BIOCHEMICAL PERTURBATIONS IN DEVELOPING EMBRYOS ASSOCIATED WITH MATERNAL EXPOSURE TO OXIDATIVE STRESS: AND ITS ATTENUATION BY BIOMOLECULES

A Thesis submitted to the
Faculty of Biochemistry
University of Mysore

for the degree of
Doctor of Philosophy

by
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DECEMBER 2007

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DECLARATION

I hereby declare that the thesis entitled '**Biochemical perturbations in developing embryos associated with maternal exposure to oxidative stress: and its attenuation by biomolecules**' 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 of Dr. Muralidhara, Scientist, Department of Biochemistry and Nutrition, Central Food Technological Research Institute during the period Oct 2002 to Nov 2005.

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

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Date: 19th December 2007

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CERTIFICATE

I hereby certify that the thesis entitled "**Biochemical perturbations in developing embryos associated with maternal exposure to oxidative stress: and its attenuation by biomolecules"** submitted by **Mr. Mahesh MS** for the degree of Doctor of Philosophy in Biochemistry, University of Mysore is the result of research work carried out by him in the Department of Biochemistry and Nutrition, CFTRI, Mysore under my guidance and supervision during the period of Oct 2002 – Nov 2005.

Dr. MURALIDHARA
Guide

Place: Mysore
Date: December 2007

Dedicated to the lotus feet of

Lord Shiva, the Omnipotent, Omniscient

and Omnipresent

ACKNOWLEDGEMENTS

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

I wish to express my deepest and warmest gratitude to :

My mentor and guide, Dr. Muralidhara, for his guidance in the scientific work and for his everlasting patience over these years. His encouraging and unrelenting attitude towards scientific work has carried my fate over minor and major problems. It has been a privilege to work under his guidance.

Dr. Salimath PV, Head, Biochemistry & Nutrition for his encouragement.

The Director, CFTRI, for giving me an opportunity to work for my PhD.

The CSIR, New Delhi, for the award of a Senior Research Fellowship.

Dr. P. S. Rajini, Scientist, FPIC Dept., for her encouragement and support throughout my stay at CFTRI.

Prof. Karuna Kumar M, Chairman, Biochemistry, University of Mysore, for his constructive criticism during the doctoral committee proceedings.

Dr. MC Varadaraj, Head, Human Resource Development and all the members of the Upgradation Committee for their suggestions.

Dr. T.P. Krishnakantha, Dr. V. Baskaran, Dr. K.S. Jagannatha Rao, Mr. A.R. Varadaraj, Dr. K. Srinivasan Mr. Jagadish L Urs, Mr. Rathina Raj, Mr. Vijay Kumar BV, Ms. Shivarajamma and other staff of the Dept. of Biochemistry and Nutrition for their timely help during the course of my work.

My colleagues at CFTRI, Dr. Shrilatha B, Mr. Doreswamy K, Mr. Thyagaraju BM, Ms. Shinomol George K, Mr. Chandrashekar KN, Mr. Ravikumar Hosmani, Mr. Santosh Sebastian, Mr. Vasudev Kamath, Mr. Kisan Jadhav, Mr. Apurva Kumar and Mr. Shashikumar for whom words would never suffice to say just thanks.

Dr. Manjunatha, Dr. Muralidhara Hegde, Ms. Anitha, Ms. Anitha Nagaraj, Ms. Chitra, Mr. Raju, Ms. Ajila, Mr. Ani V, their timely help and their great company.

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

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

Dr. Manoj Joshi, Dr. Vilas Sinkar, Dr. Shovan Ganguli of Hindusthan Unilever Ltd for their encouragement and support.

My parents, Shivananjappa and Annapurna, Godfather Basavanna, Wife Padma, Son Shivaank, Brothers {Yathish, Vishwesh, Sharath and Manjunath}, Sisters {Vasantha and Hemalatha}, Ningamma for their love, support, encouragement and constant prayers.

All those rats, unborn fetuses and embryos which have sacrificed their lives and continue to live as data in my thesis.

MS Mahesh

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LIST OF SYMBOLS AND ABBREVIATIONS

$O_2^{\cdot -}$	Superoxide radical
$^{\cdot}OH$	Hydroxyl radical
$^1O_2$	Singlet oxygen
α	Alpha
β	Beta
$^{\circ}C$	Degree Celsius
BSA	Bovine serum albumin
bw	Body weight
CDNB	1-chloro-2,4-dinitrobenzene
DCF	Dichloro fluorescein
DCFH-DA	Dichloro fluorescin diacetate
dl	Deci litre
DI	Dead implantation
DM	Diabetes mellitus
EDTA	Ethylene diamine tetraacetic acid
g	Gram
i.p.	Intra peritoneal
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione-s-transferase
G6PDH	Glucose-6-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
h	Hour(s)
Kg	Kilo gram
µg	Micro gram
µmol	Micromole
µL	Micro litre
LPO	Lipid peroxidation
LI	Live embryos
M	Molar
MDA	Malondialdehyde
min	Minutes
ml	Milliliter
mg	Milligram
ng	Nanogram
nm	Nanometer
N	Normality
n	Number
NADH	Nicotinamide adenine Dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
OS	Oxidative stress
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Standard deviation
SOD	Superoxide dismutase
STZ	Streptozotocin
TAU	Taurine
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
tbHP	Tertiary butyl hydroperoxide
v/v	Volume by volume
w/w	Weight by weight

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ABSTRACT

Pregnancy is a physiological state accompanied by a high metabolic demand and increased requirement for tissue oxygen and this increased oxygen requirement increases the rate of production of reactive oxygen species. Exposure of mother to environmental chemicals, drugs etc., can induce certain vital biochemical/physiological effects, which can result either in specific malformations in fetus or can cause maternal/fetal morbidity. Higher maternal lipid peroxide levels have been demonstrated in normal pregnancy and in pre-eclampsia. Given the understanding that LPO is high during normal pregnancy, it can be speculated that maternal and fetal tissues are likely to be more susceptible to exogenous prooxidants. Hence studies to understand the oxidative implications of maternal exposure to prooxidants during the gestation period and their impact on the developing embryos, and embryo-protective mechanisms which operate under such situations would be very relevant in animal models.

Iron deficiency and subsequent iron deficiency anemia are the most prevalent nutrient deficiency problems afflicting pregnant women. Pregnant women in developing countries are generally given daily supplements of iron in order to prevent and correct gestational iron deficiency and this dose of iron (120 mg) which is 10 times the normal daily dietary iron intake is shown to cause gastrointestinal side effects. More importantly, equivalent doses of high-iron supplements in male rats are shown to result in significant increases in LPO. Interestingly, *iron deficient* rats are also shown to be subjected to significant oxidative stress clearly suggesting that both *iron deficiency* and *iron excess* promote oxidative stress. Further, data along similar lines are also essential in animal models under experimentally-induced *iron deficient* conditions during pregnancy.

Diabetes is a chronic metabolic disorder, characterized by absolute or relative deficiency in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances in carbohydrate, lipid and protein metabolism. Uncontrolled diabetes mellitus is an etiological factor for recurrent

pregnancy loss, fetal growth disorders and major congenital malformations in the offspring. Antioxidant therapy has been advocated to overcome the oxidant–antioxidant disequilibrium inherent in diabetes and various studies have demonstrated the beneficial effects of several antioxidants during pregnancy. Currently there is an increased interest in the identification/ characterization of naturally occurring antioxidants for their potential use as dietary supplements during pregnancy.

The basic objectives envisaged were: a) To examine whether maternal exposure to *prooxidants* during specific gestational period causes differential oxidative stress among embryos/fetus b) To understand the oxidative implications of *iron supplements* on induction of oxidative stress in maternal milieu during specific gestation period and its implications on developing embryos/fetus. C) To examine if *iron deficiency during gestation* causes oxidative stress in the maternal milieu and its impact on embryonic and fetal oxidative response and d) to establish the degree of oxidative damage in diabetic dams and their correlation with oxidative implications in embryos/ fetus and assess the ameliorative propensity of garlic and selected medicinal plants.

In the first series of investigations, pregnant dams exposed to a prooxidants (t-butyl hydroperoxide or iron) at sublethal doses during specific periods of gestation (GD_{5-7} or GD_{8-10}) showed elevated oxidative induction response in the maternal tissues as evident by elevated MDA/ROS levels , depleted GSH levels, total thiols and diminished levels of antioxidant enzymes. Induction of oxidative response was of higher magnitude when prooxidant exposure occurred during GD_{5-7} compared to GD_{8-10} exposure suggesting an enhanced vulnerability of embryos to prooxidants during early gestation. Maternal exposure to prooxidants resulted in significant oxidative implications in both embryos (GD_{13}) and fetal tissues. Taken together, these findings suggested that maternal exposure to prooxidants during *early gestation* induces significant oxidative stress in maternal milieu, placenta and higher oxidative impact among embryos during early post-implantation period (GD_{5-7}) compared to GD_{8-10}.

In the second series of investigations, pregnant dams administered with oral doses (2-8 mg/rat/d) during gestation failed to show any clinical signs of toxicity, while the placental weights were increased (21-45%) at the highest dose. Differential pattern of ROS induction was evident in cytosol and mitochondria of maternal organs which was accompanied with marginal reduction in GSH levels, diminished total thiol/non-protein thiols, and elevated protein carbonyls levels. Placental tissue was also subjected to significant oxidative stress in this iron excess model. Maternal exposure to iron resulted in significant oxidative impact among embryos and fetal tissues as evidenced by elevated ROS and MDA levels in both cytosol/ mitochondria, reduction in the activities of antioxidant enzymes, marked diminution of GSH and total thiols/non protein thiols. Among iron deficient (Idd) diet fed dams, the incidence of embryolethality was significantly higher compared to the controls. Maternal tissues of Id dams were subjected to significant oxidative stress since both ROS levels and lipid peroxidation were significantly elevated. Interestingly, embryos (GD_{13}) and fetal organs of Id dams were also subjected to marked oxidative impact as evidenced by significant elevation in ROS and MDA levels, depleted GSH, total thiols, alterations in the activities of antioxidant enzymes and increased oxidative damage to proteins.

In the third phase of investigations, employing a streptozotocin diabetic pregnant rat model, we observed higher incidence of embryolethality and significant reduction in fetal weights. Diabetes induction caused significant oxidative stress in maternal organs sampled on GD 13/20. Further, embryos (GD_{13}) and fetal (GD_{20}) organs of diabetic dams exhibited significant oxidative stress as evidenced from elevated MDA/ROS levels (cytosol/mitochondria), diminished GSH/total thiols and reduced activities of antioxidant enzymes. In two parallel studies, the ameliorative efficacy of garlic supplements and dietary supplements of selected medicinal plants (viz., *Gymnema sylvestre, Tinospora cordifolia, Ipomea aquatica and Withania sominifera*) on diabetes associated embryolethality, placental weights, IUGR, oxidative impairments in maternal organs, embryonic tissue and fetal tissues have been assessed.

INTRODUCTION

Oxidative stress defined as the imbalance between oxidants and antioxidants in favour of the former is an inevitable process occurring in aerobic life. When the oxidants overwhelm the antioxidant defenses, it results in multiform cell damage specifically attacking the vital macromolecules–lipids, proteins and nucleic acids and poses a threat to the healthy survival of the cells. Severe oxidative stress can cause cell injury and cell death. There is increasing evidence that oxidative damage is associated with several age-related diseases, including cardiovascular, cancer, neuro-degenerative and hepatic diseases.

Pregnancy is a physiological state accompanied by a high metabolic demand and increased requirement for tissue oxygen which increases the rate of production of reactive oxygen species (ROS). Exposure of mother to environmental chemicals, drugs etc., during pregnancy can induce certain vital biochemical/physiological effects, which can result either in specific malformations in fetus or fetal morbidity. Several studies have found maternal lipid peroxide (LP) levels higher in normal pregnancy *per se*. Further, such increased LP levels have also been reported in preeclampsia with decreased antioxidant levels. LP's are also produced in placenta, although their pattern of change over the course of pregnancy is not clear.

Iron deficiency and subsequent iron deficiency anemia are the most prevalent nutrient deficiency problems afflicting pregnant women. Pregnant women in developing countries are generally given daily supplements of iron in order to prevent and correct gestational iron deficiency and this dose of iron (120 mg) which is 10 times the normal daily dietary iron intake is shown to cause gastrointestinal side effects. More importantly, equivalent doses of high-iron supplements in rats is shown to result in significant increases in lipid peroxidation Interestingly, iron deficient rats are also shown to be subjected to significant oxidative stress clearly suggesting that both iron deficiency and iron excess promote oxidative stress. In view of this, comprehensive understanding on the

oxidative implications of iron supplements in pregnant rats fed normo-protein and low- protein diet during pregnancy and their oxidative impact on embryos and fetus is warranted. Further, data along similar lines are also essential in animal models under experimentally- induced iron deficient conditions during pregnancy.

Normal pregnancy is associated with an increase in oxidative stress and lipid peroxidation, but simultaneously several antioxidants increase in the serum progressively as pregnancy advances. Given the understanding that lipid peroxidation is high during normal pregnancy, it can be speculated that maternal and fetal tissues are likely to be more susceptible to exogenous prooxidants. Hence studies to understand the oxidative implications of maternal exposure to various prooxidants during the gestation period as well as their impact on the developing embryos and the embryo-protective mechanisms operating under such situations would be useful. Only a thorough understanding of the underlying pathophysiology of pregnancy associated oxidative stress would help in the development of therapeutic strategies to ameliorate oxidative stress in maternal milieu and prevent fetal complications.

Uncontrolled diabetes mellitus is an etiological factor for recurrent pregnancy loss, fetal growth disorders and major congenital malformations in the offspring. Antioxidant therapy has been advocated to overcome the oxidant – antioxidant disequilibrium inherent in diabetes. Further, it is generally understood that radical excess or inadequate levels of antioxidants in the vascular endothelium in pregnancy can lead to pregnancy induced hypertension or preeclampsia. Various laboratory studies have demonstrated the beneficial effects of several antioxidants (e.g., Vitamin C, Selenium and Vit E) during pregnancy. Currently, there is an increased interest in identification and characterization of naturally occurring antioxidants for their potential use as dietary supplements during diabetic pregnancy.

The *objectives* envisaged at the beginning of the investigation were:

i) To examine whether maternal exposure to prooxidants during specific gestational period causes significant oxidative impact in embryos and fetus

ii) To understand the oxidative implications of *Iron supplements on induction of oxidative stress* in maternal milieu during specific gestation period and its implications on developing embryos and fetus.

iii) To establish whether *iron deficiency during gestation* causes oxidative stress in maternal milieu & its impact on embryonic and fetal oxidative response.

iv) To establish the degree of oxidative damage in diabetic pregnant dams, and their correlation with oxidative implications in embryos/fetus and assess the ameliorative propensity of dietary spice and selected medicinal plants.

The thesis is presented as four chapters, preceded by a general introduction.

CHAPTER 1

Materials and Methods

In this chapter, details regarding the materials used for the investigations including list of chemicals, animal care, ethical considerations, treatment protocols and various assay methods employed have been presented**.**

CHAPTER 2

Maternal exposure to prooxidants during specific periods of gestation; pattern of susceptibility of embryos and fetus to oxidative stress

Pregnancy is a condition exhibiting increased susceptibility to oxidative stress which can be described as disturbance in the prooxidant–antioxidant balance in favor of the former, leading to potential damage. There are specific evidences to show that during pregnancy a state of oxidative stress exists *in vivo.* Elevated serum levels of LPO products such as diene conjugates and TBARS levels are known to increase in normal pregnant women along with enhanced defense mechanism/s against free radical damage as pregnancy progresses. Despite this, the effects of maternal exposure to prooxidants during *specific periods of gestation* on the oxidative implications in embryos and fetus have not been comprehensively investigated. More importantly, it is not known whether

there are *critical windows* during which prooxidant exposure causes higher deleterious effect on the developing embryos /fetuses.

