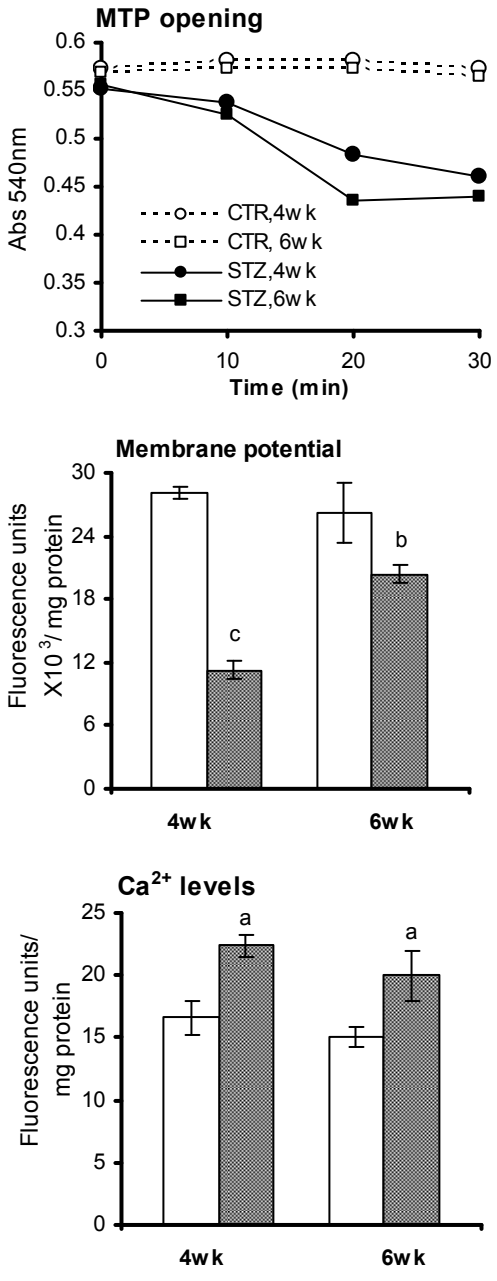


Values are mean ± SEM (n=6).

Data analyzed by student's 't' test. [a] p<0.05, [b] p<0.01, [c] p<0.001

Fig. 2.8

Alterations in mitochondrial function measured as opening of membrane transition pore, loss in membrane potential and elevations in intra-cellular Ca^{2+} levels in testis of diabetic prepubertal (4- and 6wk old) rats



Values are mean ± SEM (n=6)

Data analyzed by student's 't' test; [a] $p< 0.05$, [b] $p< 0.01$, [c] $p<0.001$

5.0 DISCUSSION

The primary objective of the present study was to examine whether testis of prepubertal rat is subjected to oxidative stress under conditions of experimentally induced diabetes. Further, the relative vulnerability of two age group rats to testicular oxidative stress was also examined. Various animal models have been employed to obtain basic insights on the potential adverse effects of diabetes upon human male reproductive system. However, chemical destruction of pancreatic β-cells (using Alloxan or Streptozotocin) is the most commonly employed, since it results in diabetes which closely mimics the human situations. It has been demonstrated that at high doses, the β- cell toxins induce insulin deficiency and T1DM with ketosis, while a lower dose that causes partial destruction of β- cells produces a state that simulates T2DM without a tendency to cause ketosis. Both chemicals are shown to preferentially accumulate in β-cells *via* the GLUT2 glucose transporter and selectively destroy them. However, Streptozotocin (STZ) is preferred because of its more specific β-cell cytotoxicity.

Based on our preliminary studies, we employed STZ at an acute dose of 90mg/kg bw to induce diabetes in prepubertal rats. At higher dosages (120mg/kg bw) STZ either failed to elicit hyperglycemia or showed significant mortality, while at a dosage of 60mg/kg bw only a mild hyperglycemia ensued. The dosage of 90mg/kg bw resulted in consistently elevated glucose levels, lower incidence of mortality and markedly reduced testis weight. Hence, this dosage was considered as the optimum dose for PP rats and employed throughout. This dosage was higher compared to that employed earlier in adult rats (Shrilatha and Muralidhara, 2007a,b). Even though the specific reason/s as to why the prepubertal rats do not develop marked hyperglycemia at a dose (60mg/kg bw) as in adult rats is not clear, it could be related to the decreased susceptibility of β-cells and or higher regenerative capacity of the pancreas.

Until recently, T2DM had been considered as an adult disorder associated with obesity, sedentary life style and a positive family history. However, during the last two decades T2DM has been reported among

adolescents and children world wide (ADA, 2000; Rami et al., 2003). The increasing prevalence of childhood diabetes is a serious new aspect to the epidemic and is considered as an emerging public health problem of significant proportions (Fagot-Campagna et al., 2000). Since, T2DM in children/ adolescents represents a health care concern world wide, a better understanding on the nature, pattern of the disease and complications are highly essential to develop specific therapeutic strategies.

Although classification of experimental animals based on age is practically difficult, the age range from 28-42days postnatal has been conservatively classified as adolescence. Owing to active metabolic changes in developing testis and higher levels of circulating growth hormone, it is likely that developing testis is rendered highly susceptible to insulin resistance and diabetes induced oxidative stress. Since our understanding on the reproductive pathologies associated with diabetes is largely known in adults, basic insight into disease pathogenesis during developmental stage is highly warranted.

Susceptibility pattern of developing PP testis

Although the main focus of this study was to assess the susceptibility pattern of prepubertal testis to STZ-induced oxidative impairments, paucity of data on vulnerability of other vital organs prompted us to determine the oxidative stress responses in liver and brain. Irrespective of the dosage, PP rats appeared to be more vulnerable as evidenced by significant reduction in body weight gain, testis weights in the first week of post STZ treatment, while such changes were apparent 3 weeks after STZ treatment in adult rats (Shrilatha and Muralidhara, 2007b). Even though the exact reason for increased testicular atrophy is not clear, it could be due to disturbances in hypothalamus – pituitary – gonadal axis which regulates development and function of testis. This appears likely, as we observed significant oxidative induction viz., increased levels of ROS, MDA and altered antioxidant defense in brain also. Secondly, it is also possible that STZ at early time points may have a direct effect on the developing testis affecting cell proliferation and inducing cell death (Bolzan and Bianchi, 2002; Kume et al., 2004). The

decrease in liver weight at the highest dose could also be due to irregularities in insulin secretion from necrotized β-cells and subsequent exhaustion of glycogen stores.

The extent of oxidative stress in testis was compared to that occurring in liver and brain by measuring the levels of both oxidative stress markers and enzymic antioxidant defenses. Of the three organs, testis and brain were equally affected as evidenced by the robust increase in ROS /MDA levels, while liver was relatively less affected. These perturbations clearly suggest the occurrence of significant disturbances in hypothalamo-gonadal axis which may culminate in hormonal imbalance and development of oxidative stress (Bacetti et al., 2002). Further, the elevated oxidative stress in testis and brain could be related to the higher degree of membrane unsaturation and lower antioxidant defenses. STZ shown to concentrate mainly in liver, kidney and pancreas, is known to be eliminated following biotransformation through the kidney. In the present study, a higher degree of peroxidation in diabetic liver mitochondria compared to cytosol suggests the higher susceptibility of the organelle to STZ treatment.

Tissue sulfhydryls such as protein thiol groups, glutathione etc., are major cellular antioxidants, whose altered levels serve as good indicators of the ongoing oxidative stress (Bauche et al., 1994). Glutathione is the predominant low molecular weight thiol in mammalian cells and demonstrated to play a major role in cellular defenses against oxidative/ nitrosative stress and against reactive electrophiles. Although relatively resistant to 'spontaneous oxidation', GSH reacts rapidly and non-enzymatically with hydroxyl radical, peroxinitrite and cytotoxic products formed by the reaction of nitric oxide and oxygen/superoxide respectively. Besides acting as a potent antioxidant, GSH also functions as a signal in the upregulation and expression of detoxification gene/s (Kirlin et al., 1999). In the present study, testicular GSH levels were marginally elevated (at 7 days) compared to marked elevation in brain cytosol, indicating the immediate need to detoxify/quench free radicals generated. In contrast, liver GSH levels were markedly diminished suggesting an exhaustive utilization of the tripeptide. Testis of

diabetic rats showed elevated levels of non-protein thiols suggesting their role as primary defense molecules to curtail free radical induced tissue damage.

Further evidences to show that PP testis is subjected to significant oxidative stress were obtained in terms of alterations in the activities of antioxidant enzymes viz., SOD, CAT, GST and GPx. The activity of SOD was less affected in all the organs. However, the effect on catalase was dose dependent in testis cytosol, which probably suggests that free radicals at higher concentrations bring about functional inactivation of the enzyme and thus defective turn over of hydrogen peroxide. Further, elevated hydrogen peroxide can also lead to inhibition in GPx activity as observed in the present study. Thus, the elevated MDA levels, together with decline in activities of CAT and GPx suggest an altered metabolism of both organic/inorganic peroxides following STZ administration. In contrast, the activities were marginally affected in both liver and brain.

STZ-induced physiological changes in testis were also discernible as significantly enhanced activity of LDH, which is often considered as a marker of mitochondrial membrane integrity. Thus, enhanced activity of cytosolic LDH probably suggests extensive membrane damage and also a possible interference in normal physiology of Sertoli cells (Pant et al., 1997). Further, a possible shunting of the metabolites of glycolytic pathway towards the lactate formation and a consequent depletion in cytosolic NADH levels may also explain the reduced activity levels. Sorbitol dehydrogenase, is the first enzyme in the polyol pathway that converts sorbitol into fructose, and significant decrease in its activity may result in the accumulation of sorbitol in the tissues and cause functional inactivation of several enzymes including catalase. The functional inactivation of the enzyme could also be due to high levels of circulating glucose and/or due to the excess accumulation of sorbitol in the tissues.

Age of PP rats as a determinant of testicular oxidative stress

In a separate study, the impact of age of PP rats on the vulnerability of testis to diabetes induced oxidative stress was investigated employing two age group rats (4- and 6wk- old). Induction of diabetes resulted in significant

reduction in testicular weights among both age groups, the degree being relatively higher among 4wk- old PP rats. This increased susceptibility could be a related to the age related differences in pituitary-gonadal axis. However, robust hyperglycemic response was discernible among PP rats of both age groups clearly suggesting extensive pancreatic β-cell death following STZ treatment. Although earlier evidences indicate hypertriglyceridemia and hypercholesterolemia during overt diabetes among experimental animals, we found significant reduction in serum triglycerides/cholesterol levels among PP diabetic rats which is likely to be due to one or several interdependent factors such as oxidative loss of membrane lipids, decreased lipid biosynthesis and/or poor nutritional status.

STZ treatment caused enhanced oxidative stress in testis (cytosol/mitochondria) of PP rats. In the present model, elevated levels of SOD, catalase and GST activities in testis (cytosol/ mitochondria) of 4wk-old PP rats (compared to 6wk- old) clearly suggests adaptive responses. In contrast, testis of 6wk old PP rats showed considerable reduction in catalase activity, besides unaltered SOD activity implicating differential regulations. Interestingly, diabetic rats also showed elevated NO levels in testis suggesting its possible role in the formation of peroxynitrite and activation of caspase mediated cell death pathways. Further, both GSH and non-protein thiol levels were uniformly elevated in mitochondria, while a differential response was evident with tissue ascorbic acid status. Testis ascorbate levels were found to be elevated among 4wk-old PP rats, while a marginal decrease was evident in 6wk- old PP rats clearly suggesting regulatory differences with age. Since enhanced ascorbate levels effectively recycle GSH/ vitamin E, these perturbations may represent an early adaptive response in the developing testis against oxidative stress (Chandrashekar and Muralidhara, 2009a)

In the present study induction of diabetes resulted in functional inactivation of testicular enzymes such as sorbitol dehydrogenase which may be attributed to high levels of circulating glucose and/or due to the excess accumulation of sorbitol in the tissues. Further, a decrease in the activity of G6PDH (as observed in the present study) becomes a limiting factor for GSH

recycling and various NADPH dependant metabolic reactions including steroidogenesis. This probably explains the observed decrease in the activity of 3β-HSD which reflects an impaired androgen biosynthesis during overt diabetes. Further, the potential contribution of stress-activated NO in regulating steroidogenesis cannot be overruled. Elevation in cytosolic LDH activity suggests an extensive seminiferous epithelial membrane damage and impaired spermiogenesis (Chandrashekar and Muralidhara, 2009a).

Unlike other organs, testicular energy requirement is dynamic with considerable changes occurring in mitochondrial enzyme activities during development. Since free radicals can potentially damage the mitochondrial integrity, it is logical to speculate that the activities of TCA and electron transport enzymes would be impaired during diabetes. In the PP model, a reduction in the activities of citrate synthase and succinate dehydrogenase suggests lowered efficiency of the cycle by possible shunting of glycolytic intermediates to other pathways. In addition, inhibition of aldehyde dehydrogenase (by stress-activated NO) emphasizes defective turnover of toxic aldehydes. Developing rat testis has growing energy demands for cellular growth and differentiation compared to adults as substantiated by the higher basal activity levels of ETC enzymes among control rats. Induction of diabetes enhanced the activities of complex I-III and II – III among rats of both age groups. A higher degree of induction in 4wk old PP testis suggests a higher demand for cellular free energy compared to 6wk old PP rats. Alteration in membrane permeability is generally considered as a critical event in the induction of apoptotic/ necrotic cell death. In the present study, a reduction in mitochondrial membrane potential together with MPT pore opening among diabetic rats clearly suggests membrane damage and leakage of matrix contents into cytosol leading to upregulation of apoptotic cell death events.

In conclusion, employing a prepubertal diabetic model, we have presented significant evidences in favor of our hypothesis that experimentally-induced diabetes causes elevated oxidative stress and mitochondrial dysfunctions in the developing testis. Further, experimental evidences of this nature are highly warranted owing to the recent epidemiological findings

describing the increased incidence of diabetes among children/adolescent males. Since a causal relationship exists between oxidative stress and male infertility, chronic testicular oxidative stress associated with diabetes beginning from prepubertal/pubertal period (as observed in this study) is likely to adversely impact the functional implications during adulthood.

6.0 SUMMARY

1. Dose determinative study indicated that administration (i.p) of STZ at a dosage of 90mg/kg bw to prepubertal (PP) rats caused a significant hyperglycemia measured at 72h. Since, the hyperglycemia was consistent at one week and beyond, and no mortality occurred, this dosage was considered as optimum.

2. The pattern of oxidative stress induction response (measured at one week of post STZ) revealed that the PP testis was relatively more prone compared to liver and brain. This may be related to the lower degree of antioxidant defenses and/or exhaustive utilization of antioxidant defenses in the testis of PP rats.

3. Determination of comparative pattern of susceptibility of testis to oxidative stress among the two age group rats (4- and 6 wk– old PP rats) clearly suggested that the 4 wk-old rats were relatively more vulnerable.

4. Analysis (at 15days) of serum lipids (triglycerides/cholesterol) in diabetic PP rats indicated significant reduction among both age group rats, which may be attributable to severe oxidative loss of membrane lipids, decreased lipid biosynthesis and/or poor nutritional status.

5. Induction of diabetes caused significant testicular oxidative stress in PP rats as evidenced by the elevated levels of ROS, MDA and hydroperoxides in both cytosol and mitochondria.

6. Consistently elevated GSH and non-protein thiols in testis of both age groups are suggests their vital role under diabetic conditions. Further,

marked enhancement of AA levels indicates its participation in recycling of GSH and Vitamin E and the differential response of the AA levels indicates differences in regulatory role of this antioxidant in developing testis.

7. Further evidence that the developing testis is subjected to oxidative stress was reflected in terms of diminished activity levels of antioxidant enzymes viz., SOD, CAT and GPx. Enhanced activity of GST is indicative of higher generation of toxic aldehydes (MDA, HNE etc.,) in testis.

8. Induction of diabetes caused significant perturbations in the activity levels of various functional enzymes which reflect altered physiology/functional status of the organ. Diminished activity of sorbitol dehydrogenase suggests the possible accumulation of sorbitol in the tissue and its associated cytotoxic implications. Further, significantly diminished G6PDH activity reveals reduced availability of NADPH for GSH recycling and steroidogenesis.

9. Marked elevation in the activity levels of LDH-X in testis of diabetic PP rats is suggestive of the significant seminiferous epithelial membrane damage and associated impaired spermatogenesis.

10. Significant reduction in the activity of 3β-HSD in testis of diabetic PP rats, indicates altered Leydig cell function and suggests the important regulatory role of oxidative stress in steroidogenic function.

11. Induction of diabetes caused various mitochondrial dysfunctions in testis of PP rats as evident by reduced activities of citrate synthase, succinate dehydrogenase implicating lowered efficiency of TCA cycle function.

12. Alterations in the activities of ETC enzymes, membrane potential and elevated intracellular Ca^{2+} levels suggest mitochondrial membrane damage and upregulation in Ca^{2+} mediated apoptotic events in diabetic testis.

1.0 INTRODUCTION

Diabetes Mellitus (DM), a state of chronic hyperglycemia, is a major cause of serious micro and macrovascular diseases, affecting nearly every system in the body. Growing body of evidence indicates that during diabetes, failure in insulin-secretion/deficiency causes glucose concentrations in blood to remain high leading to increased uptake by insulin-independent tissues. Increased glucose flux enhances both oxidant production and impairment in antioxidant defenses by multiple interacting non-enzymatic, enzymatic and mitochondrial pathways (Brownlee, 2001; Hansen, 2001; Evans et al., 2002; Ceriello, 2003; Maritim et al., 2003; Ahmed, 2005; Brownlee, 2005; Greenman et al., 2007).

A growing body of evidence supports the concept that ROS are formed within mitochondria under both physiological and pathological conditions (Droge, 2002; Turrens, 2003; Balaban et al., 2005; Murphy, 2009) resulting in decreased efficiency of antioxidant defenses that starts early and worsens over the course of the disease (Tushuizen et al., 2005). Thus it is accepted that mitochondria derived ROS plays a critical role in the development of complications (Baynes and Thorpe, 1999; Petersen et al., 2003; Di Lisa et al., 2009). Despite of intracellular protective mechanisms, excess ROS generated can damage cellular proteins, DNA and membrane lipids which turns out to be detrimental to mitochondrial dysfunction (Kim et al, 2008). Other factors such as genetic predisposition, mitochondrial biogenesis, over expression/activity of mitochondrial uncoupling proteins (UCPs) are known to contribute for considerable increase in free radical generation in mitochondria (Nishikawa et al., 2000; Kahn, 2003; Kajimoto and Kaneto, 2004; Rolo and Palmeira, 2006). Elevated free radicals inhibits glyceraldehyde-3-phosphate dehydrogenase/ cytochrome enzymes (Du et al., 2003) that upregulates diacylglycerol and PKC synthetic pathways (Brownlee, 2001; Maritim et al., 2003; Mohara et al., 2007). These pathways represent additional processes through which mitochondria accelerate, or even determine, the events of cell injury toward necrosis or apoptosis (Di Lisa et al., 2009).

Endoplasmic reticulum (ER, microsomes) is a critical intracellular organelle that plays a key role in protein synthesis/ transport and Ca^{2+} homeostasis (Chevet et al., 2001; Oshitari et al., 2008). The accumulation of unfolded proteins or disturbances in the Ca^{2+} homeostasis activate the ER stress response (Rao et al., 2004; Lindholm et al., 2006) that upregulate several cell death pathways viz., caspase-12-dependent (Nakagawa et al., 2000) and apoptosis signal-regulating kinases (JNK) pathway (Nishitoh et al., 2002; Oyadomari et al., 2002; Laybutt et al., 2007). Recently, it has been demonstrated that the ER stress-induced apoptosis is related to changes in the glucose concentration and results in the death of pericytes (Ikesugi et al., 2006). Further, evidence also suggest that ER stress-induced apoptosis is involved in the death of neurons in the brain and retina under different physiological conditions (Tobisawa et al., 2003; Imai and Takahashi, 2004; Tajiri et al., 2004; Hayashi et al., 2005; Awad et al., 2006; Shimazawa et al., 2007). Like mitochondria, microsomes are also endowed with enzymic/non-enzymic antioxidants and mixed function oxidases that are specifically targeted towards oxidation of xenobiotics. Recently, microsome specific glutathione–S-transferase has been shown to play a crucial role in toxic aldehyde metabolism during conditions of oxidative stress (Rinaldi et al., 2004).

Sexual dysfunction is a well known consequence of overt diabetes affecting at multiple levels including the endocrine control of spermatogenesis, spermatogenesis itself or impairment of penile erection and ejaculation (Sexton and Jarrow, 1997; Rehman et al., 2001; Musicki and Burnett, 2007). Additionally, there are indications that the onset and severity of diabetes is influenced by mitochondrial deficiencies or dysfunctions (Scheffler, 2001). Normal mitochondrial function is also crucial for germ cells maturation/ differentiation during the course of spermatogenesis (Meinhardt et al., 1999). It is proposed that ROS are a common mediator of several pathologies that are currently thought to afflict the reproductive function (Agarwal et al., 2008) and any impairments in mitochondrial function leads to male infertility/subfertility (Nakada et al., 2006; Amaral et al., 2008). Earlier studies showed that spermatozoa containing defective mitochondria not only produce

ATP in a less efficient way, but also generate higher oxidative stress (Wei and Kao, 2000). Considering the impact of mitochondrial dysfunctions on both diabetes and reproductive function and limited studies on testicular mitochondrial function under diabetic conditions, newer basic insights into the potential contribution of mitochondria in the development of diabetes associated complication is highly pertinent.

Recent investigations have demonstrated the mitochondrial involvement in aggravating the complications under diabetic situations employing adult rodent models (Agarwal and Said, 2005; Shrilatha and Muralidhara, 2007a,b; Doreswamy and Muralidhara, Unpublished data). However, similar studies pertaining to immature testis is non-existent as a result of which our understanding on the impact of diabetes during childhood/adolescence on testis biochemistry/physiology and its implications on spermatogenesis during later years is poor. Further, under different degree/duration of diabetic state, data on the perturbations of antioxidant levels in testis in general, subcellular organelles viz., cytosol, mitochondria and microsomes in particular and associated functional/ biochemical implications are totally lacking. Such studies assume high relevance as they would provide a better understanding of the mechanisms responsible for ROS generation and may aid in development of new intervention strategies to attenuate oxidative stress mediated testicular dysfunctions.

Accordingly, the major focus of this study was to investigate the early biochemical and oxidative dysfunctions in the testis subcellular organelles viz., cytosol, mitochondria and microsomes of prepubertal rats both during acute and progressive phase of diabetes. Further a comprehensive study was undertaken to study the nature of testicular cell type affected during diabetes over a period of one complete spermatogenic cycle (56days) employing flow cytometry. In addition, the nature of cell death responsible for testicular degeneration was ascertained by monitoring the upregulation in the activity of caspase-3 and DNA damage together with major histopathological changes were monitored in diabetic prepubertal rats. The results are presented under three sections, A, B and C.

2.0 OBJECTIVE

The primary focus of this investigation was to obtain insights into oxidative stress-mediated biochemical and functional alterations in testis subcellular organelles of prepubertal rats rendered diabetic by STZ. Further, the oxidative damage to cellular proteins, DNA and the nature of testicular cell type affected were also investigated.

SECTION – A

DIABETES INDUCED OXIDATIVE DISTURBANCES IN TESTIS CYTOSOL: ACUTE AND PROGRESSIVE PHASE

3.0 EXPERIMENTAL DESIGN

Prepubertal male rats (50± 5g) were randomly drawn from the 'Institute Animal house facility' and housed individually. The experimental animals were randomly assigned to two groups (n= 6/group): Group I designated as 'non-diabetic controls' received citrate buffer and served as 'normal controls'. Group II rats were administered an acute dose of STZ (90mg/kg b.w, dissolved in 0.1M Citrate buffer, pH 4.5). STZ treated rats were given glucose solution (5% w/v) for 24h to prevent mortality by hypoglycemic shock. During the experimental period, the animals were fed with commercial pellet diet and tap water *ad libitum*. Food intake was measured daily and the gain in body weight was monitored on alternate days throughout the experimental period.

Morphological investigations and autopsy

Both 'control' and 'diabetic' rats were sacrificed on 1, 3, 5 days (acute phase) and on 7 and 14 days (progressive phase) post STZ treatment (n=10/ group). At each sampling point, blood was collected and processed immediately for blood glucose estimation. Testis was excised, rinsed several times with ice-cold saline, blotted and weighed and was used for various biochemical investigations.

Oxidative impairments in testis cytosol

Markers of oxidative stress

Generation of ROS and the extent of lipid peroxidation were assayed in testis cytosol employing DCF-DA and 2- thiobarbituric acid. The fluorescent DCF and the TBA-MDA complex formed were subsequently measured as described earlier. Water soluble hydroperoxides, Nitric Oxide and protein carbonyl levels were measured in testis cytosol of both diabetic and non-diabetic rats.

Redox status: Effect on thiol status

Following STZ administration, alterations in the levels of total thiols, non-protein thiols and glutathione content were determined in testis cytosol at all sampling points.

Non-enzymic antioxidants

Ascorbic acid levels were estimated in testis cytosol of control and diabetic rats at all sampling points, while tocopherol levels were estimated at 7 and 14 days only.

Perturbations in the activities of antioxidant enzymes

The effect on enzymic antioxidants viz., SOD, Catalase, GPx and GST was ascertained by measuring the activity levels in testis cytosol at all sampling points.

Perturbations in the activities of functional enzymes

Alterations in the activity levels of enzymes viz., sorbitol dehydrogenase, lactate dehydrogenase, glutathione reductase, aldose reductase, glucose-6-phosphate dehydrogenase, and steroidogenic enzyme, 3β-hydroxy steroid dehydrogenase (3β-HSD), were assayed in testis cytosol of both control and diabetic rats at all sampling times.

Alterations in tissue free iron and Intra cellular Ca^{2+} ion levels

Free iron and intra cellular Ca^{2+} levels were quantified in testis cytosol of both control and diabetic rats during progressive phase (7 and 14days).

SECTION-B

OXIDATIVE DYSFUNCTIONS IN MITOCHONDRIA/ MICROSOMES

3.0 EXPERIMENTAL DESIGN

Experimental protocol was essentially similar to that outlined above in Section A of this chapter. Fresh mitochondria and microsomes prepared from prepubertal rats of control and diabetic rats sampled at all time points were used to examine the nature and progression of oxidative dysfunctions.

Markers of oxidative stress

Generation of ROS, lipid peroxidation, hydroperoxides and protein carbonyls

ROS and the extent of lipid peroxidation were quantified using DCF-DA and thiobarbituric acid respectively. Water soluble hydroperoxides and protein carbonyl levels were measured in testis mitochondria and microsomes of both the groups.

Pertubations in thiol status

Following STZ administration, alterations in the levels of total thiols, non-protein thiols and glutathione were determined in testis mitochondria/ microsomes at all sampling points.

Antioxidant enzyme activities

Activities of antioxidant enzymes viz., SOD, GST and GPx were assayed in testis mitochondria of both control and diabetic group at all sampling points. However, activity of GST was measured in testis microsomes among both control and diabetic groups.

Mitochondrial dysfunctions

Perturbations in citric acid cycle enzyme activities

Alterations in the activities of citrate synthase, succinate dehydrogenase and malate dehydrogenase were assayed in control and diabetic testis mitochondria.

Alterations in oxidative phosphorylation enzyme activities

As a measure of mitochondrial dysfunction, activities of NADH-cytochrome C reductase (complex I-III), Succinate - cytochrome C reductase (complex II-III) and Mg^{2+} -ATPase were determined in testis of both control and diabetic rats at all sampling times.

Perturbations in membrane integrity- permeability transition pore opening (MPT)

Susceptibility of diabetic testis mitochondria to MPT inducers viz., $CaCl_2$ was studied in fresh mitochondrial preparations of control and diabetic groups only during the progressive phase of diabetes.

Mitochondrial membrane potential

Alterations in mitochondrial membrane potential among both control and diabetic rats were measured as relative rhodamine123 uptake during the progressive phase.

Caspase activation assay

Testis cell suspensions were prepared from both control and diabetic rats, and were incubated with caspase substrate with/without inhibitor and the activity was measured fluorimetrically.

Alterations in mitochondrial protein profile

Mitochondrial protein profile was studied by SDS-PAGE in both control and diabetic rats only during the progressive phase.

Alterations in microsomal P450 enzyme activities

The activity levels of testicular microsomal NADPH-cytochrome p450 enzyme and ethoxyresorufin-O- dealkylase were assayed in both control and diabetic rats only during the progressive phase.

Testicular DNA damage : COMET assay

DNA damage was measured by quantifying the incidence of comets in both control and diabetic testis on day 7 and 14 post STZ treatment.

SECTION – C

FLOW CYTOMETRIC ANALYSIS OF TESTIS

3.0 EXPERIMENTAL DESIGN

Prepubertal male rats (3wk - old, 30± 5g) were randomly drawn from our 'Institute Animal house facility' and housed individually. The experimental animals were randomly assigned to two groups (n= 6 /group): Group I designated as 'non-diabetic controls' received citrate buffer and served as normal controls throughout the investigation. Group II rats were administered an acute dose of STZ (90mg/kg b.w, dissolved in 0.1M Citrate buffer, pH 4.5). STZ treated rats were given glucose solution (5% w/v) for 24h to prevent mortality by hypoglycemic shock. During the experimental period, the animals were fed with commercial pellet diet and tap water *ad libitum*. Food intake was measured daily and the gain in body weight was monitored on alternate days throughout the experimental period.

Morphological and histopathological investigations

Both 'control' and 'diabetic' rats were sacrificed at weekly intervals for five weeks (one spermatogenic cycle). One testis from both control and diabetic rats were fixed in Bouin's fluid, embedded in paraffin processed by standard histological techniques stained and examined by light microscopy.

Flow cytometric analysis

For analysis, testis was decapsulated, seminiferous tubules were gently expressed and cell suspension was prepared in phosphate buffered saline (0.1M, pH 7.4). The cells were fixed in 70% ice-cold ethanol and analyzed flow cytometrically following pepsin digestion and propidium iodide staining.

4.0 RESULTS

SECTION-A

DIABETES INDUCED OXIDATIVE DISTURBANCES IN TESTIS CYTOSOL: ACUTE AND PROGRESSIVE PHASE

Food intake, growth pattern and testis weight

Administration of streptozotocin significantly affected the growth pattern and organ weights of prepubertal rats (Table 3.1). In general, no clinical signs of mortality were observed during the experimental period, while the body weights of diabetic rats were significantly lower than those of vehicle control groups. While only a marginal decrease in the body weight was apparent during acute phase (day 3-16%; day 5-19%), the weights were significantly reduced during the progressive phase (day 7- 25%; day 14- 47%). A marginal decrease in food intake was observed during the first 3 days of STZ administration which was comparable to control on subsequent days (data not shown).

STZ administration to PP rats markedly reduced the weights of both testis and liver (data not shown). Even though a marginal decrease in testis weight was evident during the acute phase (< day5, 15%), marked decrease in the weight was evident on day 7 and 14 (59- 81%). A similar but less robust pattern was evident in liver and the liver weights were significantly decreased at day 7 and beyond (20-30%) (Fig. 3.1A, B).

Hyperglycemic response

STZ administration caused marked hyperglycemia among diabetic rats. Significant elevation in blood glucose level was evident as early as 6h which subsequently reduced below basal levels at 12h (data not shown). However, a consistently marked hyperglycemia ensued beyond 24h and remained significantly higher at all sampling times (3-4 folds) (Fig. 3.2).

Oxidative impairments in testis cytosol

Generation of ROS, formation of hydroperoxides and extent of lipid peroxidation

Administration of STZ significantly elevated the levels of oxidative stress markers in testis cytosol of prepubertal rats (Fig. 3.3). Initially, the levels of ROS and MDA were marginally reduced (15-20%) in the cytosol, while a dramatic increase was observed beyond day 3 (ROS, 30-150%; MDA, 50 - 490%) (Fig.3.3 Top panel). Further, a consistently elevated hydroperoxides (28 – 35%) and fluorescent lipid peroxides (50 – 200%) were also evident throughout the sampling times (Fig. 3.3 Mid panel).

Generation of Nitric Oxide, formation of protein carbonyls

STZ induced oxidative damage was further evident in terms of elevated levels of Nitric Oxide (25%) and formation of protein carbonyls observed beyond day 3 (23-37%) as shown in Fig. 3.3 (Lower panel).

Response of testicular antioxidant defenses

Redox status – effect on thiol status

STZ induced alterations in testis thiol status has been represented in Fig. 3.4. Induction of diabetes resulted in significant decrease in total thiol content, which was apparent on day 5 and subsequently (5d, 39%; 7d,13%; 14d, 23%). In contrast, the levels of non-protein thiols were consistently elevated throughout the sampling period with a robust increase on day 3 (107%). Despite a marginal increase (15%), the levels of cytosolic GSH remained less affected throughout the experimental period.

Antioxidant enzyme activities

Data on the effect of STZ on the activities of antioxidant enzymes in testis cytosol is presented in Fig. 3.5. A marginal to moderate increase in the activity of SOD was evident following STZ administration (3d, 31%; 5d, 26%; 7d, 17%) with little or no change on day 14. Despite a marginal to marked elevation in catalase activity during first 3 days (1d, 13%; 3d, 22%), the activity was reduced on subsequent days (30-40%). A similar trend was also apparent in the activity of glutathione peroxidase. A marked elevation in the

activities was observed during acute phase (3d, 17%; 5d, 52%), while a marginal decrease was noticeable during the progressive phase (7d, 13%; 14d, 15%). On the other hand, the activity of glutathione-S-transferase was consistently elevated at all sampling times (3d, 36%; 5d, 56%; 7d, 108%; 14d, 25%).

Alterations in the activities of functional enzymes

Data on the effect of STZ-diabetes on the activities of various functional enzymes in testicular cytosol is presented in Fig. 3.6. The activity levels of both glucose-6-phosphate dehydrogenase and glutathione reductase were elevated among diabetic rats. The activity levels of glutathione reductase were increased significantly during acute (1d, 12%; 3d, 22%; 5d, 41%) and progressive phase (7d, 12%; 14d, 31%). The activity levels of glucose-6-phosphate dehydrogenase were consistently increased throughout the sampling times (18–41%). In contrast, the activities of both lactate dehydrogenase and sorbitol dehydrogenase were significantly reduced.

Testicular ascorbate, tocopherol, free iron and calcium levels

Data on the effect of STZ administration on testis ascorbate levels, iron and calcium levels is presented in Fig. 3.7. Elevated levels of ascorbate were evident on day 1 which remained consistently elevated throughout the experimental period. Further, free iron /intra cellular calcium levels measured during progressive phase were also significantly elevated (Fe^{2+}, 50- 300%; Ca^{2+}, 50 -80%).

Activity of 3β-hydroxyl steroid dehydrogenase and serum testosterone levels

The activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) and serum testosterone levels are presented in Fig. 3.8. Administration of STZ significantly decreased (26 – 118%) the activity levels of 3β-HSD throughout the sampling times. These changes were accompanied by significantly reduced levels of serum testosterone.

SECTION-B

OXIDATIVE DYSFUNCTIONS IN MITOCHONDRIA / MICROSOMES

Oxidative stress markers in mitochondria

Generation of ROS and lipid peroxidation in testis mitochondria

Administration of STZ to prepubertal rats resulted in a significant elevation in the generation of ROS and MDA levels in testis mitochondria. Although, testis mitochondria was relatively less vulnerable during acute phase, ROS and MDA levels were significantly elevated on day 7 (45%, 45%) and day 14 (22%, 38%) respectively (Fig. 3.9 A).

Hydroperoxide and Nitric Oxide levels

A marginal induction of hydroperoxides was evident in testis mitochondria during acute phase (13%), while the levels were higher on subsequent days (23%) (Fig. 3.9). However, the levels of Nitric Oxide were less elevated at all sampling times (data not shown).

Protein carbonyl levels

Data on the oxidative damage to mitochondrial proteins measured as protein carbonyl content is presented in Fig. 3.9. While the levels were less elevated during acute phase, a marginal increase was evident on day 7 and 14 (13%, 22%).

Oxidative stress markers in microsomes

Generation of ROS and lipid peroxidation

Administration of STZ to prepubertal rats resulted in significant elevation in the generation of ROS and MDA levels in testis microsomes (Fig. 3.10) which showed consistently higher levels of ROS at all sampling times (1d,, 29%; 3d, 59%; 5d, 134%; 7d, 110% and 14d, 62%). However, a marginal to marked elevation in MDA levels were evident during both acute and progressive phase (acute phase 20 – 59%; 7d, 73%; 14d, 75%).

Protein carbonyl levels

Data on the oxidative damage to microsomal proteins measured as protein carbonyl content is presented in Fig. 3.10. STZ administration resulted in a steady increase in protein carbonyl levels in microsomal preparations beyond day 5 (3d, 12%; 5d, 21%; 7d, 33%, 14d, 31%).

Response of antioxidant defenses in mitochondria

Redox status: effect on thiol levels

Data on the total thiol, non-protein thiol and reduced glutathione status in both testis mitochondria and microsomes are presented in Fig. 3.11 and 3.12 respectively. A marginal decrease in total thiol content was apparent in testis mitochondria at all sampling times (10 -16%). Mitochondrial non-protein thiol levels were less altered during early phase, while significant reduction was apparent on day 7 (16%) and day 14 (23%). In contrast, the reduced glutathione levels were elevated throughout the study period, with a robust increase on day 5 and 7 (83%) while the degree was less severe at day14 (31%).

In testis microsomes, the thiol redox status was drastically affected at all sampling points as evidenced by lowered total thiol (23- 53%) and glutathione levels (20%).