Accordingly, in the present study, two model prooxidants *viz., t-butylhydroperoxide (tbHP)* and *Iron* were chosen in order to address the above questions. Sublethal doses of the prooxidants were administered during specific gestation days (GD) 5, 6 & 7 or 8, 9 & 10. Oxidative impact in embryos was investigated by sampling of embryos (GD_{13}) and fetal tissues (GD_{20}), while induction of oxidative stress was assessed in maternal tissues during both sampling times. The results are presented under two sections *A and B.*

Section A comprises of results of oxidative implications in pregnant dams following their exposure to t-bHP, a model organic hydroperoxide. t-bHP caused marked elevation in oxidative stress response in maternal tissues. In liver, both cytosol and mitochondria were subjected to oxidative stress as evident by elevated MDA and ROS levels which were accompanied by depleted GSH levels, total thiols, and diminished levels of antioxidant enzymes. tbHP administered during early implantation period (GD_{5-7}) induced a higher degree of oxidative stress in the maternal tissues, while induction in placenta was of similar degree irrespective of the dosing regimen. Maternal exposure to tbHP resulted in a marked elevation of oxidative perturbations among GD_{13} embryos measured in terms of ROS generation and MDA levels and were associated with reduced antioxidant enzyme activities, total/non protein thiols. Both brain and liver of fetuses (GD_{20}) obtained from prooxidant administered dams also showed enhanced ROS/MDA levels, protein carbonyls, depleted GSH and diminished activities of antioxidant enzymes suggesting a state of oxidative stress *in vivo.*

Section B comprises of results pertaining to iron -induced oxidative stress in maternal tissues, embryos and fetal tissues. Iron exposure at sublethal doses to pregnant dams during early implantation period (GD_{5-7}) or late implantation period (GD_{8-10}) caused significant oxidative impairments in maternal tissues

(liver and kidney). In liver, both cytosol and mitochondria were subjected to oxidative stress as evident by elevated MDA/ROS levels and were accompanied by depleted GSH levels, total thiols and diminished levels of antioxidant enzymes. Iron exposure during early implantation period (GD_{5-7}) resulted in a higher degree of induction in maternal tissues as well as considerable oxidative stress in placental tissue. Interestingly, maternal exposure to iron resulted in markedly elevated oxidative perturbations in GD_{13} embryos measured as ROS generation and MDA levels with concomitant reduction in antioxidant enzyme activities, total thiols and non-protein thiols. Both brain and liver of fetuses of iron administered dams showed marked enhancement of ROS/MDA levels, protein carbonyls, depleted GSH content and diminished activities of antioxidant enzymes.

CHAPTER 3

Iron excess and Iron deficiency during gestation: oxidative implications in maternal milieu and its impact on embryos and fetus

Iron induced oxidative stress has been implicated in the toxic effects of many chemicals, tissue injuries and disease processes. Iron deficiency is the most common nutritional deficiency worldwide, affecting approximately two billion people, mostly women and children. Iron deficiency is associated with an increased risk of poor pregnancy outcomes and impaired cognitive development in young children. Pregnant women in developing countries are commonly given daily supplements of iron to prevent and correct gestational iron deficiency. High iron supplements in rats result in an abnormal accumulation of intestinal, mucosal and hepatic nonheme iron and significant increase in lipid peroxidation. Recent studies demonstrated that iron deficient male rats also had markedly increased lipid peroxidation suggesting that both iron deficiency and iron excess promote oxidative stress. However, such a phenomena has not been described during pregnancy state. Accordingly, in the present study, investigations were conducted in pregnant rats employing an i) iron excess model and ii) iron deficiency model. The results are presented under two sections *A and B.*

Section A describes the results of oxidative perturbations among pregnant dams *orally administered with iron* during specific periods of gestation and their oxidative impact on embryos and fetal tissues. Iron at higher doses (4 and 8 mg/rat/d) caused a significant reduction in maternal body weight gain and increased placental weights. In maternal milieu, differential induction of ROS levels was evident in cytosol and mitochondria, the induction levels being relatively higher in mitochondria compared to the cytosol. However, the MDA levels were higher in iron administered dams during the dosing regimen GD_{5-12}. In liver, the thiol levels were uniformly diminished irrespective of the dosing regimen. In general, the activities of antioxidant enzymes were significantly decreased at higher doses. The placental ROS levels were markedly enhanced, while the induction levels of MDA were relatively of lesser magnitude.

Among embryos of iron administered dams, significant elevation in ROS levels occurred at the highest dose with concomitant enhancement in the MDA levels. Marked diminution of GSH (40%), total thiols (47%) and non protein thiols (36%), reduced activities of antioxidant enzymes were accompanied with elevated protein carbonyl content. Further, it also caused marked oxidative impact in fetal tissues as evidenced by significantly elevated ROS/MDA levels in brain, while the induction of LPO was less robust. At higher doses, significant diminutions in the levels of GSH were accompanied with elevated total thiol levels, while the protein carbonyl levels were significantly elevated. Significant reduction in the activity of antioxidant enzymes was evident at higher doses in brain, although the reductions in fetal liver were less robust.

Section B describes investigations related to the occurrence of oxidative impairments in maternal milieu associated with iron deficiency during pregnancy and its oxidative impact in embryos and fetuses. Iron deficiency was induced by following a dietary regimen consisting of feeding adult females on a diet totally devoid of iron for two weeks prior to pregnancy and throughout gestation. Significant decreases in body weight (17-25%), placental weights and increased incidence of embryolethality was evident among dams fed iron deficient diet.

Evidences such as elevated ROS levels and MDA levels in maternal organs clearly suggested that iron deficiency also induced a state of oxidative stress in the maternal milieu. Increased protein oxidation was also evident as protein carbonyl content was also enhanced. Moderate decrease in reduced GSH was accompanied with reduced activities of antioxidant enzymes in both cytosol and mitochondria of brain and liver. Placental tissue was also subjected to significant oxidative stress as revealed by elevations in ROS, MDA levels, reduced GSH levels and increased protein carbonyls. Significant oxidative impact was discernible in both embryos and fetuses. Among embryos of dams fed Iron deficient diet, marked enhancement of ROS and MDA levels were observed in both cytosol and mitochondria. Other major evidences were: diminished levels of GSH, total thiols, elevated protein carbonyls and significant perturbations in the activity of antioxidant enzymes. Among fetuses obtained from iron deficient dams, significant oxidative impairments were noted in brain and liver. Other important observations were: marked enhancement of brain ROS levels, moderate elevation in MDA levels and lowered activity of antioxidant enzymes.

CHAPTER 4

Experimentally induced diabetes during gestation: oxidative stress in mother, post-implantation embryos/fetus and its amelioration

The steadily increasing trend in the prevalence of diabetes mellitus (DM) is of global concern and women are reported to be more prone to type 1 DM than men. About 5% of all pregnancies occur among diabetic women and DM is often diagnosed in women during pregnancy. Maternal diabetes during pregnancy is associated with increased risk for growth disturbances and congenital malformations. Increased (2-3 fold) incidence of major congenital malformations is reported in the offspring of diabetic women compared to that of the general population. The malformations occur during the period of embryonic organogenesis and the nature observed in type 1, type 2 and gestational diabetes

are similar. Uncontrolled diabetes has been implicated as an etiological factor for recurrent pregnancy loss and there is an estimated incidence of 17% spontaneous abortion in DM pregnancies. Experimental studies in laboratory animals have demonstrated a consistent intrauterine growth restriction and fetoplacental abnormalities. However, the mechanisms of diabetic embryopathy are not clearly understood. Nevertheless, there is strong evidence that there is oxidant–antioxidant disequilibrium in DM. Hence, our primary objective was to establish the nature/ degree of oxidative impairments occurring among diabetic pregnant rats and to assess the ameliorative propensity of dietary components. The results are presented under two sections, *A* and *B*.

Section A comprises of basic investigations pertaining to induction of diabetes in pregnant rats (CFT-Wistar) and measurement of hyperglycemia, quantification of oxidative implications in maternal tissues (GD_{13} and GD_{20}) in order to examine the progression and pattern of oxidative stress. Administration of STZ (45 mg/kg bw, i.p.) to pregnant rats on GD_4 induced significant hyperglycemia, higher embryolethality (40%) and significant IUGR. Diabetes induction caused significant oxidative stress in maternal organs (liver, kidney) measured on GD_{13} in terms of increased generation of ROS levels, elevated lipid peroxidation, perturbations in antioxidant defenses and significant elevation in placental lipid peroxidation and alteration in antioxidant defenses. The embryos (GD_{13}) were subjected to significant oxidative stress as evident from markedly elevated MDA/ ROS levels in cytosolic/mitochondrial fractions, diminished GSH/total thiols and reduced activities of antioxidant enzymes. The oxidative impairments in maternal organs (GD_{20}) also showed more robust induction as evident by marked elevation in LPO, ROS levels, depleted GSH and total thiols antioxidant enzymes and higher protein carbonyls. The fetal (GD_{20}) tissues such as brain and liver tissues were also subjected to marked oxidative stress as measured by increased ROS/MDA levels and alterations in antioxidant defenses.

Section B comprises of studies pertaining to amelioration of diabetes induced oxidative implications in maternal tissues and their impact on embryos and fetuses by garlic and few medicinal plants. Garlic supplements to diabetic pregnant rats marginally reduced the blood glucose levels, partially restored the loss in body weight and the magnitude of polyuria. Garlic significantly reduced the incidence of embryolethality and markedly restored the fetal weights. More importantly, garlic markedly offset diabetes associated oxidative impairments in maternal organs as evident by the restoration of various oxidative markers such as MDA/ ROS levels, GSH content and enzymic antioxidants. Placental weights and oxidative stress among STZ rats was also significantly abrogated by garlic (50% protection). Other important observations were: restoration of ROS and MDA levels, GSH levels, perturbations in antioxidant defenses and thiol content.

In another study, we examined the efficacy of oral supplements of medicinal plants viz., *Gymnema sylvestre (GS), Tinospora cordifolia (TC), Ipomoea aquatica (IA), and Withania somnifera (WS)* to non-diabetic and diabetic pregnant rats. While the dietary supplements to STZ-diabetic rats marginally reduced the glucose levels, significant beneficial effects were demonstrable among diabetic rats in terms of abrogation of oxidative stress biomarkers. The incidence of embryolethality was reduced significantly by all the dietary supplements (GS>TC>IA>WS). Likewise, significant protection was evident in terms of reduction in IUGR and the supplements provided considerable protection against diabetes associated oxidative impairments among both maternal and fetal tissues.

A section titled **Conclusions** is included in which all the salient findings of this investigation have been listed. At the end of the thesis, a comprehensive list of cited **Bibliography** is presented.

Dr. Muralidhara **Mahesh MS**

GUIDE **Candidate**

MATERIALS AND METHODS

Chemicals

1,1,3,3-tetramethoxy propane (TMP), 1-chloro-2,4-dinitro benzene (CDNB), 2,4-dinitrophenylhydrazine (DNPH), 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), 2',7'-dichlorofluorescein (DCF), 2',7'-dichlorofluorescin diacetate (DCFH-DA), bovine serum albumin (BSA), glutathione reductase, hydrogen peroxide, NADH, NADP, NADPH, reduced and oxidized glutathione (GSH and GSSG), t-butyl hydroperoxide (70% aqueous CAS # 75-91-2), thiobarbituric acid, xanthine and xanthine oxidase were procured from M/s. Sigma Chemical Co., (St. Louis, MO, USA). Folin's reagent, o-pthalaldehyde, sodium dodecyl sulfate and tricholoro acetic acid were procured from M/s. Sisco Research Laboratories, Mumbai, India. All other chemicals used were of analytical grade.

Iron Dextran (ID)

Ferric hydroxide dextran complex, (100mg/ml containing 0.5% phenol as preservative) was obtained from M/s. Sigma-Aldrich Chemical Co., USA.

Diabetogenic agent

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

Medicinal Plants

Fresh leaves {*Gymnema sylvestre (GS)* and *Ipomea aquatica (IA*)}, roots {*Tinospora cordifolia (TC)* and *Withania somnifera (WS*)} and cloves {*Allium sativa (AS*)}, were procured from a reputed ayurvedic pharmacy. Dehydrated (sun dried) plant parts were finely powdered to pass through No.60 mesh sieve. The powder was stored in airtight containers at 4^{o}C.

Animals and Care

Experiments were carried out using adult rats of both sexes (CFT-wistar strain). Animals were fed on commercial pellet diet (Amruth Feeds, Sangli, India) *ad libitum*.

Adult virgin rats (10-12 wk, 200±10g), were randomly drawn from the stock colony of the 'institute animal house facility'. Rats were acclimatized for a week in polypropylene cages (2 per cage) with dust free paddy husk as the bedding material. Cages were placed on racks built of slotted angles and were housed in a controlled atmosphere with a temperature 25±5ºC and mean relative humidity of 50±5% with a 12:12h light: dark cycle.

Adult male rats (10-12 wk old, wt range 225±25g) were drawn from the 'animal house facility' of our Institute. Generally they were acclimatized for a week before use. They were housed individually in polypropylene cages with 12:12h light/dark cycles, at controlled atmosphere of temperature and humidity. For mating purpose, only proven males were used.

Ethical considerations

All procedures with animals were conducted strictly in accordance with guidelines approved by the local "Institute Animal Ethical 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. During the experiments maximum care was taken to minimize animal pain and discomfort and in addition, the number of rats used was kept at a minimum.

Mating schedule and determination of Gestation Day (GD)

Adult virgin females were paired with proven males (ratio of 1:1) and cohabited overnight. Pregnancy was ascertained by microscopic observation of vaginal flushings for the presence of spermatozoa daily. Physiological saline was used for the purpose and the procedure was conducted as gentle as possible. The day the smear was sperm positive was designated as GD '0'. The

animals which did not mate for 5 days were excluded from the study. Later pregnant rats were weighed, randomly grouped and were housed individually.

Isolation of embryos and fetuses

Briefly, uterine horns from mothers (embryos GD_{13}; fetuses: GD_{20}) were excised and were immersed in ice-cold phosphate buffer saline (PBS 0.1 M, pH 7.4). Further, whole uterine horns were pinned on to a wax base, immersed in ice cold PBS. Using surgical procedures, a small incision was made on the uterine wall so as to expose the embryos/fetuses. Later, umbilical cord was cut and embryos/fetuses were ligated. Floating embryos were gently picked up and stored in cryovials at -80^{0}C for further analysis. Fetuses were rinsed in ice-cold PBS, blotted and weighed. Further, fetuses were dissected; fetal brain and liver were excised, rinsed in ice-cold PBS, blotted and stored in cryovials at -80^{0}C until analysis.

Isolation of placenta

After excising embryos/fetuses, discoidal placentas were cleared off any remaining debris of umbilical cord and yolk sac, gently excised from the uterine horn, rinsed in ice-cold PBS, blotted, weighed and stored in cryovials at -80^0 C until analysis.

Preparation of tissue samples

Isolation of plasma

Samples of blood were collected either by cardiac puncture or from retro-orbital plexus in tubes coated with sodium fluoride (0.1%). Whole blood was centrifuged at 800Xg for 10min at 4^{o}C to obtain plasma.

Tissue homogenates

Tissues (maternal brain, liver and kidney; fetal brain and liver; embryos and placenta) excised from dams were blotted free of blood, rinsed in ice-cold saline and homogenized (10% w/v) in ice-cold phosphate buffer (0.1M, pH7.4). The homogenates were centrifuged at 800Xg at 4^{o}C for 5 min and the supernatants were used.

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

Tissues (maternal brain, liver and kidney; fetal brain and liver; embryos and placenta) were excised and used for isolating mitochondria and cytosol by differential centrifugation. Briefly, a 20% homogenate was prepared in ice-cold homogenizing buffer (2mM Tris-HCl, 250mM sucrose, pH 7.4) using a glass-teflon grinder at 4^0 C. The homogenate was centrifuged at 800Xg for 10 min at 4^0 C to obtain the nuclear pellet. Mitochondrial pellet was obtained by centrifuging the post-nuclear supernatant at 5200Xg for 20 min at 4^0 C. The mitochondrial pellet was washed twice with ice-cold MSH buffer (200mM mannitol, 70mM sucrose, 10mM HEPES, 0.1mM EDTA, pH 7.4) and was finally suspended in a known volume of MSH buffer.

Cytosol

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

Preparation of experimental diets

Iron and protein deficient diet

Composition of modified AIN-93G* diet for studies is presented in Table. Dams of iron deficient group received the same diet except for mineral mix where no ferric citrate was added. Likewise pregnant rats of protein deficient group received diet containing 100 g/kg casein (50 % of normal) compensated with corn starch so as to maintain the isocaloricity.

Supplementation of diet with medicinal plants

Commercially available pellet diet (M/s. Amruth Feeds., Sangli, India) was powdered and was supplemented with 2% {*Gymnema sylvestre, Ipomea aquatica, Tinospora cordifolia, Withania somnifera, and Allium sativa}*, (w/w) powder for studying their potency to attenuate diabetes induced oxidative damage.

Ingredient	*g/kg diet*
Corn starch	529.5
Sucrose	100.0
Casein	200.0
Fiber (Alphacel)	50.0
Refined Soybean oil	70.0
Mineral mix (AIN-93G-MX)	35.0
Vitamin mix (AIN-93-VX)	10.0
L-Cystine	3.0
Choline bitartrate	2.5
tert-Butylhydroquinone	0.014

*American Institute of Nutrition *(Reeves et al. 1993)*

ASSAY METHODS

Determination of blood glucose

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

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

LPO was quantified in tissue homogenates or supernatant fractions by measuring the thiobarbituric acid reactive substances (TBARS). To an aliquot of mitochondrial/cytosolic fractions (0.5-1mg protein) 0.2ml of 8% SDS, 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% thiobarbituric acid aqueous solution were added. The mixture was heated for 1h in a boiling water bath. After cooling, 3ml of n-butanol was added and mixed vigorously. The color extracted into butanol layer was read using a fluorescence spectrophotometer (Ex 515 and Em 553 nm). 1, 1, 3, 3-tetramethoxypropane was used as an external standard.

Reactive oxygen species *(Lebel et al., 1990)*

Mitochondrial/cytosolic fractions were diluted 1:10 in ice-cold Locke's buffer to obtain a concentration of 5mg tissue/ml. The homogenates were then pipetted into tubes and allowed to warm to room temperature (21°C) for 5 min, to which 5µl of DCFH-DA (10µM final concentration) was added to each well and the plates were pre-incubated for 15min at room temperature to allow the DCFH-DA to be incorporated into any membrane-bound vesicles and the diacetate group cleaved by esterases. After 30min, the conversion of DCFH to the fluorescent product DCF was measured using a fluorescence spectrophotometer with excitation at 485nm and emission at 530nm. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS production was quantified from a DCF standard curve and results were expressed as *p*mol DCF formed/mg protein/min.

Glutathione estimation *(Mokrasch and Teschike, 1984)*

Mitochondrial/cytosolic fractions (0.5-1mg protein equivalent) were added to 2ml of formic acid (0.1M) and centrifuged at 5200Xg for 20min. To 100µl of the supernatant, 100µl buffer A (1:4 (v/v) 37% formalin: 0.1M Na_2HPO_4) was added and after 5min, 1ml of buffer B (0.1M sodium phosphate, 5mM EDTA, pH 8.0, for reduced glutathione or 0.1M NaOH for oxidized glutathione) was added along with 100µl of o-pthalaldehyde (1mg/ml). This mixture was vortexed and incubated for 45min at room temperature and the absorbance was read at an excitation of 345nm and an emission of 425nm using a spectrofluorimeter (Shimadzu 5301 PC). The amount of total glutathione was determined from the standard curve simultaneously obtained under the same conditions with standard solutions of GSH and GSSG.

Thiols *(Seldak and Lindsay, 1968)*

Thiols (total and non-protein) in mitochondria and cytosol of both maternal (liver and kidney) and fetal organs (brain and liver) were measured according to the original method with slight modifications. Estimation of

sulfhydryls was based on the reduction of DTNB to 2-nitro-5-mercaptobenzoic acid which absorbs at 412 nm. Quantification was done using extinction coefficient of 13,600 mM^{-1}mg $protein^{-1}$.

Total thiols: 0.5mg protein was mixed with 0.5ml of 0.2M Tris-HCl (pH 8.2) containing 20mM EDTA and 0.025 ml of 10mM DTNB in methanol. The mixture was brought to 2ml with methanol. A reagent blank and a sample blank were prepared similarly. The mixture was incubated at room temperature for 30min with occasional mixing, centrifuged at 3000 g for 10min. The absorbance of the clear supernatant was read at 412nm.

Non-protein thiols: 1.0mg protein suspended in 1.0ml of distilled water, mixed with 0.5ml of 5% TCA and centrifuged at 3000g for 10min. 0.5ml of the supernatant was added to 1.5ml of Tris-HCl pH 8.9 containing 20mM EDTA and mixed with 0.025ml of 10mM DTNB. Absorbance was read within 5min of addition of DTNB at 412nm against a reagent blank.

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

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

Catalase *(Aebi, 1984)*

The enzyme activity was determined by measuring the change in absorbance in 1ml reaction mixture using final concentration of 8.8mM H_2O_2 and

phosphate buffer (50mM, pH 7.0) at 240nm, 25°C after addition of sample equivalent to 100µg protein. The enzyme activity was expressed as nmol of H_2O_2 oxidized/min/mg protein.

Glutathione peroxidase *(Flohe & Gunzler, 1984)*

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

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

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

Glutathione reductase *(Carlberg & Mannervick, 1985)*

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

Superoxide dismutase *(Flohe & Otting, 1984)*

To a micro cuvette were added 2.9ml of solution A (5μl xanthine in 0.01N NaOH + 2μl Cytochrome-c + 50mM phosphate buffer in 0.1mM EDTA) and 0.1ml of solution B (an equal volume of xanthine oxidase in 0.1mM EDTA). Reaction mixture without enzyme was used as blank. After adding various volumes of enzyme sample, inhibition of cytochrome-c reduction was monitored for 5min at 560nm. Results were expressed as Units of SOD/mg protein.

One unit was defined as the amount of enzyme that decreases the initial rate of cytochrome-c reduction to 50% of its maximal value for the particular sample being analyzed.

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

Tissue mitochondrial or cytosolic fraction (1mg equivalent) was made upto 1ml with Lowry's reagent (2% Na_2CO_3 in 0.1N NaOH with 1% copper sulfate and 2% sodium potassium tartarate) and the total volume was made up to 2 ml with distilled water. 0.1ml of Folin-Ciocalteu (1N) was added, cyclomixed and allowed to stand for 20 min at room temperature. After 20min, the color developed was measured at 750nm by using a spectrophotometer (Shimadzu 1601A). The total protein concentration was determined from the standard curve obtained under the same conditions with BSA as the standard.

Statistical analysis

In general, all the statistical calculations were done through SIGMA STAT for Windows (Version 3.5) and a *P*-value less than 0.05 was set as the minimum level of significance using Holm Sidak test.

Data in chapter 2 (tbHP and iron model), comparisons were made between control v/s GD_{5-7}, control v/s $GD_{8-10.}$ Experimental data obtained in chapter 3 (section A), comparisons were made between control and various oral doses of iron (control v/s 2, 4 and 8 mg/rat/d). However, the data obtained in section B of chapter 3, data was analyzed by students 't' test between iron sufficient (control) and iron deficient. Experimental data obtained in chapter 4 (section A), comparisons were made between non-diabetic and diabetic groups by Holm Sidak test. However, the data obtained in section B of chapter 4, comparisons were made between non-diabetic v/s diabetic groups; diabetic v/s diabetic supplemented with garlic/medicinal plants.

1.0 INTRODUCTION

In mammals, reactive oxygen species (ROS) are essential factors for cell replication, differentiation and growth, notably during gestation, but are also potentially damaging agents *(Aurousseau et al., 2006)*. Two families of radicals each including a large variety of species can be distinguished: ROS and organic radicals. Organic radicals are large molecules that are very often integral parts of cell membranes. ROS originate in the organism from various sources and their fate and effects cover wide fields. Some of the very reactive radicals such as superoxide anion ($O_2^{-\cdot}$) or hydroxyl radical ($^{\cdot}OH$) readily react with cell components only at the site of their production, while others such as hydrogen peroxide (H_2O_2) or nitric oxide (NO) can diffuse across membranes to appreciable distances where new radicals are formed (*Pryor, 1986; Halliwell & Gutteridge, 1999)*. ROS can induce cascade propagation of oxidative reactions between molecules, followed by formation of new radical species and eventually, an exponential amplification of rates of ROS production *(Porter et al., 1995; Bilodeau & Hubel, 2003; Raijmakers et al., 2004)*. The attack and alteration of cell constituents by ROS is the more commonly reported effects of radical phenomena and is considered as oxidative stress.

Extensive knowledge on radical phenomena specifically associated with healthy gestation and embryogenesis has been demonstrated both in humans and laboratory animals (*Raijmakers et al., 2004*). As in other physiological states, ROS can induce noxious effects and pathologies of gestation only when the equilibrium between the production and the elimination of ROS is disrupted *(Caniggia et al., 2002*). Increasing rates of ROS production relative to antioxidant defenses can induce increasing rates of oxidative alterations of lipids, proteins and DNA in the living organism which can lead to pathologies or even achieve a non-reversible state.

During pregnancy, ROS play a prominent role in remodeling of uterine tissues, implantation of the embryos, settlement of villi and development of blood vessels characteristic of gestation. A large set of factors can induce high steady state of ROS during pregnancy which includes: high rates of estrogen, cytokines, oncogenes and growth factor signaling, production by neutrophils and activation of NADPH oxidases by contact between tissues from the embryo and mother *(Johri & Dasgupta, 1980; Meier et al., 1989)*. In rats, high rates of $O_2^{-\cdot}$ (decreasing steadily from GD_{12} to reach a 60% lesser level on GD_{16}), H_2O_2 (with an increase between GD_{12-14} and a 63% decrease between GD_{14-16}) and $^{\cdot}OH$ (doubling between GD_{12-16}) have also been reported in *ex vivo* fetal homogenates *(Fantel et al., 1998)*. Other major sources of ROS during pregnancy which have been described are: changes in oxygen partial pressures, synthesis of hormones, escape of mitochondria of proliferating cells due to high rates of multiplication and metabolism and increased activity of mitochondrial superoxide dismutase *(Rodriguez et al., 2000; Kim et al., 2005)*. Further, in human placental mitochondria, interaction between NADPH and iron is known to produce ROS *(Milczarek et al., 2000)*.

In experimental animals (such as rat) rates of $^{\cdot}OH$ are reported to increase in the 16 day old fetus, while O_2 and H_2O_2 subside *(Fantel et al., 1998)*. H_2O_2 can diffuse across the membranes and react with redox minerals (mainly Fe) in the cytoplasm, at the nuclear membrane or within the nucleus *(Li & Byrnes, 1999)*. Direct $O_2^{\cdot -}$ production at the level of the nuclear membrane through an NAD(P)H dependent mechanism is possibly of major importance and can end in the interaction of this radical with iron to produce $^{\cdot}OH$. More means of $^{\cdot}OH$ formation can be recruited, such as interactions between H_2O_2 or $O_2^{\cdot -}$ and iron or interactions between various peroxides of proteins, lipids or catechols and redox minerals. At different stages of pregnancy, a high energy metabolic rate is observed, but is automatically followed by high flux of ROS, originating notably from the mitochondria *(Fantel et al., 1998; Kim et al., 2005)*.

The exposure of the blastocyst or the embryo to oxygen induces risks of oxidative stress and of gestation failure or pathologies. In normal pregnancy, high physiological rates of ROS production have been reported in the uterine wall. The placenta and fetus must be protected to avoid any lethal effect of the high rates of ROS production. GSH production and metabolism are of the utmost importance to prevent pathologies of gestation, both for radical scavenging properties of GSH and for its role in the control of the redox state inside the cells and control of gene expression. Protection of cell structures is also afforded by a set of specialized antioxidant defenses against ROS.

While free radicals and ROS play a number of beneficial roles during gestation, excess ROS can be associated with reproductive dysfunctions. That normal pregnancy is associated with an increase in oxidative stress and lipid peroxidation, clearly suggests that some of the vital maternal organs may be relatively more vulnerable to exogenous prooxidants during gestation. More importantly, these oxidative perturbations are likely to have a substantial impact on the developing embryo and fetus. The existence of *critical windows* during which exposure to xenobiotics results in embryopathy and malformations have been documented *(Wells et al., 2005)*. However, the existence of such 'critical vulnerable windows' to prooxidant exposure during gestation are rather limited.

Accordingly, in the current study, two prooxidants viz., *t-butyl hydroperoxide (tbHP)* and *iron* were chosen as model compounds to induce oxidative stress in the maternal milieu and their oxidative implications on growing embryos and fetus were investigated. tbHP has been earlier used as a prototypic inducer of oxidative stress in a variety of *in vitro* and *in vivo* systems *(Rajesh Kumar et al., 2002; Kumar & Muralidhara 2007)*. Prior to this study, tbHP has never been reported to be employed to induce oxidative stress in pregnant rodents and hence we consider this as a new *in vivo* model. However, iron exposure has often been used and we employed this approach mainly due to its practical relevance. The data obtained in these models are presented as two separate *sections A and B*.

SECTION A

HYDROPEROXIDE MODEL: OXIDATIVE STRESS INDUCTION IN MOTHER AND EMBRYOS

2.0 OBJECTIVE

The primary objective of this study was to examine (i) If there are 'critical windows' during gestation which are vulnerable to prooxidant exposure (ii) The extent of oxidative implications among the early post implantation embryos following maternal prooxidant exposure. This was achieved by administering pregnant dams with a model organic hydroperoxide viz., t-butyl hydroperoxide at sublethal doses during two specific periods of gestation (either on GD_{5-7} or GD_{8-10}) and assessment of oxidative response in maternal tissues and their implications in embryos obtained on gestation day 13 (GD_{13}).