Perturbations in antioxidant enzymes

Data on the effect of STZ administration on selected antioxidant enzymes are presented in Fig. 3.13. Administration of STZ significantly elevated the activity levels of Mn-SOD (20 – 60%) throughout the sampling times. No measurable alterations were apparent in the activities of GPx and GR in the early phase, while an obvious reduction in the activity levels of both enzymes were observed at day 7 (15%, 37%) and day14 (30%, 31%). In contrast, the activity levels of GST showed a robust increase on day 3 (80%) followed by a steady levels (55%) in the rest of the sampling times. A similar trend was also evident in the activity of GST in testis microsomes.

Effect on mitochondrial TCA cycle enzyme activities

Data on the effect of STZ administration on the activity levels of selected TCA cycle enzymes are presented in Fig. 3.14. Even though the activity of citrate synthase was less affected during the first three days, a marked reduction was evident on day 5 onwards (5d, 30%; 7d, 74%, 15d, 72%). The activity of succinate dehydrogenase was less altered during acute phase, while the activity was significantly reduced on day 7 (35%) and day 14 (42%). Similarly, the activity of malate dehydrogenase was found to be less altered during the acute phase, while a marginal decrease was evident on day 7 and day 14 (18%).

Effect on oxidative phosphorylation enzymes

Data on the activities of oxidative phosphorylation enzymes are presented in Fig 3.15. The activity of NADH-cytochrome c reductase remained less altered during first 3 days, while the activity levels increased consistently on subsequent days (5d, 23%; 7d, 24%; 14d, 18%). On the other hand, a marginal decrease in the activity levels of succinate-cytochrome c reductase was evident in mitochondria of diabetic testis beyond day 5 (18%). The activity of Mg^{2+}- ATPase was decreased by 32 and 34% on day 7 and 14 of post STZ treatment.

Membrane permeability transition (MPT) and mitochondrial membrane potential

MPT induction as assessed by opening of permeability transition pore in presence of an inducer viz., $CaCl_2$ during progressive phase is presented in Fig. 3.16. The susceptibility of mitochondria to MPT pore induction was higher among diabetic testis compared to the controls.

Mitochondrial membrane potential as measured by the uptake of Rhodamine 123 by freshly prepared mitochondria of control and diabetic testis is presented in Fig. 3.16. A large drop in the membrane potential was evident in the diabetic testis mitochondria on days 7 and 14 (45%) compared to controls.

Response of antioxidant defenses in microsomes

Redox status: effect on thiol levels

Data on the total thiol, non-protein thiol and reduced glutathione status in testis microsomes is presented in Fig. 3.12. STZ administration significantly reduced the total thiol content (23- 53%) and glutathione levels (20%) at all sampling times.

Microsomal enzyme activities

Data on the effect of STZ administration on testis microsomal enzyme activities eg., NADPH-cytochrome-c reductase and ethoxy-resorufin–O-dealkylase (EROD) on day 7 and 14 is presented in Fig. 3.17. Induction of diabetes markedly reduced the activities of both the enzymes significantly.

Caspase 3 activity

Caspase 3 activity measured in testicular cell suspension prepared from both control and diabetic testis sampled at 7 and 14 days post STZ treatment is presented in Fig. 3.18. The activity of Caspase 3 was enhanced significantly at both the sampling points.

Testis protein profile – SDS PAGE

The protein profile of diabetic testis cytosol and mitochondria sampled on 7 and 14d is illustrated in Plate 3.1. It is evident from the electrophorograms that STZ treatment significantly affected the expression of majority of protein/s in both cytosol and mitochondria of prepubertal testis.

DNA – fragmentation – COMET Assay

DNA damage was measured *in situ* in testicular cells of diabetic rats at day 14 post STZ. Slides were prepared from each group and 100 cells were counted from each slide to study the incidence of comets. Typical microphotographs of testicular cells showing comets with mild to marked single strand breaks are presented in Plate 3.2. The incidence of comets were significantly higher in diabetic rats (53%) compared to control.

SECTION – C

FLOW CYTOMETRIC ANALYSIS OF TESTIS

Diabetic rats showed a progressive reduction in body weight (24 – 59%) accompanied by severe testicular atrophy (68 – 93%) over a period of five weeks (Fig.3.19). Subsequent flow cytometric studies were performed with testis sampled each week. Flow cytometric analysis of non-diabetic testis showed three major peaks that represents haploid, diploid and tetraploid cell population with unique PI staining properties. The haploid cell population was split into two peaks that represent matured elongated spermatids and immature/elongating spermatids. A progressive increase in the peak area of mature elongated spermatids (n) with subsequent reduction in immature haploid cell population was evident in control PP testis. The appearance of first matured haploid spermatid was observed at day 49 post natal suggesting germ cell maturation and onset of maturity. Further, the peak that represents diploid (primary spermatocytes, Sertoli/Leydig cells) and tetraploid cell population decreased throughout the sampling times. However, flow cytometric data obtained from PP diabetic testis showed preferential loss of tetraploid (4n cells) and haploid (both immature/mature) cell population, with subsequent increase in diploid cell population (Figures. 3.20, 3.21 and 3.22).

Testicular pathology

Histopathological lesions were studied in the testis at various sampling weeks. The pathological alterations were obvious in testis sampled on day 28 post STZ administration and were persistent throughout. Some of the typical lesions in the testis of diabetic rats sampled at 28, 35, 42, 49, 56 days post STZ treatment is presented in Plates 3.3 – 3.7. There was obvious shrinkage of seminiferous tubules with reduced tubular diameter, Sertoli cell vacuolization and absence of sperm in the lumen. There was extensive desquamation of germinal epithelium and degenerating spermatids were also evident.

Table 3.1

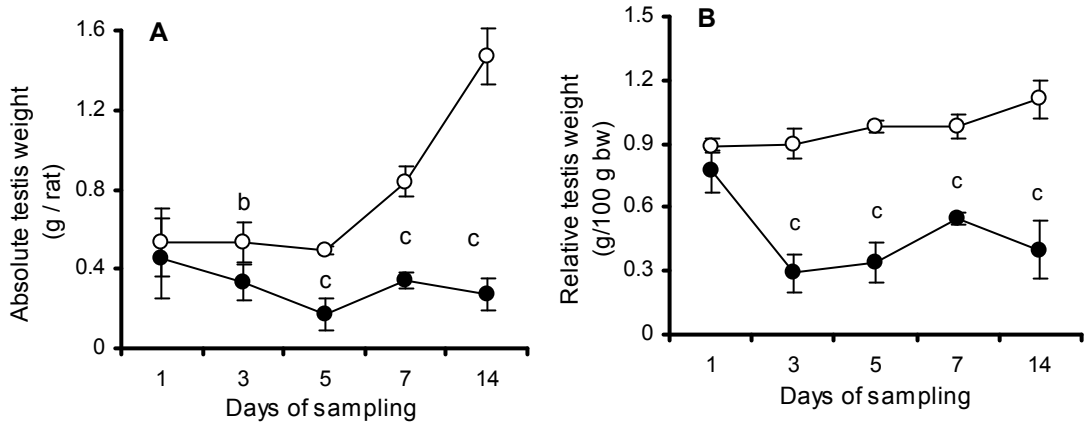
Body weights of prepubertal rats rendered diabetic by an acute dose of Streptozotocin (STZ)

Body weight (g)		Days after STZ administration				
		1	3	5	7	14
CTR	Initial	56.0 ± 2.0	49.6 ± 0.4	44.8 ± 3.4	54.0 ± 1.7	60.0 ± 1.0
	Final	59.0 ± 1.7	59.0 ± 1.1	69.2 ± 1.1	84.3 ± 3.1	132.0 ±1.7
STZ	Initial	49.6 ± 0.4	51.0 ± 0.9	45.8 ± 3.0	53.6 ± 2.0	53.0 ±1.2
	Final	59.0 ± 1.1	49.6 ± 0.2	51.1 ± 1.2[a]	59.7 ± 4.6[a]	70.2 ±2.2[b]

Values are mean ± SEM (n=6).
Data analyzed by Student's 't' test; [a] p< 0.05, [b] p< 0.01

Fig. 3.1

Absolute (A) and relative (B) testis weights of prepubertal rats rendered diabetic by an acute dose of Streptozotocin

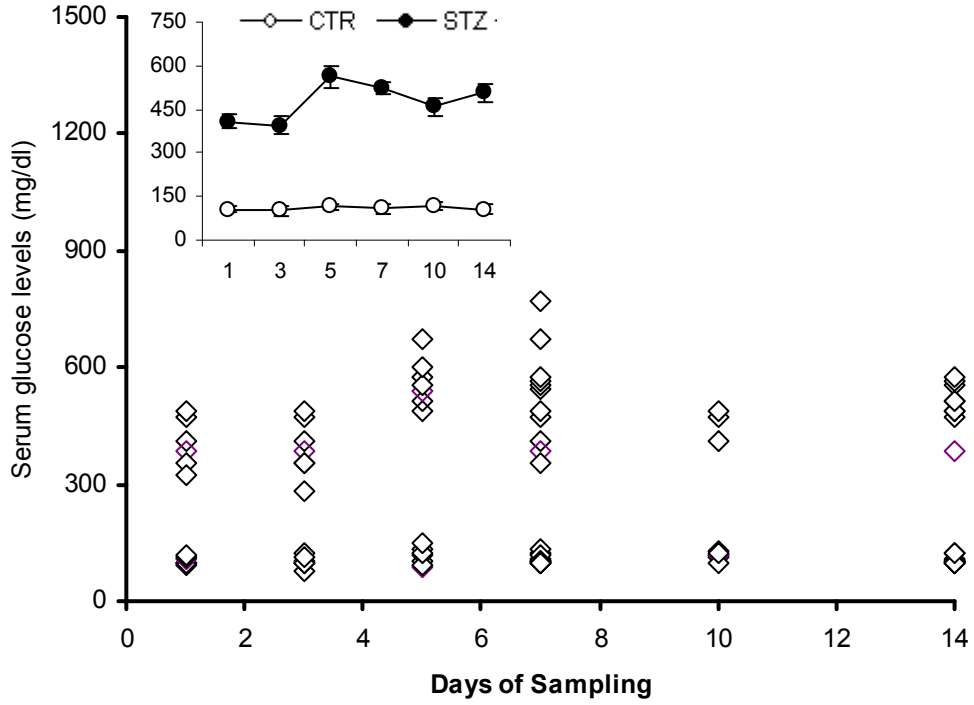


Values are mean ± SEM (n=6). —○— Control —●— STZ

Data analyzed by Student's 't' test; [b] p< 0.01, [c] p< 0.05

Fig. 3.2

Scattered pooled diagram of changes in blood glucose levels. Inset shows the average glucose levels in prepubertal rats administered an acute dose of STZ

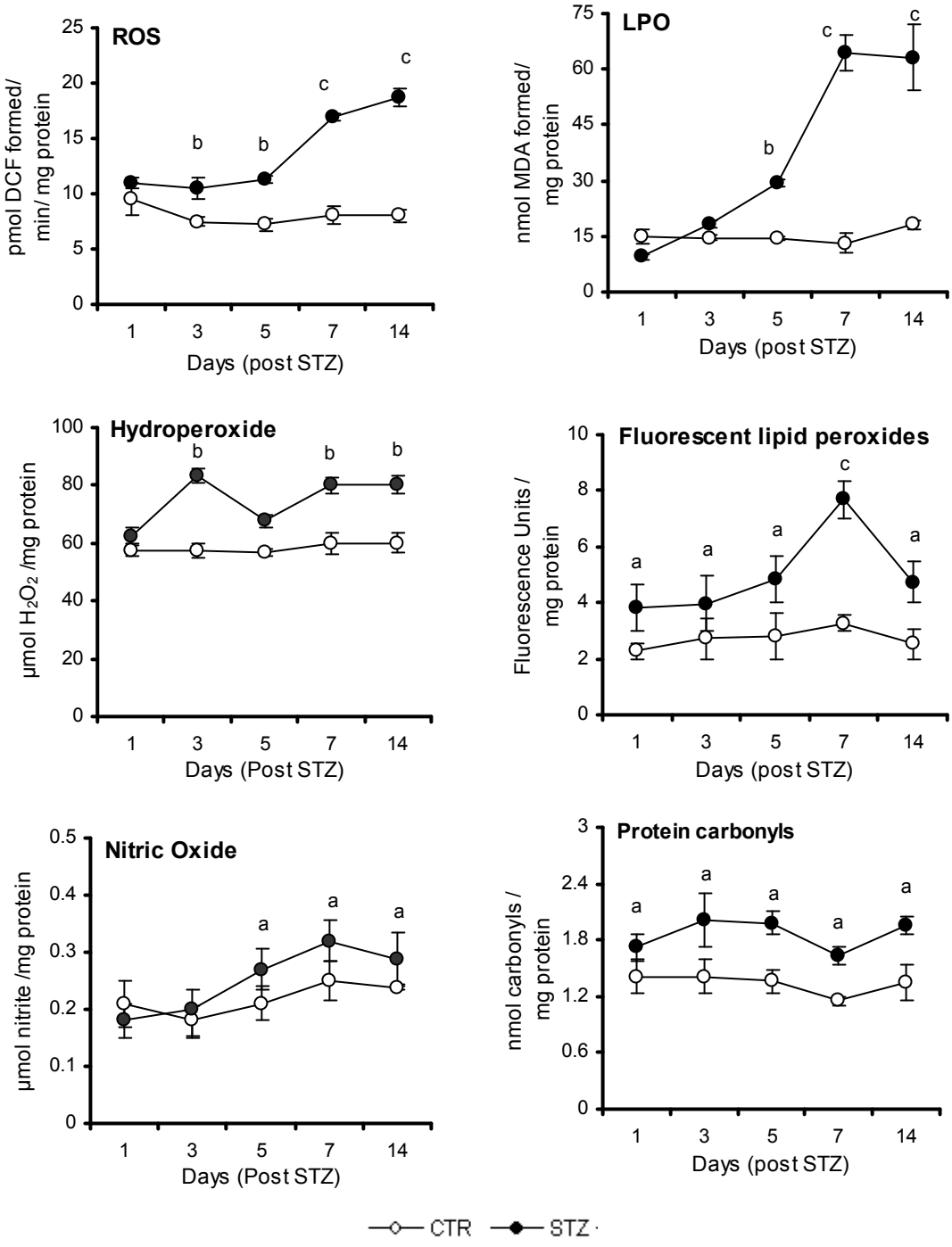


Values are mean ± SEM (n=10).

Data analyzed by Student's 't' test.

Fig. 3.3

Status of oxidative stress markers in testis cytosol of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

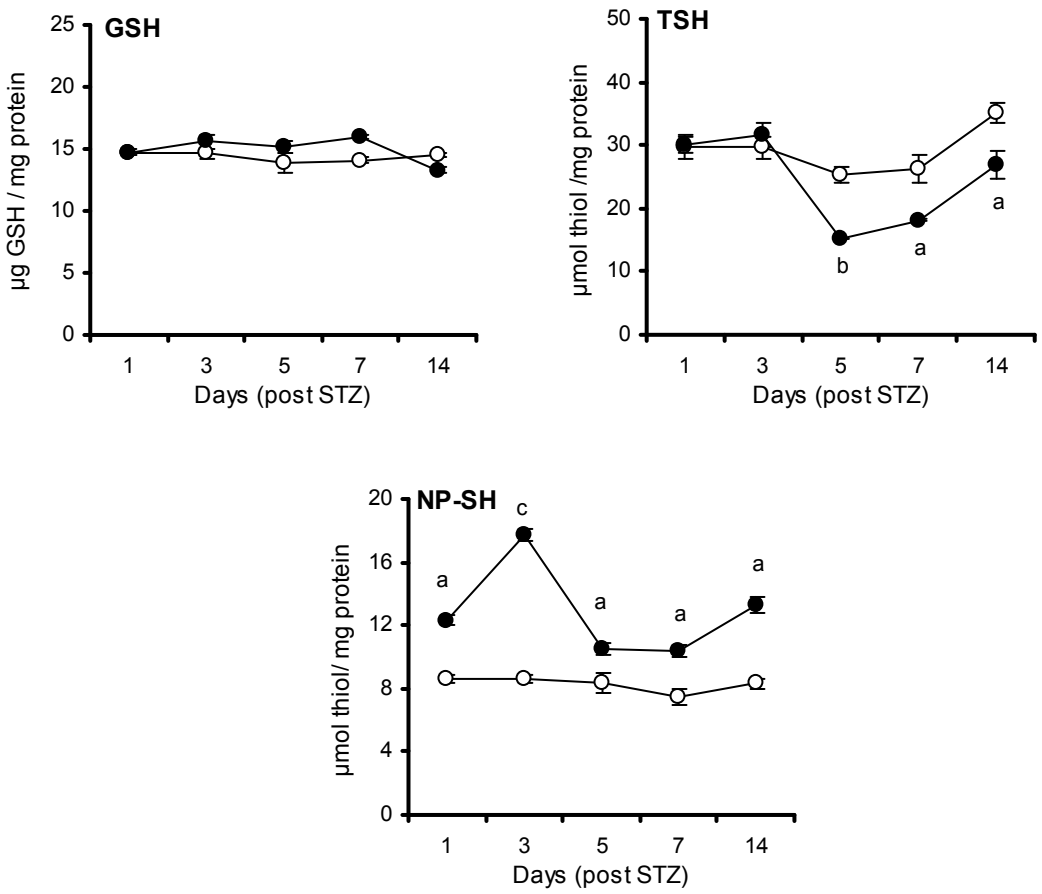


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p< 0.05$, [b] $p< 0.01$, [c] $p<0.001$.

Fig. 3.4

Alterations in thiol status in testis cytosol of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

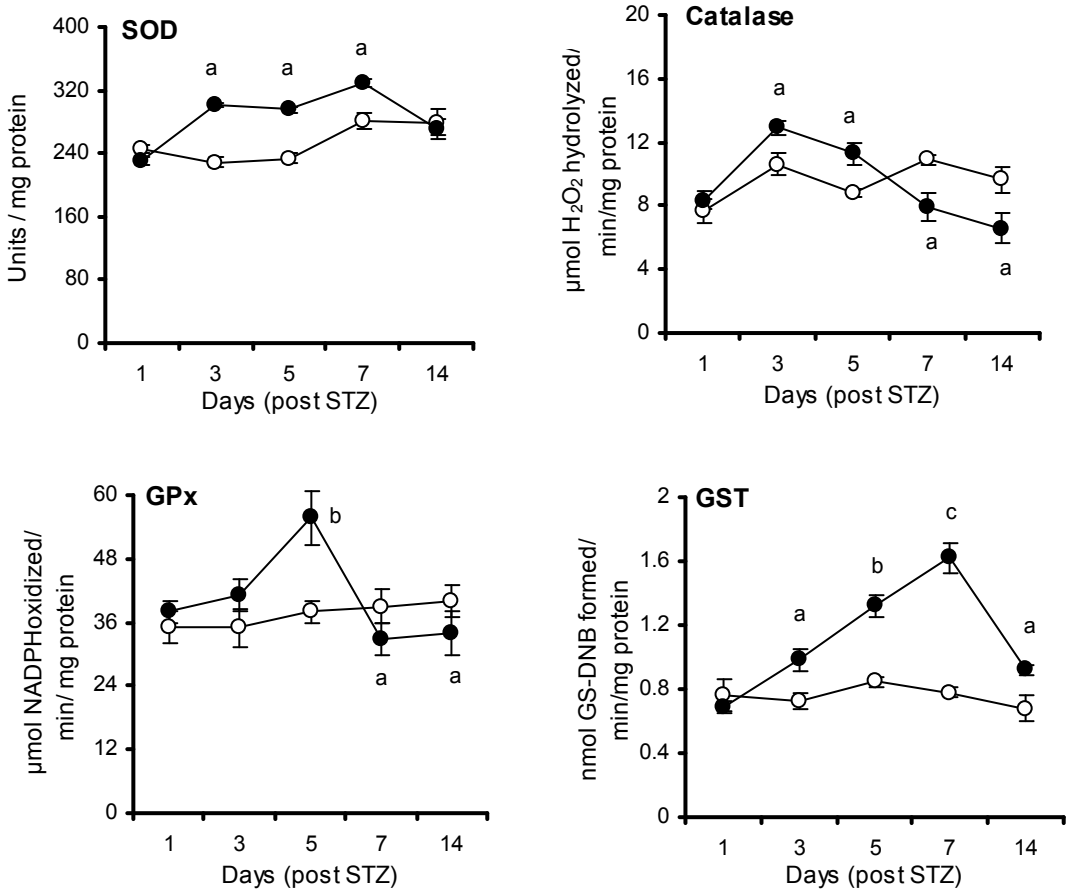


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Fig. 3.5

Alterations in the activities of antioxidant enzymes in testis cytosol of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

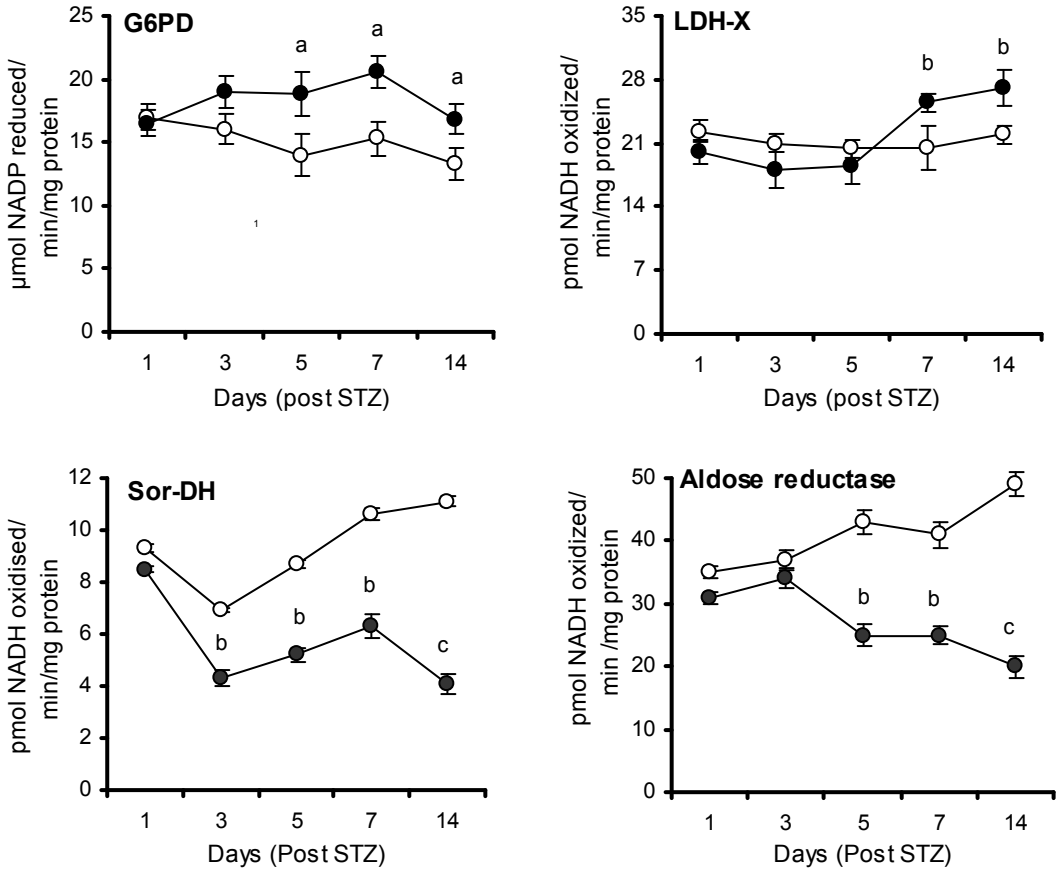


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p< 0.05$, [b] $p< 0.01$, [c] $p<0.001$.

Fig. 3.6

Alterations in the activities of testis cytosolic functional enzymes in prepubertal rats rendered diabetic by an acute dose of STZ measured during both acute and progressive phase

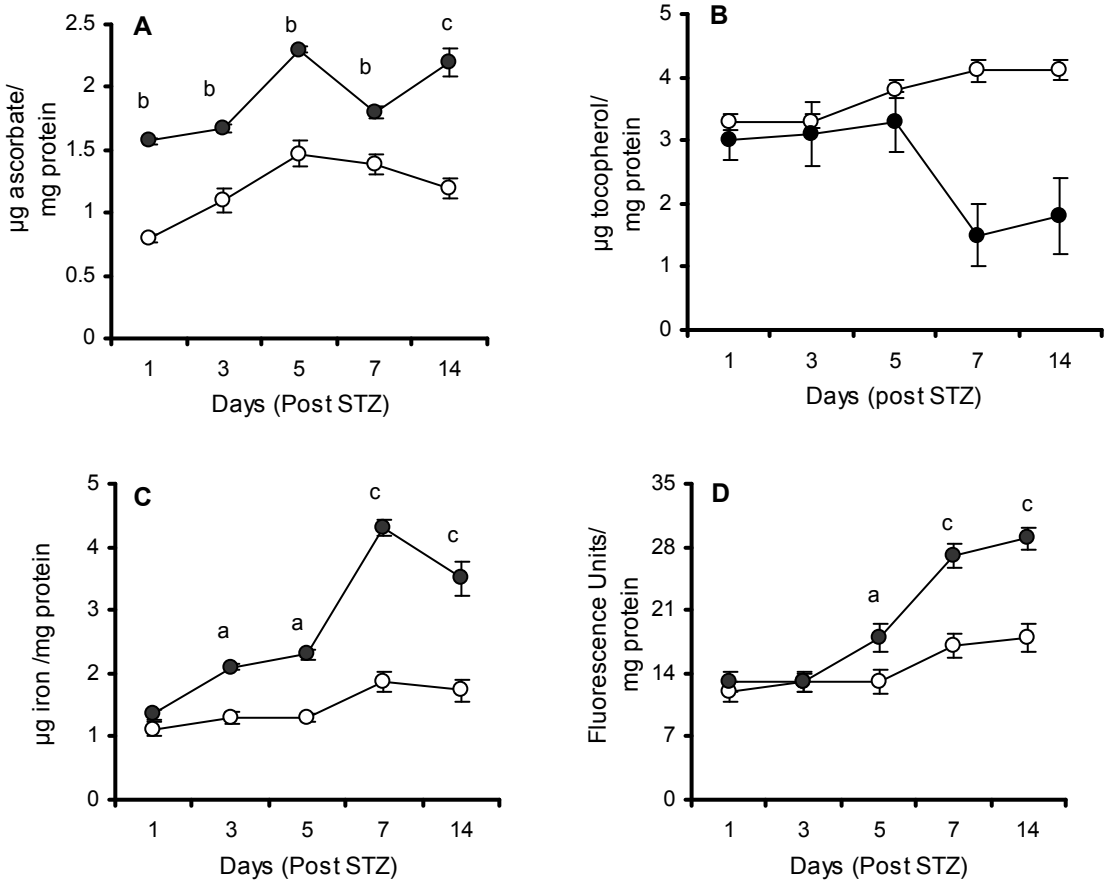


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$, [c] $p<0.001$.

Fig. 3.7

Alterations in testicular ascorbate (A), Free iron (B) and intracellular calcium (C) and tocopherol (E) level in testis cytosol of prepubertal rats rendered diabetic by an acute dose of STZ measured during both acute and progressive phase



Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$.

Fig. 3.8 A

Alterations in the activity of 3β- hydroxyl steroid dehydrogenase in testis cytosol of prepubertal rats rendered diabetic by an acute dose of STZ measured during both acute and progressive phase

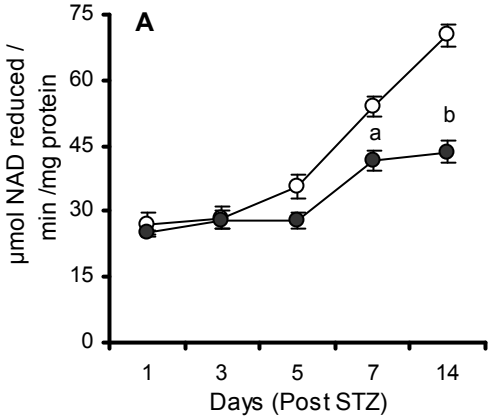
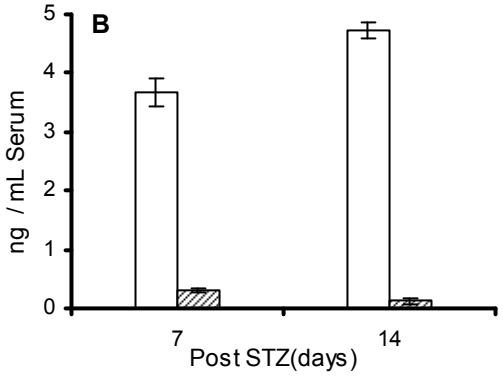


Fig. 3.8 B

Effect of STZ administration on serum testosterone levels in prepubertal rats measured on day 7 and 14.

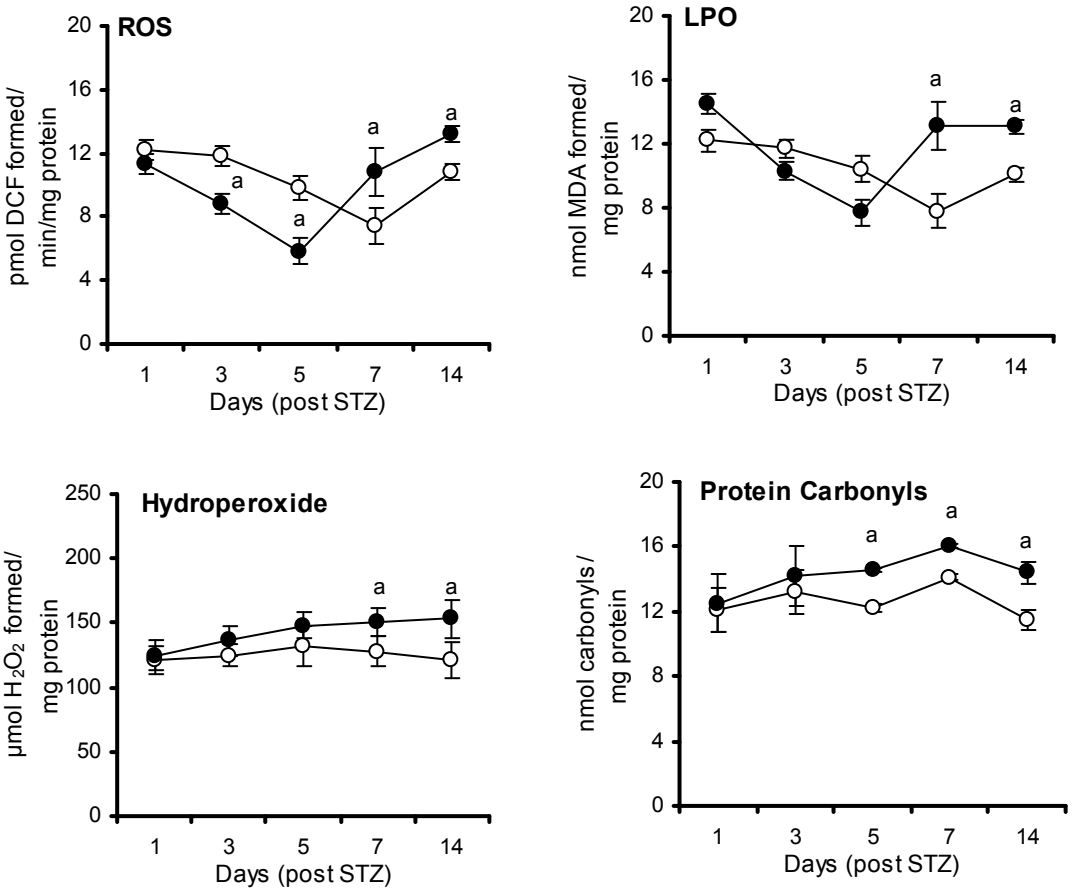


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p < 0.05$, [b] $p < 0.01$.

Fig. 3.9

Status of oxidative stress markers in testis mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

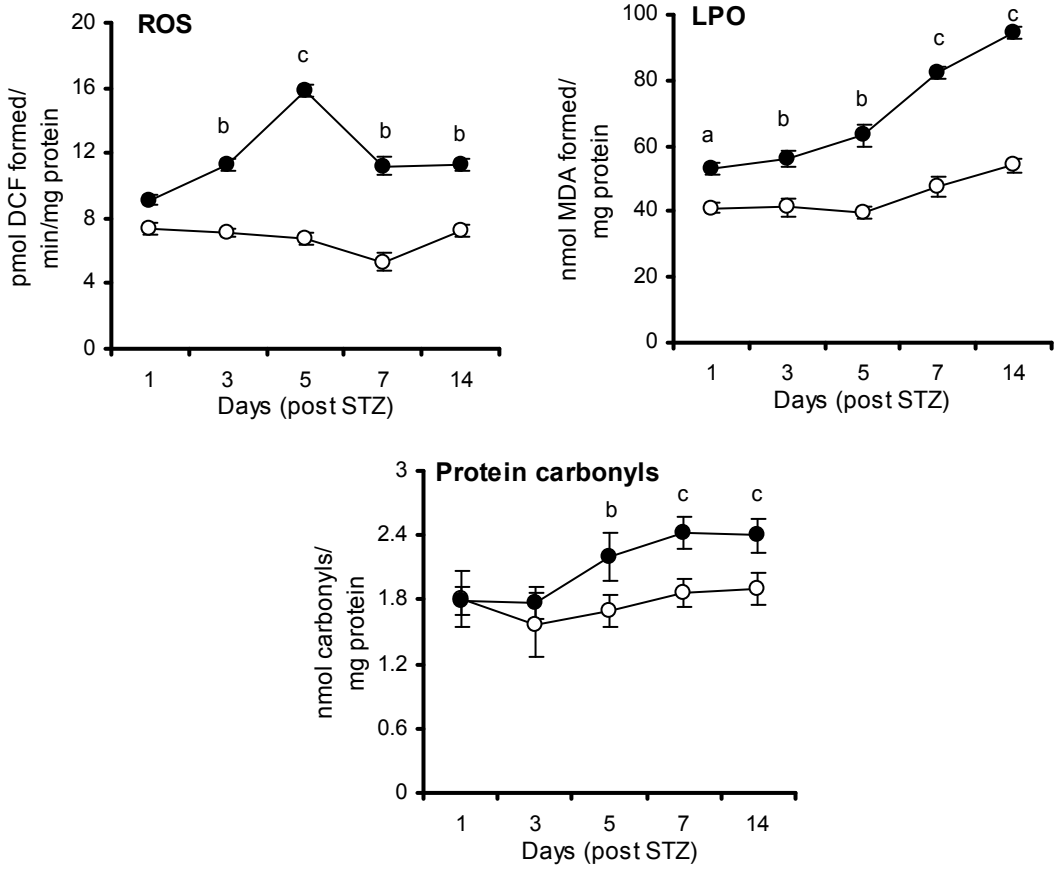


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p<0.05$

Fig 3.10

Status of oxidative stress markers in testis microsomes of prepubertal rats rendered diabetic by an acute dose of STZ measured during both acute and progressive phase

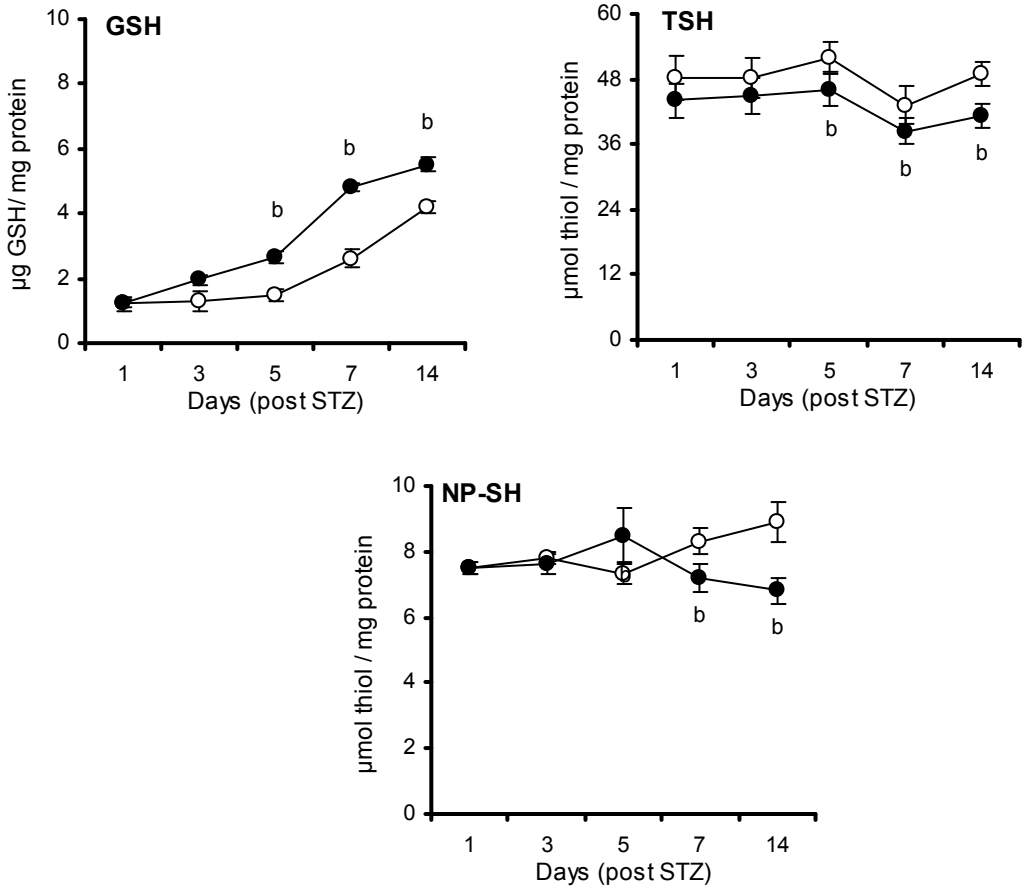


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a] $p<0.05$,[b] $p<0.01$,[c] $p<0.001$.