3.0 EXPERIMENTAL DESIGN

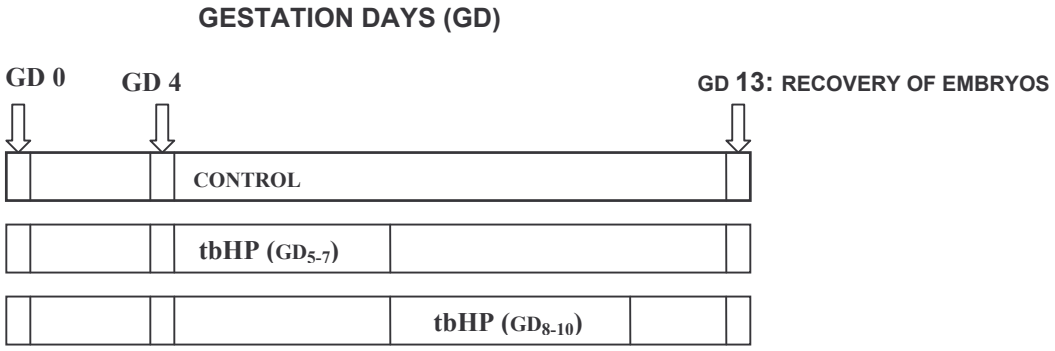
3.1 Preliminary toxicity study: Selection of dosages

A preliminary study was conducted to determine the median lethal dose (LD_{50}, intraperitoneal) of the model prooxidant, tertiary butyl hydroperoxide (tbHP) in adult female rats. Acute graded doses of tbHP were administered at dosages ranging from 100-500 µmoles/100g bw. The mortality data obtained was subjected to probit regression analysis to compute the LD_{50} value. Since the statistically computed LD_{50} value for tbHP was 400 µmoles/100g bw, two dosages viz., 1/10 and 1/5 of LD_{50} were selected for repeated dosing experiments.

3.2 Determinative study

In this study, the basic experimental protocol comprised of administering (i.p) two dosages of tbHP (equivalent to 1/10th and 1/5th LD_{50}) to pregnant rats during specific periods of gestation (two critical periods of gestation early post-implantation GD_{5-7} or GD_{8-10}). The rats were maintained upto GD_{13}.

Both control and tbHP treated dams were provided with known amounts of diet and the food intake was monitored by weighing the residual diet each day. Body weight gain was monitored regularly throughout the experiment. Rats were sacrificed on GD_{13} after mild diethyl ether anesthesia. Blood was drawn by cardiac puncture for measuring serum MDA levels. Maternal organs (liver & kidney) were excised, rinsed in ice-cold phosphate buffered saline (PBS 0.1 mol/L, pH 7.4), blotted, weighed and stored at -80^{0}C for further analysis. Uterine horns were excised and pinned to wax base immersed in ice cold PBS. Using surgical procedures, embryos and placenta were excised and weighed.



Treatment regimens employed to ascertain the effect of maternal oxidative stress on embryos.

3.3 Post- implantation embryonic lethality and placental weights

Pregnant rats were sacrificed on GD_{13} and uterine contents were analyzed for post-implantation losses. Total implantations, live and dead implantations were scored from each dam and the data was pooled for the group. Further, placenta and embryos were excised, trimmed, rinsed in ice-cold PBS, blotted weighed and stored at -80^{0}C for further analysis.

3.4 Quantification of oxidative impairments in maternal organs and embryos

3.4.1 Status of lipid peroxidation and generation of ROS

Pregnant rats administered tbHP were sacrificed along with untreated normal controls to measure the degree of oxidative damage (levels of MDA, ROS and protein carbonyls) in mitochondrial and cytosolic fractions of embryonic homogenates. In order to obtain a comparative picture, LPO and ROS were determined in placental homogenates and maternal tissues (liver and kidney).

3.4.2 Alterations in redox state

The amount of reduced glutathione, total thiols and non-protein thiols in maternal tissues, placenta and embryos were determined in both control and tbHP administered rats.

3.4.3 Perturbations in antioxidant defenses

Activities of antioxidant enzymes viz., CAT, GPX, GR and GST in embryos, placenta and maternal tissues were determined in both control and prooxidant administered rats.

SECTION B

IRON EXCESS MODEL: OXIDATIVE IMPLICATIONS IN EMBRYOS/FETUSES

2.0 OBJECTIVE

The primary objective of this study was to examine (i) if there are 'critical windows' during gestation which are susceptible to maternal iron exposure and (ii) subsequent oxidative impact on the growing embryos and fetus. This was studied by administration of a well known model prooxidant viz., iron at sublethal doses during two specific periods of gestation (viz., GD_{5-7} or GD_{8-10}) and ascertaining the oxidative response in maternal tissues and their implications in embryos and fetal tissues.

3.0 EXPERIMENTAL DESIGN

3.1 Preliminary Study: dose selection of Iron Dextran (ID)

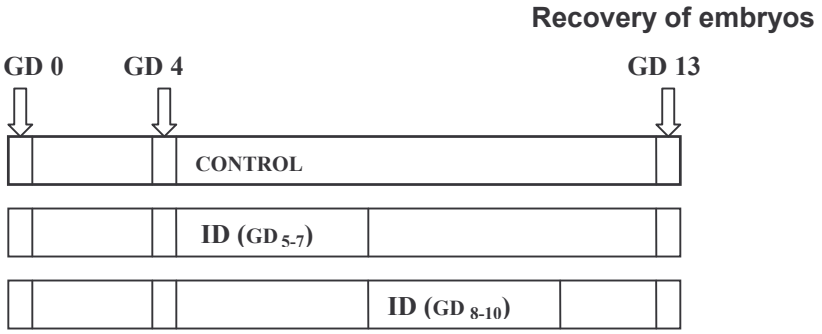
With an objective of determining the sublethal dosages of iron dextran (ID), initially pregnant rats (n=4) were administered (i.p) with ID at dosages ranging from 10–100 mg/kg bw either during gestation days (GD) 5-7 or 8-10. Subsequently dams were sacrificed on GD_{13} or GD_{20} to assess the degree of post implantation embryolethality. Based on the results, three dosages viz., 10, 25 and 50 mg/kg bw were selected for further experimentation.

3.2 Protocol and design

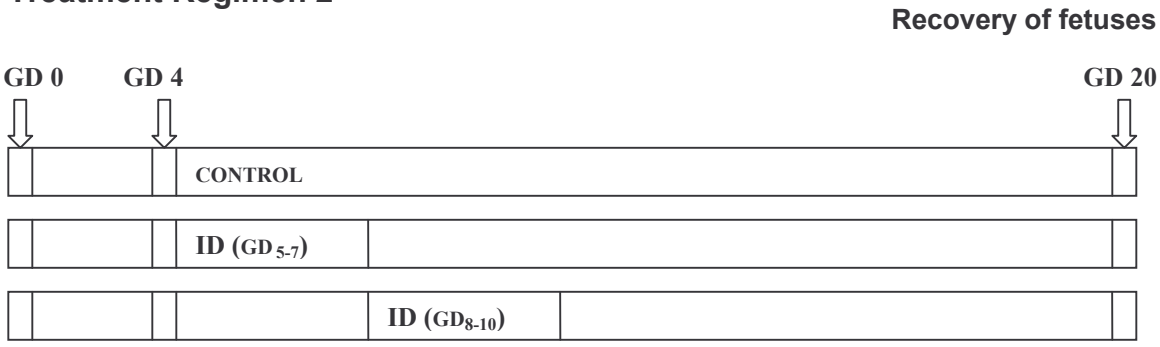
Two sets of experiments were conducted in pregnant rats to assess the susceptibility of the mother to iron induced oxidative stress and its oxidative implications in embryos and fetuses. In order to study the effects on embryos, dams were sacrificed on GD_{13}, while to assess the effects on fetus, dams were sacrificed on GD_{20}. Further, to assess the vulnerability or resiliency of growing embryos to prooxidant exposure, pregnant dams were administered ID either during GD_{5-7} (early post implantation exposure) or GD_{8-10} (late post-implantation exposure).

The following selected markers of oxidative stress were quantified to study the effect of maternal iron exposure: TBARS, ROS levels, reduced glutathione, protein carbonyls, total thiols and activities of selected enzymic antioxidants.

Treatment Regimen 1



Treatment Regimen 2



Treatment regimen employed to ascertain the effect of maternal iron overload on embryos and fetuses.

4.0 RESULTS

SECTION A

HYDROPEROXIDE MODEL: OXIDATIVE STRESS INDUCTION IN MOTHER AND EMBRYOS

Toxicity profile

The LD_{50} value of tbHP computed by probit regression analysis was 400 µmoles /100g bw.

4.1 Effects on growth, placental weights and embryolethality

4.1.1 Food consumption and growth pattern

There were no significant changes in the food intake among pregnant rats administered with tbHP during the experimental period (data not shown). Further, no appreciable alterations were evident with respect to the body weight gain among treated rats (Table 2.1).

4.1.2 Placental weights

The placental weights of tbHP treated rats showed only a marginal increase (Table 2.1). However, at the lower dosage, tbHP did not alter the placental weights significantly irrespective of the dosing regimen.

4.1.3 Incidence of embryolethality

The incidence of embryolethality among tbHP administered rats was significantly elevated at both doses compared to that in normal controls (Table 2.2). The percent dead implantations (%DI) calculated as a percentage of total implantations were elevated by 4-6 folds with respect to the normal controls.

4.2. Evidences of oxidative damage in maternal milieu

4.2.1 Generation of ROS in maternal organs

The generation of ROS in maternal liver and kidney of both control and tbHP treated rats are presented in Table 2.3. In general, the lower dose did not significantly alter the ROS levels, while the higher dose induced a significant increase. Interestingly, the induction levels were higher when tbHP was administered during GD_{5-7} compared to GD_{8-10}. In liver, both fractions showed higher ROS levels (GD_{5-7}: cytosol, 45%; mitochondria, 68% and GD_{8-10}: cytosol, 5%; mitochondria, 31%). A similar trend of enhancement in ROS levels was also evident in the maternal kidney at the higher dose of tbHP.

4.2.2 Status of lipid peroxidation in maternal organs

The lipid peroxidation status measured as MDA levels in liver and kidney of both control and tbHP treated rats are also presented in Table 2.3. At the higher dose, tbHP induced noticeable increase in hepatic tissue, the induction levels being higher during GD_{5-7} and marginal among dams administered on GD_{8-10}. A similar trend of induction was also evident in maternal kidney, and the induction levels were more robust compared to the liver tissue.

4.2.3 Status of reduced glutathione and protein carbonyls in maternal organs

Data on the GSH and protein carbonyl levels determined in liver and kidney of rats administered tbHP is presented in Table 2.4. Significant depletion of GSH levels was observed at both doses and there was no difference in the response among two dosing regimens. In liver, the GSH levels were significantly depleted (by 25-30%), while the magnitude of depletion was relatively higher in kidney (35%).

With higher dose of tbHP, the protein carbonyl levels in both liver and kidney were significantly elevated. While liver showed an increase of 27-37% over the basal levels, the increase was nearly 37% in the kidney.

4.3 Susceptibility of placenta to oxidative stress

4.3.1 Oxidative markers

Data on the status of MDA, ROS levels and glutathione levels measured in placenta of control and tbHP treated rats are presented in Table 2.5. In general, the ROS levels were significantly enhanced in placenta of tbHP treated dams. The levels were relatively higher (irrespective of the dose) when administered during GD_{5-7} than during GD_{8-10}. ROS levels were more robust at the higher dose in both cytosol and mitochondria (GD_{5-7}: cytosol, 135%; mitochondria, 146%). The induction levels were relatively lower when tbHP was administered during the late post-implantation days (GD_{8-10}: cytosol, 73%; mitochondria, 77%). The placental MDA levels also showed a similar trend in increase. At the higher dose, tbHP caused significantly elevated MDA levels in both dosing regimens (GD_{5-7}; cytosol, 81%; mitochondria, 108% and GD_{8-10}; cytosol, 65%; mitochondria, 95%). Concomitantly, the GSH levels in placenta were diminished at the higher dose (19-25%) compared to the controls.

4.3.2 Response of antioxidant enzymes in maternal organs and placenta

Data on the activities of antioxidant enzymes in liver, kidney and placenta of control and tbHP treated rats. The activities of catalase and GST were relatively diminished in all organs (Fig 2.1). The reduction appeared to be of higher magnitude when tbHP was administered during GD_{5-7} compared to GD_{8-10}. The catalase activity was significantly reduced during GD_{5-7} (liver, 33 and 21%; kidney, 30%; placenta, 31-47%). While hepatic and kidney GST levels were moderately reduced (20-28%) at the higher dose, the placental GST activity was markedly reduced (GD_{5-7}: 42 % and GD_{8-10}: 34%).

4.4 Oxidative implications among GD_{13} embryos

4.4.1 Status of ROS levels and induction of lipid peroxidation

Data on the generation of ROS and MDA levels among embryos recovered on GD_{13} from control and tbHP treated dams are presented in Table 2.6. The lower dose of tbHP failed to increase the ROS generation in embryos irrespective of the dosing regimen. However, at higher dose, significant elevation in ROS levels were evident, the induction levels were more robust when tbHP was administered during GD_{5-7} (45%) compared to that during GD_{8-10} (21%). On the other hand, the MDA levels were dramatically elevated among embryos of tbHP treated dams. The levels were markedly elevated during both dosing regimens (GD_{5-7}: 6 folds; GD_{8-10}: 4-6 folds) suggesting the increased susceptibility of early embryos to lipid peroxidation.

4.4.2 Alterations in GSH and thiol levels in embryos

Data on the GSH, total thiols and protein carbonyls among embryos recovered on GD_{13} from control and tbHP treated dams is presented in Table 2.7. The GSH levels were decreased only at the higher dose during both dosing regimens (24-30%). A similar decrease was also evident in total thiol content of embryos of tbHP treated dams (24-17%). The protein carbonyl content of embryos were significantly elevated (56 and 49%) during both the dosing regimens.

4.4.3 Effect on antioxidant enzyme activities in embryos

The activities of both catalase (GD_{5-7}, 49%; GD_{8-10}: 56%) and GST (GD_{5-7}: 34% and GD_{8-10}: 41%) were markedly reduced among embryos of tbHP treated dams (Fig 2.2).

4.0 RESULTS

SECTION B

IRON EXCESS: OXIDATIVE IMPLICATIONS IN MOTHER, EMBRYOS/FETUSES

Dosage selection

The criteria for selection of iron dextran doses were based on the incidence of post-implantation embryonic losses induced by iron. The selected doses were: 10, 25 and 50mg/kg bw/d with two treatment regimens: GD_{5-7} and GD_{8-10}.

4.1 General effects of iron administration

4.1.1 Food consumption and growth pattern

There were no significant changes in the food intake among dams administered with ID during the experimental period (data not shown). Further, no significant alterations were evident with respect to the body weight gain among treated rats (Table 2.8) excepting for a marginal decrease in the maternal body weight gain among rats administered the higher dose of ID.

4.1.2 Placental and fetal weights

Data on the placental weights of rats sampled at two gestational time points viz., GD_{13} and GD_{20} are presented in Table 2.8 and 2.9. The placental weights of ID treated dams on GD_{13} were marginally increased (8-11%) (Table 2.8). On the other hand, the placental weights on GD_{20} were significantly elevated at the higher dose of ID (GD_{5-7}: 30-51% and GD_{8-10}: 30-43%). The weights of fetuses monitored on GD_{20} were significantly reduced among ID treated dams during both dosing regimens (GD_{5-7}: 23-40 %; GD_{8-10}: 19-32%).