Fig. 3.11

Alteration in thiol status in testis mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

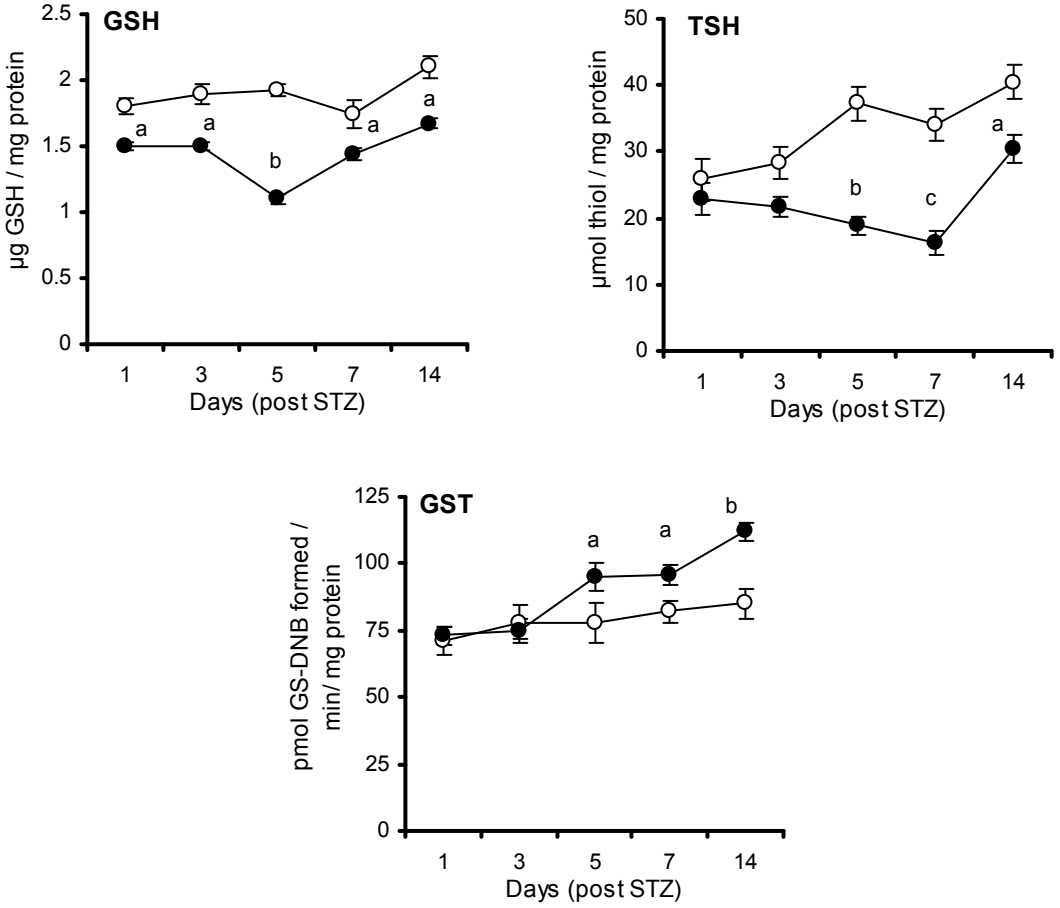


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [b] $p < 0.01$

Fig. 3.12

Alteration in levels of glutathione (GSH), total thiols (TSH) and in the activity of glutathione S- transferase (GST) in testis microsomes of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

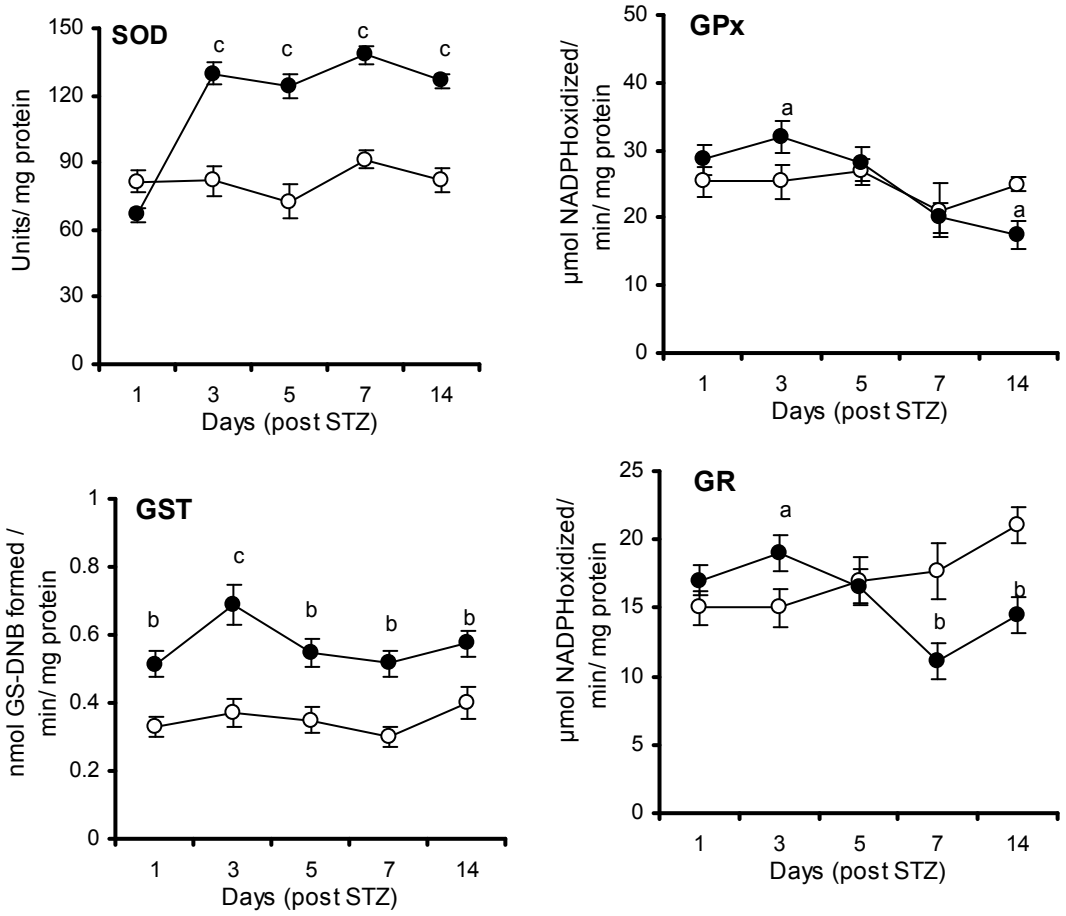


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a]p<0.05, [b] p< 0.01, [c] p< 0.001

Fig. 3.13

Alterations in the activities of antioxidant enzymes in testis mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

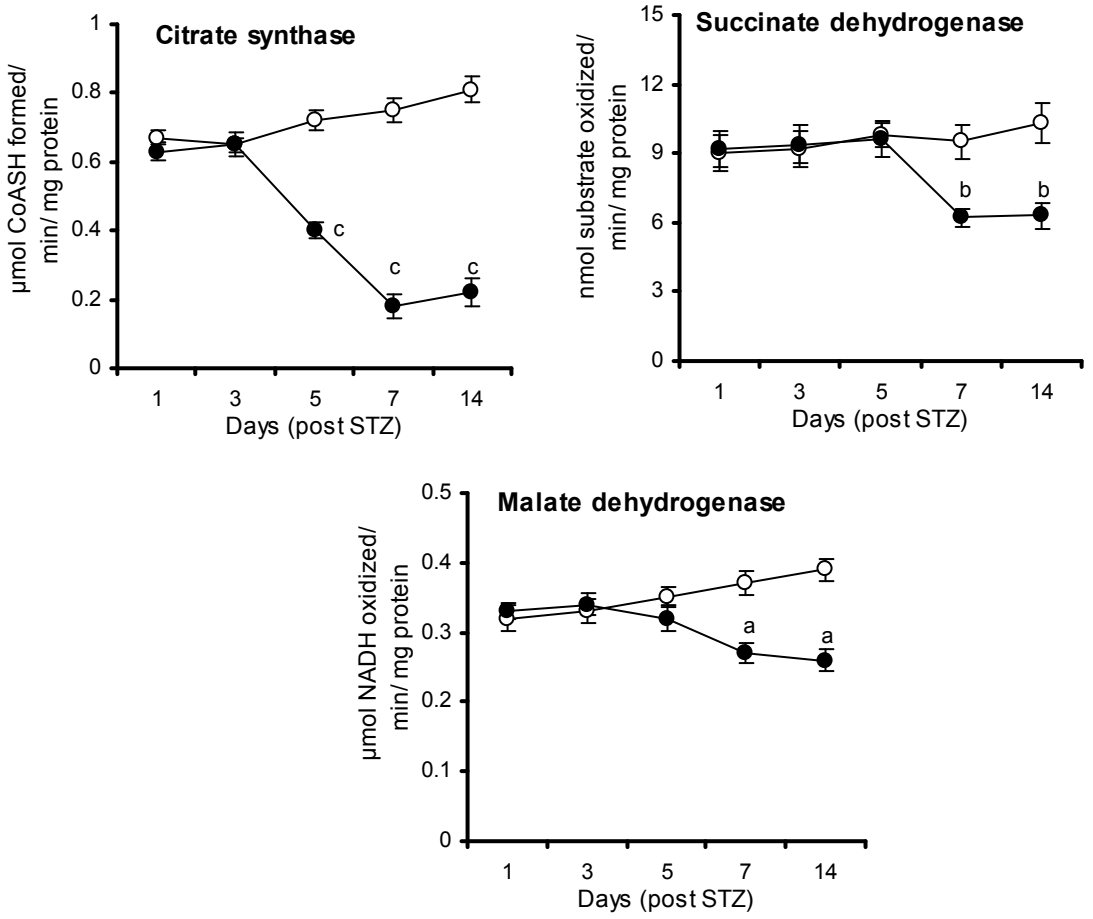


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a]p<0.05, [b] p< 0.01, [c] p< 0.001

Fig 3.14

Alterations in the activities of citric acid cycle enzymes in testis mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

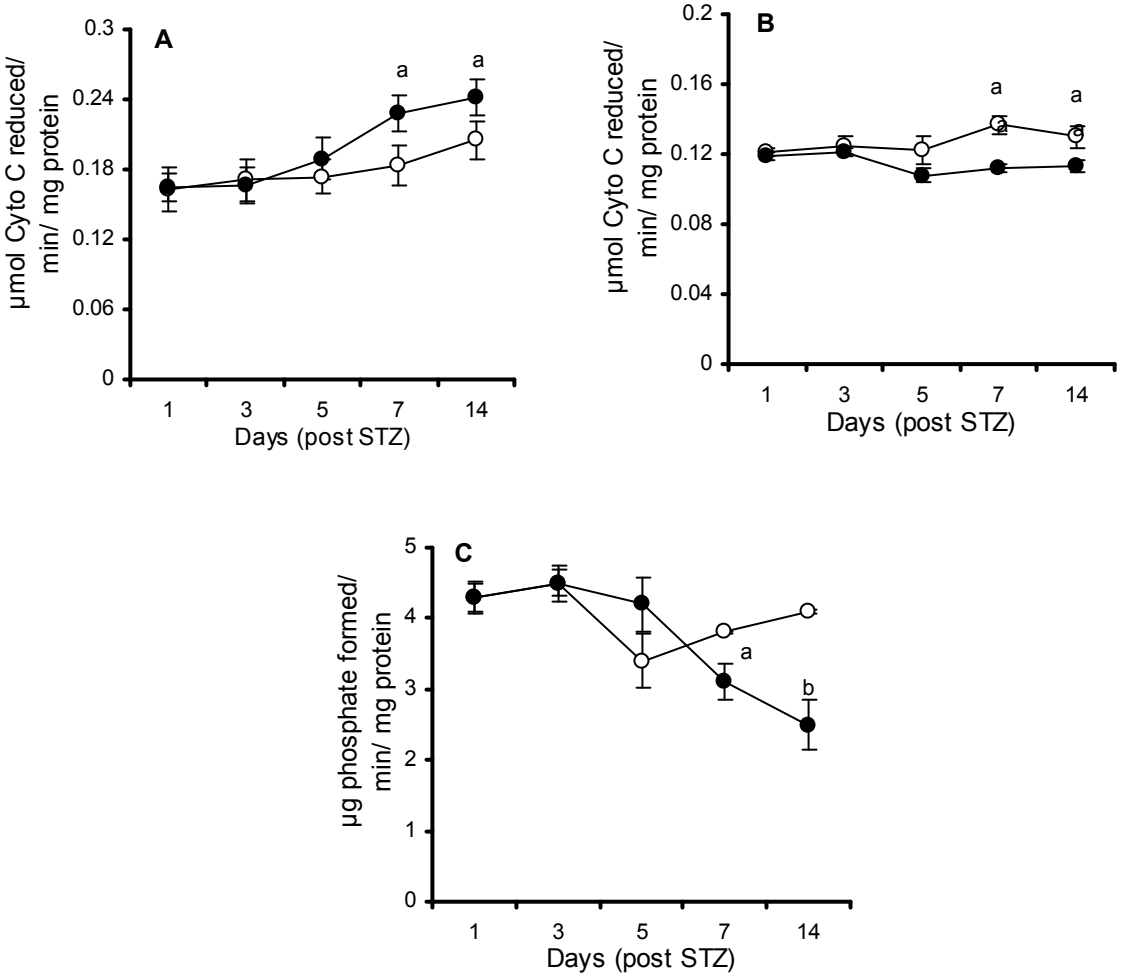


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a]p<0.05, [b] p< 0.01, [c] p< 0.001

Fig. 3.15

Alterations in the activities of NADH- cytochrome C reductase (A), succinate- cytochrome C reductase (B) and Mg- ATPase (C) in testis mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

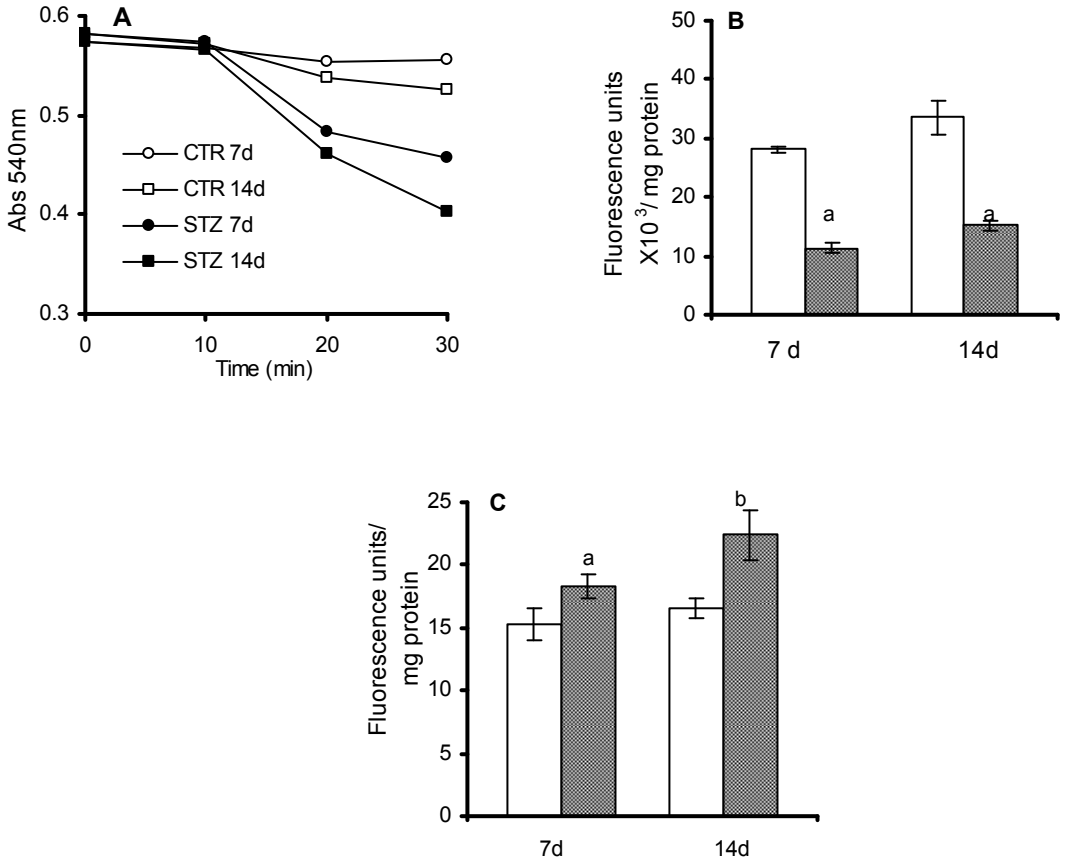


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a]p<0.05, [b] p< 0.01

Fig. 3.16

Opening of permeability transition pore (A), membrane potential (B) and alterations in the activity of aldehyde dehydrogenase (C) in testis mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

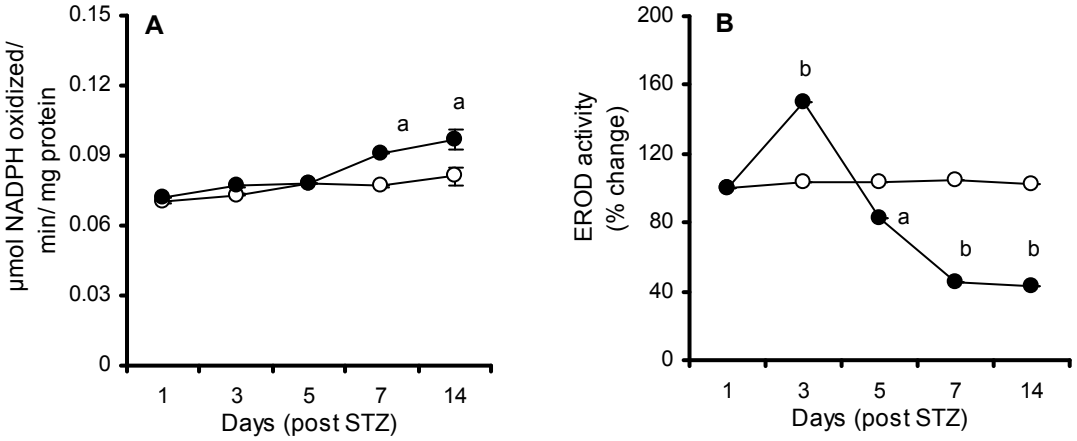


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a]p<0.05, [b] p< 0.01

Fig. 3.17

Alterations in the activities of NADPH- cytochrome C P450 reductase (A) and ethoxy-resorufin–O-dealkylase (EROD) (B) in testis microsomes of prepubertal rats rendered diabetic by an acute dose of STZ measured during acute and progressive phase

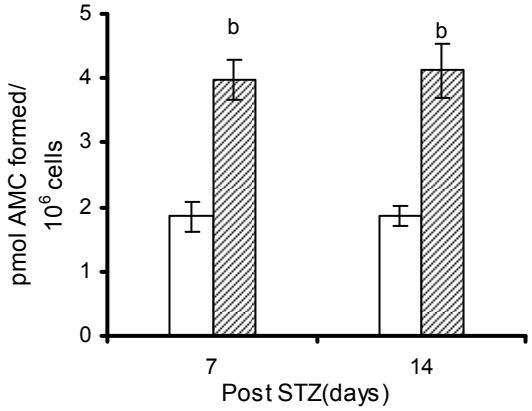


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [a]p<0.05, [b] p< 0.01.

Fig 3.18

Effect of STZ administration on the activity of Caspase-3

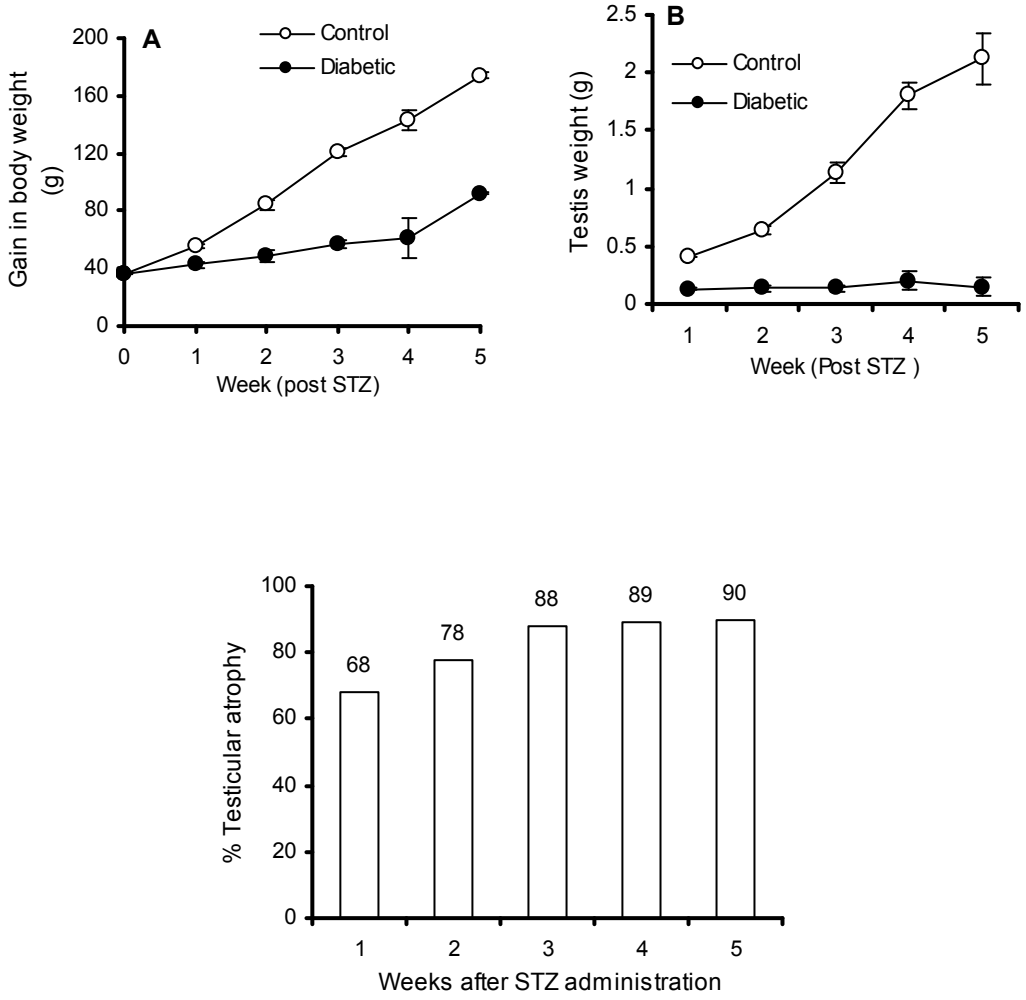


Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test; [b] p< 0.01

Fig. 3.19

Body weight (A) and testis weights (B) of prepubertal rats rendered diabetic by an acute dose of STZ



Values are mean ± SEM (n=6).

Data analyzed by Student's 't' test. Values assigned with different alphabets differ significantly

Fig. 3.20

Flow cytometric analysis of testis of diabetic prepubertal rats sampled on 28 (A), 35 (B) and 42 (C) days

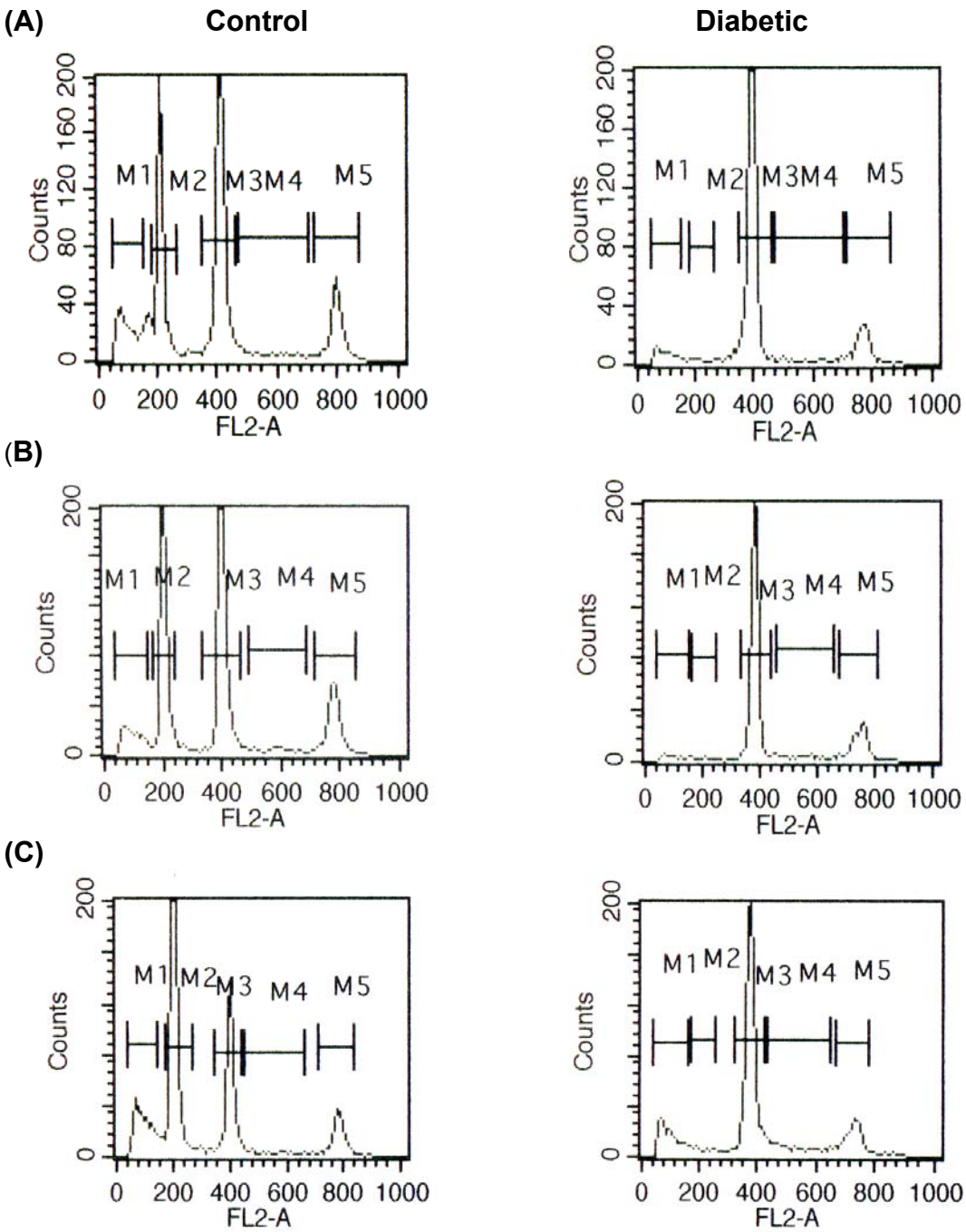


Fig. 3.21

Flow cytometric analysis of testis of diabetic prepubertal rats sampled on 49 (A) and 56 (B) days

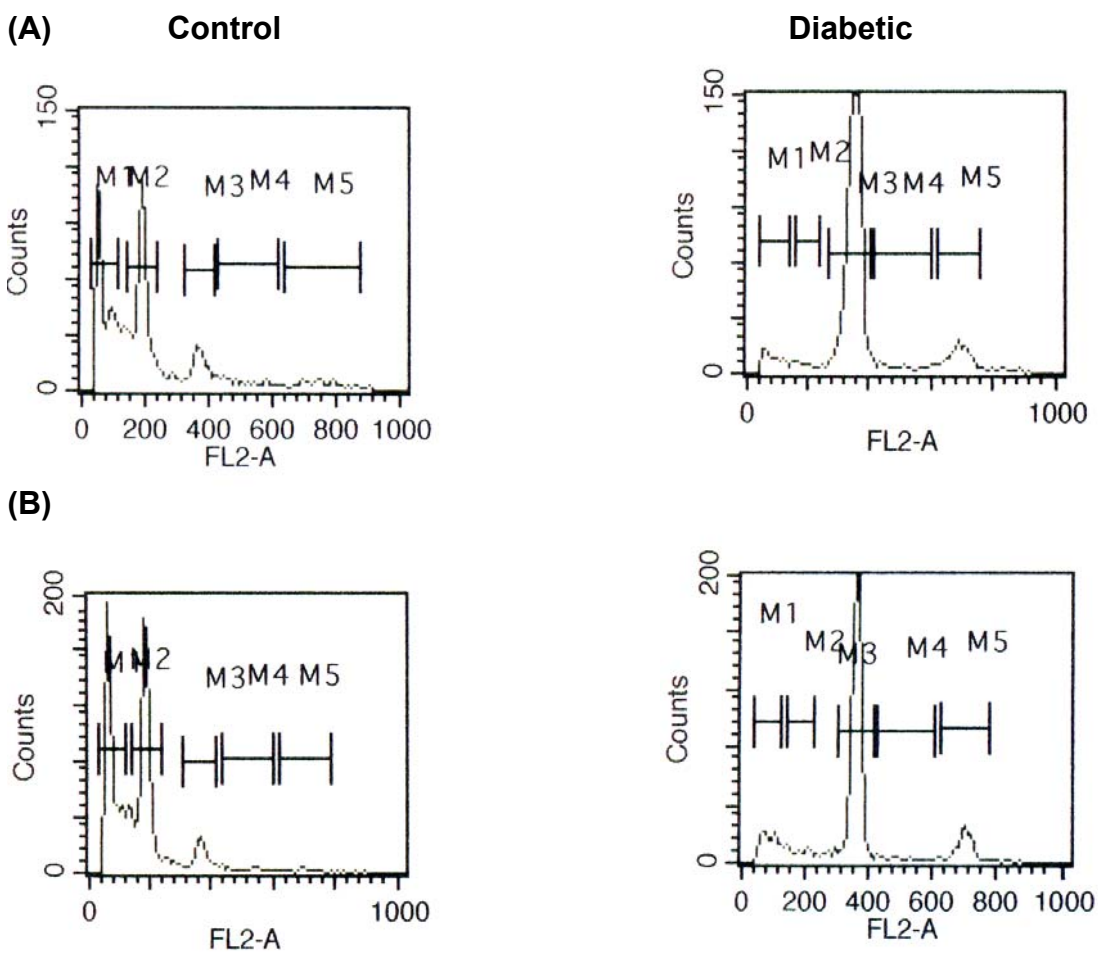


Fig. 3.22

Flow cytometric analysis of testis of prepubertal rats rendered diabetic by an acute dose of STZ

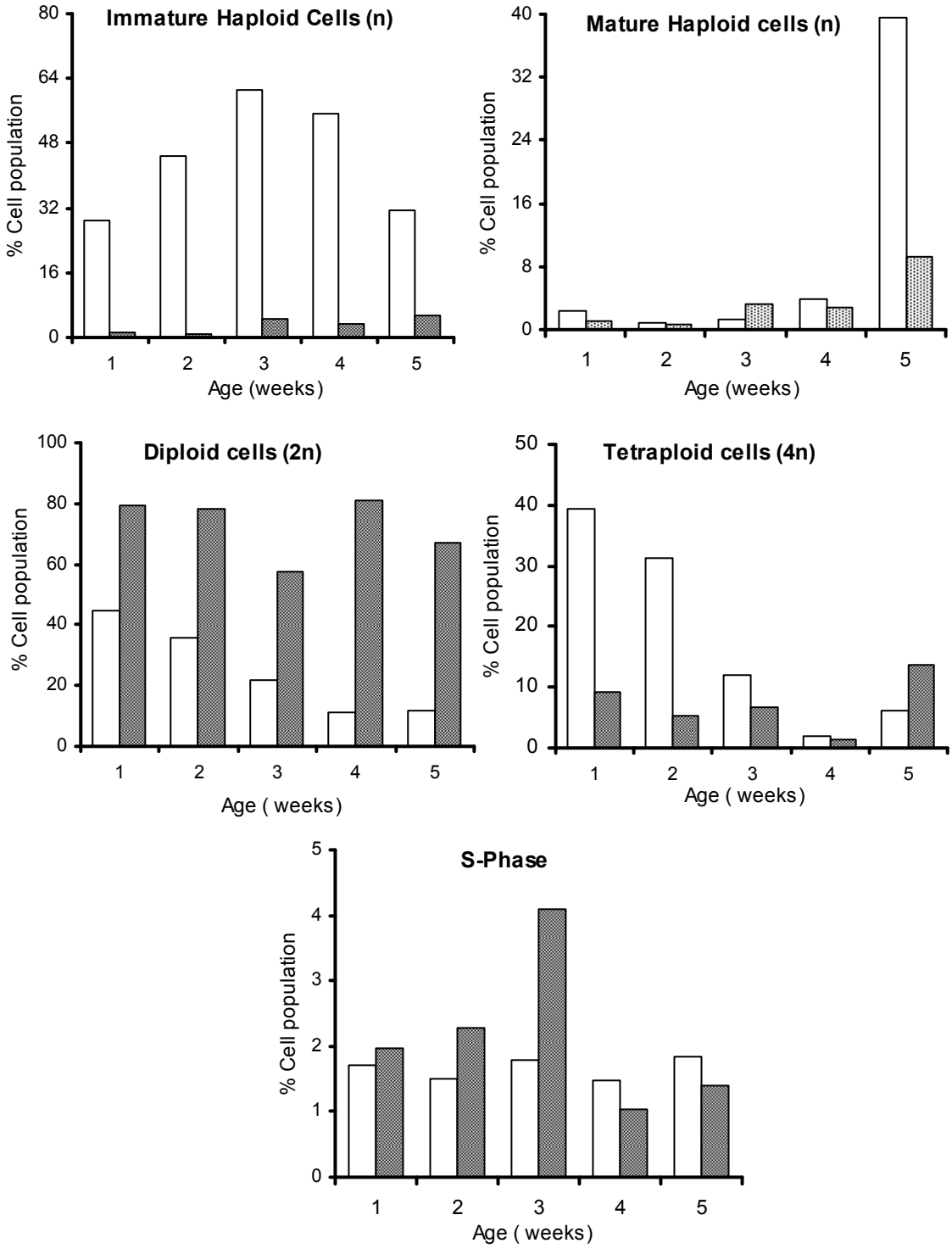


Plate 3.1

Protein profile of testis cytosol (A) and mitochondria (B) at day 7 and 14

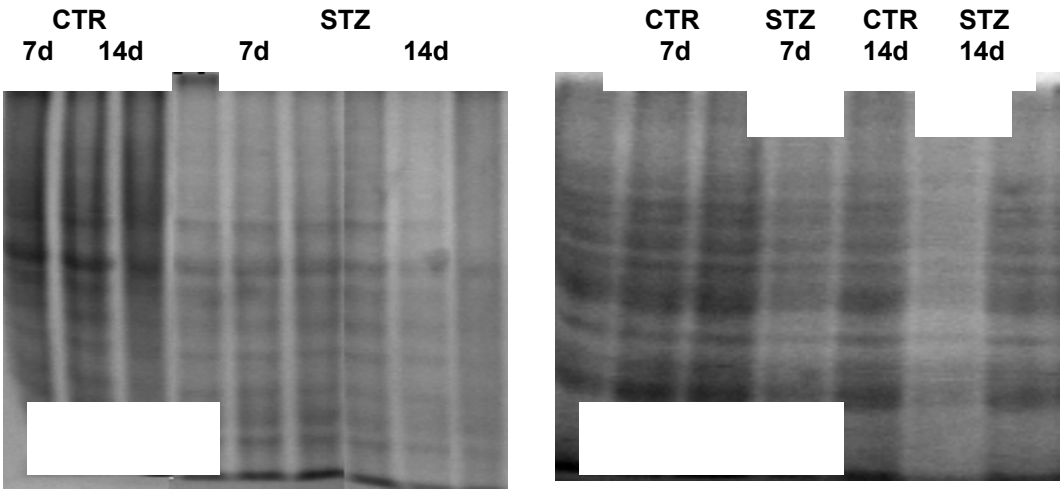
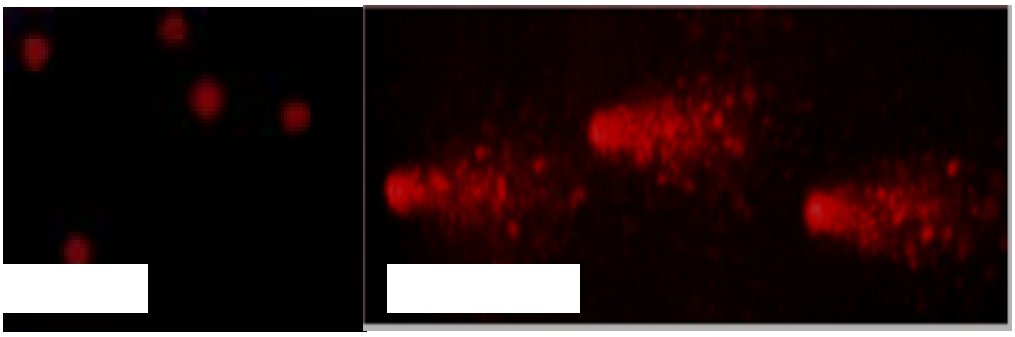


Plate 3.2

DNA fragmentation measured as incidence of comets in testis of diabetic prepubertal rats on day 14



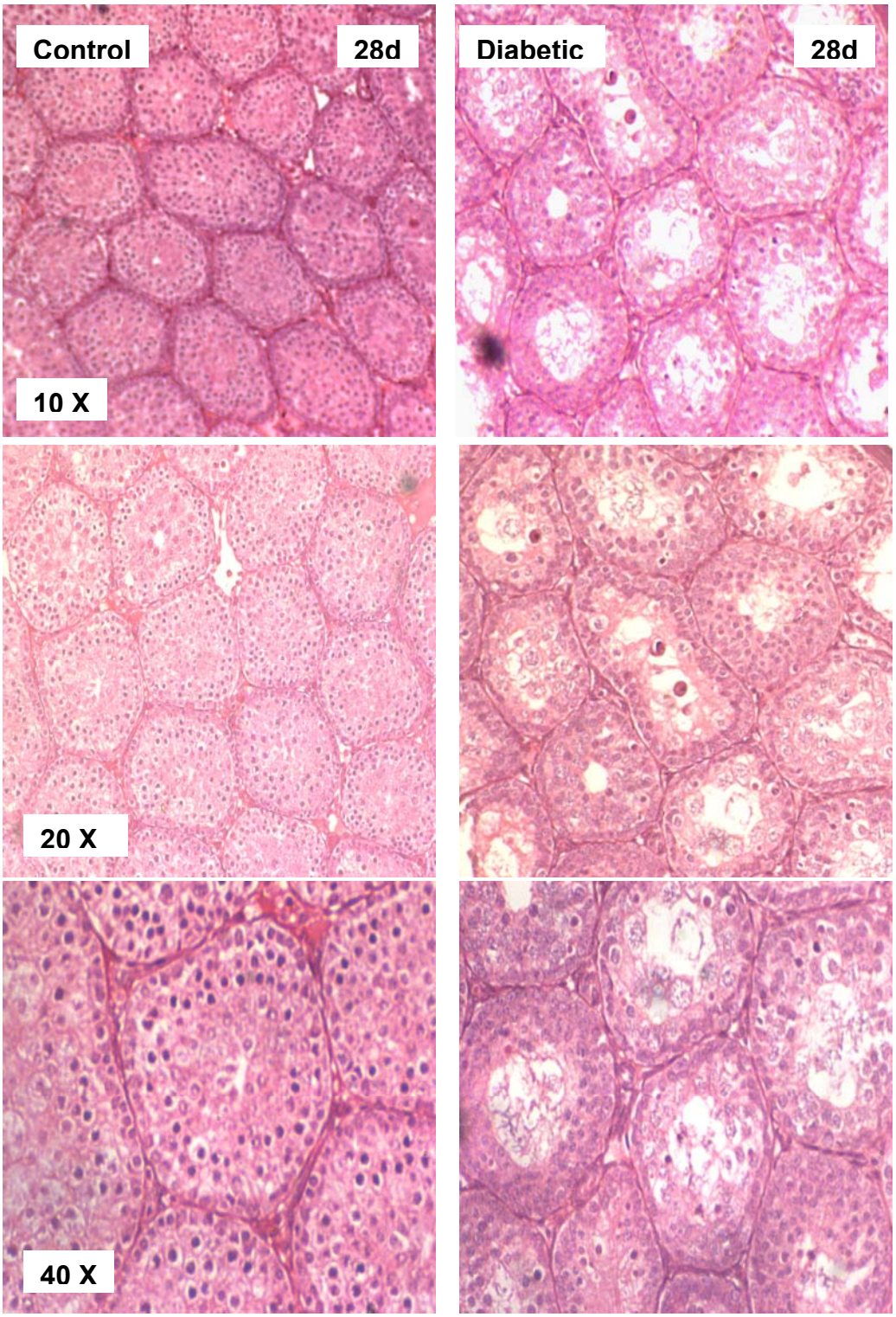


Plate 3.3: Photomicrographs (TS) of prepubertal rat testis sampled at week I post STZ treatment (H&E); **Control** (Left panel): Normal histoarchitecture: Typical appearance of seminiferous tubules;
STZ (Right panel): showing reduction in seminiferous tubule size and extensive Sertoli cell vacuolization (Magnification: 10X; 20X and 40X).

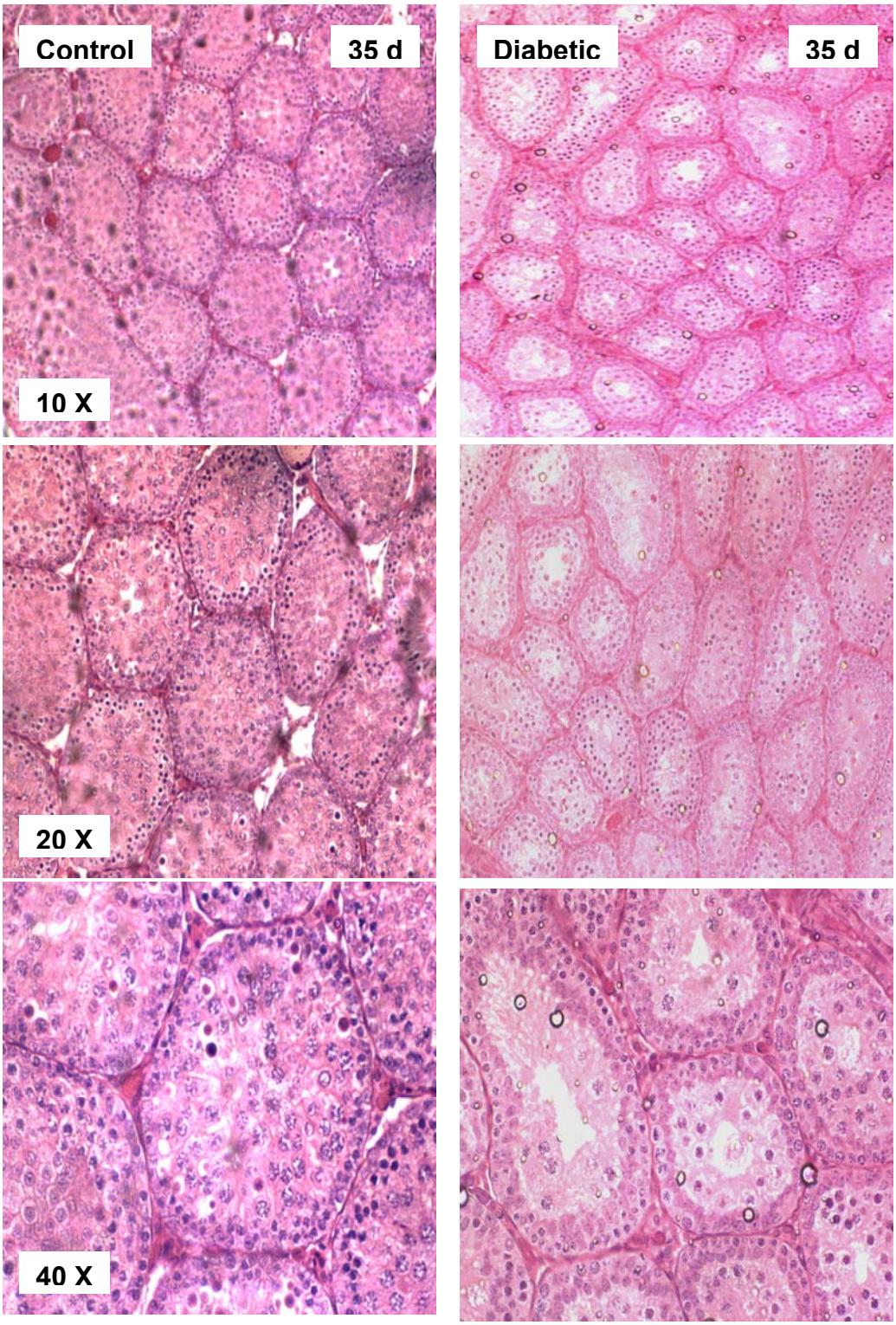


Plate 3.4: Photomicrographs (TS) of PP testis sampled at week II post STZ (H&E): **Control** (Left panel): Normal histoarchitecture showing different cellular associations: **STZ** (Right panel): showing higher degree of seminiferous tubular shrinkage.

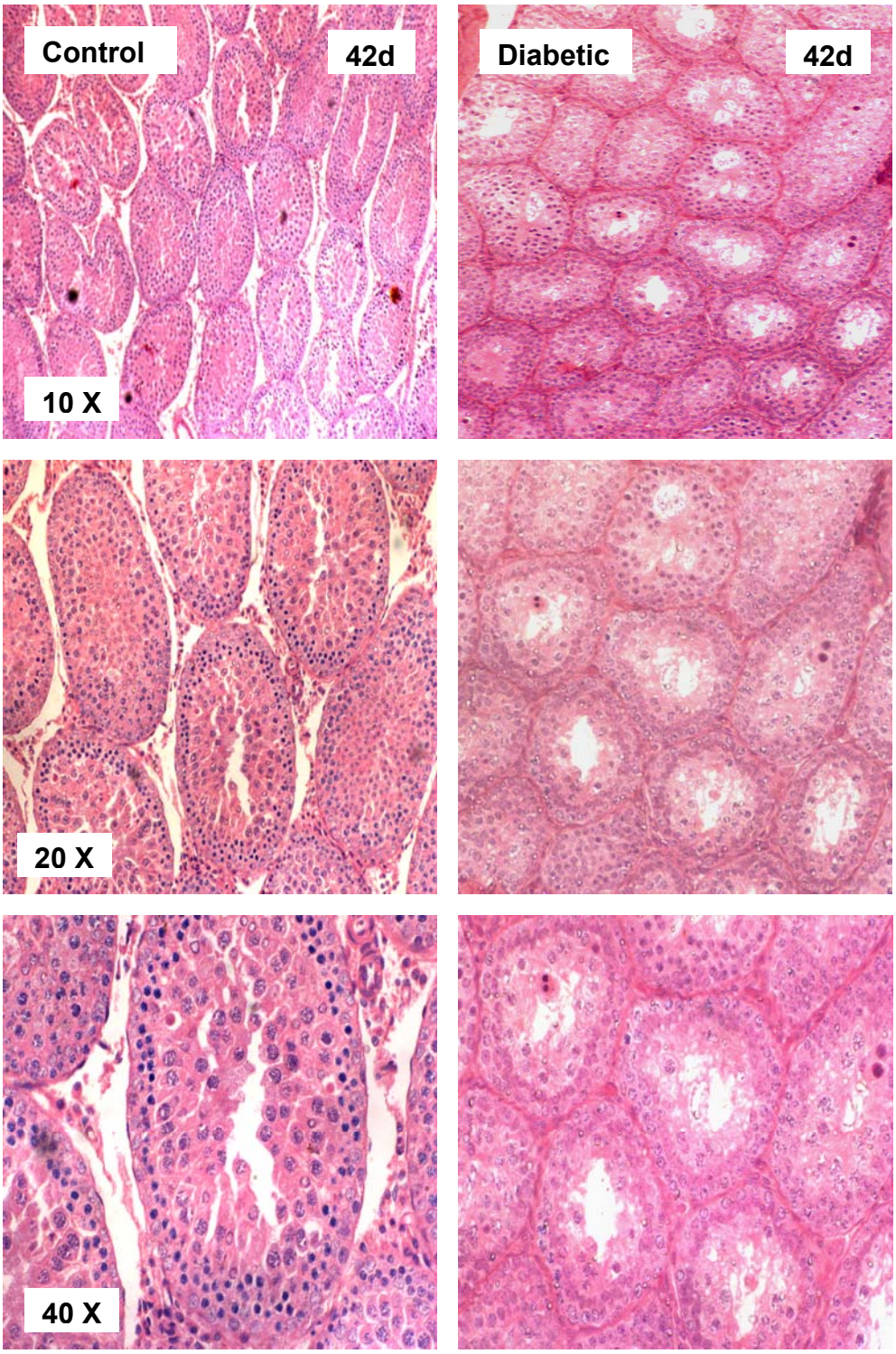


Plate 3.5: Photomicrographs of PP testis sampled at week III post STZ (TS, H&E): **Control** (Left panel): Normal histoarchitecture: with germinal cell differentiation; **STZ** (Right panel): Reduced tubular size, loss of interstitial cells, germinal cell death.

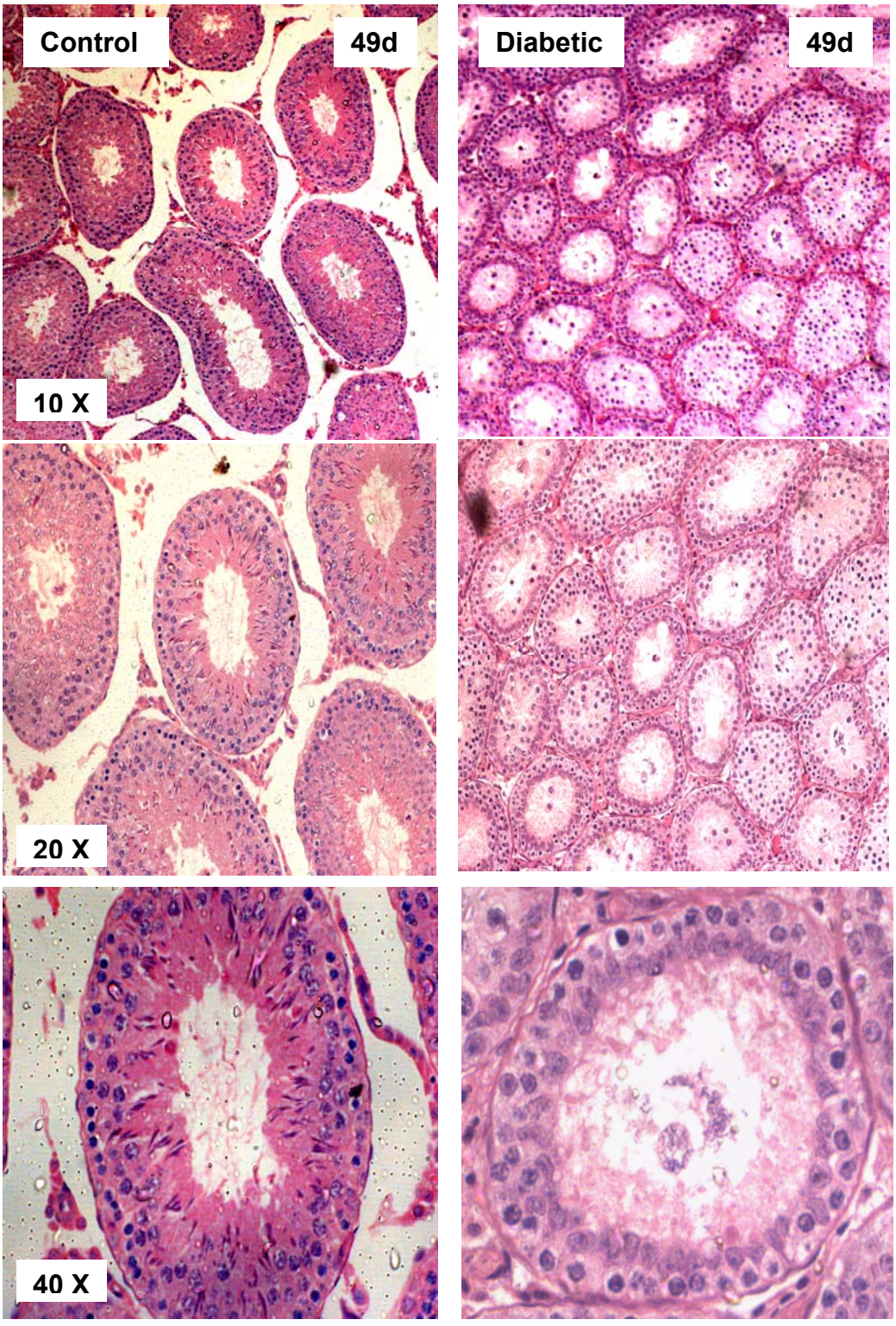


Plate 3.6 : Photo micrographs of testis of PP rats rendered diabetic sampled at week IV post STZ treatment (TS, H&E). **Control** (Left panel): Active spermatogenesis and appearance of matured spermatids in the lumen; **STZ** (Right panel) Complete loss in germinal cell population and absence of spermatozoa in the lumen.

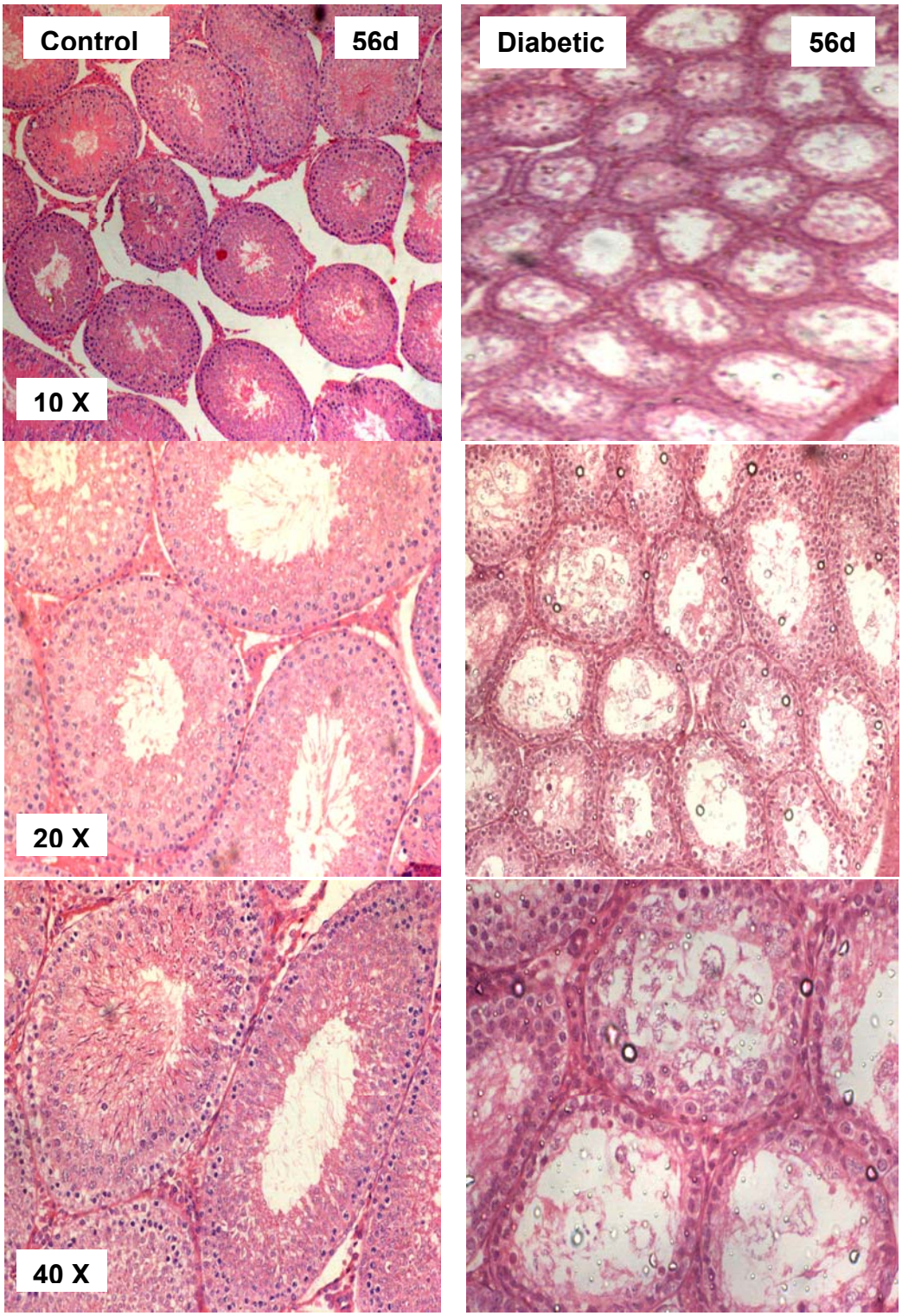


Plate 3.7: Photo micrographs of testis of prepubertal rats sampled at week IV post STZ treatment (TS, H&E). **Control** (Left panel): Active spermatogenesis and appearance of matured spermatids in the lumen; **STZ group** (Right panel) Complete loss in germinal cell population and absence of spermatozoa in the lumen.

5.0 DISCUSSION

Data obtained earlier (results of Chapter 2) in PP rats clearly demonstrated the vulnerability of testis to oxidative stress under diabetic conditions. Since testes of 4wk old rats were relatively more vulnerable, these were selected to further elucidate diabetes associated biochemical and functional alterations in PP testis. The emphasis was to obtain data during two phases of diabetes viz., acute and progressive. Acute phase comprised of sampling of testis during 1-5 days, while the progressive phase consisted of sampling at 7 and 14 days of post STZ administration.

STZ administration to PP rats induced significant hyperglycemia which stabilized on day 3 and beyond. Earlier studies have reported that hyperglycemia occurs in three phases- (i) a transient hyperglycemic phase (<1h post STZ), accompanied by a decrease in plasma insulin caused due to morphological changes in pancreas viz., intracellular vacuolization dilation of the rough endoplasmic reticulum, decreased golgi area, reduced secretory granules and insulin content leading to inhibition of insulin secretion. (ii) the hypoglycemic phase, which typically occurs 4-8h post STZ administration and lasts for several hours. It may be so severe that it causes convulsion, and may even be fatal without glucose administration. During this phase, the liver glycogen stores are depleted exhaustively through starvation. This severe transitional hypoglycemia is produced by the flooding of the circulation with insulin probably due to increased proliferation of insulin producing β-cells. (iii) The third phase is the permanent diabetic hyperglycemia, which is evident within 48h, where there will be complete degranulation and loss of β-cell integrity (Lenzen, 2008). Our findings are consistent with earlier reports where a stable hyperglycemia was evident at 48hr post STZ treatment in adult mice (Kume et al., 2004).

Oxidative stress has been implicated to be the general mechanism underlying the progression of diabetes associated complications (Brownlee, 2001; Rolo and Palmeira, 2003; Obrosova, 2008). The increased blood glucose level during early stages of diabetes becomes a chief source of free radicals in the chronic stage damaging secondary target organs including

testis. In the present study, hyperglycemia induced oxidative impairments were evident in all the testicular subcellular fractions (cytosol, mitochondria and microsomes) as reflected by the enhanced ROS generation, hydroperoxide and MDA levels. The oxidative damage was more robust in testis cytosol and microsomes during both acute and progressive phases. Although less affected during the acute phase, testis mitochondria showed a marked oxidative response during the progressive phase. This differential susceptibility could be related to one or several factors viz., upregulation in free radical synthesizing machineries via., xanthine oxidase, NADPH oxidase etc., and /or down regulation of antioxidant defenses that result in enormous amount of free radical generation. Since the potential target sites for these reactive intermediates are the cellular membranes which are rich in polyunsaturated fatty acids. Thus both microsomes and mitochondria are affected to a greater extent resulting in loss of membrane integrity (Shin et al., 1995). Further, the free radicals may dislodge iron from iron-sulfur centres in mitochondria thus amplifying the effect. Consistent with earlier reports (Campenhout et al., 2006), data obtained in the present study suggests that increased lipid peroxidation significantly contributes to diabetic complications.

Microsomes are organelles actively involved in a variety of metabolic process including steroidogenesis, fatty acid oxidation, xenobiotics metabolism etc., which are shown to generate free radicals. Reactive dicarbonyls viz., MDA, 4-HNE formed by the autoxidation of carbohydrates, nucleic acids, unsaturated lipids or amino acids bind covalently to proteins and undergo further oxidation to form advanced glycation end products (AGE) and advanced lipid end products (ALE). The prolonged dyslipidemia and hyperglycemia in diabetes increases AGE that contributes to the development of diabetes complications (Obrein et al., 2005; Thomas et al., 2005). Further, chromolipids fluorescent (at ex 360nm / em 430) are related to the formation of HNE-phospholipid adducts. The increase of the development of fluorescent chromolipids in testis cytosol of diabetic rats is suggestive of an enhanced formation of HNE-phospholipid adducts in the testis (Traverso et al.1999).

Nitric Oxide (NO), a free radical metabolite of L-arginine is highly lipophilic, readily diffusible molecule which is demonstrated to possess a dual

regulatory role under physiological and pathological conditions (Choi et al., 2002). It is synthesized from L-arginine by the catalytic activity of three different isoforms of nitric oxide synthase (NOS): the constitutive Ca^{2+}/calmodulin-dependent neuronal (nNOS), endothelial (eNOS), and the inducible (iNOS) isoforms. NO under physiological condition is known to act as an intracellular messenger and regulates various biological processes including neurotransmission (Monacada et al., 1991; Lownstein et al., 1994). In addition, NO has been speculated to act as a pro-oxidant at high concentrations and reacts with superoxides to form highly reactive peroxynitrite ($ONOO^-$) (Joshi et al., 1999). Conversely, NO· can inhibit oxidation and terminate chain reactions during lipid peroxidation (Radi, 2004). More importantly, NO is shown to participate in modulation of testicular cell function, since it acts as an inhibitor of steroidogenesis. Earlier evidences in rats indicate elevated serum testosterone levels following the administration of a NOS inhibitor and are suppressed by agents which promote NO production (Delpunta et al., 1996; Kostic et al., 1999; Fujisawa, 2000). The elevated levels of NO observed in the present study can be attributed to the increased oxidative stress, lowered steroidogeneic activities and activation of caspase mediated apoptotic pathways (Choi et al., 2002).

Antioxidant enzymes constitute the first line of defense against ROS (Halliwell and Gutteridge, 1999). Under normal physiological conditions, a delicate balance exists between the rate of formation of hydrogen peroxide (via dismutation of superoxide as a result of SOD activity) and the rate of removal of hydrogen peroxide by CAT and GPx. Therefore, any impairment caused to any member will influence the activities of other enzymes in the cascade. A reduction in the activity of SOD will result in an increased level of Superoxide, which is known to inhibit CAT activity. On the otherhand, impairment of the activities of CAT and GPx will lead to the accumulation of hydrogen peroxide in the cell causing inactivation of SOD (Halliwell, 2002, 2006). In the present investigation, during the acute phase, the activities of cytosolic SOD, CAT and GPx were increased suggesting an elevated free radical generation, while significant reduction in the activities ensued during the progressive phase. The plausible explanation for these perturbations

could be linked directly to oxidative stress-induced inactivation and/or exhaustion of antioxidant enzymes. However, the potential inhibitory effects of stress induced Nitric Oxide also cannot be overlooked (Cooper, 1999). On the contrary, testis mitochondria showed elevated SOD activity, accompanied by reduced activity levels of both GPx and GR, implicating increased generation of superoxide from electron transport chain. Further, the elevated levels may also cause functional inactivation of GPx and amplify the oxidative cascade in cytosol. These perturbations were reflected by a transient increase in the activity of GR during the acute phase, followed by reduction during progressive phase. This may be due to limited supply of NADPH or functional inactivation of the enzyme. Further, glycosylation of proteins inactivates enzymes (eg. the anti-oxidant enzymes) and also affects the functions of binding, transport and protein structure (Yan and Harding, 1997).

Protein oxidative modifications viz., protein carbonyls, side chain oxidation etc., are induced either directly by ROS or indirectly by reactions of secondary by-products of oxidative stress (Stadtman, 2001, 2006) resulting in fragmentation of the polypeptide chain, oxidation of amino acid side chains, and formation of protein-protein cross linkages. Considerable evidence indicate that the maintenance of protein redox status is a fundamental requisite for normal cell function and thus structural changes in proteins are considered to be vital among several mechanisms underlying the progression of disease complications (Stadtman and Levine, 2000). The consistently elevated levels of protein carbonyls during both acute and progressive phase of diabetes in testis (cytosol/mitochondria/microsomes) clearly implicate the altered function of membrane proteins.

The GSH and glutathione related enzyme system, in a quantitative sense are the most important protective systems which are involved in the metabolism and detoxification of cytotoxic/carcinogenic compounds as well as ROS in mammalian tissues. GST, a phase II enzyme, catalyses the conjugation of GSH to a wide variety of xenobiotics and oxidative products such as 4-HNE there by making a toxin more water soluble and less biologically active. In the present study, the activity of GST was elevated in all subcellular organelles of testis clearly implicating the formation of toxic

aldehydes *in vivo* during both phases. GSH, is the predominant low molecular weight thiol in mammalian cells which plays a major role in cellular defenses against oxidative/nitrosative stress and against electrophiles. Although, relatively resistant to 'spontaneous oxidation', GSH reacts rapidly and quenches hydroxyl radicals and cytotoxic products of NO (Griffith, 1999) and gets converted to oxidized glutathione (GSSG), a species that is reduced intracellularly to GSH by GSSG-reductase in a NADPH-dependent reaction. At normal levels of oxidative and nitrosative stress, GR activity and NADPH availability are sufficient to maintain GSH/GSSG ratio >1. Under these circumstances there is essentially no net loss of GSH through oxidation. However, during stress conditions due to limitations in NADPH supply, GSSG accumulates, resulting in shift in the redox balance of the cell and secretion of GSSG from the cell, as it is not taken up by the intact cells, but is rather degraded extrracellularly. Loss of GSSG from cells under conditions of oxidative or nitrosative stress increases cellular requirements for *denovo* synthesis of GSH. In the present study, the levels of GSH were elevated in cytosol and mitochondria, but significantly reduced in microsomes. The elevated levels of GSH may be explained as a primary response elicited against oxidative stress. On the other hand, decreased levels in testis microsomes during diabetes can be attributed to increased utilization of GSH due to oxidative stress (Obrasova et al., 2003). Earlier investigators support the hypothesis that, in diabetes, chronic hyperglycemia increases the polyol pathways, as well as ROS generation, leading to increased GSH oxidation. Besides GSH, other non-protein thiols viz., α-lipoic acid, cysteine, methionine, taurine etc., are also shown to ameliorate diabetes induced oxidative stress (Maritim et al., 2003).

Ascorbic acid (AA) is one of the major water soluble antioxidant capable of scavenging reactive oxygen / nitrogen species which prevents oxidative damage to important biological macromolecules. AA also reduces redox active transition metal ions in the active sites of specific biosynthetic enzymes. However, during conditions of oxidative stress, AA can interact with 'free catalytically active metal ions and contribute to oxidative damage through the production of hydroxyl and alkoxyl radicals. During normal physiological

conditions, the requirement of AA during prepubertal stage is very high as this period comprises of rapid structural changes and metabolic activity in preparation for the onset of puberty. In the present study, testis of diabetic rats showed consistently elevated ascorbate levels during both acute and progressive phase. Although the exact reason for the elevated AA levels is difficult to explain, a parallel increase in free iron in diabetic testis may be regarded as one of the several factors aggravating the disease complications. Earlier, AA administration has been shown to elevate steroidogenic activities and testosterone levels (Murugesan et al., 2007). However, the activity of 3β-HSD and testosterone levels was significantly decreased in diabetic testis implicating the prooxidant role of ascorbic acid.

Interestingly, among non-diabetic PP rats, the ascorbic acid levels in testis showed a spurt on day 5 and beyond. This may be due to the increased requirements of AA for the animal while is attaining reproductive maturation and this observation is consistent with earlier reports (Chinoy et al., 1982). In addition to scavenging ROS and RNS, AA can regenerate other small molecule antioxidants, such as α-tocopherol, GSH, urate and β-carotene, from their respective radical species. Interaction of ascorbate with α-tocopheroxyl radical to regenerate α-tocopherol moves radicals from the lipid phase into the aqueous phase and hence prevent tocopherol-mediated peroxidation. Although ascorbate acts as a co-antioxidant for α -tocopherol, it is uncertain whether ascorbate recycles, or rather spares, α -tocopherol *in vivo*. In contrast, ascorbate has been shown to spare GSH under conditions of increased oxidative stress *in vivo* (Padayatty et al., 2003). This clearly explains the lowered levels of vitamin E and unaffected levels of GSH observed in the present study.

In the present study, functional impairments in diabetic testis were ascertained by determining the activities of sorbitol dehydrogenase and lactate dehydrogenase which are shown to play a vital role in the disease pathogenesis. Sorbitol dehydrogenase and aldose reductase are two members of the polyol pathway, which play vital role in the development of various diabetic complications (Brownlee, 2001). Aldose reductase catalyzes the conversion of glucose into sorbitol by NADH dependent process, while the

later formed is oxidized to fructose by sorbitol dehydrogenase. Sorbitol dehydrogenase in testis augments the synthesis of fructose, the chief energy source for developing spermatozoa. Previous workers have demonstrated abnormal sorbitol accumulation in male reproductive tract under hyperglycemic conditions (Paz et al., 1980) due to either functional inactivation of the enzyme by high levels of circulating glucose/ fructose (Kobayashi et al., 2002) and/or the excess accumulation of sorbitol in the tissues. Further, these changes were accompanied by significant decrease in aldose reductase activity implicating the possible posttranslational modification (glycosylation) of both the enzymes by elevated levels of glucose rendering them inactive. This type of modification has been shown to decrease the activities SOD, aldehyde reductase, alcohol dehydrogenase, Na^+-K^+ATPase and carbonic anhydrase (Kobayashi et al., 2002).

Glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway, is a major intracellular source of NADPH generation. NADPH is the central component of both pro- and antioxidant processes. NADPH serves as a cofactor for the reduction of GSSG (oxidized glutathione) to its reduced form GSH by glutathione reductase, which scavenges ROS. NADPH is also required for ROS production by pro-oxidant enzymes such as iNOS and NADPH oxidase. Therefore, the intracellular NADPH-to-NADP+ ratio appear to be essential for the determination of cellular redox potential. Recently, in adipocytes G6PD overexpression was shown to promote the expression of pro-oxidative enzymes, including inducible nitric oxide synthase and NADPH oxidase, and the activation of nuclear factor-κB signaling, which eventually leads to insulin resistance (Spolarics, 1999). The consistently elevated levels of G6PD, implicate increased formation of NADPH, which besides being used for recycling of oxidized glutathione is also utilized by other NADPH dependent oxidases which generate free radicals eventually resulting in oxidative stress. However, the result obtained is in contrast with that of earlier reports, since induction of diabetes is reported to cause inhibition of G6PDH resulting in activation of PKA and oxidative stress conditions in rat kidney.

Low testosterone levels are common among men with T2DM and may be associated with insulin resistance (Grossman et al., 2008). Recent studies have also demonstrated that low testosterone levels during diabetes affect semen parameters and impair distinct phases of spermatogenesis in male rats (Arikawe et al., 2006). Induction of diabetes in prepubertal rats caused reduced activities of 3β-HSD, a rate limiting enzyme in testosterone biosynthesis and this corroborates earlier results (Doreswamy and Muralidhara, unpublished) .The decrease in the activity could be due to elevated oxidative stress in microsomes, which are the primary sites of steroidogenesis.

Testicular mitochondrial dysfunctions

Mitochondria harbor a bulk of oxidative pathways and are packed with various redox carriers that can potentially leak single electrons to oxygen and convert it into Superoxide anion, a progenitor of ROS. Therefore, mitochondria accumulate oxidative damage more rapidly than the rest of the cell, contributing to mitochondrial dysfunction (Lenaz, 1998). Normally associated with generation of ATP through oxidative phosphorylation mitochondria lose their membrane integrity following increase in free radical production resulting in peroxide leakage into the cytosol, decline in the activities of membrane bound enzymes and ultimately death (Enns, 2003). The reducing equivalents for the oxidative phosphorylation are supplied by TCA cycle, which lodges various dehydrogenases, which are also capable of producing ROS (Andreyev et al., 2005). Under normal physiological conditions, Acetyl coA that enters TCA cycle combines with oxaloacetate to form citrate which spontaneously loses the inherent energy either in terms of NADH or $FADH_2$. Therefore any impairment caused to any member will influence the activities of other enzymes in the cascade. Accordingly, reduced activities of Citrate synthase, Succinate dehydrogenase (SDH) and malate dehydrogenase observed in testis mitochondria of diabetic rats suggests a downward shift in the efficiency of TCA cycle and electron transfer reactions. Further, the potential of these enzymes to generate ROS cannot be overruled (Zhang et al., 1998).

NADH dehydrogenase, a flavin linked dehydrogenase (complex I) of electron transport chain, harvests electrons from NADH and transfers to coenzyme Q. In the present study, a moderate increase in the activity of NADH- cytochrome C reductase (Complex I –III, coupled) was observed in testis mitochondria. The increase in the activity could be considered as an adaptive response to harvest electrons from available reducing equivalents for synthesis of ATP. In contrast, the activity of Succinate-Cytochrome C reductase (Complex II- III, coupled) was significantly diminished, which is consistent with the functional inactivation of SDH during the progressive phase of diabetes. Although speculative, this differential activity suggests early changes in the expression of mitochondrial proteins prior to the development of dysfunctions. A decline in the mitochondrial ATP production was also evident in terms of reduction in the activities of Mg^{2+}- ATPase during the progressive phase.

Mitochondria are normally associated with generation of ATP through oxidative phosphorylation. However these organelles also participate in a wide variety of essential cellular functions such as homeostasis of calcium and iron, steroid biosynthesis etc., by releasing several proteins that incite programmed cell death, mitochondria act as the ‘executioners’ in apoptosis. Mitochondria also act as high-capacity Ca^{2+} -sinks by transporting calcium from cytosol for the regulation of key enzymes (dehydrogenases) of the TCA cycle, and they also act as temporary stores of this ion (Nicholls and Budd, 2000; Nicholls, 2005; Norenberg and Ramarao, 2007). Calcium from the cytosol is normally transported into mitochondria by an electrogenic uniporter while its efflux from mitochondria is mediated by the Na^{+}/Ca^{2+} exchanger (Gunter and Gunter, 2001). However, mitochondrial dysfunctions and associated bioenergetic failure can lead to abnormal cellular ion homeostasis, as a result of which cells undergo swelling and cellular disruptions, eventually leading to necrotic death (Nieminen, 2003). The outermembrane of mitochondria is permeable to small solutes and ions, while the inner membrane is virtually impermeable and forms a barrier between the cytosol and mitochondrial matrix. Electrons are transferred from reduced nucleotides to various intermediates of the electron transport chain during which protons

are pumped across the inner membrane from the matrix into the inter membrane space. This proton transport creates a transmembrane potential which provides the motive force required for ATP synthesis, as well as for facilitating the selective entry of ions such as Ca^{2+}. Under conditions of increased Ca^{2+} loading especially when accompanied by oxidative stress and a fall in adenine nucleotides, mitochondria undergo a phenomenon referred to as the permeability transition (mPT). The mPT is traditionally defined as a phenomenon associated with the opening of a proteinaceous permeability transition located in the inner mitochondrial membrane allowing solutes with molecular masses of up to 1500 Da to enter or exit the mitochondrial matrix. This opening results in osmotic swelling of the mitochondrial matrix, dissipation of membrane potential, cessation of the ATP synthesis, and the release of cytochrome c and other apoptogenic factors (Zoratti and Szabo, 1995). In the present study, testis mitochondria of PP rats showed elevated Ca^{2+} levels, exogenous Ca^{2+} induced opening of membrane permeability transition and decrease in membrane potential clearly indicating the vulnerability of testis mitochondria to oxidative dysfunctions during progression of diabetes.