4.2 Evidences of Oxidative Damage in maternal organs (GD_{13} and GD_{20})

4.2.1 Generation of ROS in maternal Organs

Data on the generation of ROS in maternal liver of both control and ID treated rats sampled on GD_{13} and GD_{20} are presented in Fig 2.3. In general, the lowest dose (10mg/kg bw) did not significantly alter the ROS levels, whereas higher doses induced marked elevation irrespective of the dosing regimen. At higher doses, ID caused significant increase in ROS levels in both cytosol and mitochondrial fractions. In cytosol, the increase was more robust during GD_{5-7} (25, 42%) compared to GD_{8-10} (12, 33%) whereas in mitochondria, the magnitude of elevation in ROS levels at the highest dose was relatively higher (GD_{5-7}: 58% and GD_{8-10}: 51%).

4.2.2 Status of lipid peroxidation in maternal organs

The lipid peroxidation status in liver measured as MDA levels among both control and ID treated rats is depicted in Fig 2.3. ID at higher doses induced a marked increase in MDA levels irrespective of the dosing regimens with the cytosolic fraction showing higher levels compared to mitochondria. At the highest dose, the cytosol MDA levels were elevated by 82 and 60% when ID was administered during GD_{5-7}, while the levels were enhanced by 86 and 78% during GD_{8-10}. Likewise, the mitochondrial levels were elevated by 56 and 46% (GD_{5-7}) and 58 and 40% (GD_{8-10}) respectively. The status of LPO and ROS levels monitored in kidney (Fig 2.4) cytosol and mitochondria also showed a similar response.

4.2.3 GSH levels, thiol content and protein carbonyls in maternal liver

Data on the alterations in reduced glutathione levels, thiol content and protein carbonyl in maternal liver of rats administered ID is presented in Fig 2.5. While the lowest dose had no effect, at higher doses, the hepatic GSH levels were marginally (12–28%) diminished irrespective of the dosing regimen. Similar diminutions were also evident in total thiols and non-protein thiols in liver at higher doses of ID (Fig 2.6). Oxidative damage to proteins measured as protein

carbonyls in maternal liver of ID administered dams resulted in significant dose-dependent increase during both dosing regimens.

4.2.4. Antioxidant enzyme activity in maternal liver

In general, varying degrees of reduction in the activities of three enzymes viz., catalase, GST and GPX were observed (data not shown). At the highest dose, the percent decrease in the activity of catalase was: GD_{13}, 12-27%; and GD_{20}; 15-18%). The hepatic GST levels were markedly reduced during both dosing regimens. At the highest dose the percent decrease in the activity was (GD_{13}: 30-45% and GD_{20}: 18-31%). Significant decrease in hepatic GPX levels was also evident among ID treated rats among both dosing regimens. At the highest dose, the percent reduction in the activity was (GD_{13}: 35-24% and GD_{20}: 29-24%).

4.3 Induction of oxidative stress in placenta

Data on the ROS and MDA levels measured in placenta of control and ID treated dams on GD_{13} and GD_{20} are presented in Fig 2.7. The lowest dose caused no significant elevation in both markers of oxidative stress. In general, the placenta obtained from GD_{13} dams showed higher elevation in ROS levels both in cytosol and mitochondrial fractions compared to those from GD_{20} dams.

4.4 Oxidative implications in embryos recovered on GD_{13}

4.4.1 Generation of ROS and MDA levels

Data on the generation of ROS and oxidative damage to lipids in the embryos of ID treated dams are presented in Table 2.10. While there was a moderate increase in cytosol (MDA and ROS: 38%), mitochondrial levels were highly elevated at the highest dose (MDA-62%; ROS-44%) during GD_{5-7}. Similar increase in the ROS and MDA levels was also evident during GD_{8-10}.

4.4.2 Reduced glutathione and thiols

Data on the GSH, total thiols and protein carbonyls among embryos recovered on GD_{13} from control and iron treated dams are presented in Table 2.11. The GSH levels were decreased significantly only at higher dose during both dosing regimens (25-35%). However, the total thiols content in embryos of ID treated dams were significantly decreased (GD_{5-7}: 40-46%: GD_{8-10} 20-28%) at the higher doses. Similar decrease was also evident with non-protein thiols (GD_{5-7}: 28-48%, GD_{8-10}: 30-47%). Further, the protein carbonyl content were significantly elevated (GD_{5-7}: 33-53%; GD_{8-10}: 25-35%).

4.5 Evidences of oxidative damage in fetuses

4.5.1 Status of lipid peroxidation and generation of ROS in fetal organs

Data on the oxidative implications in brain and liver of fetuses following maternal exposure to ID (i.p) determined in terms of ROS and LPO are presented in the Figs 2.8 and 2.9. A dose-related increase was evident in both cytosol and mitochondria of whole brain irrespective of the dosing regimen. At higher doses of ID (25 & 50 mg/kg bw) the ROS levels were elevated significantly, the magnitude of increase being higher in cytosol and marginally higher during dosing regimen GD I. In brain, the elevations in ROS levels were: cytosol GD_{5-7}: 47-70%; GD_{8-10}: 38-60% and mitochondria GD_{5-7}: 48-69%; GD_{8-10}: 36-56%. A similar induction of MDA levels was evident in the brain and the percent increases were: cytosol GD_{5-7}: 55-74%; GD_{8-10}: 53-66% and mitochondria GD_{5-7}: 28-48%; GD_{8-10}: 23-37%. In liver, the mitochondria showed marginal alterations in ROS levels, while the cytosol showed significant elevations (GD_{5-7}: 65-74% and GD_{8-10}). However, the MDA levels were higher in both cytosol and mitochondria.

4.5.2 GSH and protein carbonyls in fetal organs

GSH levels were diminished (41-52%) in both liver and brain at the higher ID dose irrespective of the dosing regimen (Fig 2.10). The protein carbonyls were also significantly higher among ID treated dams during both dosing regimens.

4.5.3 Antioxidant enzymes in fetal organs

The levels of antioxidant enzymes measured in brain and liver of fetal tissues of ID treated dams showed varying degrees of reduction in the activities of catalase, GST and GPX at higher doses irrespective of dosing regimen (Fig 2.11).

Table 2.1 Maternal body weight gain, placental weights of pregnant rats administered t-butyl hydroperoxide (tbHP)

GD	Group	Body wt gain (g)	Mean placental wt (mg)
5-7	CTR	44.50 ± 4.5	255 ± 06
	$tbHP_1$	42.50 ± 5.0	265 ± 05
	$tbHP_2$	39.50 ± 2.5	288 ± 08[a]
8-10	$tbHP_1$	42.00 ± 3.5	263 ± 07
	$tbHP_2$	40.50 ± 2.0	281 ± 06[b]

Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}
Data analysed by Holm-Sidak method; [a]*P<0.001,* [b]*P<0.01,*

Table 2.2 Effect of tbHP administration on the frequency of embryonic deaths determined on GD_{13}

GD	Group	Implantations			% DI*
		Total	Live	Dead	
5-7	CTR	72	70	2	2.7 ± 1.2
	$tbHP_1$	66	60	6	9.09 ± 1.5[a]
	$tbHP_2$	68	58	10	14.7 ± 1.7[a]
8-10	$tbHP_1$	70	63	7	10.0 ± 1.4[a]
	$tbHP_2$	65	57	8	12.3 ± 2.3[a]

Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}
* Expressed as a percentage of total implantations
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 2.3 Pattern of ROS generation and status of lipid peroxidation in liver and kidney of pregnant rats administered tbHP

GD	Group	Liver		Kidney	
		Cytosol	Mitochondria	Cytosol	Mitochondria
	Reactive oxygen Species[1]				
5-7	CTR	6.05 ± 0.38	3.36 ± 0.31	5.40 ± 0.47	4.53 ± 0.33
	$tbHP_1$	6.41 ± 0.10	4.13 ± 0.49[a]	5.73 ± 0.67	4.40 ± 0.51
	$tbHP_2$	8.75 ± 0.59[a]	5.66 ± 0.53[a]	6.66 ± 0.52[b]	6.51 ± 0.57
8-10	$tbHP_1$	6.51 ± 0.21	3.60 ± 0.75	5.60 ± 0.85	4.80 ± 0.60
	$tbHP_2$	6.83 ± 0.62[c]	4.71 ± 0.63[b]	5.86 ± 0.69	5.51 ± 0.64
	Malondialdehyde[2]				
5-7	CTR	5.71 ± 0.48	6.54 ± 0.31	5.21 ± 0.37	5.04 ± 0.53
	$tbHP_1$	6.11 ± 0.30	6.37 ± 0.45	5.37 ± 0.64	5.40 ± 0.71
	$tbHP_2$	7.83 ± 0.47[a]	8.66 ± 0.38[a]	7.56 ± 0.58[a]	8.51 ± 0.68[a]
8-10	$tbHP_1$	6.51 ± 0.21[b]	6.20 ± 0.75	5.60 ± 0.73	5.80 ± 0.60[a]
	$tbHP_2$	6.95 ± 0.73[b]	7.86 ± 0.65[a]	6.48 ± 0.54[b]	7.76 ± 0.34[a]

Values are mean ± SD (n=6); $tbHP_1$: 1/10th LD_{50}; $tbHP_2$: 1/5th LD_{50}
[1]pmol/min/mg protein; [2]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001,* [b]*P<0.01,* [c]*P<0.05*

Table 2.4 Status of glutathione and protein carbonyls in liver and kidney of rats administered tbHP

GD	Group	Liver	Kidney
	Glutathione[1]		
5-7	CTR	27.07 ± 1.35	4.45 ± 0.35
	$tbHP_1$	21.81 ± 2.76[a]	3.73 ± 0.37[a]
	$tbHP_2$	18.73 ± 2.47[a]	2.66 ± 0.47[a]
8-10	$tbHP_1$	28.01 ± 2.66	3.81 ± 0.30[a]
	$tbHP_2$	21.43 ± 3.57[a]	2.48 ± 0.54[a]
	Protein carbonyls[1]		
5-7	CTR	5.38 ± 0.35	3.41 ± 0.35
	$tbHP_1$	5.53 ± 0.59	3.63 ± 0.23
	$tbHP_2$	7.37 ± 1.03[a]	4.76 ± 0.37[a]
8-10	$tbHP_1$	6.01 ± 0.21	3.50 ± 0.45
	$tbHP_2$	6.83 ± 0.91[a]	4.68 ± 0.57[a]

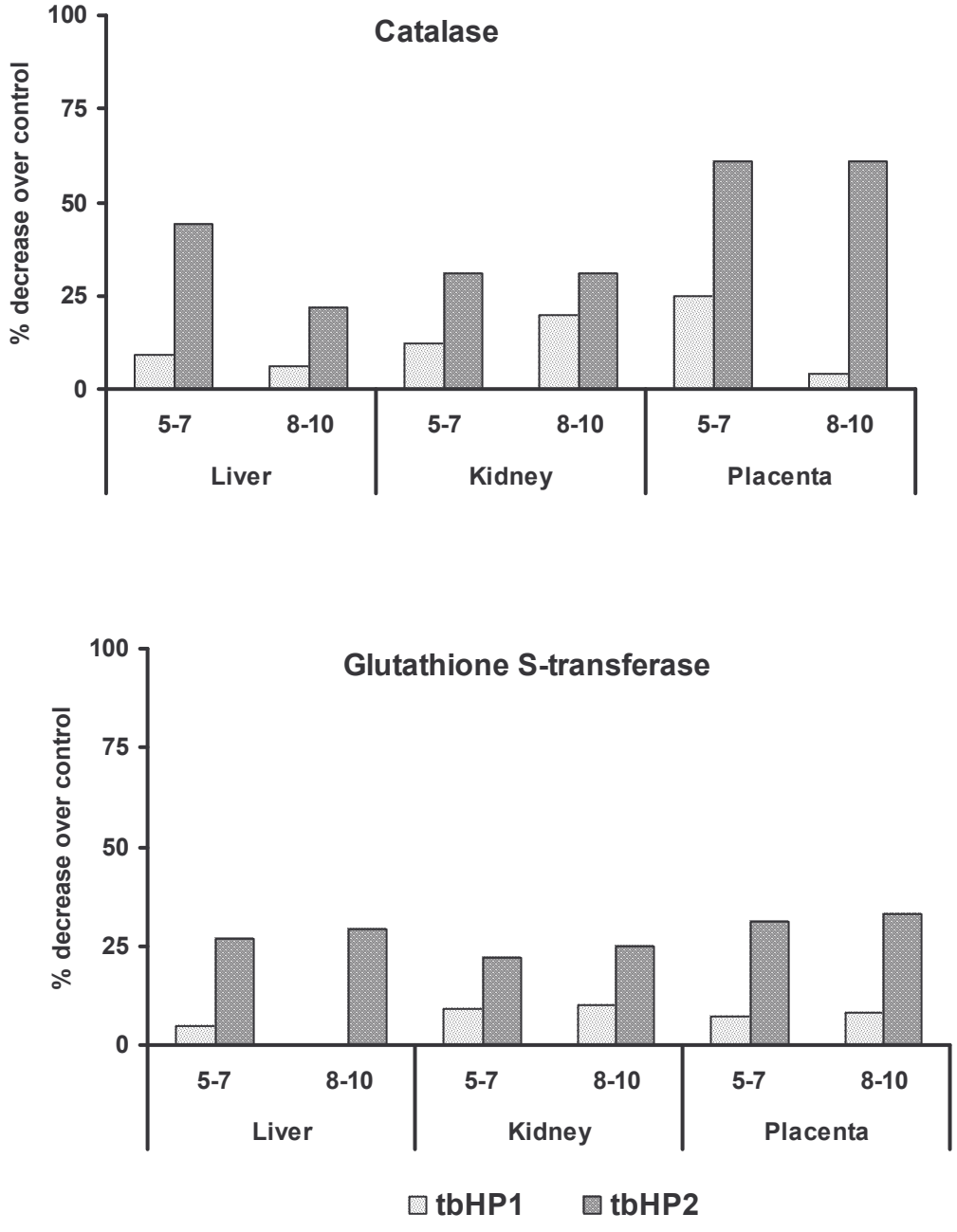
Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}
[1]nmol/mg protein;
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 2.5 Effect of tbHP administration on ROS generation, lipid peroxidation and reduced glutathione levels in placental tissue

GD	Group	Cytosol	Mitochondria
		Reactive oxygen species[1]	
5-7	CTR	0.48 ± 0.08	0.43 ± 0.06
	$tbHP_1$	0.61 ± 0.10[a]	0.37 ± 0.09
	$tbHP_2$	1.13 ± 0.09[a]	1.06 ± 0.03[a]
8-10	$tbHP_1$	0.51 ± 0.02	0.60 ± 0.07[a]
	$tbHP_2$	0.83 ± 0.05[a]	0.76 ± 0.03[a]
		Malondialdehyde[2]	
5-7	CTR	3.12 ± 0.35	2.16 ± 0.54
	$tbHP_1$	3.37 ± 0.29	3.40 ± 0.67[a]
	$tbHP_2$	5.66 ± 0.63[a]	4.51 ± 0.72[a]
8-10	$tbHP_1$	3.60 ± 0.75	3.80 ± 0.60[a]
	$tbHP_2$	5.16 ± 0.53[a]	4.21 ± 0.57[a]
		Glutathione[2]	
5-7	CTR	6.04 ± 0.64	-
	$tbHP_1$	5.47 ± 0.70	-
	$tbHP_2$	4.55 ± 0.53[b]	-
8-10	$tbHP_1$	5.73 ± 0.30	-
	$tbHP_2$	4.86 ± 0.59[b]	-

Values are mean ± SD (n=6); $tbHP_1$: 1/10th LD_{50}; $tbHP_2$: 1/5th LD_{50}
[1]pmol/min/mg protein; [2]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001,* [b]*P<0.01*

Fig 2.1 Activities of catalase and glutathione transferase in liver, kidney and placenta of rats administered tbHP



Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}

Table 2.6 Pattern of ROS generation and status of lipid peroxidation in embryos of rats administered tbHP

GD	Group	ROS[1]	LPO[2]
5-7	CTR	2.02 ± 0.03	0.15 ± 0.01
	$tbHP_1$	1.87 ± 0.08[a]	0.89 ± 0.18[a]
	$tbHP_2$	2.19 ± 0.06[a]	0.93 ± 0.25[a]
8-10	$tbHP_1$	1.47 ± 0.18[a]	0.61 ± 0.15[a]
	$tbHP_2$	1.69 ± 0.23[a]	0.95 ± 0.29[a]

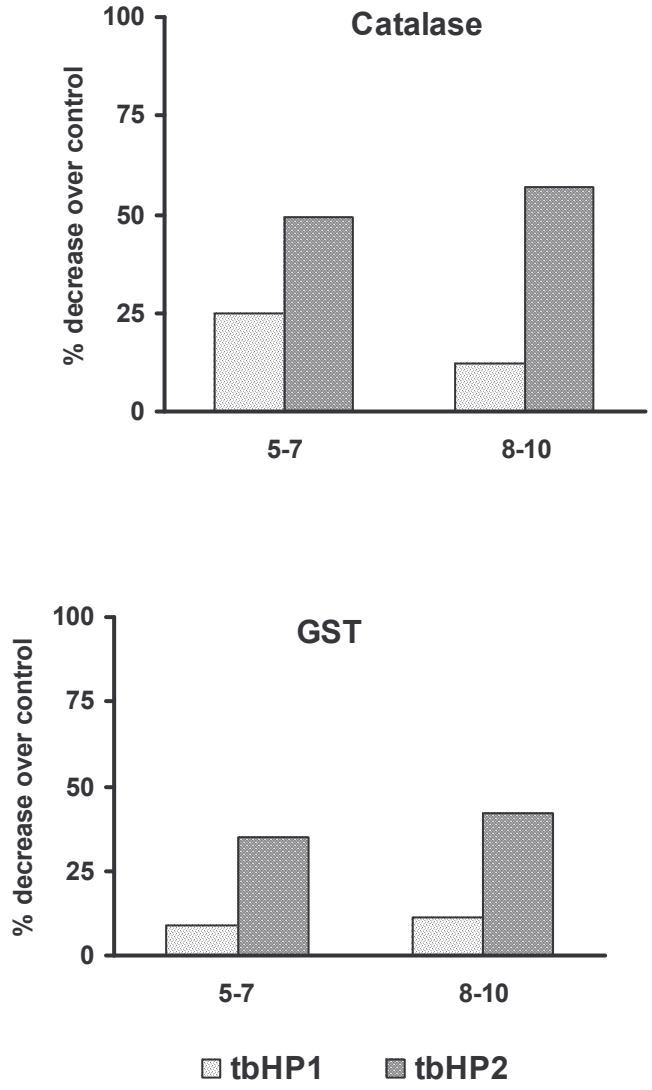
Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}
[1]pmol/min/mg protein; [2]LPO: nmol MDA/mg protein
Data analysed by Holm-Sidak method; [a]*P< 0.001*

Table 2.7 Status of glutathione, protein carbonyls (PC) and total thiols in embryos of rats administered tbHP

GD	Group	Glutathione[1]	Total thiols[1]	PC[1]
5-7	CTR	4.87 ± 1.19	21.25 ± 1.25	1.89 ± 0.22
	$tbHP_1$	5.75 ± 1.57	21.87 ± 2.08	1.39 ± 0.35[a]
	$tbHP_2$	3.70 ± 0.83	15.99 ± 1.06[a]	2.95 ± 0.19[a]
8-10	$tbHP_1$	5.70 ± 2.35	20.47 ± 1.68	1.65 ± 0.37
	$tbHP_2$	3.39 ± 1.46	17.69 ± 1.73[b]	2.81 ± 0.47[a]

Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}
[1]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P< 0.001,* [b]*P< 0.01*

Fig 2.2 Activities of catalase and glutathione transferase (GST) in embryos of rats administered tbHP



Values are mean ± SD (n=6); $tbHP_1$: $1/10^{th}$ LD_{50}; $tbHP_2$: $1/5^{th}$ LD_{50}

Table 2.8 Body weight gain, placental weights and embryolethality in pregnant rats administered (i.p) iron dextran (ID) during specific periods of gestation (GD_{13})

GD	ID (mg / kg bw)	Body wt gain (g)	Placental wt (mg)	Embryolethality (%DI)
5- 7	0	42.15 ± 4.0	0.26 ± 0.06	5.05 ± 0.48
	10	40.05 ± 3.5	0.26 ± 0.05	10.85 ± 1.36[a]
	25	38.50 ± 2.5	0.27 ± 0.04	18.65 ± 0.41[a]
	50	36.25 ± 4.2	0.28 ± 0.06	26.55 ± 1.59[a]
8- 10	10	39.00 ± 4.0	0.27 ± 0.08	8.55 ± 0.32[a]
	25	41.25 ± 1.0	0.28 ± 0.03	15.10 ± 0.52[a]
	50	37.50 ± 3.5	0.30 ± 0.08	20.75 ± 1.85[a]

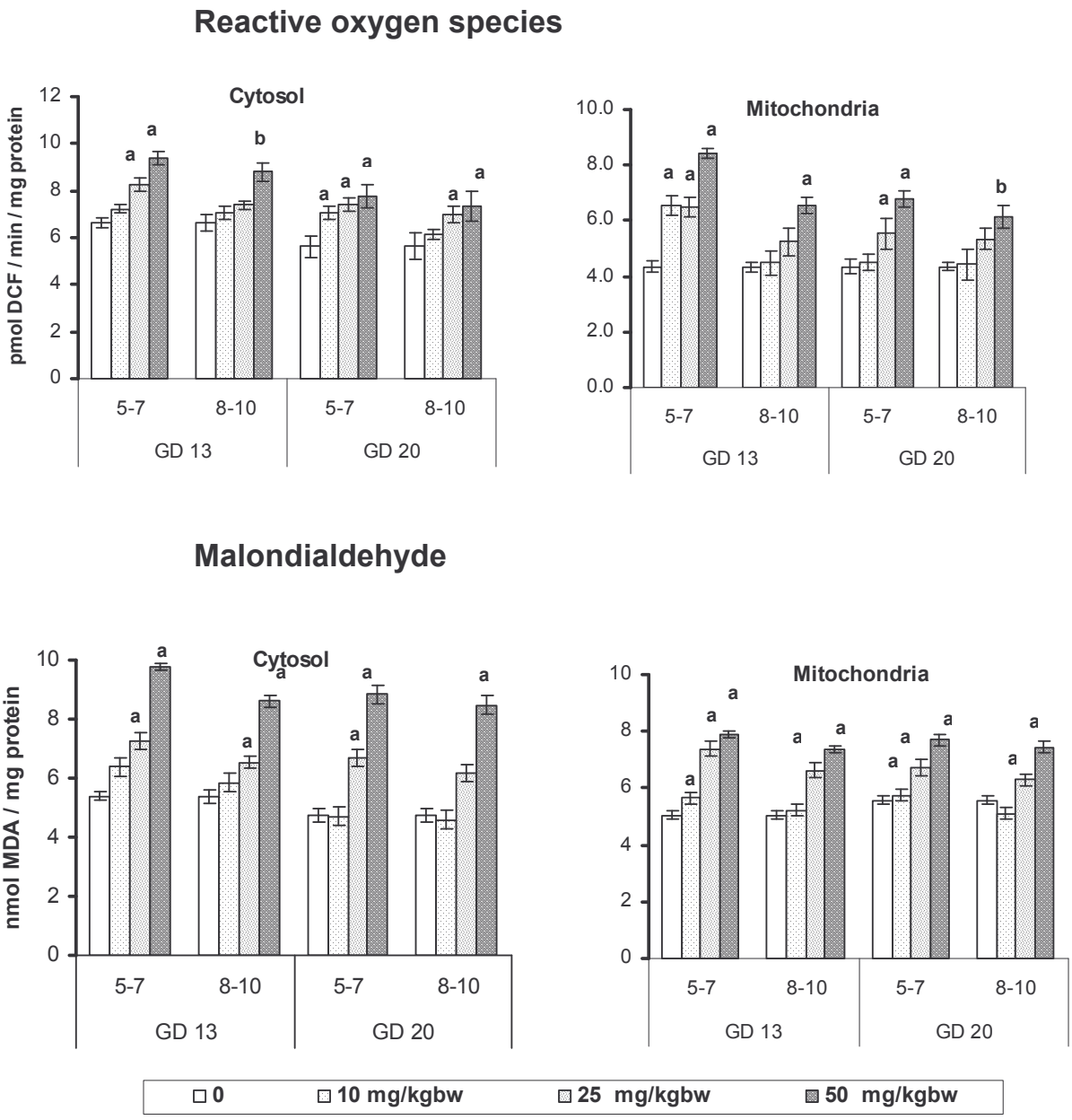
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 2.9 Body weight gain, placental/fetal weight and embryolethality in pregnant rats administered (i.p) iron dextran (ID) during specific periods of gestation (GD_{20})

GD	ID (mg / kg bw)	Body wt gain (g)	Placental wt (mg)	Fetal wt (g)	Embryolethality (%DI)
5-7	0	96.50 ± 5.0	0.47 ± 0.06	3.51 ± 0.15	6.20 ± 0.35
	10	95.65 ± 5.5	0.61 ± 0.06[a]	3.40 ± 0.08	12.20 ± 0.95[a]
	25	88.55 ± 4.5[a]	0.66 ± 0.07[a]	2.70 ± 0.20[a]	20.45 ± 0.75[a]
	50	82.65 ± 4.5[a]	0.71 ± 0.08[a]	2.09 ± 0.18[a]	27.50 ± 0.99[a]
8-10	10	90.50 ± 4.0[b]	0.61 ± 0.04[a]	3.54 ± 0.16[a]	8.01 ± 0.70[a]
	25	88.50 ± 3.5[b]	0.63 ± 0.03[a]	2.83 ± 0.10[a]	19.50 ± 0.55[a]
	50	86.50 ± 5.5[b]	0.67 ± 0.10[a]	2.37 ± 0.15[a]	23.65 ± 1.55[a]

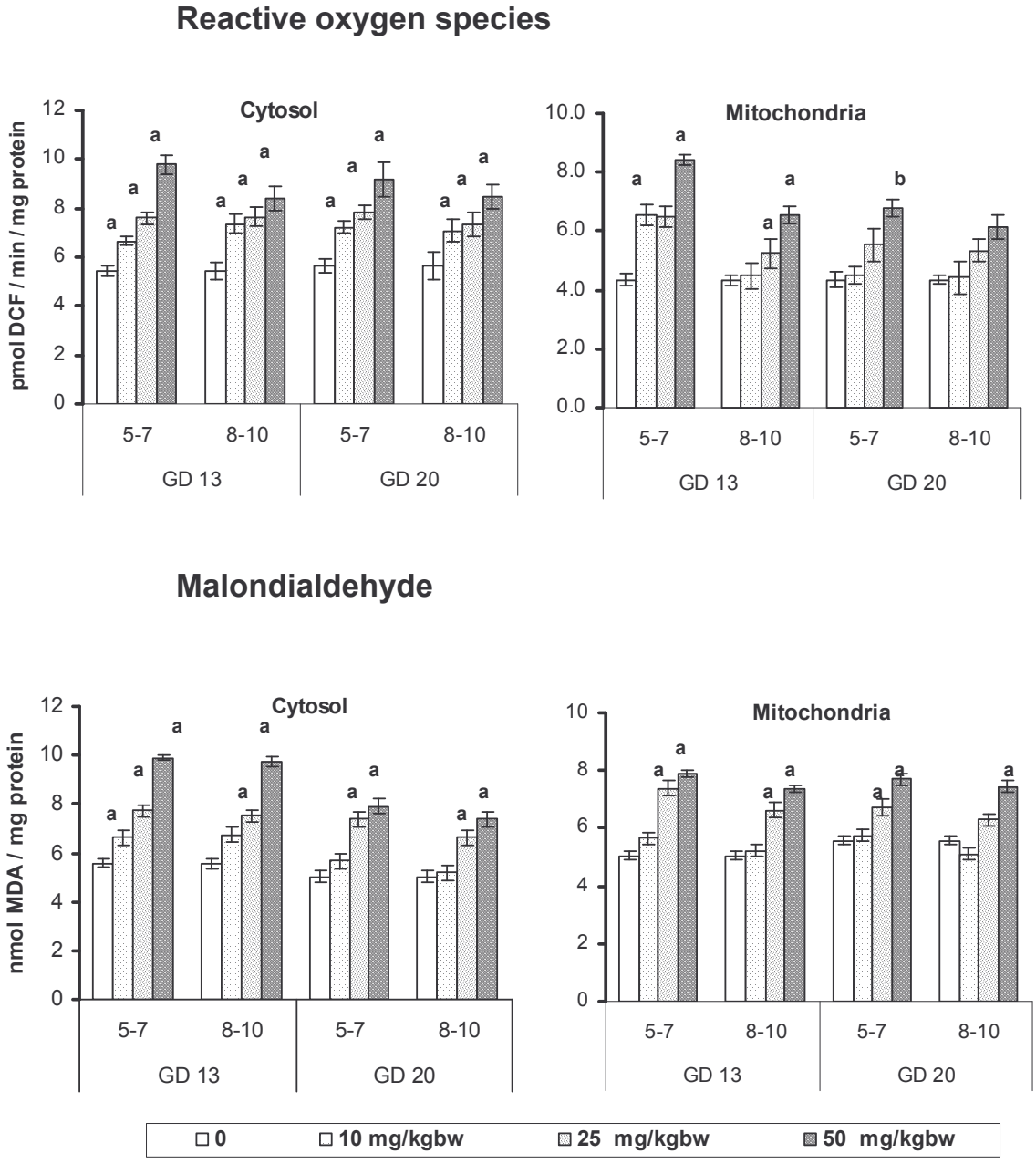
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001,* [b]*P<0.01*

Fig 2.3 Pattern of ROS generation and status of lipid peroxidation in liver of pregnant rats administered iron dextran