Cytochrome P450 and cytochrome P-450 reductase are components of the monooxygenase enzyme system. Being localized in the endoplasmic reticulum membranes of the cells it plays an important role in the biotransformation of steroids, fatty acids, drugs, carcinogens and other xenobiotics (Silig and Cetinkaya, 1998). In the present study, the cytoP450 reductase activity in the diabetic group was significantly increased during the progressive phase, implicating detoxification of STZ induced metabolites. However, the activity of Ethoxy-Resorufin-O-Dethylase was reduced suggesting disturbances in microsomal membrane integrity and functional inactivation of the enzyme.

Caspase-3, (also known as CPP-32, Yama or Apopain), is an intracellular cysteine protease that exists as a proenzyme, and gets activated during the cascade of events associated with apoptosis. Caspase-3 cleaves a variety of cellular molecules that contain the amino acid motif DEVD such as poly ADP-ribose polymerase (PARP), the 70 kD protein of the U1-

ribonucleoprotein and a subunit of the DNA dependent protein kinase. The presence of caspase-3 in cells of different lineages suggests that caspase-3 is a key enzyme required for the execution of apoptosis (Fernandes-Alnemri et al., 1994). Caspases are aspartate-directed cysteine proteases that cleave a diverse group of intracellular substrates to contribute to various manifestations of apoptosis. These proteases are synthesized as inactive precursors and are activated as a consequence of signaling induced by a wide range of physiological and pathological stimuli (Scott et al., 2008). In the present study, diabetic testis during progressive phase showed elevated activities of caspase-3 implicating upregulation of apoptotic death events following diabetes. The increased activities best explains the increased incidence of DNA fragmentation measured as increased frequency of comets and testicular atrophy observed in the current study.

The importance of mitochondria in apoptosis has been suggested by studies in which spontaneous bcl-2 inhibition caused by nuclear condensation and DNA fragmentation using a cell free system, and the inhibitory effects of bcl-2 were found to be dependent on the presence of mitochondria. Subsequently, studies in other cell-free systems showed that activation of caspase by the addition of dATP depended on the cytochrome c release from the mitochondria. The mitochondria sequester a potent cocktail of proapoptotic proteins. Most prominent among these is cytochrome c, one of the factors required for activation of caspase 9 in the cytosol. During apoptosis, cytochrome c is released from mitochondria and forms an apoptosome with Apaf-1 and procaspase 9. This results in the activation of caspase-9 which in turn, activates other downstream caspases, leading to cell death. In the present study, chronic glucose exposure to significant apoptosis in the rat testes, and signs of STZ-induced apoptosis were evident as early as 7 days of treatment. However, the cellular and molecular mechanisms by which STZ induces testicular damage need further investigation. The present study demonstrated the release of cytochrome c from the mitochondria to cytosol, and these results also provide a direct link between the mitochondria and diabetes-induced apoptosis in the testis.

Caspase 8 is the key initiator caspase in the death-receptor pathway. On ligand binding, death receptors (such as Fas and TNF receptor) aggregate and recruit procaspase 8 by way of the adapter protein. This results in activation of caspase 8 and the apoptosis-signalling pathway (Andreyev et al., 1998). The proapoptic signals regulate cytochrome c release from mitochondria and further activate caspase 9 through interaction with Apaf-1. Both death receptor and mitochondria pathways converge at the level of caspase 3 activation. To support the role of cytochrome c and its subsequent activation of the caspase cascade in diabetes induced apoptosis in testes, we further determined the hyperglycemia induced activation of caspase 3. Our findings clearly showed marked increase in the activity of caspase 3 in testes during the progressive phase of diabetes. Taken together, these findings suggest that diabetes-induced apoptosis in the testes may be mediated by the mitochondrial pathway and release of cytochrome c and subsequent activation of caspase 3 plays a key role.

Flow cytometric analysis of testis

The term puberty in mammals, including man, may be defined as the period of time characterized by a cascade of morphological, physiological, and behavioural sequalae of increased gonadal activity (Plant, 1988). This major developmental phase usually occurs in normal individuals within a specified time window. However, under conditions of hormonal imbalance (Gonadotropin deficiency, growth hormone deficiency, androgen deficiency) and in certain pathologies (eg., chronic renal failure) a constitutional delay in the development occurs that ultimately affects fertility. However, the neural, anatomical, physiological, or biochemical mechanism/s that correlate the cause of the delay has not been comprehensively studied (Kulin, 1993; Krishnamurthy et al., 2001). Owing to active metabolic changes in developing testis and higher levels of circulating growth hormone, the age range from 28-42 days post natal has been conservatively classified as adolescence (Spear, 2007). However, a clear definition of the endocrine or other mechanism during progression to puberty in these animals remains inadequate.

Mammalian spermatogenesis is a finely tuned complex process involving intimate interactions among cells in the two compartments of the testis. In this highly organized event, the development of an undifferentiated diploid germ cell into a fully differentiated and mature spermatozoon is orchestrated in a time frame unique for each species. The rat testis has 14 stages in the development cycle of the seminiferous epithelium during which germ cells proliferate and transform into different cell types each having different DNA content. DNA flow cytometry is employed as a tool to analyze stage-specific changes that occur in absolute cell numbers in testes. Quantification of numbers of different cell types in specific stages of the seminiferous epithelium is often employed to characterize testicular pathologies. The seminiferous tubules contain three distinct cell populations, the tetraploid -spermatogonial G2 cells and primary spermatocytes, diploid - secondary spermatocytes and spermatogonial G1 cells and haploid- spermatids and spermatozoa are 1C. As spermatogenesis proceeds, there will be an increase in the 1C cell population which finally becomes an active sperm ready for fertilization (Gangolli and Philips, 1998).

While, diabetes associated testicular complications leading to male infertility are well known, there are no comprehensive reports which explain the exact cause for the testicular degeneration/ pathophysiological changes. Further, no study has been attempted to identify the nature of testicular cell type affected and its subsequent consequences leading to infertility. Accordingly, in the present study, the nature of cell types affected under conditions of diabetes in PP rat testis was monitored from day 21 to day 56 post natal which represents onset of puberty and first complete spermatogenic cycle using flow cytometry.

During spermatogenesis, germ cells proliferate and transform into various cells with different DNA content. In this study, flow cytometric analysis of PP testis showed three major peaks which represent haploid, diploid and tetraploid cell population distinguished clearly by comparing the fluorescent properties of Propidium Iodide-stained testicular cell populations. Further, the haploid (1n) region was split into two peaks because of differential uptake of stain by elongated and round/elongating spermatids. This may reflect

progressive condensation of chromatin structure. The nuclear packaging is known to reduce the number of DNA sites available for fluorochrome binding, thus resulting in apparently sub-haploid DNA content. In the present study, control PP rat testis showed a gradual increase in 1C population (immature haploid cells) from day 21 to day 35 beyond which the immature cell population gradually decreased with subsequent increase in matured haploid cell population. The appearance of round spermatids in 7 week old rats suggests the beginning of the first wave of spermiogenesis, germ cell maturation and finally onset of puberty.

Further, the relative proportions of diploid cells (primary spermatocytes, Sertoli/Leydig cells) decreased throughout the sampling times (upto 8wk). Thus, a decrease in diploid cell population and an accompanying increase in haploid cell number may represent the meiosis of secondary spermatocytes. The appearance of tetraploid (4n) cells followed much the same pattern of the diploid cells. However, there was a marginal increase in the tetraploid cell population at 8wk which could be due to accumulation of primary or secondary spermatocytes before the onset of the first or secondary meiotic division. The proliferation of Sertoli cells supporting the development of germ cells may stop at the age of 13 and 84 days.

Although decreased fertility among diabetic men has been proven beyond doubt, the exact reason is still not completely understood. However, DNA flow cytometry performed on testicular tissue of diabetic PP rats primarily showed complete loss of immature/matured haploid cell population which could be explained on the basis of severe depletion in testosterone levels. It is well established that testosterone plays a vital role in differentiation and function of various testicular cell types including germ cells. Further, diabetic testis showed increased diploid cell population throughout the sampling times (evident from 4wk and beyond) suggesting the possible cell cycle arrest at spermatogonia/pre-leptotene meiotic stage. In the present study, collagenase was not used to liberate testicular cells; therefore, it is assumed that 2n and 4n cell populations contains primarily seminiferous epithelial cells which are more easily released by mechanical disruption than are somatic interstitial cells. Further, testicular cells in S-Phase (DNA

synthesizing) were consistently decreased in control testis, while their population was higher till 6wk which thereafter subsided gradually.

Our findings clearly demonstrate the differential susceptibility of testis subcellular organelles to oxidative stress under experimentally induced diabetes. Robust oxidative dysfunctions in cytosol, mitochondria and microsomes clearly suggest both physiological and functional perturbations which occur *in vivo* in the developing testis. It is hypothesized that the progressive mitochondrial dysfunctions occurring during the vital developmental stage of testis are responsible for the impaired steroidogenesis and spermatogenesis and cause infertility.

6.0 SUMMARY

1. Administration of STZ (i.p.) at a dosage of 90mg/kg bw caused significant, consistent hyperglycemia at all the sampling times both during acute and progressive phase (day 1 and beyond)

2. Diabetes-induced oxidative alterations were discernable in testis cytosol and microsomes as early as 24h which remained consistently elevated at all sampling times. Further, mitochondrial dysfunctions were evident only during progressive phase suggesting differential susceptibility subcellular organelles in testis to oxidative insult.

3. Perturbations in GSH and total/non-protein thiol content in testis of diabetic rats suggest altered redox status. In addition, consistently elevated ascorbic acid levels suggest its participation in the recycling of GSH and Vitamin E. In contrast, reduction in testicular vitamin E levels implicates enhanced quenching of free radicals formed during diabetes.

4. A marginal to moderate increase in the activity of SOD in both testis cytosol/mitochondria was accompanied by reduced activity levels of CAT and GPX implicating generation of free radicals (O_2^{2-} and H_2O_2) which mediate peroxidative damage.

5. Moderate to marked increase in the activity of GST in the testis subcellular organelles (cytosol/mitochondria/ microsomes) suggests the formation of toxic aldehydes and its subsequent elimination by GSH conjugation.

6. Significant increase in protein carbonyl levels in testis cytosol/mitochondria of diabetic rats is suggestive of increased protein oxidation in vivo.

7. Marked elevation in the activities of G6PDH/ glutathione reductase during the acute phase of diabetes implicates their participation in reductive biosynthesis of biomolecules by recycling GSH and NADPH. In contrast, lowered activity levels during progressive phase reflect complete exhaustion of the substrates during chronic diabetic conditions.

8. Consistently decreased activity of sorbitol dehydrogenase at all the sampling times suggests altered testicular physiology probably by accumulation of sorbitol in testis, which enhances further free radical generation. An increase in the activity of LDH-X during progressive phase implicates severe damage to cellular mitochondria and in the process of spermatogenesis.

9. Altered activity levels of mitochondrial TCA cycle enzymes suggests lowered efficiency and possible shunting of glycolytic intermediates to other minor pathways. Further, enhanced activity levels of ETC enzymes strongly correlates with the increased demand for ATP, while reduced activity of Mg^{2+}ATPase implicates dysfunction in oxidative phosphorylation due to altered membrane structure and function.

10. Diabetes induced mitochondrial impairments were further demonstrated by opening of membrane transition pore, collapse in membrane potential and release of cytochrome C into cytosol which activates Caspase 3 mediated cell death events.

11. Perturbations in the activities of cytochrome P450 and ethoxy-resorufin-O-dealkylase suggests ongoing oxidative stress in testis microsomes.

12. Higher incidence of testicular atrophy observed during diabetes could be attributed mainly to irreversible depletion in various testicular cell population viz., germinal cells (both immature/mature spermatids) and tetraploid cells (Sertoli/Leydig cells). These changes were accompanied by severe DNA damage as evidenced by fragmentation of DNA measured as comets.

1.0 INTRODUCTION

It is becoming increasingly clear that oxidative stress, mediated mainly by hyperglycemia-induced generation of free radicals is the primary cause for the development/ progression of diabetes and its related complications (Lipinski, 2001; Rolo and Palmeira, 2006; Oborosova, 2008). The prevention and treatment of diabetes, as well as its various complications such as neuropathy, retinopathy and nephropathy is considered to be of utmost clinical importance. A growing body of evidence suggests the only promising approach for the treatment is to control blood glucose levels. Further, oxidative stress developed secondarily to hyperglycemia (Baynes, 1991) accelerates oxidative damage to biomolecules such as lipids, proteins and DNA, resulting in cellular and tissue injury (Kakimoto et al., 2002). Therefore, attenuation of oxidative stress may hinder/delay the pathological events associated with development of diabetic complications (Mohara et al., 2007).

The potential ameliorative effects of classical antioxidants such as vitamin E (Oliveira et al., 2004), vitamin C (Mekinova et al., 1995), lipoic acid (Doreswamy and Muralidhara, unpublished data), Taurine (Doreswamy and Muralidhara, 2009c) and aminoguanidine (El-Khatib et al., 2001) have been shown to prevent hyperglycemica-induced insulin resistance *in vivo*. Further, taking into consideration the relative insufficiency of insulin/ lowered response of cells to insulin many different drugs have been developed that either enhance glucose uptake by peripheral cells (eg., biguanides), inhibit glucose absorption (eg., glucosidase inhibitors such as acarbose, miglitol and voglibose) or induce the release insulin. Although, several therapies are in use for the treatment of diabetes, the strategies employed have certain limitations in terms of response to antioxidants in the prevention of complications (Dey et al., 2002; Mohara et al., 2007).

Male infertility associated with reproductive dysfunctions viz., retrograde ejaculation, premature ejaculation, decreased libido, delayed sexual maturation and compromised semen quality etc., are commonly observed during chronic diabetes (Musicki and Burnett, 2007). It is well established that high circulating levels of blood glucose and severe depletion

in testosterone levels are the prime causative factors for the increased testicular dysfunctions. Testosterone deficiency common among men with diabetes is also influenced by insulin resistance, obesity and cardiovascular disease which are considered to be the risk factors for the development of diabetes. Based on these findings, various therapeutic strategies have been employed to modulate glycemic response and improve testosterone levels. Gonadal steroids/ mimetics and compounds that modulate androgen biosynthesis have been employed to treat testicular dysfunctions. Androgens, particularly testosterone is long known to influence normal physiology/function of male reproductive organs and is often employed to improve impaired sexual functions during pathological conditions including diabetes (Rice et al., 2008; Stanworth and Jones, 2009). However, studies pertaining to testosterone mediated amelioration of diabetes induced testicular dysfunctions have not been studied extensively either in adults or in prepubertal rodents.

D-Asp, an endogenous amino acid present in various vegetables /marine animals has been shown to facilitate the synthesis of testosterone by upregulating the expression of Steroid Acute Regulatory protein (StAR). Besides its wide occurrence, it is also demonstrated to be synthesized/ secreted by seminiferous tubules of testis whose precise function in testis is beginning to be understood (Fuchs et al., 2005). In adult testis, D-Asp has been shown to be taken up by Leydig cells (D'Aniello et al., 1998) *via* NMDA receptors (Storto et al., 2001) and interact with DNA or nuclear protein(s) and that eventually express StAR protein facilitating testosterone biosynthesis (Nagata et al. 1999). Further, the potential of exogenous D-Asp to accumulate in neuro-endocrine organs and induce the synthesis/ release of hypothalamo-pituitary hormones has been reported (D'Aniello et al. 1996, 2000; Fukushima et al., 1998). Since exogenous administration of D-Asp to experimental animals induces elevated testosterone levels, it is quite likely that it may ameliorate testosterone deficiencies observed during diabetes. However, studies pertaining to the potential of D-Asp to improve testosterone deficiencies and its interactive effects on testis under conditions of diabetes in prepubertal rats have not been extensively studied.

There has been a growing interest in investigating phytochemicals for their antidiabetic and protective properties against diabetes induced oxidative dysfunctions due to the side effects of conventional therapy (Modak et al., 2007). *Withania somnifera*, a traditional herb has been used in several ayurvedic preparations for its antioxidant and hypoglycemic effects. *Withania somnifera* (Family: Solanaceae) commonly known as 'Indian ginseng' has been used in folk medicine as an aphrodisiac and a geriatric tonic. Further it is reported to have several pharmacological effects including antihelminthic, narcotic, radiosensitizer, antistressor, adaptogenic, hepatoprotective, cardioprotective, anti-inflammatory, antibacterial and antifungal properties (Dhuley, 1998, 2000; Archana and Namasivayam, 1999). Earlier workers have demonstrated that aqueous extract of *Withania somnifera* to regulate pituitary gonadotrophins coupled with enhancement in epididymal sperm pattern in adult male rats and folliculogenesis in immature female rats (Al-Qarawi et al., 2000). However, the potential hypoglycemic and ameliorative effect on diabetes induced testicular dysfunctions in prepubertal rats has not been comprehensively studied.

Accordingly, the present study primarily aims to understand the attenuating influence of phytochemicals/ bioactives that enhance testosterone levels in diabetic prepubertal rats. The present chapter has been presented in three sections, viz., A, B and C. Section A comprises of the results pertaining to the abrogation of diabetes induced testicular impairments by testosterone. *Section B* describes the interactive effects of D-Aspartic acid, an endogenous amino acid known to induce synthesis/secretion of testosterone on diabetes induced testicular oxidative stress. *Section C* describes the effect of oral administration of aqueous extract of *Withania somnifera* on diabetes induced testicular oxidative stress.

2.0 OBJECTIVE

The focus of the present investigation is to assess the modulatory potency of D-Aspartic acid, testosterone and bioactive principles of *Withania somnifera*, on diabetes induced testicular dysfunctions in prepubertal rats.

SECTION – A

INTERACTIVE EFFECTS OF D-ASPARTIC ACID (D-Asp)

3.0 EXPERIMENTAL DESIGN

Effect of D-Aspartic acid *per se* on PP rat testis

Prior to the assessment of interactive effects of D-Asp in diabetic model, its potential to induce oxidative stress in testis of PP rats was studied both *in vitro* and *in vivo*. The *in vitro* oxidative response was investigated employing testicular preparations viz., homogenates, mitochondria, cell suspension and explants. The *in vivo* effect was studied employing a short term exposure approach.

Susceptibility of PP testis to D-Aspartic acid exposure *in vitro*

Experimental protocol

Fresh testes of PP rats (4wk old) were employed for *in vitro* investigations. A 10% homogenate was prepared using KRBS, centrifuged and the supernatant served as testicular homogenates. Testis mitochondria was prepared by differential centrifugation as described earlier (Chapter 1). For the preparation of testicular explants, 50mg of seminiferous tubules were dispensed into culture wells, teased and cut into 3-5mm fragments. The explants thus prepared were suspended in a known volume of KRBS and used. Testicular cell suspensions were prepared by digesting seminiferous tubules with collagenase followed by trypsin at 37ºC for 15min. Finally, a known number of cells were used for incubations.

D-Aspartic acid exposure – Concentration response

Testicular homogenate/explants/cells were exposed to D-Asp at various concentrations (0.25, 0.5 and 1mM) for 30min at 37˚C. Terminally, the induction of oxidative response was determined by quantifying ROS, MDA and hydroperoxide levels.

Effect of enzyme inhibitors on D-Asp induced oxidative response

In order to examine the modulatory effect of specific enzyme inhibitors on D-Asp induced oxidative stress, testicular homogenates were pre-incubated with either mercaptosuccinate (MS-50μM) or 3-Amino triazole (AT-50mM) and subsequently exposed to D-Asp for 30min at 37˚ C. The induction of peroxidation was quantified as MDA levels.

Effect of Ferrous iron on D-Asp induced oxidative response

The effect of iron (as ferrous sulphate, 5μM) on D-Asp induced oxidative stress was studied in PP testis. Testis homogenates were pre-incubated with D-Asp at various concentrations (0.25, 0.5 and 1mM) for 30 min at 37˚C and subsequently exposed to ferrous sulphate (5μM) for a period of 30min. The induction of peroxidation was quantified by measuring MDA levels.

Interactive effect of L-Arginine on D-Asp induced oxidative response

To assess the interactive effect of L-Arginine on D-Asp induced oxidative response, testis cell suspension/homogenates were pre-incubated with L-Arginine (0.5, 1.0mM) for 30 min and then exposed to various concentration of D-Asp for a period of 30 min. The degree of lipid peroxidation was quantified as MDA levels.

Potential of D-Aspartic acid to induce testicular oxidative stress *in vivo*

D-Aspartic acid was dissolved in 0.1% sodium bicarbonate and administered to PP rats (n=6) at two dosage viz., 100 and 500mg/kg bw /d for 7days. The animals were maintained on a commercial pellet diet throughout the experimental period. Food intake and body weights were monitored daily. Terminally, the animals were sacrificed under light ether anesthesia. Testis

was excised and used for biochemical analysis. The markers of testicular oxidative damage were determined in both cytosol and mitochondria.

Modulatory effect of D-Aspartic acid on diabetes associated testicular oxidative stress

PP male rats were assigned into four groups, Group I, Control; Group II, STZ *per se*; Group III, D-Asp *per se* ; Group IV, STZ + D-Asp. Rats of Group II and IV were rendered diabetic by an acute dose of STZ (90mg/kg b.w, i.p). To prevent hypoglycemic shock, STZ administered rats were given glucose (5% w/v) for 48hours. To group III and IV, D-Asp dissolved in alkaline saline (100mg/kg b.w) was administered (i.p) each day between 9.00- 9.30 AM for 7days. All the animals were maintained on commercial pellet diet throughout the experimental period. Food intake, body weights were recorded every alternate day. Terminally, modulatory effects of D-Asp were measured in terms of status of oxidative stress markers, redox status, alterations in antioxidant/functional enzyme activities in testis subcellular organelles viz., cytosol and mitochondria.

SECTION – B

MODULATORY EFFECTS OF TESTOSTERONE AGAINST TESTICULAR OXIDATIVE STRESS IN DIABETIC PP RATS

Testosterone Dosage selection

Testosterone propionate (TP) was administered to PP rats at the dosage of 5mg/kg bw/d (i.p) for 5 and 10 days. The dosage selected was based on a preliminary study and also on published literature (Fitts et al., 2004).

Experimental protocol

PP male rats were assigned into four groups, Group I, Control; Group II, STZ *per se*; Group III, STZ + TP. Rats of Group II and III were rendered diabetic by an acute dose of streptozotocin (90mg/kg b.w, i.p). To prevent hypoglycemic shock, STZ administered rats were given glucose (5% w/v) for

48 hours. For group III rats TP (5mg/kg b.w) was administered (i.p) each day (between 9.00- 9.30 AM) for 5 and 10 days. All the animals were maintained on commercial pellet diet throughout the experimental period. Food intake and body weights were recorded every alternate day. Animals of all groups were sacrificed as described earlier. The following biochemical investigations were made in fresh testis samples, status of oxidative stress markers, redox status, activities of antioxidant enzymes/functional enzymes in testis cytosol and mitochondria.

SECTION – C

PROTECTIVE EFFECTS OF *WITHANIA SOMNIFERA* AGAINST TESTICULAR OXIDATIVE STRESS IN DIABETIC PP RATS

Preparation of *Withania somnifera* extract

Withania somnifera root powder (WS) was procured from M/s Natural Remedies, Bengaluru, and administered orally (in saline) to PP rats at the dosage of 500mg/kg bw/d for 15 days. The selection of dosage was made based on our preliminary studies and also on background literature (Abdel-Magied, 2001; Hemalatha et al., 2004).

Experimental protocol

Prepubertal male rats were rendered assigned into four groups, Group I, Control; Group II, STZ *per se*; Group III, STZ + WS. Rats of Group II and III were rendered diabetic by a single acute dose of streptozotocin (90mg/kg b.w, i.p). To prevent hypoglycemic shock, STZ administered rats were given glucose (5% w/v) for 48hours. To group III, WS (500 mg/kg b.w) was administered (orally) each day (between 9.00- 9.30 AM) for 15 days. All the animals were maintained on commercial pellet diet throughout the experimental period. Food intake, body weights were recorded every alternate day. Animals of all groups were sacrificed as described earlier. The following biochemical investigations were made in fresh testis samples, status of oxidative stress markers, redox status, activities of antioxidant enzymes/functional enzymes in testis cytosol and mitochondria.

4.0 RESULTS

SECTION – A

INTERACTIVE EFFECTS OF D-ASPARTIC ACID

In vitro Susceptibility of PP rat testis to D-Aspartic acid

D-aspartic acid induced oxidative response measured as generation of ROS, MDA and HP levels in testis homogenates and explants of PP ats is presented in Table 4.1. Higher concentrations of D-Asp (0.5 and 1.0mM) caused a significant oxidative induction in homogenates and explants. Of the three endpoints, the elevation in MDA levels was the most robust compared with less robust (16-33%) increase in HP levels, with no appreciable change in ROS levels.

Although, in testicular cell suspension, D-Asp at lower concentrations (0.05 and 0.1mM) failed to induce any significant increase in oxidative response (data not shown), marked induction in ROS (1- 2.6 folds) and peroxidation (0.6 – 3.6 folds) was evident at concentrations beyond 0.1mM (Table 4.2).

Modulation of D-Asp induced oxidative response by enzyme inhibitors

The effect of enzyme inhibitors on D-Asp induced oxidative response is presented in Fig. 4.1A,B. A dramatic elevation in the peroxidation levels was evident in testis homogenates following exposure to either aminotriazole (AT, 6.5 folds) or mercapto-succinate (MS, 7.4 folds). Interestingly, the peroxidation levels were further enhanced on co-incubation of AT with D-Asp (7,12 and 10 folds) at the three concentrations respectively. Co-incubation of MS with D-Asp resulted in a higher degree of oxidative response, as evidenced (by 8, 16 and 37-folds) dramatic increase in peroxidation at the three concentrations.

Effect of iron exposure on D-Asp induced oxidative response

The effect of iron exposure on D-Asp induced oxidative response is presented in Fig. 4.2. Iron (5μM) *per se* induced significant lipid peroxidation and caused nearly 8.6 fold increase over basal levels. Interestingly, the extent

of peroxidation was further enhanced on co-exposure of D-Asp with iron (5μM) at all concentrations (12 - 20 folds)

Attenuation of D-Asp induced oxidative response by L-Arginine

Effect of L-Arginine (LA) *per se* on the endogenous levels of oxidative markers and attenuation of D-Asp induced oxidative response in testis homogenates of PP rats is presented in Figures 4.3 and 4.4. Incubation of testicular cells with LA (0.5mM) significantly induced the release of Nitric Oxide (92 nmol/12X10 ^{6} cells). However, pre-incubation with N-nitro-L-arginine methyl ester (0.1mM) followed by exposure to LA (0.5mM), completely inhibited NO release and the levels were comparable to control.

LA *per se* significantly decreased the endogenous levels of ROS, lipid peroxidation and hydroperoxide formation. Co-incubation of testis homogenates with LA significantly attenuated the D-Asp induced oxidative induction. At a conc. of 0.5mM, LA markedly decreased (67-78%) the degree of D-Asp induced production of ROS. Interestingly, at the highest conc. of D-Asp (0.1mM) the effect was less pronounced (55%). However, incubation of LA with D-Asp at a lower conc. did not drastically decrease peroxidation, compared to conc. dependent decreases at higher conc. (28-50%). Levels of lipid hydroperoxides were decreased by 40% when incubated at lower concentrations of D-Asp, compared to higher conc., where the response was comparable to that of the control.

Potential of D-Asp to induce testicular oxidative stress in PP rats *in vivo*

Effect of D-Asp on markers of oxidative stress

Data on the effect of D-aspartic acid (D-Asp) administration on the generation of ROS, hydroperoxide and MDA levels in testis cytosol and mitochondria of prepubertal rats is presented in Fig. 4.5. Marked enhancement in the levels of oxidative stress markers was discernible in the testis of D-Asp administered rats. D-Asp caused a robust elevation in ROS generation in mitochondria (74, 85%) as compared to the cytosol (30, 46%). Further, the MDA levels were uniformly enhanced in both cytosol (30, 55%)

and mitochondria (33, 50%). Likewise, the hydroperoxide levels were also significantly increased in cytosol (20, 41%) and mitochondria (28, 40%).

Nitric Oxide release, thiol status and D-Aspartate oxidase activity (D-AspO)

D-Asp induced release of NO and alterations in the activities of D-AspO are illustrated in Fig. 4.6. D-Asp administration significantly increased (21, 31%) the activity of D-AspO, while the nitric oxide synthase activity (NOS) measured as NO levels were enhanced (30%) only in testis cytosol at the lowest dose. Data on the effect of D-Asp administration on glutathione levels in both testis cytosol and mitochondria is presented in Fig. 4.6. In testis of D-Asp treated rats, the GSH levels were markedly enhanced in both cytosol (45, 50%) and mitochondria (14, 30%), while the total thiol levels were unaltered (data not shown)

Activities of antioxidant/ functional enzymes

Data on the specific activities of antioxidant enzymes measured in testis cytosol and mitochondria among different groups are presented in Table 4.3. A dose related increase (58, 75%) in catalase activity was accompanied with marked elevations in GPx levels in cytosol (32, 40%) and mitochondria (25, 37%). Concomitantly, the activity of GST was elevated in cytosol (47, 40%) and mitochondria (10, 29 %), while the activity of SOD was unaltered. D-Asp also caused significant increase in the activities of cytosolic LDH (27, 30%) and 3β-HSD (12,20%), while the activity of MDH was marginally (15%) reduced.

Effect of D-Asp on mitochondrial enzyme activities

Data on the effect of D-Asp on testis mitochondrial enzymes are presented in Table 4.4. The activity of aldehyde dehydrogenase was significantly decreased (34, 20%), compared to activity levels of SDH and MDH. However, the activity of citrate synthase was consistently elevated (15, 43%).

Effect of D-Asp on mitochondrial complex enzymes

D-Asp administration appeared to differentially regulate the activity levels of ETC enzymes. While D-Asp had no significant effect on NADH-Cyt C reductase, the activity of Succinate-Cyt C reductase was significantly elevated (38, 51%) along with a marginal (16%) increase in the activity of Mg^{2+} ATPase at the highest dose (Table 4.4).

Effect of D-Asp on mitochondrial membrane integrity

D-Asp induced alterations in mitochondrial membrane integrity are presented in Fig. 4.7. D-Asp induced alterations in mitochondrial enzyme activities were accompanied by enhanced rate (43, 36%) of MTT reduction, increased susceptibility to membrane damage and leakage as evidenced by opening of the membrane transition pore and loss in membrane potential. This was associated with elevated intracellular Ca^{2+} levels.

Modulatory effects of D-Aspartic acid on testicular oxidative impairments

Growth pattern, organ weights and plasma glucose levels

As anticipated, significant reduction in body weight (40%) and testis weight (48%) were evident among STZ- diabetic rats. D-Asp treatment had no marked effect on either body weight gain or testicular weights in both *per se* and diabetic rats (Table 4.5). Plasma glucose levels were significantly elevated among STZ- diabetic rats (2.4 folds) compared to control and D-Asp control group. Interestingly, administration of D-Asp to diabetic rats markedly reduced the hyperglycemic levels (Fig. 4.8).

Effect D-Asp on ROS generation and lipid peroxidation in testis

Data on the effect of D-Asp administration on the generation of ROS and MDA levels in testis cytosol and mitochondria of both diabetic and non-diabetic rats is presented in Fig. 4.9. In general, administration of D-Asp to non-diabetic rats marginally increased the ROS levels both in cytosol (15%) and mitochondria (23%). Among diabetic rats, the ROS levels were markedly elevated in both cytosol (33%) and mitochondria (68%) of testis. Interestingly, D-Asp administration to diabetic rats marginally decreased the testis cytosolic

ROS levels (15%), while the levels were further elevated in mitochondria (85%).

D-Asp administration significantly enhanced the MDA levels in both cytosol (48%) and mitochondria (20%) in non-diabetic rats., Testis of diabetic rats showed marked increase in MDA levels in both cytosol (242%) and mitochondria (60%). In contrast, D-Asp administration to diabetic rats caused significant diminution in the levels of MDA in cytosol (24%), while the levels were remained unaffected in mitochondria (Fig. 4.9).

Effect of D-Asp on protein carbonyls and glutathione content

D-Asp treatment *per se* had no effect on protein carbonyl levels in testis cytosol and mitochondria of non-diabetic rats. Testis of diabetic rats exhibited higher levels (43%) of protein carbonyls in both cytosol and mitochondria. D-Asp administration to diabetic rats had little or no effect on the levels in both cytosol and mitochondria.

D-Asp administration to non-diabetic rats marginally elevated the glutathione levels in testis cytosol (20%). Testis of diabetic rats showed elevated GSH levels in mitochondria (39%), the levels were unaltered in cytosol. Interestingly, D-Asp administration to diabetic rats significantly elevated the GSH levels both in cytosol and mitochondria (22-25%) (Fig. 4.10).

Activities of antioxidant enzymes

The specific activities of antioxidant enzymes measured in testis cytosol and mitochondria among various groups are presented in Figures 4.11 and 4.12. D-Asp *per se* elevated the activities of SOD (cytosol, 12%; mitochondria, 66%), Catalase (cytosol, 24%), GPx (cytosol, 40%; mitochondria, 71%) and GST (cytosol, 17%) in non-diabetic rats. However D-Asp treatment to diabetic rats, further elevated the activities of SOD (cytosol, 16%; mitochondria, 151%), GPx (cytosol, 18%; mitochondria, 36%) and GST (cytosol, 41%; mitochondria, 73%). Further, D-Asp administration restored the activity levels of catalase to near to normalcy in diabetic testis.

*Activity of 3β-hydroxy steroid dehydrogenase (*3β-HSD)

The effect of D-Asp administration on 3β-HSD is presented in the Fig. 4.13. D-Asp *per se* elevated the activity levels marginally (13%), while significant reduction was evident in diabetic testis (23%). Interestingly, D-Asp treatment to diabetic rats restored the activity levels to normalcy.

SECTION-B

MODULATORY EFFECTS OF TESTOSTERONE AGAINST DIABETES INDUCED TESTICULAR OXIDATIVE STRESS IN PP- RATS

Growth pattern, testis weight and plasma glucose levels

The effect of testosterone administration on growth pattern measured as gain in body weight, testis weight and plasma glucose levels are presented in the Table 4.6. A marginal to marked decrease in body weight (15-32%), testis weight (47–53%) was evident among diabetic prepubertal rats on day 5 and 10 respectively. TP administration to diabetic rats had no effect on gain in body weight, while testis weights were on par with control. Plasma glucose levels in STZ rats were significantly elevated (3 folds) compared to non-diabetic controls at both sampling times. Interestingly, TP administration completely restored the elevated glucose levels to normalcy among diabetic rats.

Effect on oxidative stress markers

Data on the effect of TP administration on ROS and MDA levels in testis cytosol and mitochondria of prepubertal rats is presented in Fig. 4.15. Diabetes induced testicular oxidative stress was evident in both testis cytosol and mitochondria. Testis cytosol of diabetic rats showed a consistently elevated levels of ROS and MDA levels on both the sampling times (5d, 76%, 75%; 10d, 35%, 328% increase over control). However, no measurable changes were evident in mitochondria at day 5, while significant increase in ROS and MDA levels were apparent on day 10 (ROS, 34%; MDA, 43%).

Interestingly, TP administration to diabetic rats significantly offset STZ-induced elevations in ROS and MDA levels in both cytosol and mitochondria.

Testis of diabetic rats showed elevated protein carbonyls in both cytosol (5d, 46%; 10d, 37%) and mitochondria (5d, 81%, 10d, 72%). Interestingly, TP administration to diabetic rats brought the levels near to normalcy.

Effect on testis thiol status

Data on the effect of TP administration on thiol status is presented in Fig. 4.16. Diabetic testis showed marginal to moderate decrease in total thiol content, which was (5d, 29%; 10d, 17%) in both cytosol and mitochondria. In contrast, the levels of non-protein thiol were consistently elevated in cytosol (5d, 50%; 10d, 36%), whereas in mitochondria the levels were elevated only on day 5 (5d, 33%). The GSH levels were less affected in cytosol, in contrast to elevated levels in testis mitochondria (5d, 22%; 10d, 36%). However, TP failed to bring any appreciable change in thiol status in diabetic rats.

Activities of antioxidant enzymes

The effect of TP administration on selected antioxidant enzyme activities is presented in the Fig. 4.17. Testis of diabetic rats, showed elevated activity levels of SOD in both cytosol (5d, 35%; 10d, 69%), and mitochondria (5d, 50%; 10d, 59%) which were restored to normalcy by TP treatment at both the sampling points. Similarly, TP administration to diabetic rats significantly offset the changes in the activity levels of Catalase, GST, GPx and GR in both cytosol and mitochondria (Fig. 4.18).

Effect on activities of functional enzymes

Data on the effect of TP administration on testicular functional enzyme activities are presented in Fig. 4.19. Among diabetic rats, the activities of LDH, G6PDH and 3β-HSD were relatively less affected on day 5, while the levels were significantly elevated at 10d (LDH, 86%; G6PDH, 31%) except for the activity of 3β-HSD which was significantly reduced (25%). Interestingly, the activities were normalized upon TP administration.

Effect of TP on mitochondrial complex enzymes

A marginal to marked increase in the activities of NADH –cytochrome c reductase and succinate –cytochrome c reductase were evident among diabetic rats (18-23%) at both sampling times. Further, TP administration restored the activity levels near to normalcy (Fig. 4.20).