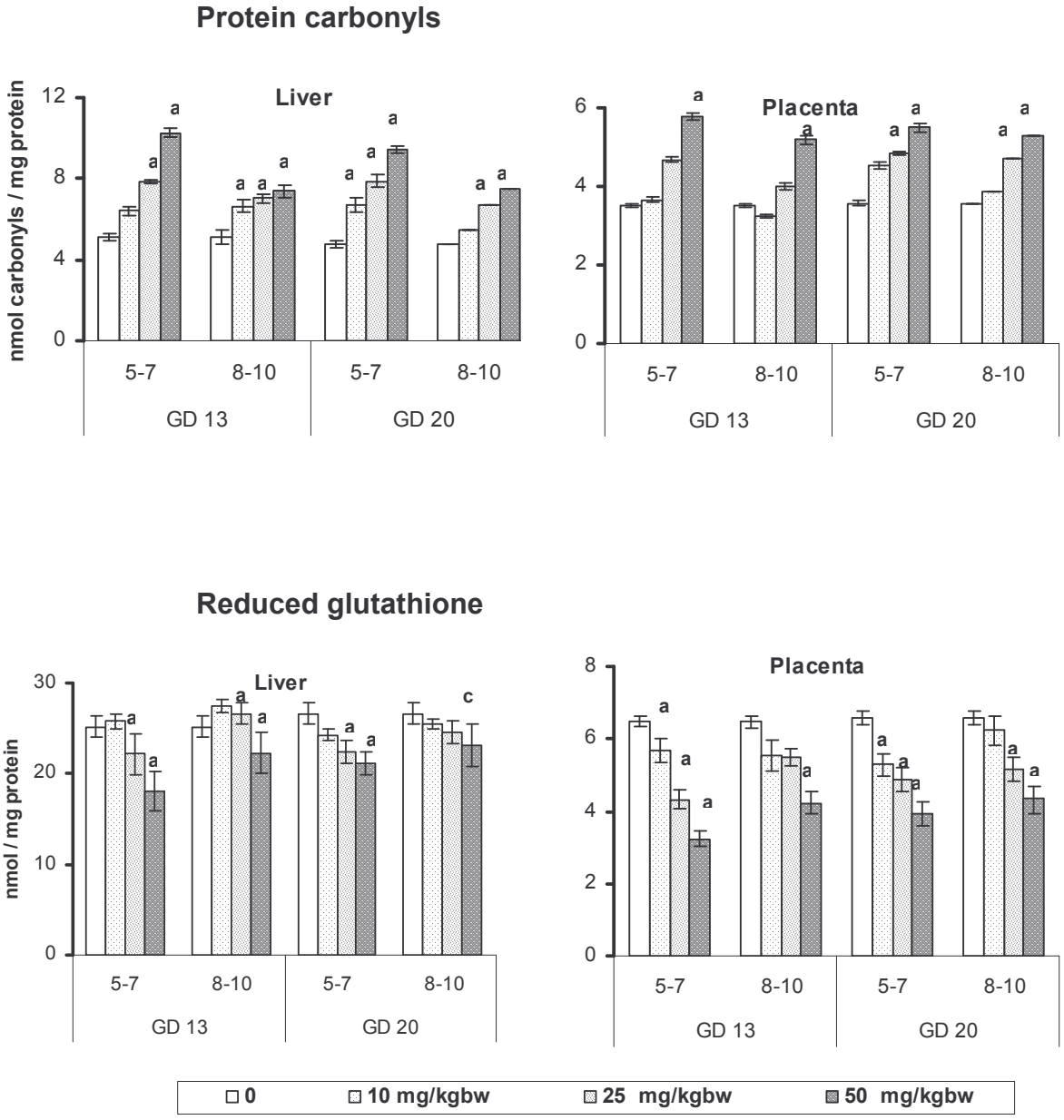
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$, $^{b}P<0.01$

Fig 2.4 **Pattern of generation of reactive oxygen species and status of lipid peroxidation in kidney of pregnant rats administered iron dextran**



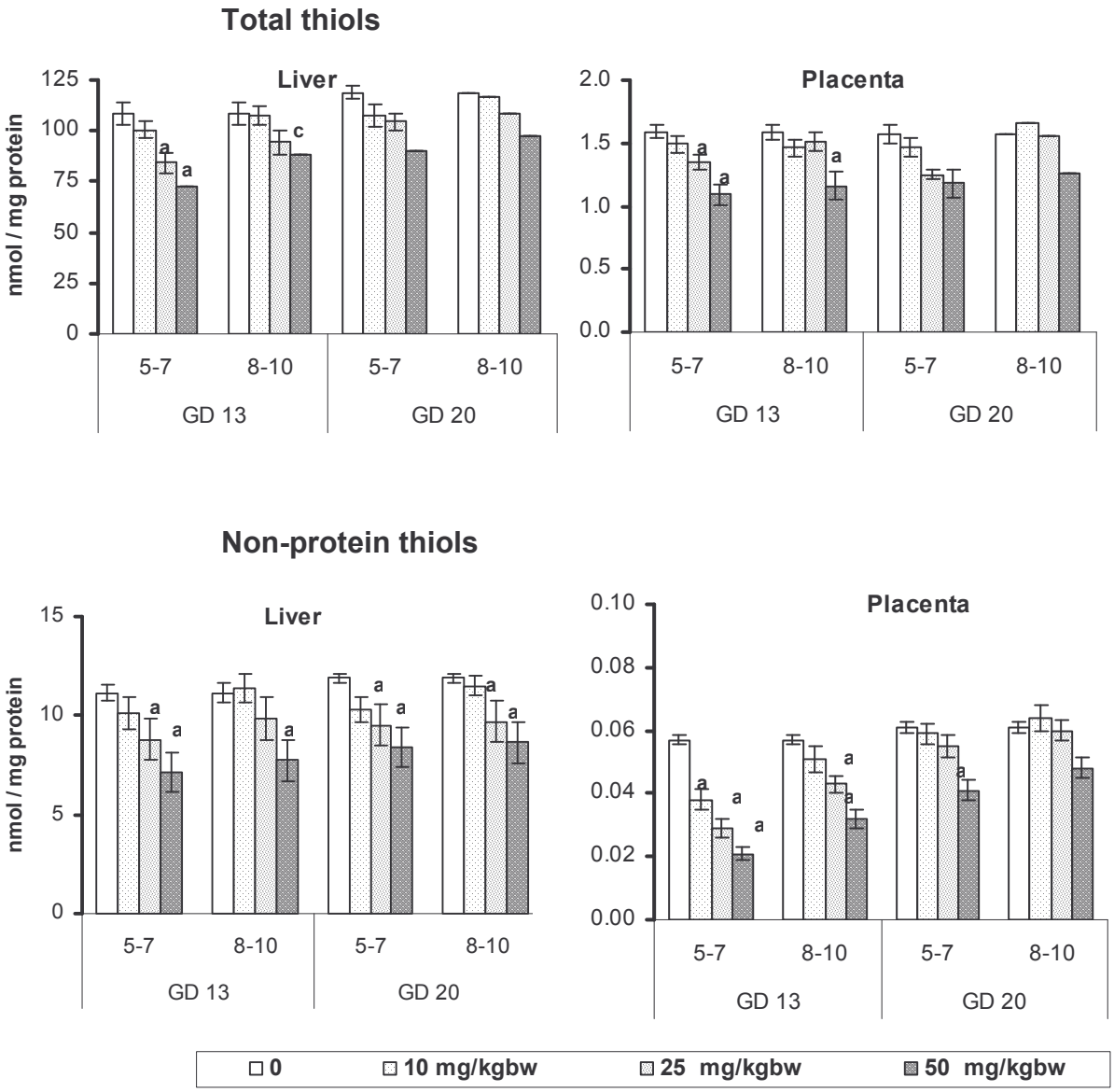
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$

Fig 2.5 Status of protein carbonyls and reduced glutathione in maternal liver and placenta of rats administered iron dextran



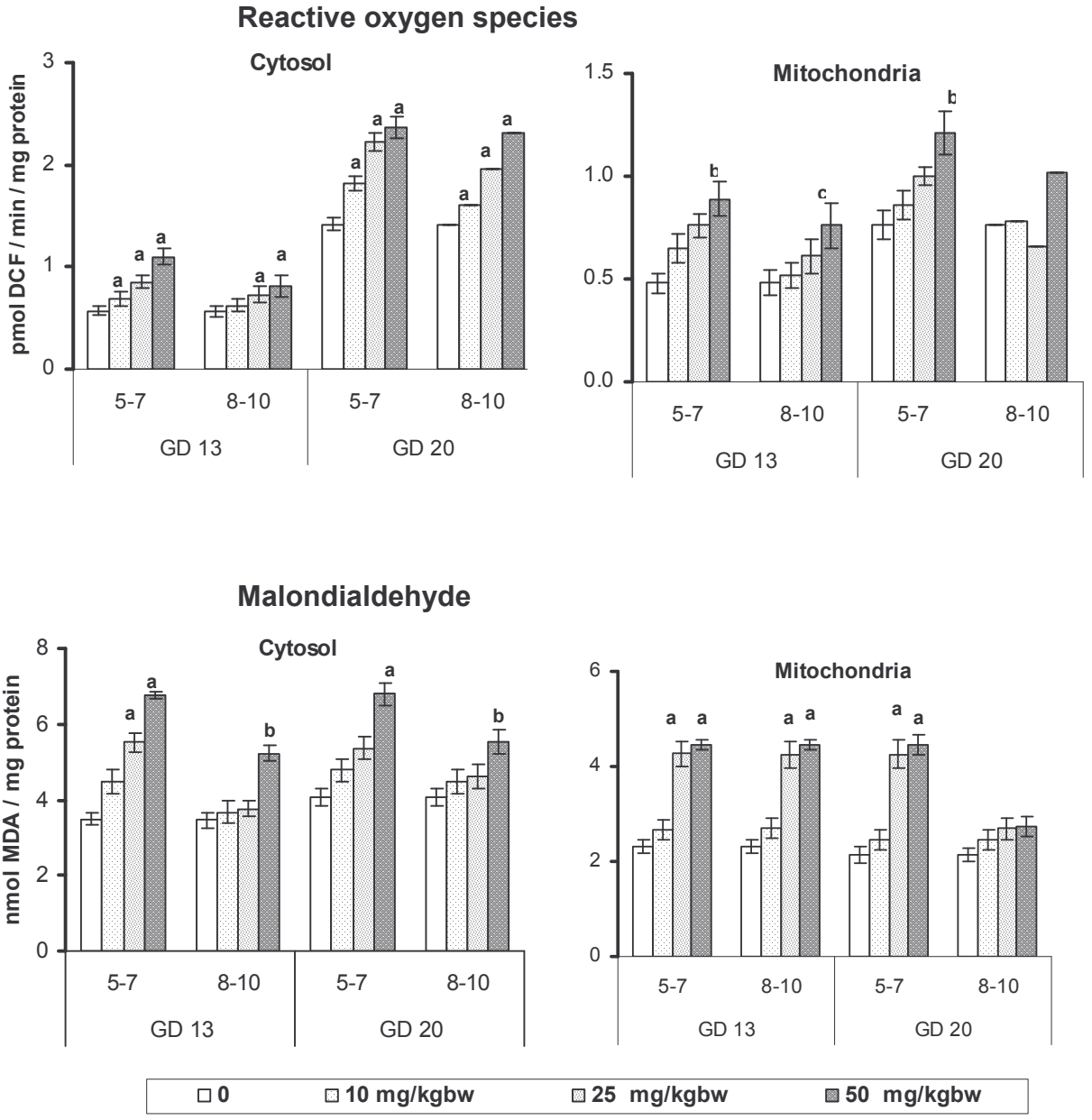
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$, $^{c}P<0.05$

Fig 2.6 Status of total thiols and non protein thiols in maternal liver and placenta of rats administered iron dextran



Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$, $^{c}P<0.050$

Fig 2.7 Effect of maternal iron exposure on placental oxidative stress markers



Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$

Table 2.10 Effect of maternal iron (ID) exposure on the status of lipid peroxidation and ROS generation among embryos (GD_{13})

	ID (mg / kg bw)	Cytosol		Mitochondria	
		5-7	8-10	5-7	8-10
ROS[1]	0	2.27 ± 0.13	2.27 ± 0.13	1.29 ± 0.14	1.29 ± 0.14
	10	2.16 ± 0.12[a]	2.31 ± 0.14	1.34 ± 0.15	1.26 ± 0.16
	25	2.66 ± 0.16[a]	2.68 ± 0.13[a]	1.57 ± 0.17[a]	1.39 ± 0.19
	50	3.14 ± 0.23[a]	2.92 ± 0.17[a]	1.86 ± 0.12[a]	1.68 ± 0.15[a]
MDA[2]	0	0.13 ± 0.01	0.13 ± 0.01	0.16 ± 0.09	0.16 ± 0.09
	10	0.12 ± 0.04	0.11 ± 0.06	0.17± 0.03	0.18 ± 0.07
	25	0.15 ± 0.07	0.16 ± 0.08	0.19 ± 0.06	0.21 ± 0.04
	50	0.18 ± 0.05	0.19 ± 0.10	0.26 ± 0.02[c]	0.25 ± 0.08

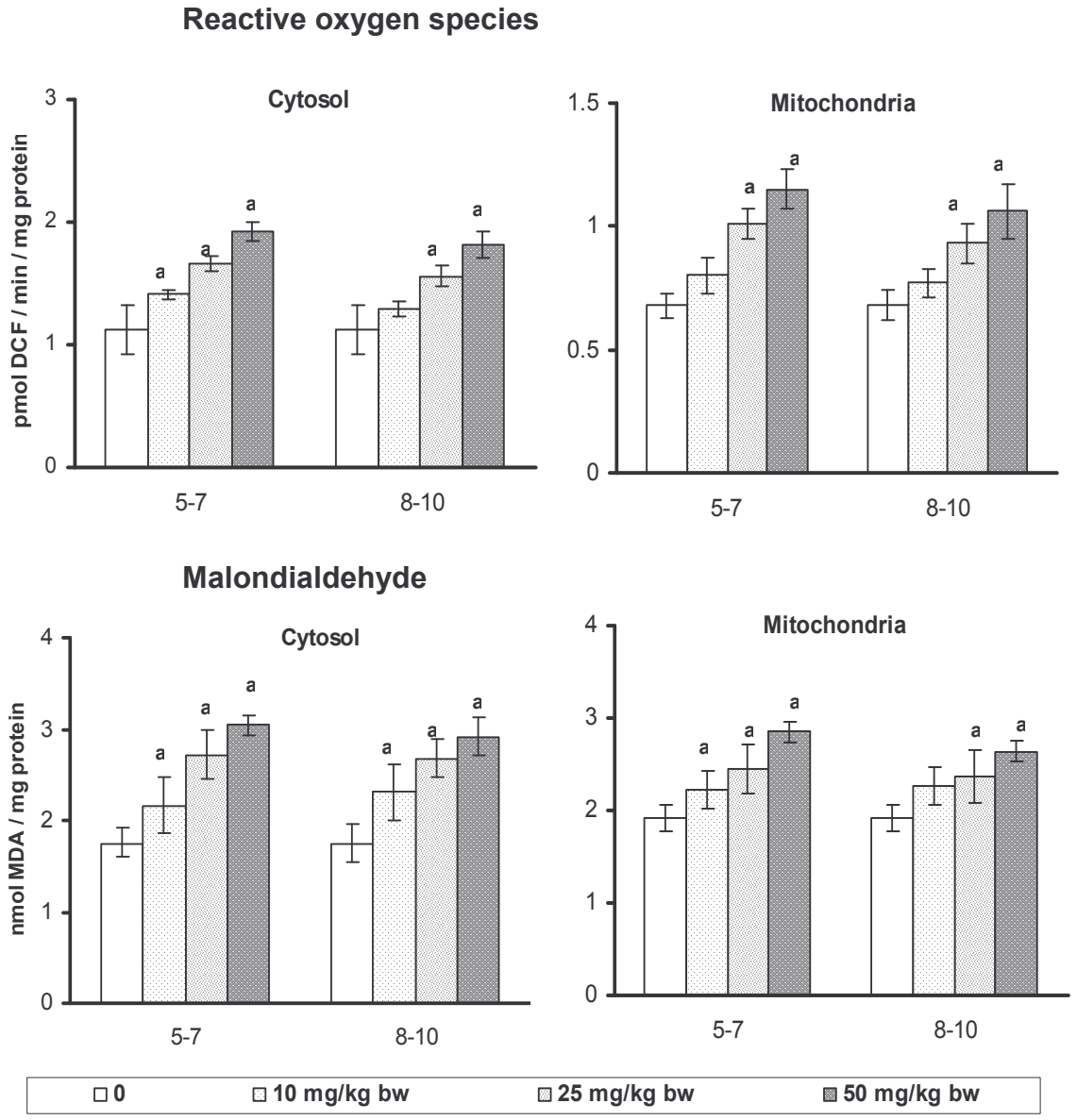
Values are mean ± SD (n=10)
[1]pmol/min/mg protein; [2]nmol/mg protein;
Data analysed by Holm-Sidak method; [a]*P<0.001,* [c]*P<0.05*