SECTION - C

PROTECTIVE EFFECTS OF *WITHANIA SOMNIFERA* AGAINST DIABETES-INDUCED TESTICULAR OXIDATIVE STRESS

Growth pattern, organ weights and plasma glucose levels

Terminally, significant reduction in body weight (33%) and testis weight (48%) were evident among diabetic rats (Table 4.7). However, WS treatment appreciably increased the body weight (17%) and testicular weight (56%) among diabetic rats. While, plasma glucose levels were significantly elevated in diabetic rats (3.5 folds). Administration of WS to diabetic rats markedly reduced the hyperglycemic levels to near normalcy (Fig. 4.21).

Effect on oxidative stress markers

In general, ROS levels were markedly elevated in cytosol (120%) and mitochondria (66%) among diabetic rats (Fig. 4.22). Interestingly, WS administration to diabetic rats significantly lowered (25%) the levels in both the compartments. Like wise the MDA levels which were markedly enhanced in cytosol and mitochondria among diabetic rats were significantly attenuated following WS administration (cytosol, 38%; mitochondria, 24%).

Effect on testis thiol status

Diabetic testis showed significant decrease in total thiol content, which was apparent in both cytosol (43%) and mitochondrial compartments (16%) (Fig. 4.23). In addition, non-protein thiol levels were elevated in cytosol (36%) in contrast to significantly lowered levels in testis mitochondria. However, no appreciable change was evident in cytosolic GSH levels in diabetic rats, while a marginal increase (22%) was evident in testis mitochondria.

WS supplementation to diabetic rats elevated the thiols status in both the compartments.

Activities of antioxidant enzymes in testis

The specific activities of antioxidant enzymes measured in testis cytosol and mitochondria among various groups are presented in the figures 4.24 and 4.25. In diabetic rats, the activity levels of SOD were unaffected in cytosol, while a marked elevation in the activity was evident in mitochondrial compartment (53%). Further, diabetic testis showed reduced activity levels of Catalase, GPx in both the compartments. However, the activities of GST and GR were consistently elevated in both cytosol and mitochondria of diabetic testis. WS supplementation significantly offset the perturbation in enzymic antioxidants and restored the activities to normalcy.

Activities of functional enzymes

Data on the effect of WS supplementation on testicular functional enzyme activities are presented in the Fig. 4.26. Among diabetic rats, the activities of LDH and G6PDH were significantly (26%) elevated except for the activity of 3β-HSD which was diminished (25%). Interestingly, WS administration to diabetic rats offered significant protection against these perturbations and restored the activities to near normalcy.

Table 4.1

D-aspartic acid induced oxidative response in testis homogenates and explants of prepubertal (PP) rats *in vitro*

	D-Aspartic acid (mM)			
	0	0.25	0.5	1.0
Homogenates				
ROS [1]	14.4 ± 1.6	15.4 ± 2.0	17.8 ± 1.1[a]	34.9 ± 3.0[c]
LPO [2]	4.6 ± 1.0	8.8 ± 1.2[c]	10.4 ± 1.8[c]	19.9 ± 1.4[c]
HP [3]	21.3 ± 1.8	24.8 ± 3.0	35.0 ± 2.2[c]	38.3 ± 1.5[c]
Explants				
ROS [1]	1.8 ± 0.05	1.9 ± 0.1	1.9 ± 0.02	1.9 ± 0.4
LPO [2]	0.51 ± 0.08	0.79 ± 0.05 [b]	0.9 ± 0.15[c]	1.2 ± 0.1[c]
HP [3]	0.36 ± 0.02	0.40 ± 0.1	0.42 ± 0.1	0.48 ± 0.2[b]

Values are mean ± SE of three separate determinations. Data analysed by student 't' test.
$p<0.05$; [b,c,d] $p<0.001$ vs control.

[1] ROS - pmol DCF formed/min/ $12X10^6$ cells ; [2] LPO -nmol MDA formed/mg protein ;

[3] Hydroperoxides - μmol H_2O_2 formed/mg protein

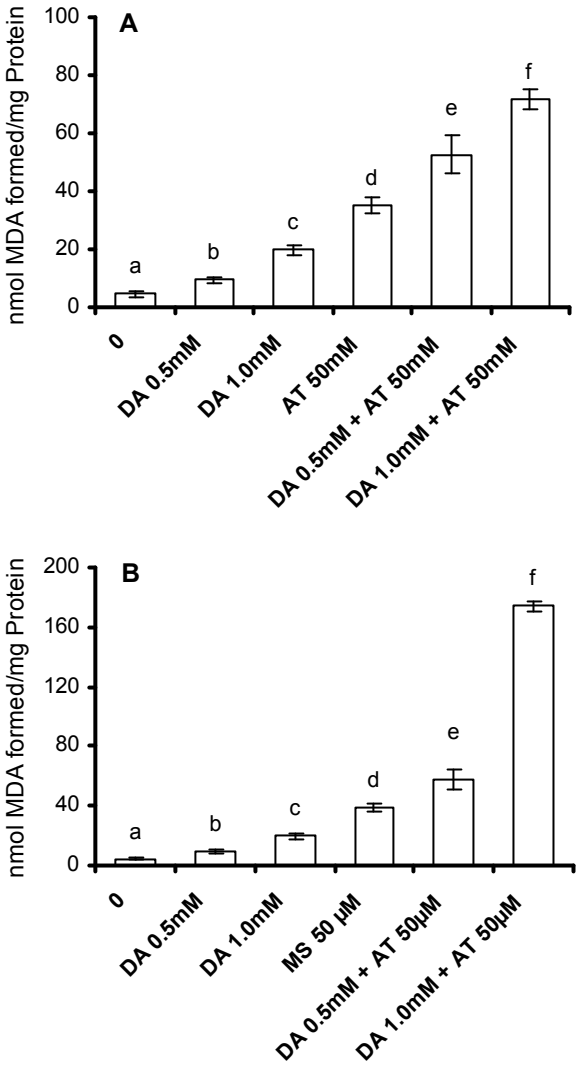
Table 4.2

Concentration related increase in oxidative stress markers in testis cell suspensions of prepubertal rats exposed *in vitro* to D-aspartic acid

D-Asp (mM)	ROS (pmol DCF formed/ $12X10^6$ cells)	LPO (n mol MDA formed/ $12X10^6$ cells)
0	0.38 ± 0.02	24.1 ± 1.8
0.25	0.78 ± 0.05 [b]	38.6 ± 2.5 [b]
0.5	1.12 ± 0.05 [c]	72.3 ± 5.0 [c]
1.0	1.37 ± 0.10 [c]	110.8 ± 8.3 [c]

Fig. 4.1

Effect of enzyme inhibitors, amino triazole (A) and mercaptosucinate (B) on D-aspartic acid induced lipid peroxidation in testis homogenates of PP rats



DA, D-Aspartic acid; AT, Amino triazole; MS, Mercaptosuccinic acid

Values are mean ± SE of three separate determinations.

Data analysed by student 't' test. $p<0.05$; ^{b,c,d} $p<0.001$ vs control.

Fig. 4.2

Effect of ferrous iron (5µM) on D-aspartic acid induced lipid peroxidation response in testis homogenates of PP rats

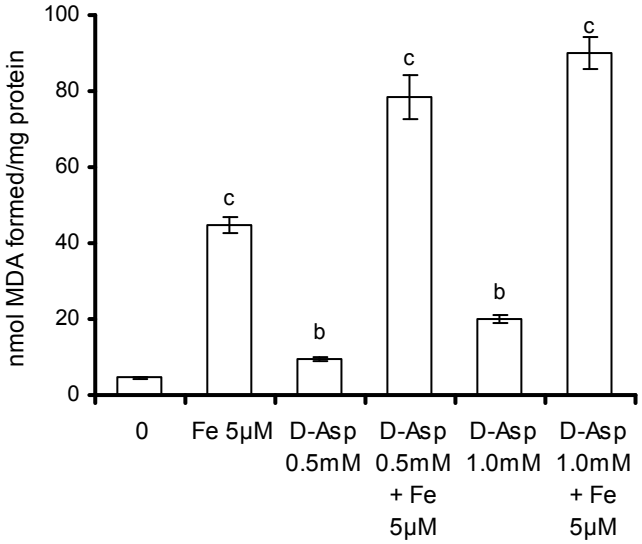
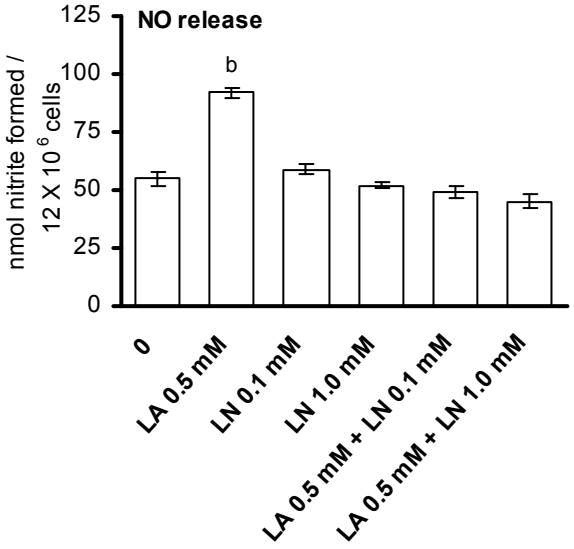


Fig. 4.3

Nitric Oxide release in testis cell suspensions (prepubertal rats) exposed to L-arginine and subsequently to L-nitro-arginine methyl ester (L-NAME) *in vitro*



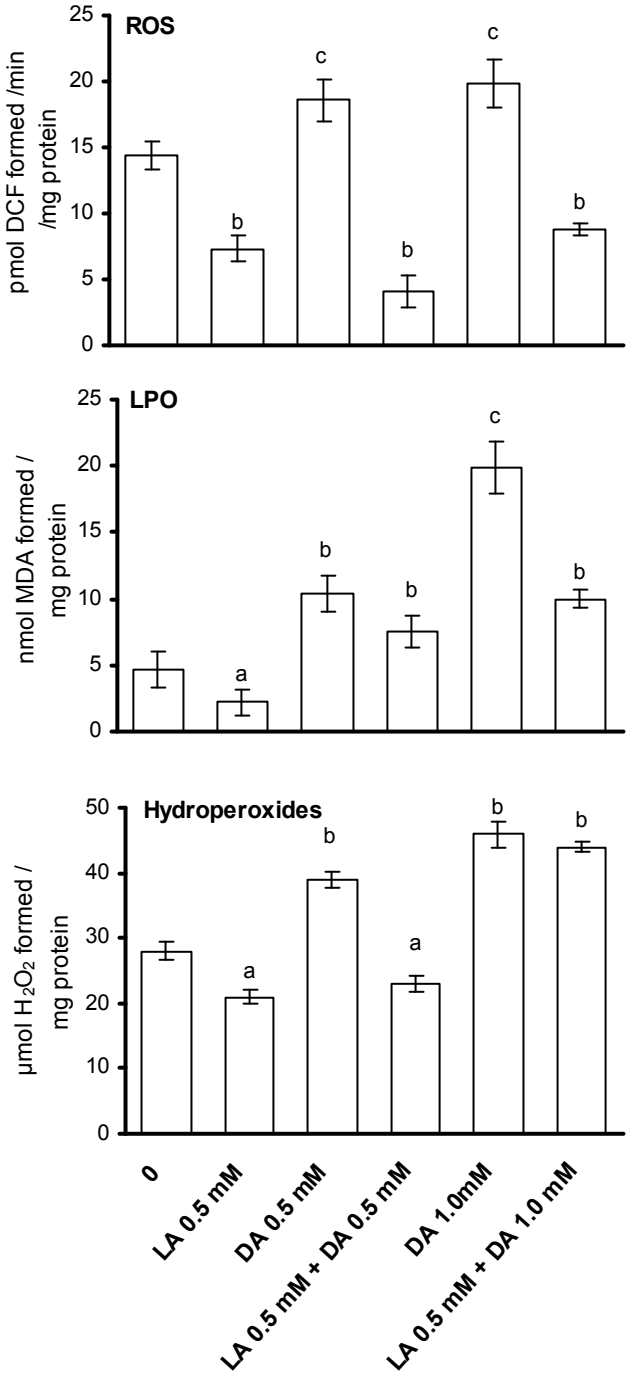
LA, L-Arginine; LN, L- nitro- arginine methyl ester

Values are mean ± SE of three separate determinations.

Data analysed by student 't' test. [b] $p<0.01$; [c] $p<0.001$ vs control

Fig. 4.4

Effect of L-Arginine (LA) *per se* on the endogenous levels of oxidative markers and attenuation of D-aspartic acid (DA) induced generation of reactive oxygen species (ROS), lipid peroxidation (MDA) and hydroperoxide levels in testis homogenates of PP rats.

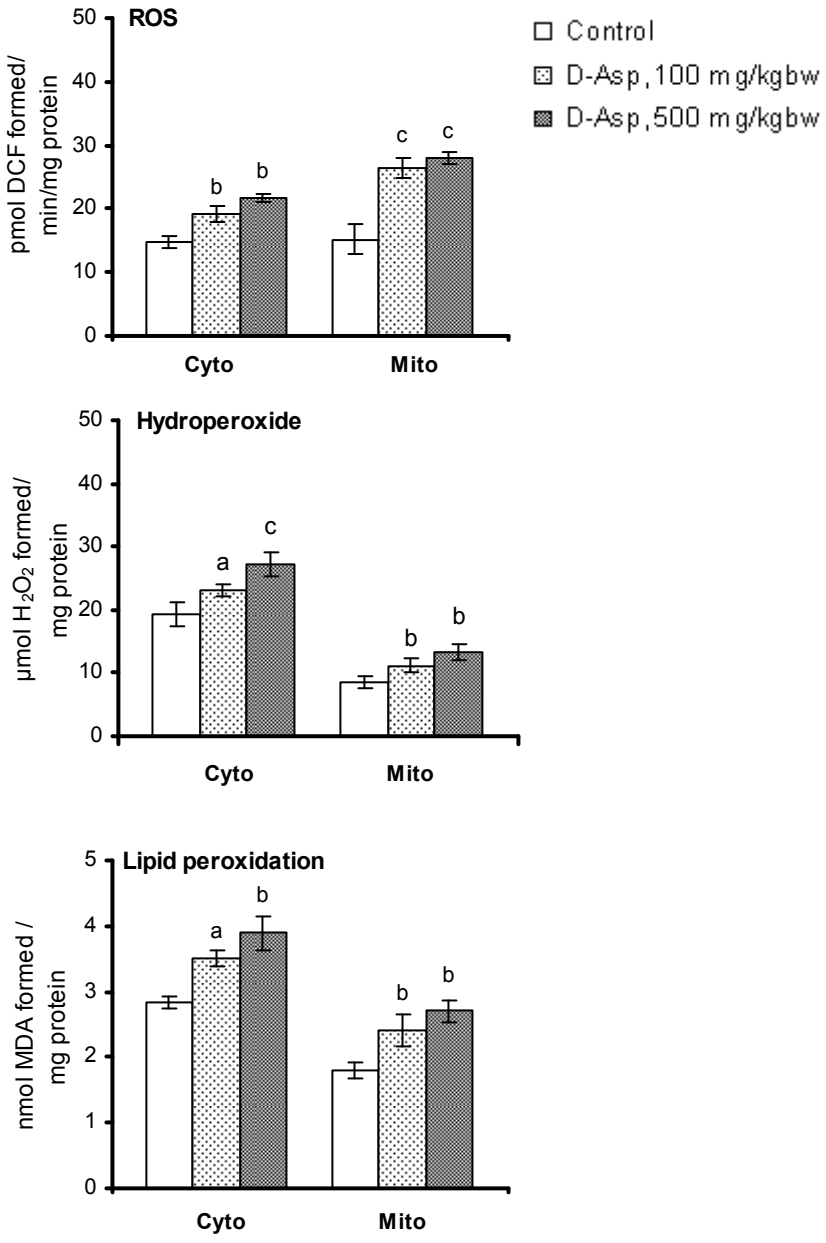


Values are mean ± SE of three separate determinations.

Data analysed by student's 't' test; [a] $p<0.05$; [b]$p<0.01$; [c]$p<0.001$ vs control.

Fig. 4.5

Effect of D-aspartic acid administration on oxidative stress markers in cytosol (cyto) and mitochondria (mito) of PP rat testis

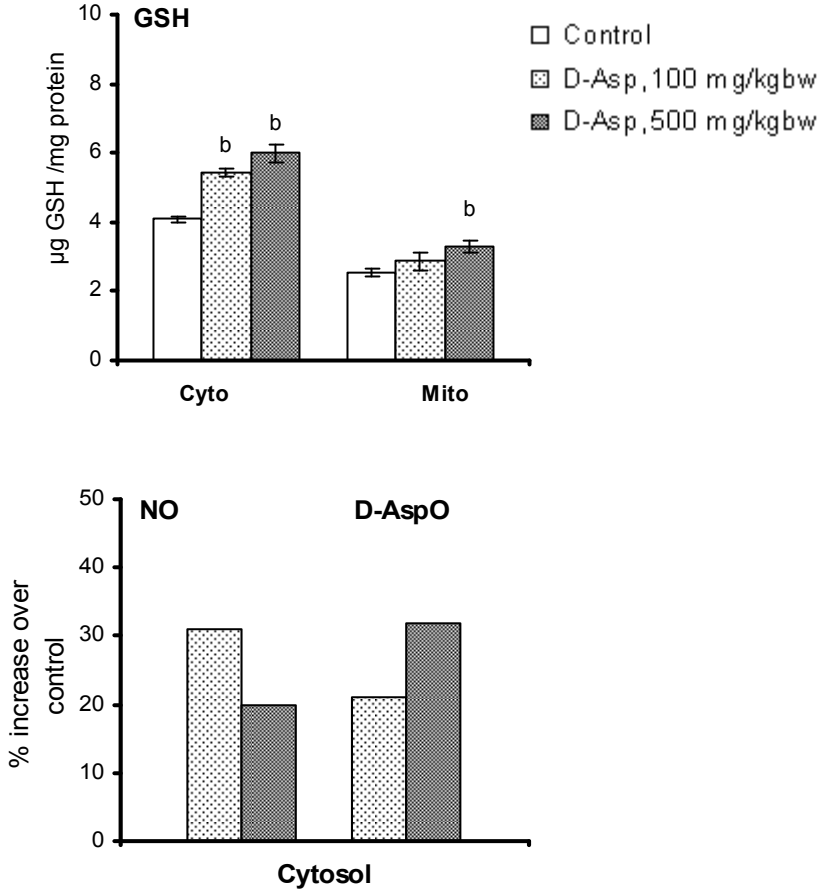


Values are mean ± SE of three separate determinations.

Data analysed by student's 't' test; [a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs control.

Fig. 4.6

Alterations in glutathione (GSH), nitric oxide (NO) release and in the activity of D-aspartate oxidase (DAspO) in testis of PP rats administered with D-aspartic acid (D-Asp)



Values are mean ± SE of three separate determinations.

Data analysed by student 't' test; [a]p<0.05; [b] p<0.01 vs control.

Table 4.3

Effect of exogenous D-Aspartic acid on the activities of antioxidant and functional enzymes in testis cytosol and mitochondrial fractions of prepubertal rats

	D-Aspartic acid (mg/kg bw/d)		
	0	100	500
Catalase [1]	0.012 ± 0.02	0.019±0.02 [b]	0.021 ± 0.03 [b]
Glutathione peroxidase (cyto)[2]	1.06 ± 0.02	1.40 ± 0.03 [b]	1.49 ± 0.02 [b]
Glutathione peroxidase(mito)[2]	0.08 ± 0.01	0.10 ± 0.02 [a]	0.11 ± 0.01[a]
Glutathione transferase (cyto)[3]	1.23 ± 0.08	1.81 ± 0.11 [b]	1.78 ± 0.05 [b]
Glutathione transferase(mito)[3]	0.72 ± 0.02	0.73 ± 0.04	0.93 ± 0.13 [b]
Lactate dehydrogenase (cyto)[4]	0.044 ± 0.01	0.056 ± 0.0 [a]	0.057 ± 0.01 [a]
3β-hydroxy steroid dehydrogenase [5]	0.29 ± 0.01	0.33 ± 0.05 [a]	0.35 ± 0.02 [a]

Value are mean ± SE of three separate determinations;

Values within row with no common superscripts are statistically significant at $p < 0.05$

[a] $p<0.05$; [b] $p<0.01$ vs control.

D-Aspartic acid administered for 7 consecutive days

[1] µmol H_2O_2 decomposed/min/mg protein;

[2] µ mol NADPH oxidized/min/mg protein;

[3] µ mol GS-DNB conjugate formed/min/mg protein ;

[4] µ mol NADH oxidized/min/mg protein;

[5] µ mol NADH formed/min/mg protein.

Table 4.4

Alterations in the activities of mitochondrial enzymes in testis of prepubertal rats administered D-Aspartic acid

	D-Aspartic acid (mg/kg bw/d)		
	0	100	500
Aldehyde dehydrogenase [1]	5.4 ± 0.7	3.6 ± 0.2 [b]	4.3 ± 0.9 [a]
Citrate synthase [2]	19.5 ± 1.2	22.5 ± 0.6	28.0 ± 2.0 [b]
Succinate dehydrogenase [3]	7.9 ± 0.2	7.6 ± 0.11	7.4 ± 0.03
Malate dehydrogenase [4]	0.55 ± 0.04	0.50 ± 0.03	0.49 ± 0.06
NADH – Cyt C reductase[5]	0.074 ± 0.01	0.079 ± 0.05	0.078 ± 0.02
Succinate – Cyt C reductase[4]	0.098 ± 0.01	0.135 ± 0.05 [b]	0.148 ± 0.02 [b]
Mg^{2+} ATPase (Complex V)[5]	46.7 ± 1.8	45.7 ± 0.8	54.0 ± 0.3 [b]

Values are mean ± SE of three separate determinations.

Values within row with no common superscripts are statistically significant at $p< 0.05$

[a] $p<0.05$; [b]$p<0.01$ vs control.

[1]- nmol NADH oxidized /min/mg protein;

[2] -nmol DTNB oxidized/min/mg protein;

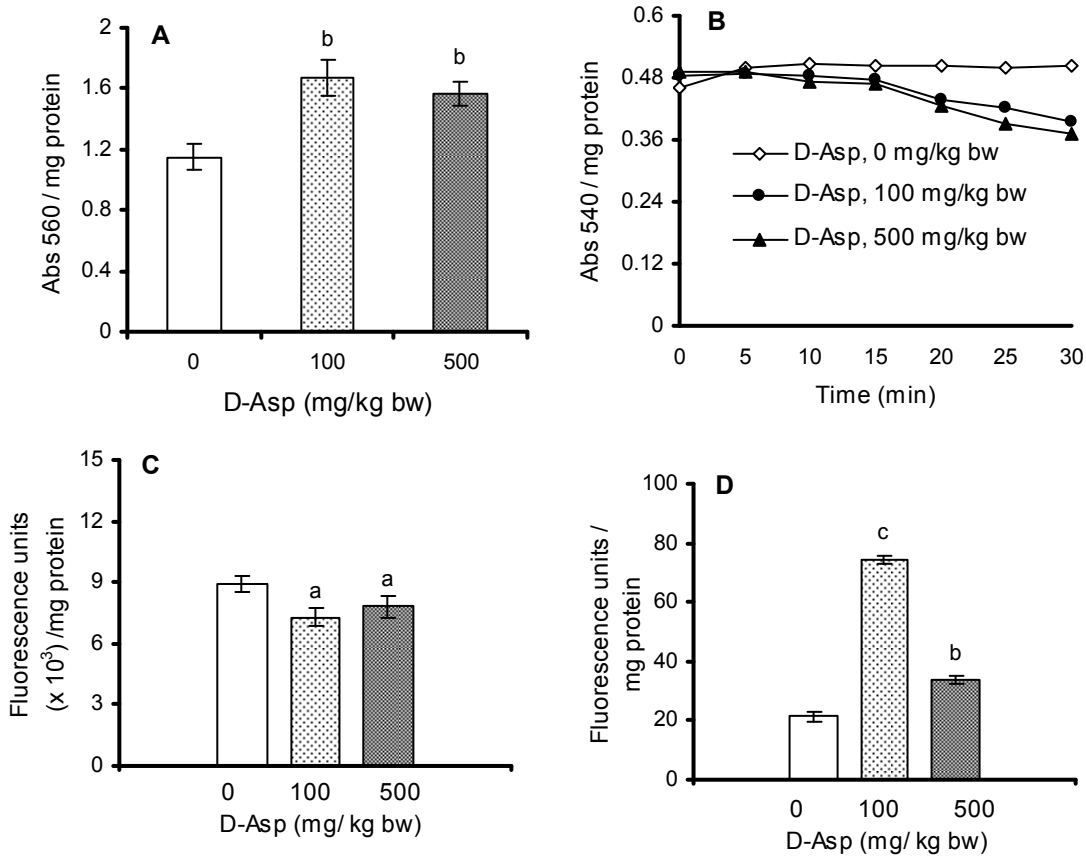
[3]- mmol substrate oxidized /mg protein;

[4]- µmol Cytochrome C reduced/min/mg protein;

[5] -µg inorganic phosphate formed/min/mg protein.

Fig. 4.7

Alterations in mitochondrial function measured as increase in MTT reduction (A), mitochondrial membrane pore opening (B), membrane potential (C) and cytosolic Ca^{2+} levels (D) in testis of PP rats administered with D-Aspartic acid



Values are mean ± SE of three separate determinations.

Data analysed by student 't' test. [a]p<0.05; [b] p<0.01; [c] p<0.001.

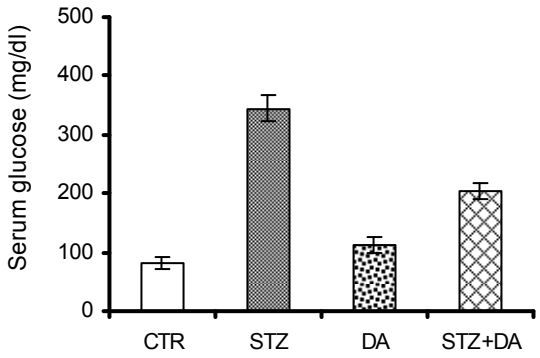
Table 4.5

Effect of D-Aspartic acid administration on the body weight and testis weights of diabetic prepubertal rats

	Body weight (g)		Testis wt (g)	
	Initial	Final	Absolute	Relative
Control	54.3 ± 1.1	69.0 ± 2.3	0.39 ± 0.05	0.58 ± 0.07
STZ	55.8 ± 0.8	40.3 ± 1.0[b]	0.13 ± 0.05[c]	0.30 ± 0.06[b]
DA	57.8 ± 0.3	72.8 ± 0.8	0.45 ± 0.03	0.62 ± 0.04
STZ+ DA	53.0 ± 1.2	45.0 ± 0.9[b]	0.25 ± 0.04[b]	0.47 ± 0.05[a]

Fig. 4.8

Modulatory effects of D-Aspartic acid on blood glucose levels in diabetic PP rats

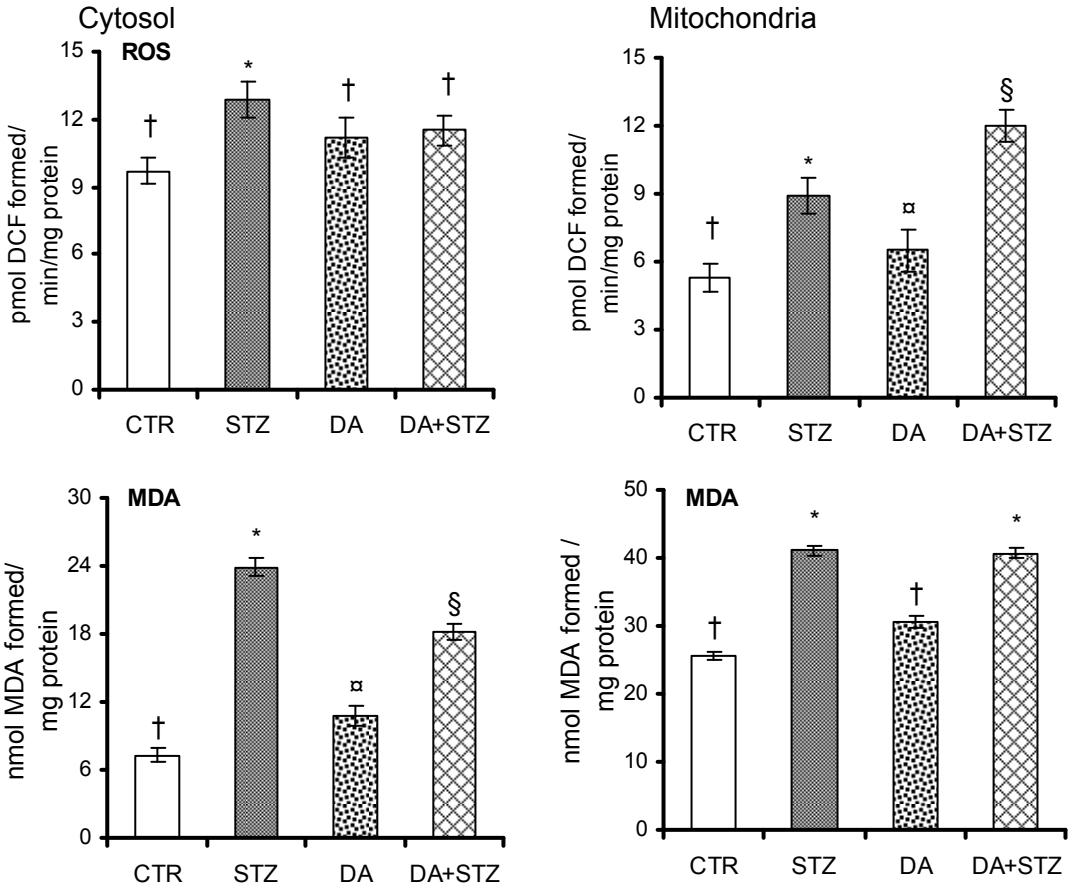


Values are mean ± SEM (n=6).

Data analysed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.9

Modulatory effects of D-aspartic acid (D-Asp) on generation of reactive oxygen species (ROS) and extent of lipid peroxidation (MDA) in testis (cytosol and mitochondria) of non-diabetic and diabetic PP rats

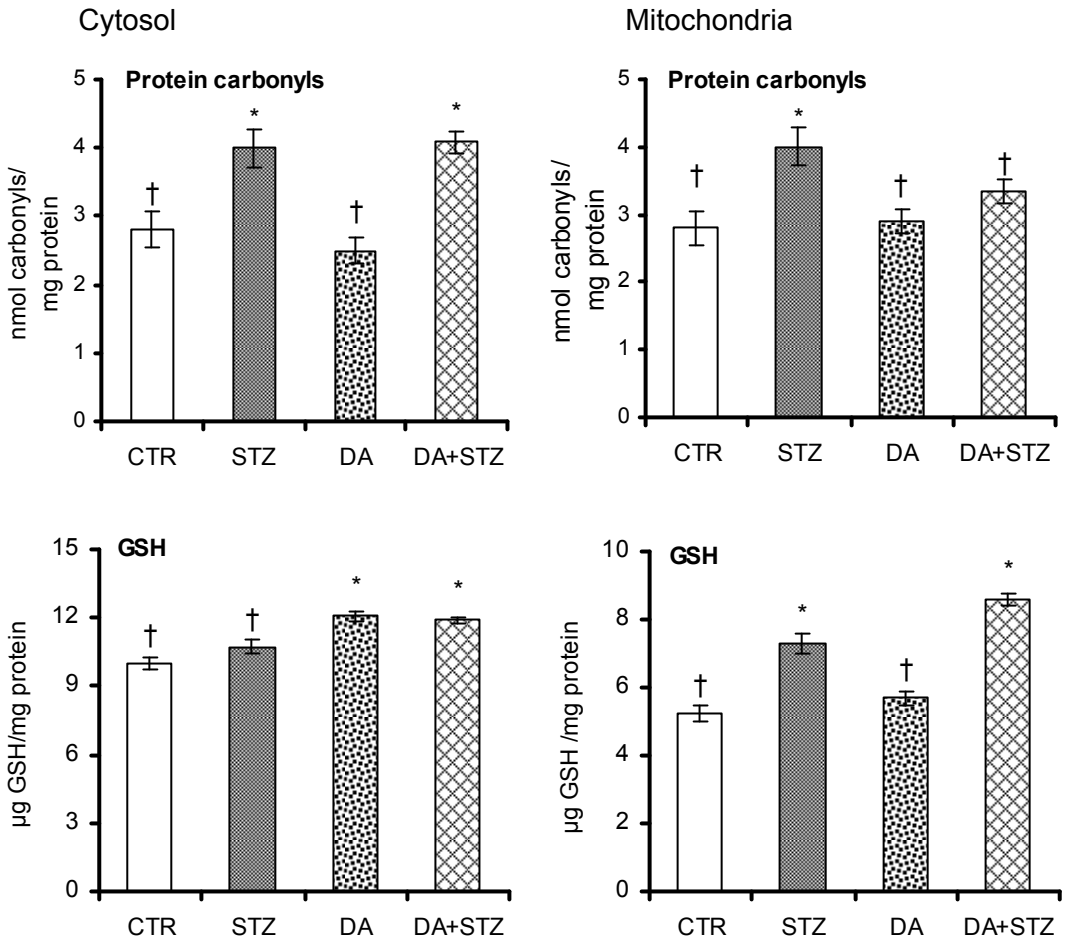


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.10

Effect of D-aspartic acid (D-Asp) administration on protein carbonyls and reduced glutathione in testis cytosol /mitochondria of STZ-diabetic PP rats

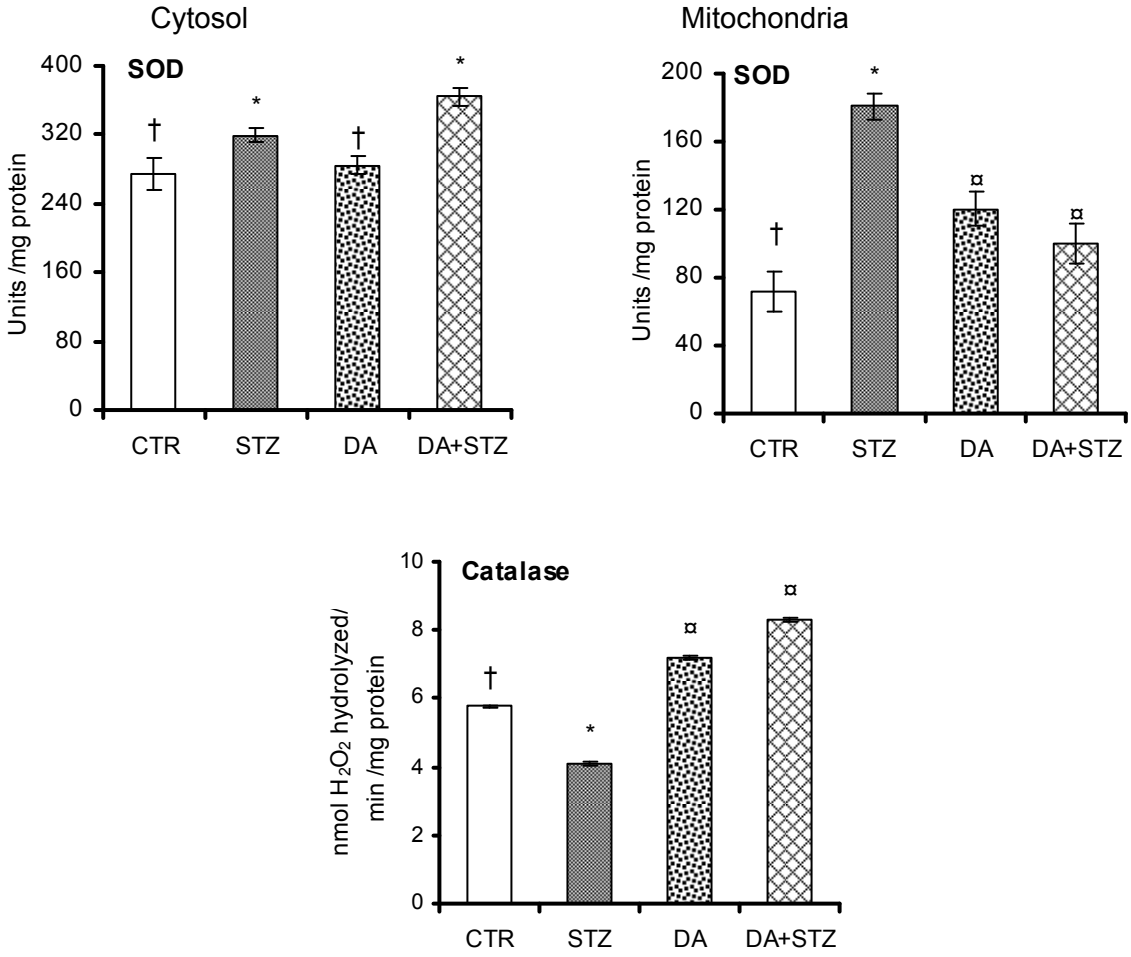


Values are mean ± SEM (n=6).

Data analysed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.11

Effect of D-aspartic acid administration on the activity levels of superoxide dismutase (SOD) and catalase in testis cytosol /mitochondria of both non-diabetic and diabetic PP rats

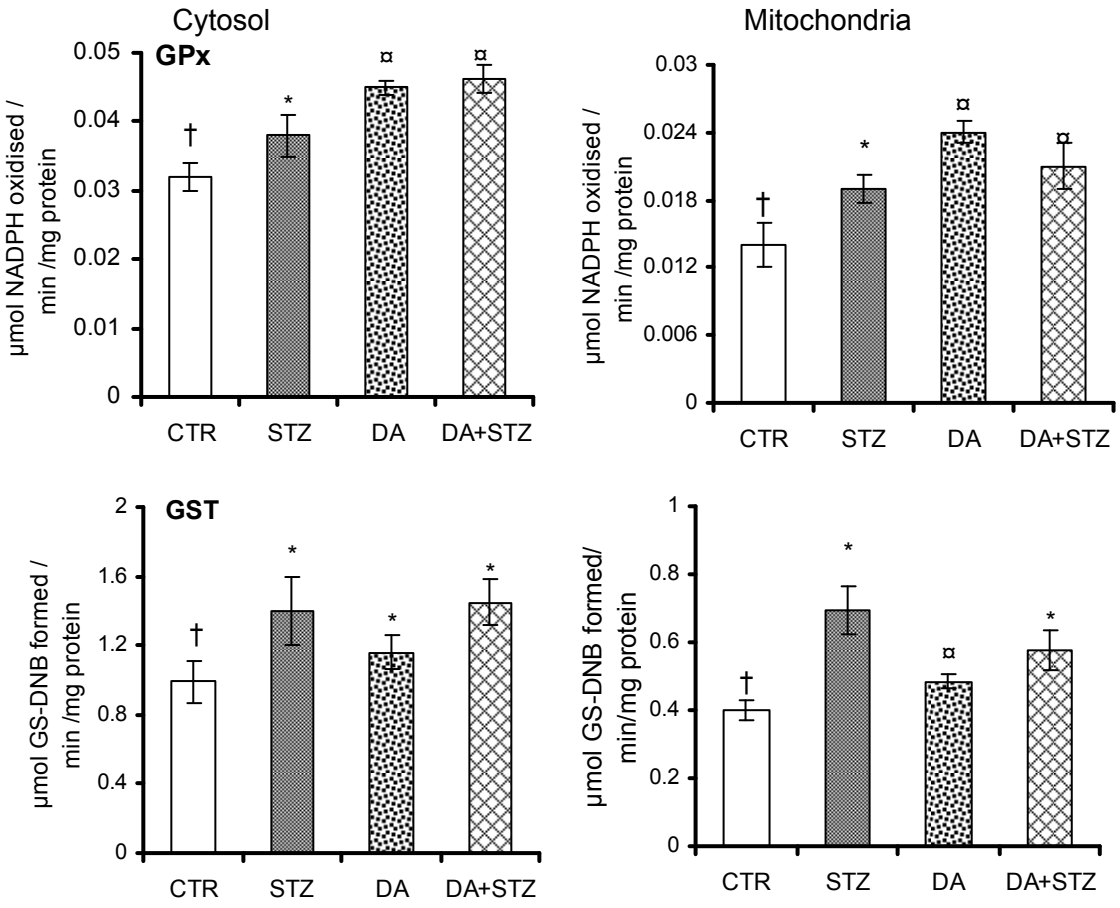


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.12

Effect of D-aspartic acid administration on the activities of glutathione peroxidase and glutathione–S- transferase in testis cytosol and mitochondria of diabetic PP rats

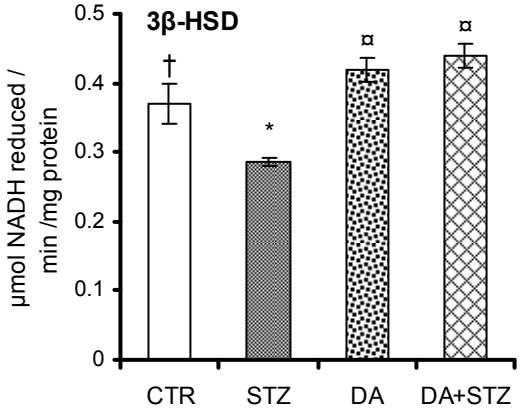


Values are mean ± SEM (n=6).

Data analysed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.13

Effect of D-Aspartic acid on the activity of 3β-hydroxy steroid dehydrogenase in testis cytosol of STZ-diabetic PP rats



Values are mean ± SEM (n=6).

Data analysed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$)

Table 4.6

Effect of testosterone administration on the body weight, testis weight of prepubertal rats rendered diabetic by an acute dose of streptozotocin

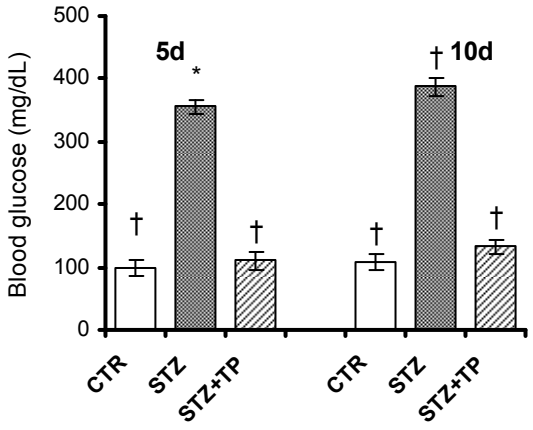
	Body weight (g)		Testis wt (g)	Body weight (g)		Testis wt (g)
	Initial	5d		Initial	10d	
Control	44.3 ± 1.4	60.0 ± 0.6	0.44 ± 0.09	47.3 ± 1.7	80.5 ± 1.3	0.57 ± 0.1
STZ	45.0 ± 1.0	51.0 ± 3.0	0.17 ± 0.02[c]	43.3 ± 0.9	54.0 ± 4.7[b]	0.19 ± 0.1[c]
STZ+TP	45.3 ± 2.2	52.0 ± 4.3	0.36 ± 0.05[a]	43.2 ± 1.1	54.2 ± 1.1[b]	0.36± 0.07[b]

Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.14

Modulatory effects of Testosterone against STZ –induced hyperglycemia

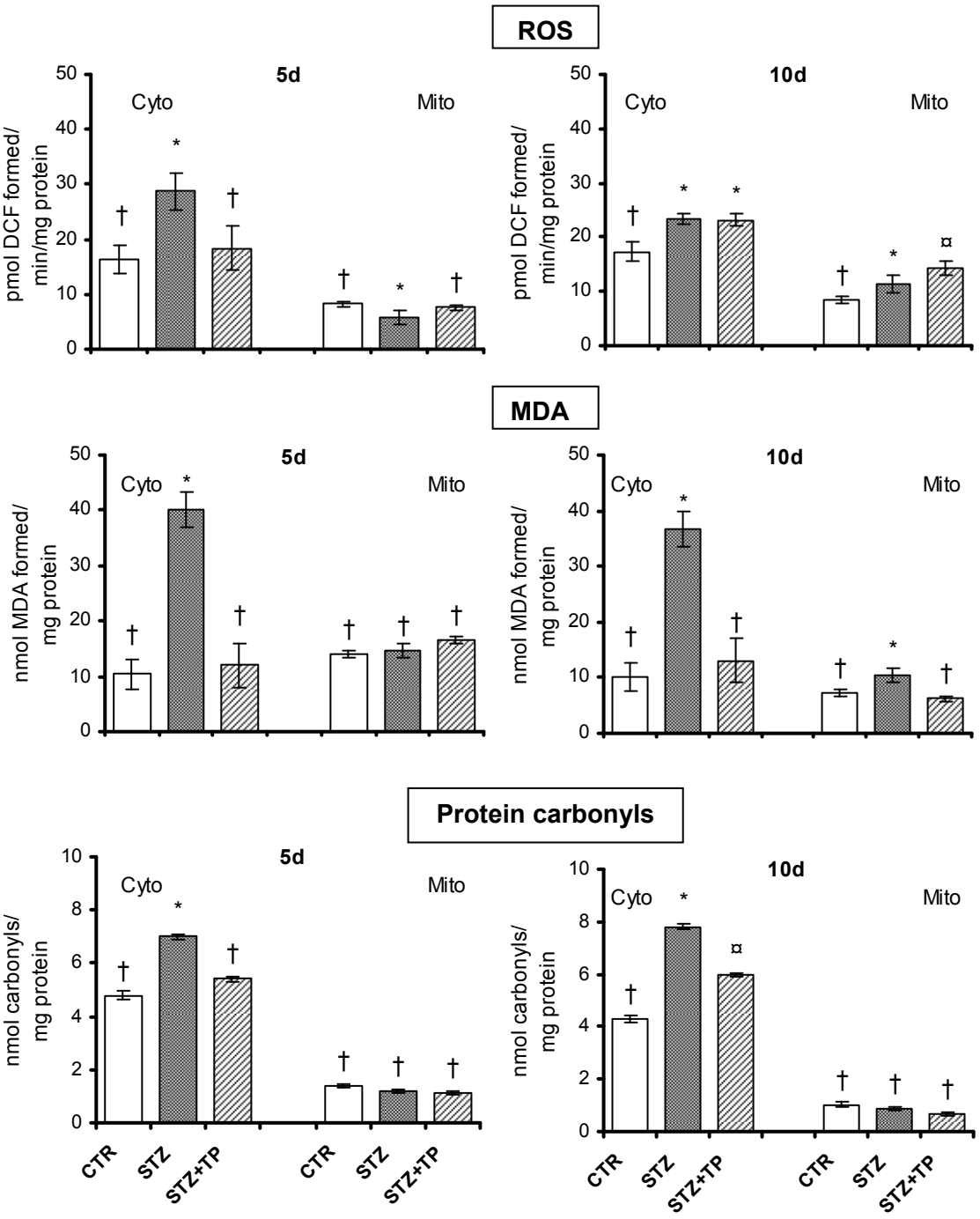


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.15

Modulatory effect of testosterone (TP) on oxidative stress markers in testis cytosol (cyto) and mitochondria (mito) of diabetic PP rats

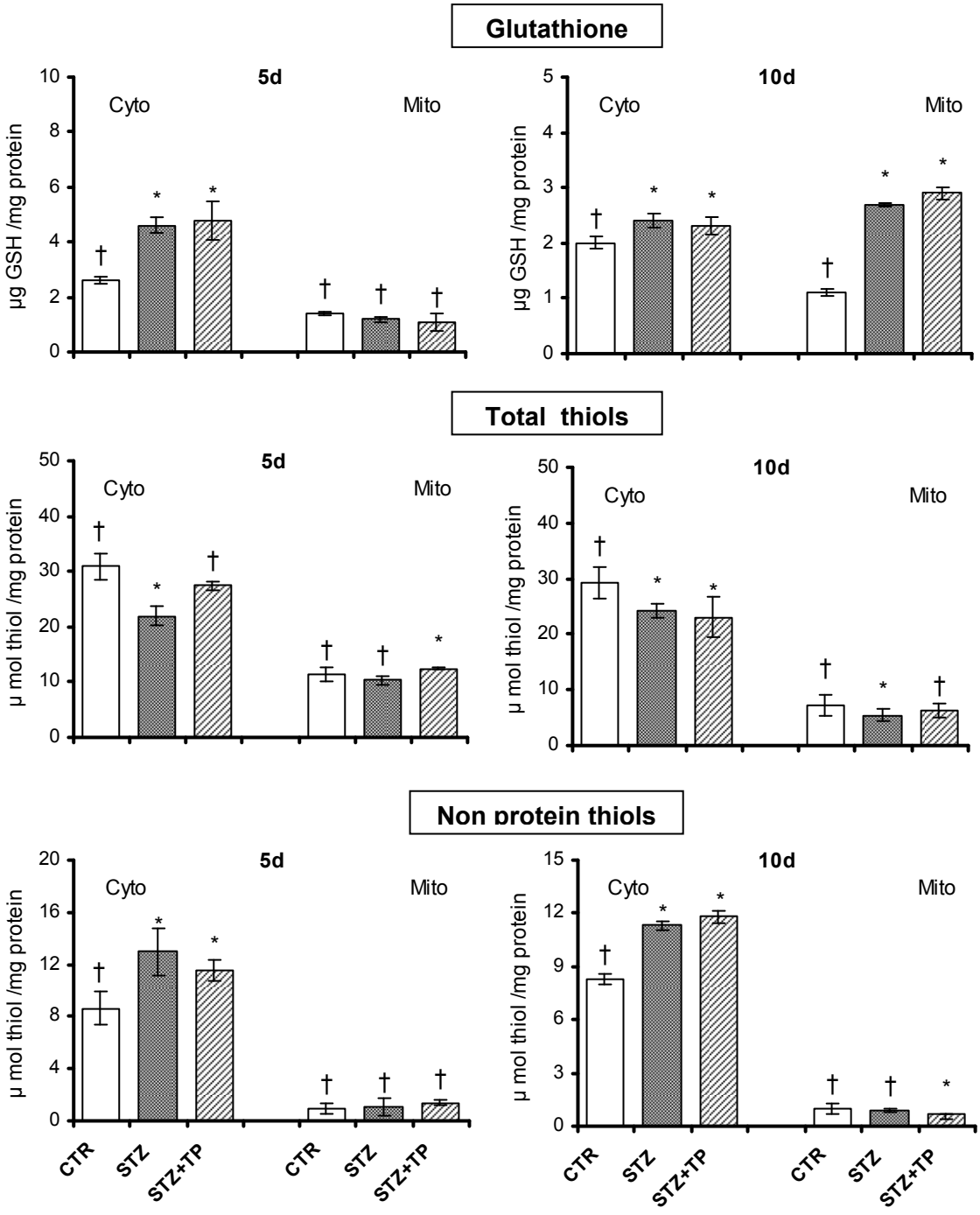


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.16

Effect of testosterone administration on thiol status in testis cytosol and mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ

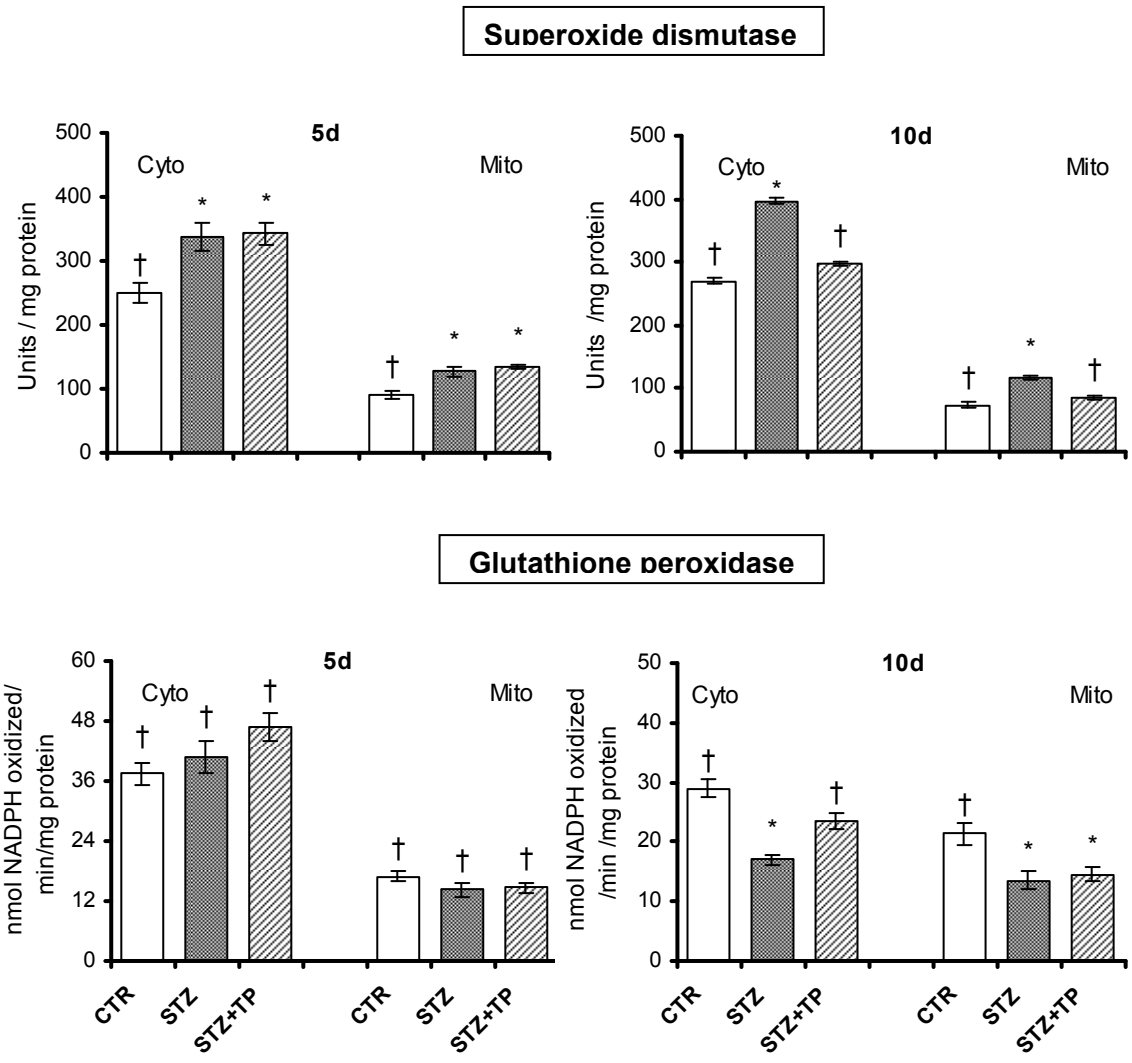


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.17

Effect of testosterone administration on the activity of antioxidant enzyme in testis cytosol and mitochondria of prepubertal rats rendered diabetic by an acute dose of STZ

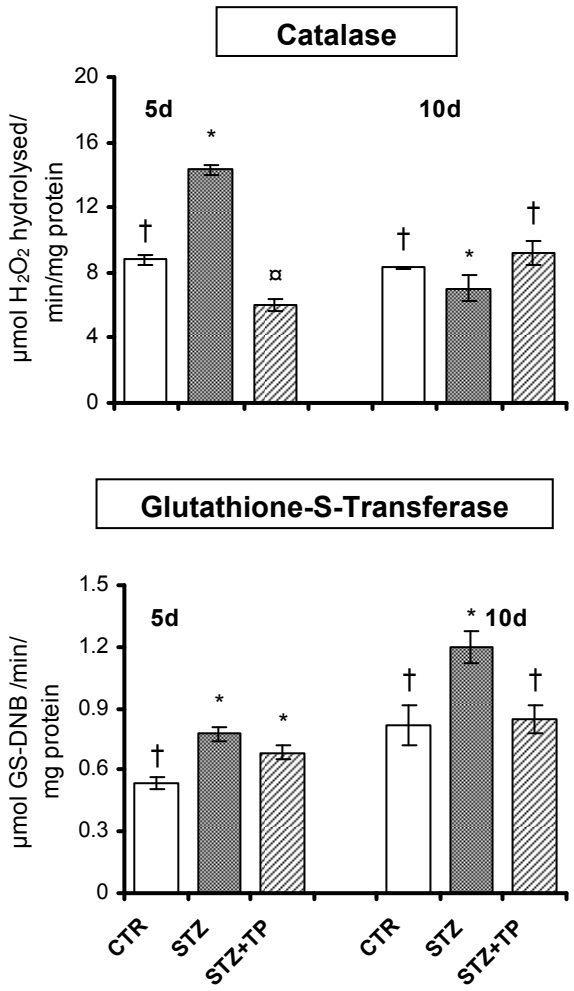


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.18

Effect of testosterone administration on the activity of antioxidant enzyme in testis cytosol of diabetic PP rats

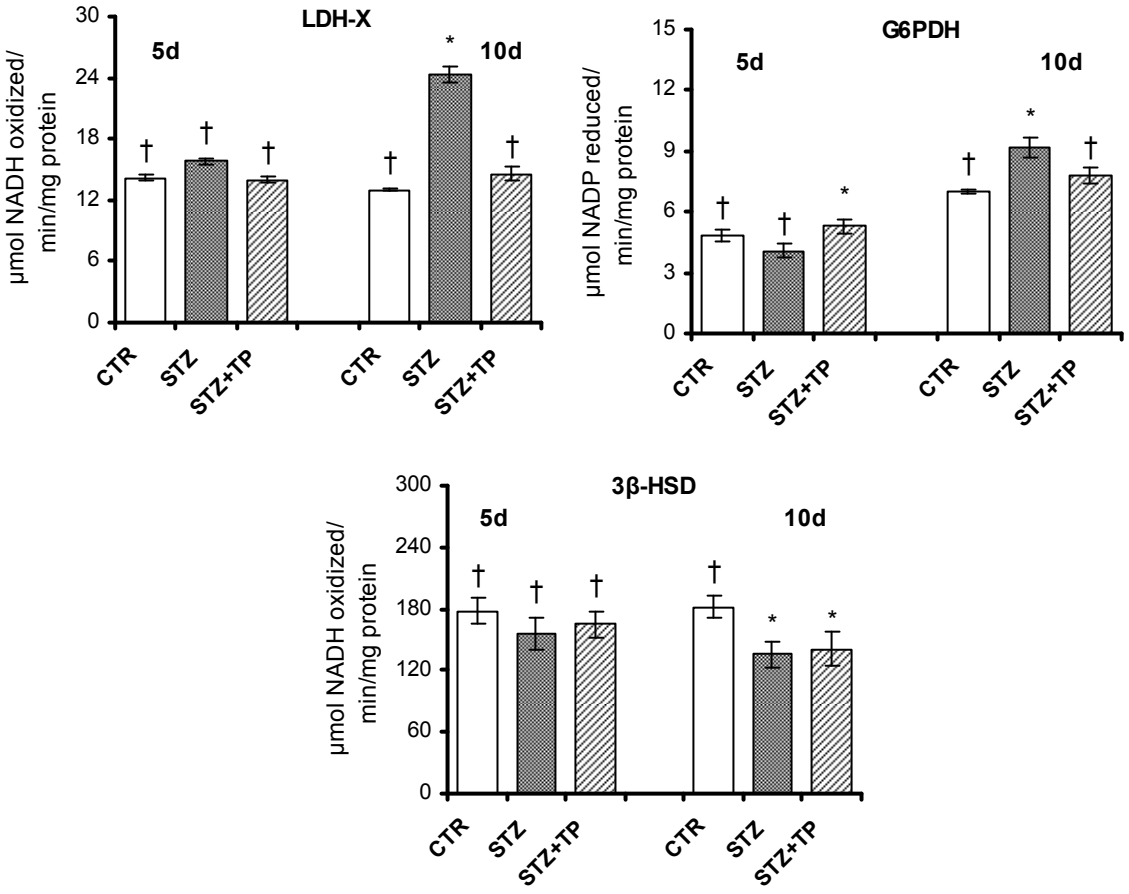


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.19

Effect of testosterone administration on functional enzyme activities in testis cytosol of diabetic PP rats

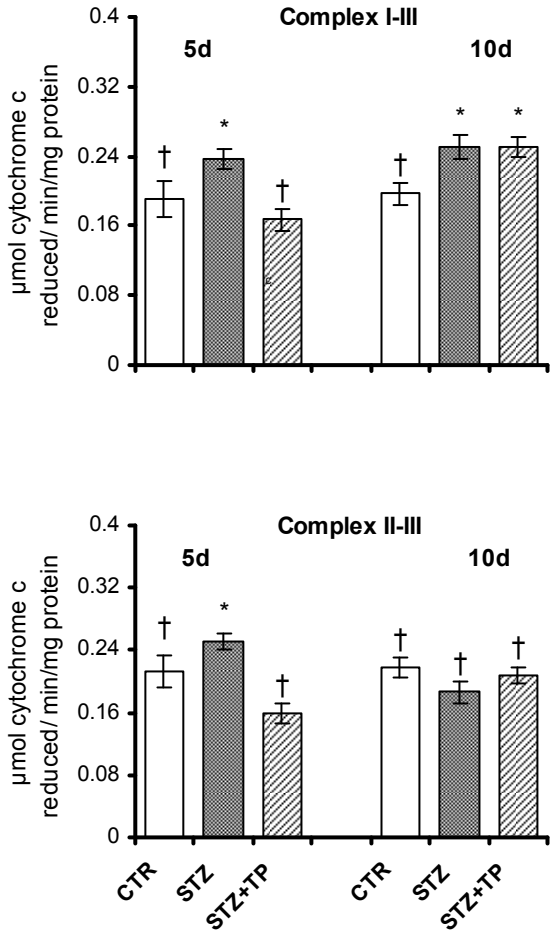


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.20

Effect of testosterone (TP) administration on the activities of NADH-cytochrome C reductase and succinate-cytochrome C reductase in testis mitochondria of diabetic PP rats



Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Table 4.7

Effect of oral administration of aqueous extract of *W*ithania somnifera (*500mg/kgbw*) for 15days on body and organ weights of prepubertal rats rendered diabetic by an acute dose of STZ.

	Body weight (g)		Testis wt (g)		Liver wt (g)
	Initial	Final	Absolute	Relative	
Control	37.2 ± 1.1	67.0 ± 2.3	0.39 ± 0.05	0.58 ± 0.07	3.0 ± 0.2
STZ	37.3 ± 1.9	45.3 ± 5.0*	0.13 ± 0.05*	0.30 ± 0.06*	1.9 ± 0.2*
STZ+WS	39.0 ± 0.8	53.0 ± 3.1†	0.25 ± 0.04†	0.47 ± 0.05†	2.3 ± 0.1†

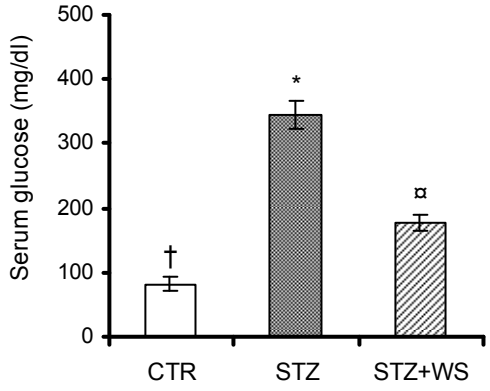
Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Withania Somnifera: Aqueous extract was orally administered for 15 consecutive days

Fig. 4.21

Modulatory effects of *Withania somnifera* on blood glucose levels in diabetic PP rats

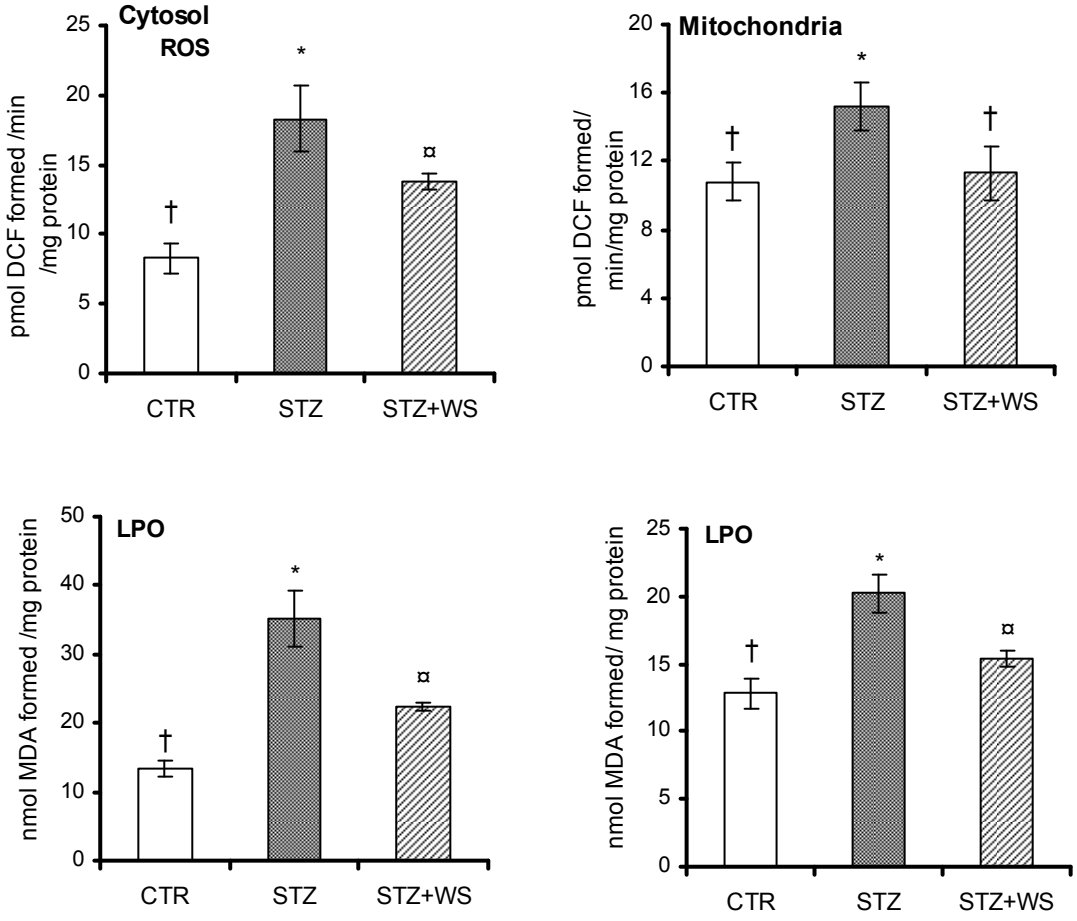


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.22

Effect of oral *Withania somnifera (WS)* supplements on generation of reactive oxygen species (ROS), malondialdehyde (MDA) levels in testis cytosol and mitochondria of diabetic PP rats

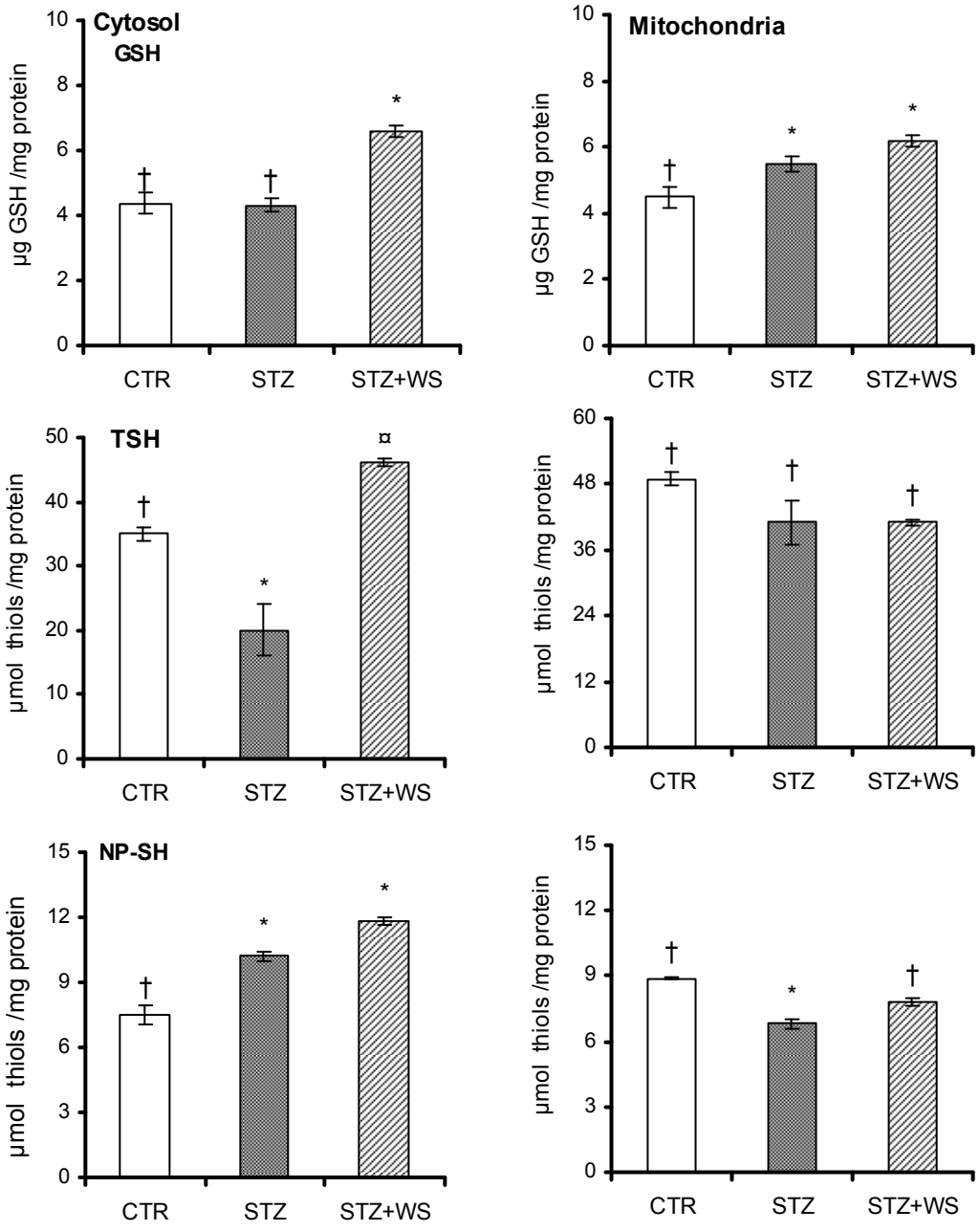


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.23

Effect of oral supplementation of *Withania somnifera (WS)* supplements on thiol status in testis cytosol and mitochondria of diabetic PP rats

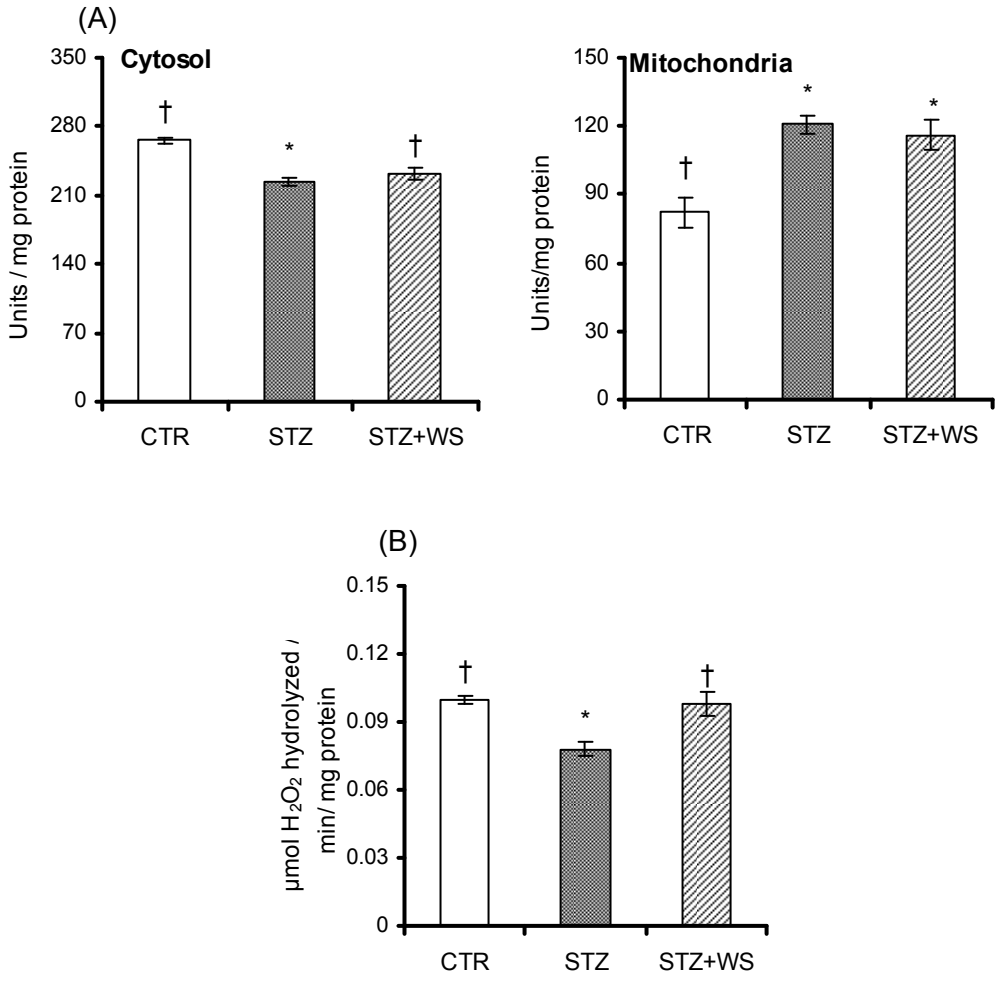


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.24

Effect of oral *Withania somnifera (WS)* supplements on the activities of superoxide dismustase (A) and catalase (B) in testis cytosol and mitochondria of PP rats

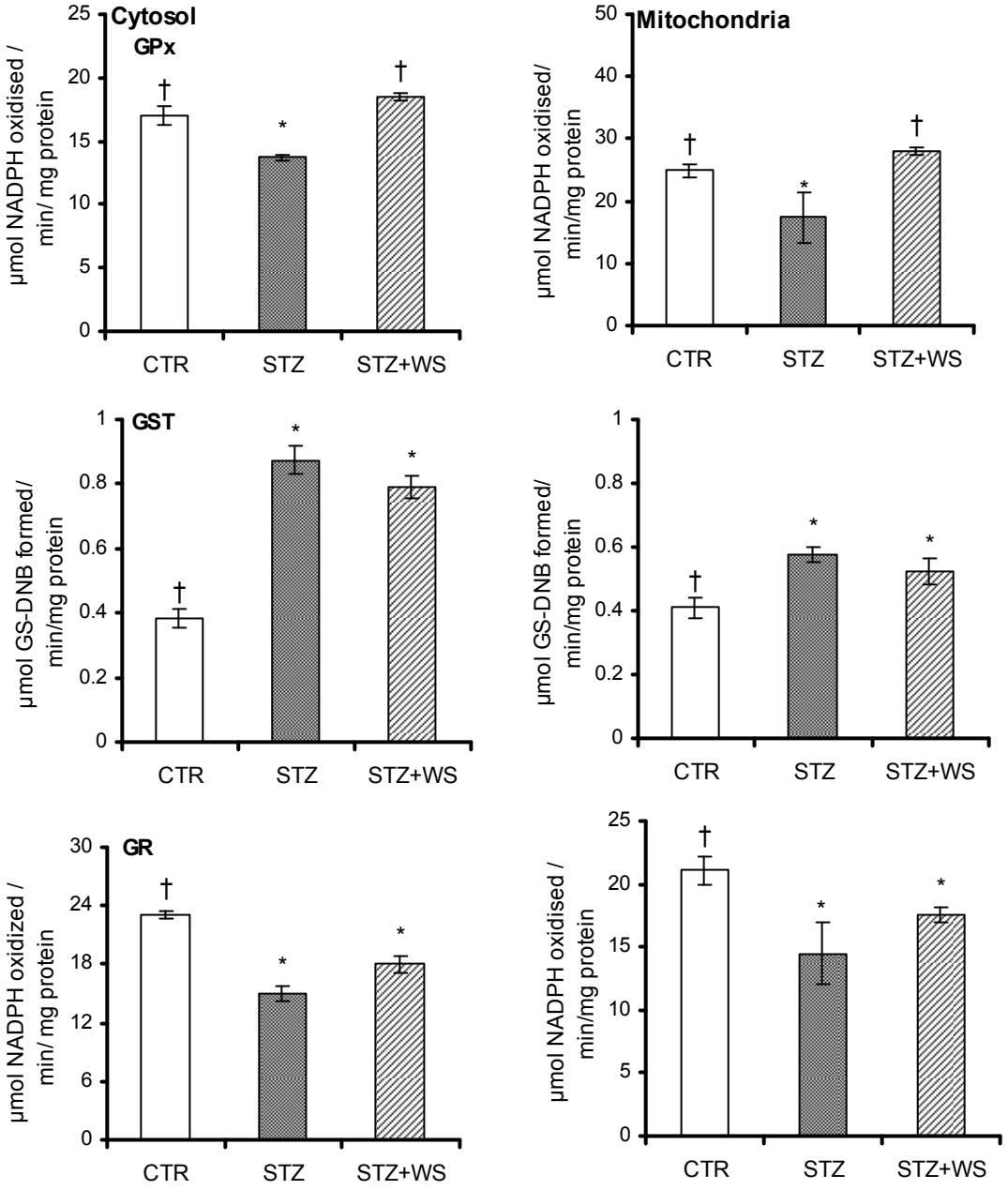


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig. 4.25

Effect of oral *Withania somnifera* (WS) supplements on the activities of glutathione peroxidase (GPx), glutathione- S- transferase (GST) and glutathione reductase (GR) in testis (cytosol and mitochondria) of diabetic PP rats

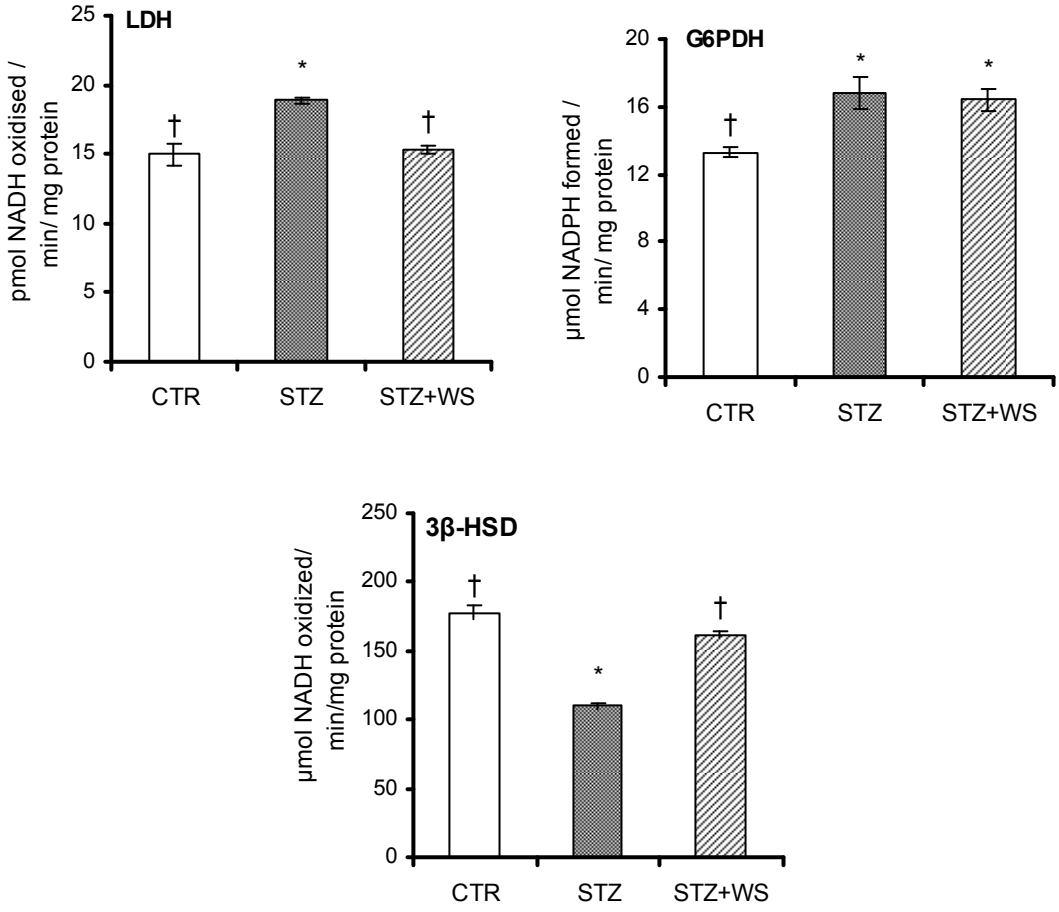


Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

Fig 4.26

Effect of oral *Withania somnifera* (WS) supplements on the activities of lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH) and 3β-hydroxysteroid dehydrogenase in testis cytosol of diabetic prepubertal rats



Values are mean ± SEM (n=6).

Data analyzed by One-way ANOVA; the means not sharing the common symbol are significantly different between groups ($p<0.05$).

5.0 DISCUSSION

Several experimental evidences suggest that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models as well as reducing the severity of diabetic complications (Maritim et al., 2003). Hence numerous focussede efforts are underway to find suitable antidiabetic and antioxidant therapies. Medicinal plants/phytochemicals are being looked up once again for the treatment of diabetes owing to their hypoglycemic, antioxidant and tissue protective properties. The WHO Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated. Major hindrance in amalgamation of herbal medicine in modern medical practices is the lack of scientific and clinical data proving their efficacy and safety (Takei and Kasatani, 2004). It is in this context, these investigations were conducted in prepubertal diabetic male rats.

Interactive effects of D-Aspartic acid- Effects on testis in vitro

D-Aspartic acid is an endogenous amino acid synthesized by the intestinal micro flora and also shown to occur in various vegetables and sea foods. The concentrations of free D-Asp is shown to increase in several regions during different developmental period suggesting that the amino acid plays an important role in the regulation of developmental processes. In rat testis, D-Asp concentrations are shown to increase in early fetal life and during sexual maturity (D'Aniello et al., 1996) with simultaneous increase in steroidogenesis and/or testosterone secretion (Homma and Furuchi, 2005). On exposure to D-Asp, PP testis showed a concentration-dependent oxidative induction, manifested as increased generation of ROS, formation of malondialdehyde and hydroperoxides. The elevated oxidative response could be due to its cellular uptake followed by metabolism (by inducible D-AspO) resulting in the formation of hydrogen peroxide. Hydrogen peroxide alone or in presence of other free radicals/ transition metal ions, may execute the peroxidation events on D-Asp exposure in PP testis. This thinking is consistent with our finding of significantly potentiated oxidative induction by D-

Asp in the presence of iron. This interactive phenomenon may partly explain, the enhanced oxidative stress in brain under various neuropathological conditions reported to contain higher levels of circulating D-Asp and iron (Fisher et al., 1994).

The consequences of D-Asp exposure under conditions of compromised enzyme activities were also investigated by employing specific inhibitors of two major peroxide detoxifying enzymes viz., catalase (Aminotriazole, AT) and glutathione peroxidase (Mercaptosuccinate, MS). As anticipated, the degree of peroxidation was enhanced in testicular homogenates incubated with AT or MS clearly suggesting that partial or complete inhibition of CAT and or GPx can lead to elevated oxidative stress. Further, pretreatment with AT or MS followed by D-Asp exposure yielded higher oxidative response, the degree being particularly very high following inhibition of GPx suggesting the formation of a variety of hydroperoxides following D-Asp exposure (Chandrashekar and Muralidhara, 2008).

Nitric Oxide (NO), a free radical metabolite of L-Arg metabolism is shown to regulate D-Asp mediated testicular functions (Lamanna et al., 2007). In the present study, L-Arg at concentrations tested significantly mitigated D-Asp induced oxidative response suggesting attenuating influence of L-Arg derived NO against D-Asp induced tissue damage either by direct quenching of free radicals (Wink et al., 1993), or inhibiting the free-radical generating processes.

In vivo Effect of D-Asp on prepubertal testis

Several vegetables, marine foods and beverages contain exceptionally high quantities of D-Asp (Friedman, 1999) and their consumption is likely to result in an increased accumulation in various tissues including testis. Previously, it has been demonstrated that exogenously administered D-Asp accumulate in testis of rats with concomitant increase in testosterone and luteinizing hormone levels in blood (D'Aniello, 1996). Recent studies have shown remarkably augmented D-Asp levels in the pineal and pituitary glands following its intravenous administration suggesting a possible accumulation of D-Asp derived from intestinal bacteria or food in specific tissues (Morikawa et

al., 2007). However, *in vivo* implications of excess D-Asp intake on testis physiology have not been comprehensively investigated (Hashimoto and Chiba, 2004).

In order to ascertain the effect of D-Asp on endogenous levels of testicular oxidative stress, PP rats were administered with D-Asp at two dosage levels (100 and 500mg/kg bw, i.p. 15days). The criteria of dosage selection have been explained earlier (Chandrashekar and Muralidhara, 2009b). Our principal findings suggest that exogenous D-Asp induces a marked elevation in oxidative stress markers in testis of PP rats which could be primarily related to its metabolism by inducible D-aspartate oxidase (DAspO). Under normal physiological conditions, D-Asp is metabolized by DAspO into oxaloacetate, ammonia and hydrogen peroxide. Hydrogen peroxide is then immediately hydrolyzed either by catalase and/or glutathione peroxidase thereby minimizing the peroxidative injuries. This is consistent with the enhanced activities of CAT and GPx enzymes suggesting their importance under D-Asp exposure. Further these observations corroborate with our *in vitro* findings (Chandrashekar and Muralidhara, 2008). Further, alterations in the activity of D-AspO explain the potential tissue damaging effects of D-Asp (D'Aniello et al., 1993).

In the present study, both *in vitro* and *in vivo* exposure to D-Asp caused significant oxidative dysfunctions in testis mitochondria as revealed by elevated ROS generation, hydroperoxide levels and MDA levels implicating the higher vulnerability of PP testis mitochondria to D-Asp. These findings are consistent with earlier reports regarding the involvement of oxidative stress mediated mechanism/s in mitochondrial dysfunctions observed following D-amino acid exposure (Gonzalez-Hernandez et al., 2003; Cortes-Rojo et al., 2007; Orozco-Ibarra et al., 2007).

Testis is endowed with a variety of antioxidant enzymes capable of metabolizing toxic aldehydes (MDA/ HNE) generated during oxidative stress conditions. GST and aldehyde dehydrogenase are particularly involved in the catalysis of the conjugation of HNE to glutathione and NAD^+ dependant oxidation of HNE to 4-hydroxy-2-nonenoic acid (Traverso et al., 2002). In the present study, irrespective of the dosage, testis mitochondria of D-Asp

exposed rats exhibited reduced ALDH activity and enhanced GST activity, a finding which suggests a preferential regulation of aldehyde metabolizing enzymes. Further, D-Asp treatment significantly enhanced glutathione levels with concomitant increase in the activities of glutathione dependant enzymes which favors the detoxification of free radicals generated upon D-Asp metabolism. Additionally, higher ROS levels in a tissue can subsequently generate toxic reactive intermediates such as glutathione sulfenic acid and glutathiyl radicals which can form mixed disulfides with cellular proteins and alter their function (Ying et al., 2007)

In the present study, a significant increase in the NO levels was discernable in testis cytosol following D-Asp exposure. NO, a free radical metabolite of L-arginine is highly lipophilic, readily diffusible molecule which is demonstrated to possess a dual regulatory role under physiological and pathological conditions (Choi et al., 2002). Although the precise mechanism by which D-Asp causes enhanced NO levels is not clear, it may be directly related to the D-Asp-mediated upregulation of NO Synthase activity. The results of a recent report on induction of nitric oxide synthase by D-Asp in boar testis support such a possibility (Lamanna et al., 2007a). Further, over-production of NO can also be potentially deleterious by activating caspase mediated apoptotic pathways (Choi et al., 2002).

Although earlier evidences suggest the involvement of D-Asp in upregulating testosterone biosynthetic pathways (D'Aniello et al., 1996; Lamanna et al., 2007b) we observed only a marginal increase in the activity of 3β-HSD. This could be attributed to the age of rats employed for the study and down regulation of steroidogenesis by high NO levels (Kostic et al., 1998). D-Asp induced physiological changes in testis were also evidenced by significant elevation in the activity of LDH which is often considered as a marker of mitochondrial membrane integrity. Thus, enhanced activity of cytosolic LDH probably suggests extensive membrane damage and also a possible interference in normal physiology of Sertoli cells (Pant et al., 1997). Further, this effect is also suggestive of a possible shunting of metabolites of glycolytic pathway towards the lactate formation and a consequent depletion

in cytosolic NADH levels. This finding is consistent with reduced activity of MDH, which catalyzes the entry of cytosolic NADH into mitochondria.

D-Asp treatment had no significant effect on the activities of TCA cycle enzymes in testis excepting for the elevated activity of citrate synthase. D-Asp elicited differential effects on the ETC enzyme activities particularly succinate–cytochrome C reductase and Mg^{2+}ATPase which were markedly enhanced in contrast to the unaltered levels of NADH-cytochrome C reductase. This was further reflected by the increased rate of MTT reduction in testis mitochondria of D-Asp treated rats. However, the reasons for the differential effects on ETC enzymes are not clear. Mitochondrial dysfunctions were studied by further examining two interrelated events such as transmembrane potential and opening membrane transition pore (MPT) opening in the presence of Ca^{2+} (Kroemer et al., 1997). Marked reduction in the membrane potential and associated increased MPT opening is suggestive of the potential of D-Asp to cause perturbations in mitochondrial membrane integrity. This finding in testis mitochondria corroborates well with previous observations of D-amino acid- induced hepatic mitochondrial dysfunctions *in vitro* (Gonzalez-Hernandez et al., 2003). Induction of MPT is shown to occur due to the dissipation of the difference in voltage between the mitochondrial membrane possibly due to free radical-mediated increase in intracellular concentration of calcium (Armstrong et al., 2004; Gunter et al., 2004). Further, the ETC may also generate more free radicals due to loss of specific components through the pore opening (Luetjens et al., 2000). Thus, it is likely that D-Asp induced specific mitochondrial membrane alterations in testis may probably result from altered calcium homeostasis.

Modulatory effects of D-Asp against diabetes induced testicular dysfunctions

Our earlier data (Chapter 2 and 3) demonstrated that PP testes are highly susceptible to diabetes induced oxidative insults. Since, testicular function is largely controlled by the hypothalamo-pituitary-gondal axis, any compound that interacts positively with the axis is likely to bring improvement in testicular dysfunctions during diabetes. Since, D-Asp has a demonstrated neuroendocrine regulatory role, it was hypothesized that D-Asp can modulate

the impaired HP-axis and offer protection against diabetes induced testicular dysfunctions.

Our salient findings such as reduction in body weight gain, marked hyperglycemia, testicular atrophy, enhanced ROS/MDA levels, compromised levels of antioxidant enzymes, altered redox status suggests the development of oxidative stress in testis of diabetic PP rats (Chapter 3). Although, D-Asp administration to diabetic rats failed to bring any improvement in body weight gain, it significantly lowered elevated blood glucose levels and offered significant protection against diabetes induced testicular atrophy. These results suggest the propensity of D-Asp to maintain tissue integrity during preexisting oxidative stress conditions. This line of thinking is consistent with recent findings that suggests besides having pro-oxidant propensity, several D-aminoacids show protective effects during specific pathological conditions (Cortes-Rojo et al., 2007). Hence our results suggest a possible physiological interaction between D-Asp and pancreas that lead to increased secretion of insulin resulting in lowering of blood glucose levels. Further, the propensity of D-Asp to induce synthesis /secretion of testosterone indirectly explains the observed anti-hyperglycemic effects of D-Asp.

D-Asp administration to diabetic rats lowered the levels of ROS, MDA (in both cytosol and mitochondria), which is attributable to the inherent potency of D-Asp to increase GSH levels as shown earlier. Further, the relative oxidative stress lowering effects of D-Asp during pathological conditions is evidenced by upregulation in the activities of SOD, Catalase, GPx and GST in testis implicating immediate/efficient turnover of free radicals. In the current model of oxidative stress, the activity of testicular 3β-HSD was significantly reduced among diabetic rats suggesting impairments in reactions leading to testosterone biosynthesis. However, D-Asp administration to diabetic rats elevated the activity of the enzyme suggesting a probable upregulation of StAR mRNA expression and subsequent protective effects on Leydig cells.

Ameliorative effects of testosterone

Androgen deficiency has recently come to the forefront in the medical literature after being ignored for decades and important associations are being developed and confirmed between androgen deficiency and metabolic disorders such as insulin resistance (IR), T2DM and erectile dysfunction (Dhindsa et al., 2004; Pitteloud et al., 2005; Rhoden et al., 2005; Kapoor et al, 2007; Selvin et al., 2007, Grossman et al., 2008). Being globally recognized phenomenon, both diabetes and IR are associated with a severe decline in serum testosterone which has been adjudged as the key factor for hypogonadism observed during chronic diabetes (Steinbrook, 2006). Several studies have suggested that men with low testosterone are at a greater risk of developing T2DM, and that low levels may even predict the onset of diabetes (Stellato et al., 2000; Rhoden et al., 2005; Selvin et al., 2007). Further, a low sex hormone-binding globulin (SHBG) and testosterone predict a higher glucose and increased obesity (Haffner et al., 1996). Several clinical studies have demonstrated that a potential correlation exists between serum testosterone levels and IR (Kapoor et al., 2006), further testosterone supplementation in such hypogonadal men could decrease IR and its consequences. Collectively, these findings raise the possibility of attenuation of diabetes induced testicular dysfunctions by testosterone supplementation.

Administration of testosterone to diabetic PP rats failed to bring any improvement in the body weight gain, while testicular weights were restored towards normalcy implicating protective effects of testosterone on Leydig/Sertoli cells of seminiferous epithelium. Further, testosterone normalized the hyper-glycemia in diabetic rats, which is suggestive of its stimulating or regenerating effect on pancreatic β-cells. Recent evidences demonstrate a strong relationship that exists between sex steroids and pancreatic biology (Robles- Diaz and Duarte-Rojo, 2001). Pancreatic tissue is shown to express steroid specific receptors that respond to steroid hormones (Morales- Miranda et al., 2007) and release insulin. Furthermore, the pancreatic protective action of testosterone was demonstrated in a recent study in which Testosterone supplements reversed STZ-induced early apoptotic damage in castrated animals. The sex steroid mediated glucose

oxidation in extrapancreatic organs was also reported in Chang liver cells, where testosterone exposure enhanced insulin receptor mRNA expression, insulin binding and facilitated glucose oxidation (Parthasarathy et al., 2009).

The ameliorative effects of testosterone were evidenced by significantly lowered levels of oxidative stress markers in both testis cytosol and mitochondria of diabetic rats at both the sampling times. However, the effect was more prominent on day5 raising the safety issues on long term application of testosterone on other bodily functions. However, testosterone administration to diabetic rats did not have any appreciable effect on GSH/non-protein thiols implicating the lesser probability of testosterone to affect thiol modifications under diabetic conditions.

Increased production of oxygen free radicals in diabetes is suggested to be the primary mechanism/s involved in the altered antioxidant enzyme activities in various tissues. Superoxide dismutase (Cu-Zn/Mn-SOD) scavenges superoxide anions into hydrogen peroxide whose altered activities in both cytosol/ mitochondria of diabetic testis reflect increased production of free radicals. A subsequent increase in the activity of catalase, suggests an immediate turnover of these reactive intermediates in order to terminate the propagation of peroxidation events. However, a differential decrease in the activity of catalase on day 10 of post STZ may represent functional inactivation of the enzyme by high levels of blood glucose and/or loss of enzyme cofactors viz., copper and zinc (El Missiry, 1999) which were reversed by Testosterone administration. Further, elevation in GPx activities in diabetic group, on day 5 of post STZ administration suggests its possible upregulation to eliminate organic/inorganic hydroperoxides formed during STZ-induced oxidative insult. However, down regulation in the activity levels at day10 post STZ suggests potential adverse effects of free radicals generated on enzyme activity. Testosterone treatment considerably restored the activity to normalcy suggesting its potential to upregulate antioxidant defenses during pathological conditions as in diabetes.

Alterations in the activities of enzymes viz., G6PDH, LDH and 3β-HSD in testis are often considered as markers of testicular physiology/function. Induction of diabetes elevated the activities of G6PDH and LDH reflecting the

ongoing oxidative stress and extensive membrane damage which were restored to normalcy by testosterone. However, testosterone failed to improve diabetes induced reduction in the activity of 3β-HSD implicating either complete destruction of the enzyme by high circulating glucose and/or functional inactivation of the enzyme by high levels of circulating testosterone. While a constant supply of energy is an essential requirement for the optimum testicular function. Under diabetic conditions, due to elevated oxidative stress, it is likely that the end products of lipid peroxidation may inactivate the enzymes of electron transport chain and deplete cellular energy levels. However, in the present study, elevated activities of NADH/succinate – cytochrome c reductase, implicates a higher requirement for ATP to meet cellular demands.

In conclusion, Testosterone supplements during the acute phase of diabetes significantly abrogate testicular oxidative stress by upregulating enzymic antioxidants.

Modulatory effects of Withania somnifera (WS)

Withania somnifera (WS), popularly known as Ashwagandha is widely used in Ayurvedic formulations to promote physical and mental health, rejuvenate the body in debilitated conditions and increase longevity. Having wide range of activity, it is used to treat almost all disorders that affect the human health. Several of its claimed medicinal properties have been shown to be associated with specific secondary metabolites known as Withanolides. Several Withanolides and alkaloids extracted from WS have demonstrated pharmacological properties including antioxidant, antistressor, aphrodisiac properties (Mishra et al., 2000; Subbaraju et al., 2006). A growing number of reports confirm the potential applications of WS for the treatment/ management of T2DM and associated complications (Andallu and Radhika, 2000; Anwer et al., 2008). Although the pituitary hormone stimulating and spermatogenic activity of WS is well known (Al-Qarawi et al., 2000), no systematic studies have been carried to understand the potential ameliorative effects of WS on diabetes induced testicular dysfunctions.

Oral supplementation of WS reduced the elevated levels of blood glucose in diabetic PP rats suggesting its hypoglycemic effect possibly by its insulin like action and or improving insulin sensitivity. It is also likely that additionally, bioactives of WS may possibly increase the uptake of glucose by peripheral tissues and thus decrease the blood glucose levels (Anwer et al., 2008).

In the present study, diabetes induced testicular oxidative stress was consistent with our previous findings (Chapter 2 and 3). Administration of WS (500mg/kg bw) to diabetic rats significantly lowered the oxidative response evidenced by lowered levels of ROS and lipid peroxidation suggesting its free radical scavenging/ antiperoxidative properties of WS. Further, oral supplementation of WS markedly restored the activities of various antioxidant enzymes among diabetic rats. Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. Because antioxidant enzymes are known to scavenge ROS any change in enzyme activities should provide indirect information about ROS production (Valls et al., 2005). A differential regulation in the activity of cytosolic and mitochondrial forms of SOD clearly suggests the relative vulnerability of the enzyme to diabetes induced free radical damage. A decrease in the activity of cytosolic Cu,Zn-SOD reflects the functional inactivation of the enzyme and subsequent increase in free radical generation, while an increase in mitochondrial Mn-SOD represents an adaptive response under diabetic condition. Hydrogen peroxide, formed as a result of SOD activity, is immediately detoxified by either catalase/glutathione peroxidase. A down regulation in the activity of catalase observed in the present study also suggests the functional inactivation of the enzyme by higher levels of hydrogen peroxide. WS supplementation to diabetic rats failed to reverse the altered activities of SOD, while the activity of catalase was restored to near normalcy implicating differential regulatory effect of WS on antioxidant defenses.

Glutathione peroxidase plays a vital role in testicular mitochondrial function since they lack catalase, and accordingly peroxides formed during stress conditions depend largely on its function (Isabella et al., 2003). Further,

the activity of GPx is determined by continuous flux of the substrate, GSH and its recycling enzyme GR. WS supplementation significantly offset the diabetes induced perturbations in the activities of Catalase, GPx and GR in both cytosol and mitochondria of PP testis suggesting the potential of WS to reverse the inhibitory effects caused due to hyperglycemia and or enhance the gene expression of antioxidant proteins to meet the cellular needs. However, the elevated GST activity in diabetic testis was not modulated by WS supplementation. These results are consistent with earlier report on the hepatoprotective effect of WS glycowithanolides on Iron – induced hepatotoxicity in adult rats (Bhattacharya et al., 1997, 2000). Alterations in testicular thiol levels among diabetic PP rats are consistent with our earlier observation (Chapter 3). Diabetes induced elevation in non-protein thiols and reduction in total thiol levels were restored to normalcy levels by WS, suggesting a probable thiol protecting properties of the extract.

In the present study, activities of enzymes such as G6PDH and LDH were measured in diabetic testis as marker of altered physiology/ membrane damage. The activity of GPx/ GR largely depends on the supply of the NADPH obtained from G6PDH catalyzed reactions. An elevation in the activity levels of G6PDH suggests increased metabolism of free radicals by the GSH dependent enzymes utilizing NADPH as a cofactor. However, elevated NADPH may also upregulate a variety of NADPH dependent oxidases viz., NADPH oxidase, NADPH-cytochrome c reductase etc. that eventually result in enhanced free radical formation. WS supplementation to diabetic PP rats had no measurable effect on the elevated G6PDH activity. Further, elevation in LDH activity in testis cytosol of diabetic rats suggests extensive seminiferous epithelial membrane damage and a possible interference in normal physiology of Sertoli cells/spermatogenesis (Pant et al. 1997) which were restored to normalcy following WS supplementation. The protective potential of WS was further discernable in terms of significant improvement in the activity of 3β-HSD, a regulatory enzyme in testosterone biosynthesis, which was significantly down regulated during diabetes.

In conclusion, we have obtained significant evidence for the first time that in diabetic PP rats, oral supplements of WS has the potential to

significantly mitigate testicular oxidative stress. It is speculated that WS may attenuate oxidative stress *in vivo* through scavenging of free radicals or enhance the level of GSH and antioxidant enzymes that can effectively detoxify free radicals.

6.0 SUMMARY

1. D-Aspartic acid (D-Asp) exposure elicited significant oxidative response in testicular preparations (homogenates/cell suspension) of PP rats *in vitro* which was potentiated in the presence of iron (μM) and under conditions of compromised enzyme activities (GPx and CAT).

2. D-Asp induced oxidative response was significantly abrogated by L-Arginine *in vitro* implicating a regulatory role of L-Arginine.

3. D-Asp (100 & 500mg/kg bw, for 7d)elicited significant oxidative stress response when administered *in vivo* in testis (cytosol/ mitochondria) of PP rats.
4. D-Asp treatment caused enhanced activities of CAT, GPx, Aldehyde dehydrogenase and GST in testis (cytosol/mitochondria) of PP rats suggesting increased generation of free radicals and possibly toxic aldehydes.

5. Enhanced levels of testicular GSH in (cytosol/mitochondria) are suggestive of the ongoing oxidative stress and increased requirement of GSH to quench the free radicals generated under D-Asp exposure.

6. D-Asp induced mitochondrial dysfunctions were discernable by altered activity levels of ETC enzymes, disturbances in membrane integrity/leakage and subsequent increase in intracellular calcium levels.

7. However, administration of D-Asp to diabetic PP rats significantly reduced the glucose levels probably by increasing the insulin sensitivity.

8. Diabetes induced oxidative dysfunctions in testis cytosol were significantly offset by D-Asp administration as evident by decreased level of oxidative markers and restoration of antioxidant enzyme to normal levels.

9. D-Asp significantly elevated the activity of 3β-HSD among both non-diabetic and diabetic rats suggesting its potential steroidogenic role.

10. Administration of Testosterone propionate (TP, 5mg/kg bw/d for 5 and 10days) significantly lowered blood glucose levels in diabetic rats suggesting its antihyperglycemic effect.

11. Protective effects of TP among diabetic PP rats were discernable in terms of reduction in the incidence/ extent of testicular atrophy, implicating its role in maintaining the cellular integrity of seminiferous epithelium.

12. TP supplements significantly abrogated diabetes induced testis oxidative impairments as evidenced by reduced levels of oxidative markers in cytosol and mitochondria. The degree of protection was relatively higher on day 5.

13. TP supplementation resulted in a dramatic restoration of ROS and MDA levels in testis cytosol and mitochondria and lowered the extent of protein oxidation.

14. Pertubation in the thiol redox status, alterations in the activities of major antioxidant activities were restored to normalcy following TP supplementation suggesting an efficient turnover of free radicals generated.

15. Protective effects of TP were also evidenced by lowered activities of LDH-X and ETC enzymes implicating its mitochondrial membrane stabilizing effect.

16. TP supplementation to diabetic PP rats had no appreciable effect on the activity of 3β-HSD suggesting the probable inhibition of the enzyme by high levels of circulating testosterone.

17. Induction of diabetes caused significant hyperglycemia among diabetic PP rats and oral administration of WS supplements (500 mg/kg bw/d, 15d) significantly lowered blood glucose levels suggesting its antihyperglycemic potential.

18. Supplementation of WS to diabetic PP rats markedly improved body weight gain and testicular weights suggesting its tissue protective effects.

19. Testis of diabetic PP rats exhibited an increased free radical mediated oxidative damage and supplementation of WS significantly attenuated the oxidative damage as evidenced by lowered ROS/MDA levels possibly by scavenging free radicals.

20. WS supplementation significantly restored the thiol redox status in testis of diabetic PP rats implicating enhanced thiol regeneration.

21. Diabetes induced perturbations in antioxidant enzyme activities were restored to normalcy with WS supplementation clearly suggesting its propensity to upregulate defense mechanism during oxidative stress.

22. Enhanced activities of GPx and GR in diabetic testis with WS supplements suggest an increased turnover of free radicals and efficient recycling of GSH.

23. WS supplementation significantly restored the activity of LDH-X to normalcy in testis of diabetic PP rats implicating its protective influence on seminiferous membrane and normal physiology of testis.

24. WS supplementation moderately elevated the activity of 3β-HSD in testis of diabetic PP rats suggesting its potential to upregulate steroidogenic machinery/ pathways that lead to testosterone biosynthesis.

CONCLUSIONS

1. A dosage of STZ (90mg/kg bw) in prepubertal rats, resulted in nearly 4-5 fold increase in plasma glucose levels indicating induction of consistent hyperglycemia was designated as ‘optimum dose’. The oxidative stress induction response was relatively higher in testis compared to other somatic organs viz., liver and brain as evidenced by enhanced generation of ROS and MDA levels suggesting higher vulnerability of PP testis to diabetes induced oxidative damage. The increased susceptibility reflects lower degree of antioxidant defenses and/or exhaustive utilization of antioxidant defenses in the testis of PP rats.

2. Age associated vulnerability studies showed increased susceptibility of 4wk-old PP rats to diabetes induced oxidative stress compared to 6wk-old PP rats. The increased susceptibility was discernable in terms of elevated ROS, MDA, hydroperoxides and NO levels, perturbations in enzymic/non-enzymic antioxidants and altered activities of major functional enzymes as sorbitol dehydrogenase, lactate dehydrogenase in testicular milieu diabetic PP rats clearly suggesting the ongoing oxidative disturbances *in vivo*.

3. Elevated redox thiol status and ascorbate levels among 4wk-old PP rats suggestive of the vital role played by these antioxidants to combat the oxidants generated during diabetic conditions. A differential upregulation in testis ascorbic acid among diabetic PP rats suggest regulatory differences in antioxidant systems in developing testis.

4. In testis mitochondria of diabetic rats of both the age groups, the activities of citrate synthase and succinate dehydrogenase were consistently lower. These changes were accompanied by altered activities of oxidative phosphorylation enzymes, loss in electrochemical gradient, membrane transition pore opening and swelling.

5. An acute dose of STZ (90mg/kg bw) in prepubertal rats resulted in consistently elevated glucose levels in both acute and progressive phase. In testis cytosol, salient findings viz., enhanced generation of ROS and MDA levels at day 1 clearly suggests that they are subjected to significant oxidative stress under STZ-induced diabetes. Consistent, progressive increase in lipid peroxidation, hydroperoxide and NO levels in testis cytosol suggests clearly the involvement of oxidative stress in the development of testicular dysfunctions.

6. In testis mitochondria of diabetic PP rats, elevated oxidative stress response was discernable only during progressive phase, while the response was consistent in both acute and progressive phase in testis microsomes.

7. Perturbations in major non- enzymic antioxidants viz., GSH, vitamin E and ascorbic acid in testis cytosol/mitochondria were accompanied with significant alterations in the activities of antioxidant enzymes such as SOD, CAT, GPx implicating disturbances in free radical detoxification machinery subsequently elevating peroxidative damage. Diabetes induced oxidative damage was further evident in terms of consistently elevated activity of GST, that is actively involved in elimination of toxic aldehydes formed during peroxidative injury.

8. Oxidative damage to protein was evident in terms of significant increase in protein carbonyl levels in both testis cytosol/mitochondria of diabetic rats.

9. Alterations in the activities of major functional enzymes such as G6PDH, SorDH, LDH-X suggests increased tissue damage by the oxidative products of glucose metabolism eventually leading to impaired spermatogenesis. Significant reduction in the activity of 3β-HSD in testis of diabetic PP rats, indicates altered Leydig cell function and suggests the important regulatory role of oxidative stress in steroidogenic function.

10. The testis mitochondria of diabetic rats showed significant reduction in the activities of citrate synthase and succinate dehydrogenase suggesting lowered efficiency and probable reduced flux of glycolytic intermediates into TCA cycle. Further, elevated activites of oxidative phosphorylation enzymes implicates increased demand for cellular energy to maintain the normal mitochondrial integrity and metabolism.

11. Testis mitochondria from diabetic PP rats exhibited relatively higher susceptibility to $CaCl_2$ induced membrane permeability transition that resulted in loss of transmembrane potential and leakage of cytochrome C into cytosol that activates cell death cascade (Caspase mediated).

12. Testis microsomes from diabetic PP rats also exhibited significant perturbations in the activities of cytochrome P450 and Ethoxy-Resorufin-O- dealkylase suggesting ongoing oxidative stress in testis microsomes.

13. Increased incidence of testicular atrophy discernable during diabetic conditions is primarily be accounted to irreversible loss of germ cell population that constitutes major portion of testicular cell types during developmental stages. The increased testicular degeneration was accompanied by fragmentation of nuclear DNA measured as comets.

14. Exposure of PP testis to D-Aspartic acid (D-Asp) resulted in significant oxidative stress response induction which was markedly attenuated by L-Arginine *in vitro*. D-Asp induced oxidative response was further potentiated in presence of iron and under conditions of compromised enzyme activities (GPx and CAT) *in vitro*.

15. D-Asp induced oxidative stress response was also demonstrable in testis of PP rats *in vivo* as evidenced by enhanced ROS, MDA and hydroperoxide levels, perturbations in both enzymic/non-enzymic antioxidants in both cytosol and mitochondria. Enhanced levels of GSH in (cytosol/mitochondria) are suggestive of the ongoing oxidative stress

and increased requirement to quench the free radicals generated following D-Asp exposure.

16. In testis mitochondria of D-Asp treated rats, salient findings viz., altered activity levels of ETC enzymes, disturbances in membrane integrity unequivocally suggest mitochondria are the principal target for the observed prooxidant activity of D-Asp.

17. Administration of D-Asp to diabetic PP rats resulted in significant reduced hyperglycemia probably by increasing insulin sensitivity. Further, D-Asp administration significantly offset diabetes induced oxidative dysfunctions in testis cytosol as evidenced by decreased level of oxidative markers and restoration of antioxidant enzyme activities.

18. D-Asp administration to diabetic rats exhibited marked elevation in the activity levels of 3β-hydroxy steroid dehydrogenase suggesting its potential steroidogenic role.

19. Administration of Testosterone propionate (TP, 5mg/kg bw/d for 5 and 10days) lowered blood glucose levels and reduced the incidence/ extent of testicular atrophy among diabetic rats.

20. TP administration to diabetic rats resulted in dramatic restoration of both ROS/ MDA and protein carbonyl levels, activities of antioxidant defenses/enzymes in both testis cytosol and mitochondria.

21. TP administration significantly offset the elevated activities of LDH-x and ETC enzymes implicating its mitochondrial membrane protective effects. effect. However, TP administration failed to show any improvement in the activity of 3β-HSD suggesting the probable destruction of the enzyme by high levels of circulating glucose during diabetic conditions.

22. Oral supplementation of WS to diabetic rats (500 mg/kg bw/d, 15d) lowered blood glucose levels, significantly improved body/testicular

weights suggesting its antihyperglycemic, antistress and tissue protective properties.

23. WS supplements significantly attenuated diabetes induced oxidative damage in PP testis as evidenced by lowered ROS/MDA levels, restored thiol redox status and activities of antioxidant enzymes suggesting its free radical scavenging, propensity to upregulate antioxidant defense mechanism during conditions of oxidative stress.

24. WS supplementation significantly restored the activities of LDH-x and 3β-HSD in testis of diabetic PP rats implicating its protective influence on seminiferous epithelial membrane which maintain normal physiology of testis and spermatogenesis.

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