Table 2.11 Status of reduced glutathione, protein carbonyls, total thiols (TSH) and non protein thiols (NPSH) in embryos of pregnant rats administered iron dextran

	ID (mg/kg bw)	Gestation Days	
		5-7	8-10
GSH[1]	0	5.47 ± 1.19	5.47 ± 1.19
	10	5.14 ± 0.72	5.39 ± 0.79
	25	4.62 ± 0.55	4.81 ± 0.69
	50	3.51 ± 0.77[b]	4.12 ± 0.90
TSH[1]	0	25.27 ± 1.25	25.27 ± 1.25
	10	20.45 ± 1.36	25.34 ± 1.81
	25	15.28 ± 1.64[a]	20.43 ± 1.76[a]
	50	13.76 ± 1.58[a]	18.26 ± 1.42[a]
NPSH[1]	0	5.99 ± 0.41	5.99 ± 0.41
	10	5.26 ± 0.30	5.82 ± 0.42
	25	4.34 ± 0.24[a]	4.24 ± 0.45[a]
	50	3.15 ± 0.52[a]	3.44 ± 0.14[a]
PC[2]	0	3.34 ± 0.36	3.34 ± 0.36
	10	3.72 ± 0.71	3.41 ± 0.41
	25	4.47 ± 0.54[b]	4.15 ± 0.87
	50	5.12 ± 1.16[b]	3.51 ± 1.17

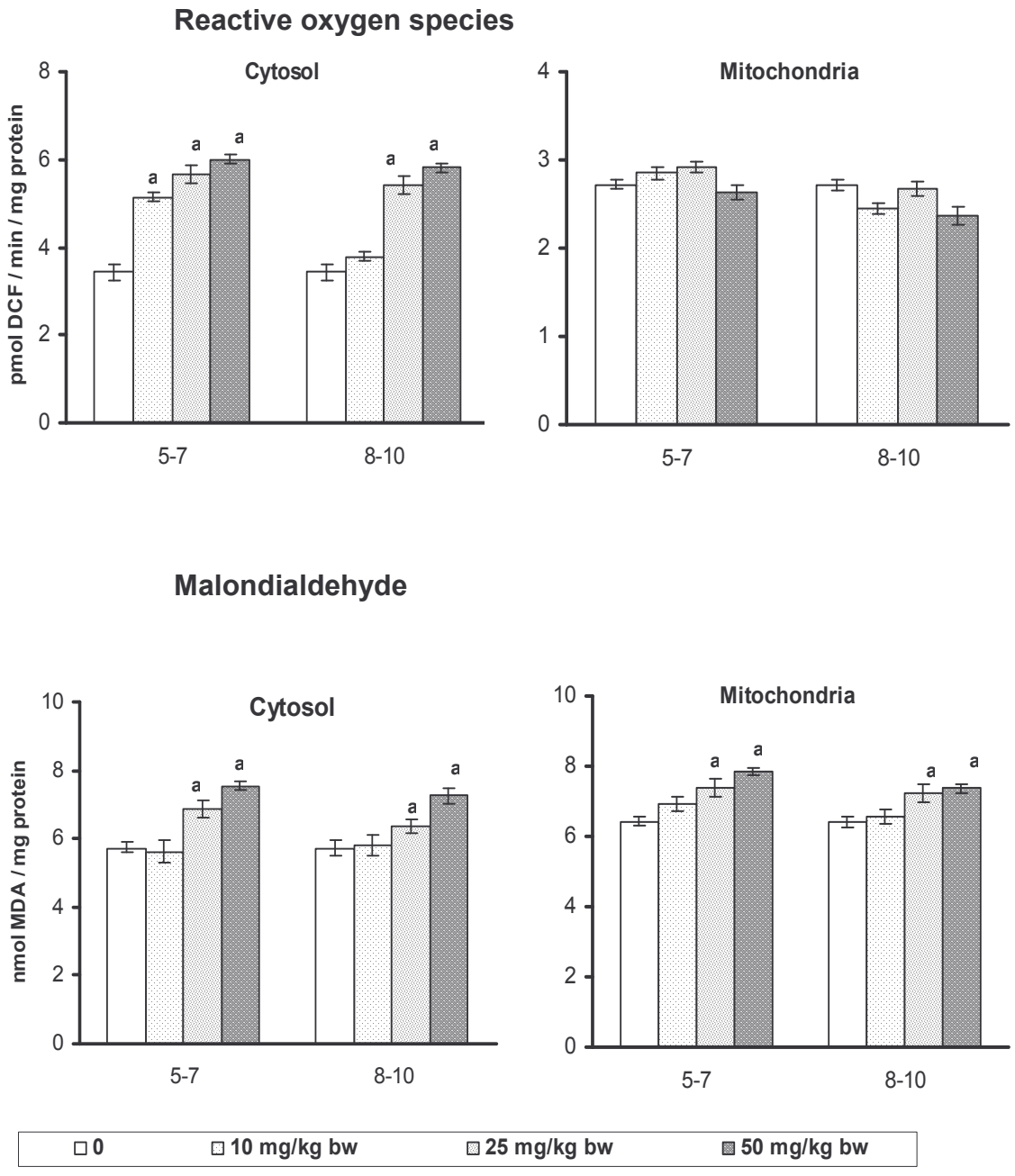
Values are mean ± SD (n=10)
[1]nmol/mg protein; [2]nmol carbonyls/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001,* [b]*P<0.01*

Fig 2.8 Alterations in reactive oxygen species and malondialdehyde levels in fetal brain obtained from iron administered pregnant rats



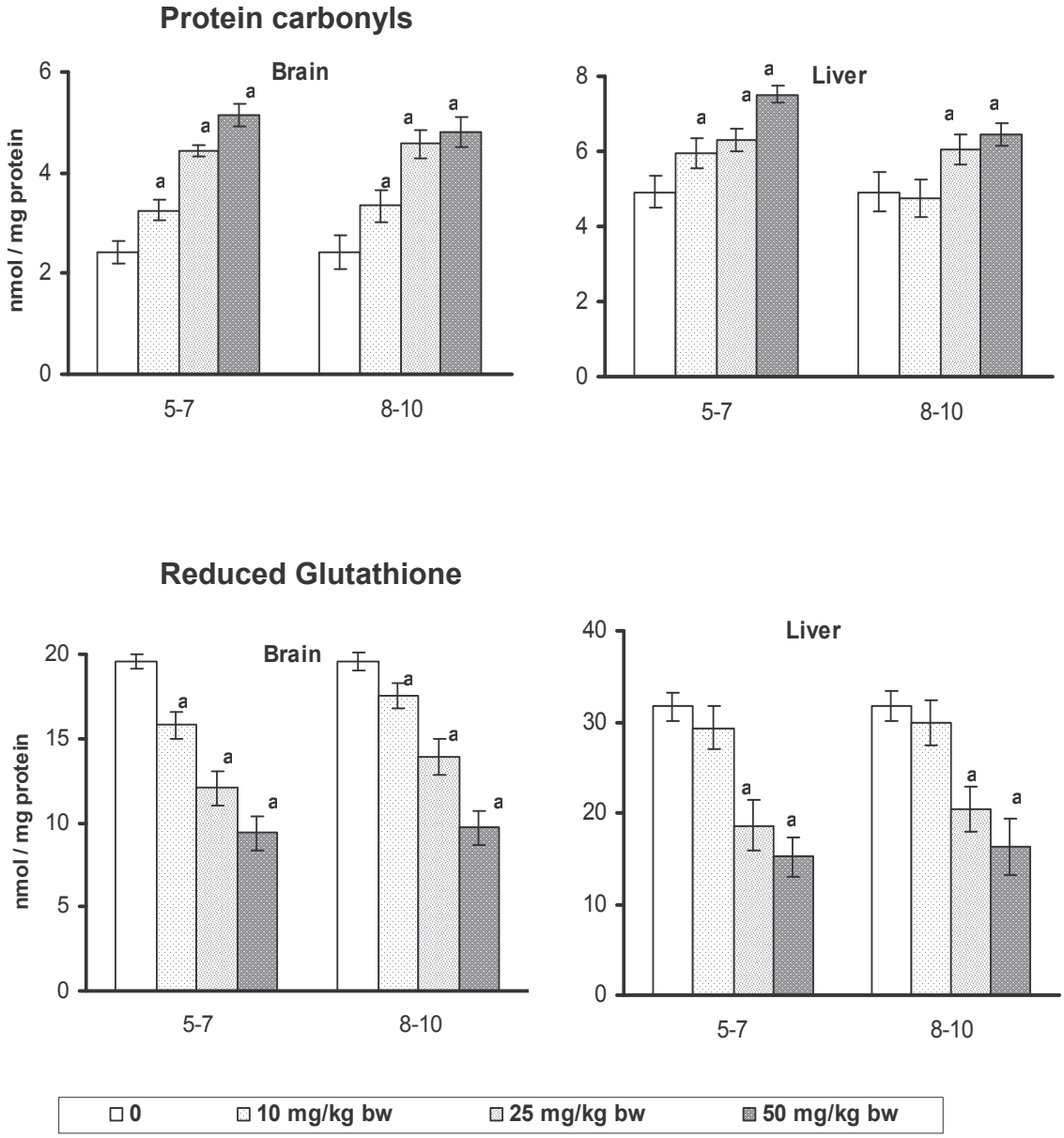
Values are mean ± SD (n=12)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Fig 2.9 **Alterations in reactive oxygen species and malondialdehyde levels in fetal liver obtained from iron administered pregnant rats**



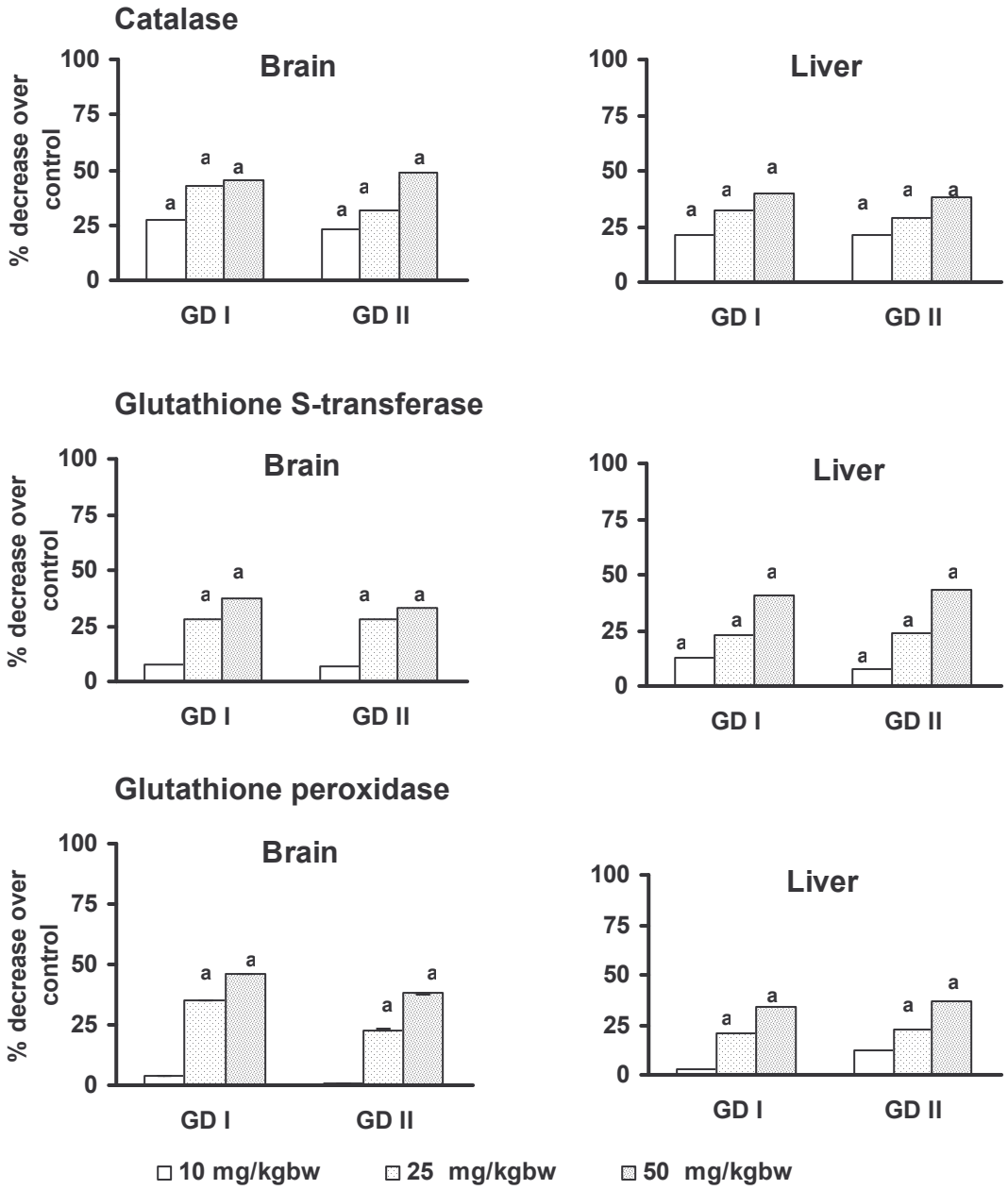
Values are mean ± SD (n=12)
Data analysed by Holm-Sidak method; $^{a}P<0.001$

Fig 2.10 Status of protein carbonyls and reduced glutathione in fetal brain and liver obtained from iron administered pregnant rats



Values are mean ± SD (n=12)
Data analysed by Holm-Sidak method; $^{a}P<0.001$

Fig 2.11 Activities of catalase, glutathione transferase and glutathione peroxidase in fetal brain and liver obtained from iron administered pregnant rats



Values are mean ± SD (n=12)
Data analysed by Holm-Sidak method; [a]*P<0.001*

4.0 DISCUSSION

Pregnancy is a condition exhibiting increased susceptibility to oxidative stress, defined here as a disturbance in the prooxidant-oxidant balance in favour of the former, leading to potential damage *(Sies, 1991; Page, 1993).* The primary objective of the current study was to examine whether gestational exposure to specific prooxidants such as a t-butyl hydroperoxide (a model prooxidant) and iron (a well known prooxidant) induces any significant oxidative impact in embryos and fetuses. Further, we examined the correlations between maternal, placental, and embryonic oxidative stress in the tbHP model, while we also examined the fetal oxidative impact in the iron model. More importantly, a basic question "whether there are any *critical windows* to oxidative stress during gestation" was also addressed in these two prooxidant models.

That normal pregnancy is associated with an increase in oxidative stress and lipid peroxidation, clearly suggests that some of the vital maternal organs may be relatively more vulnerable to exogenous prooxidants during gestation. More importantly, these oxidative perturbations are likely to have a substantial impact on the developing embryo and fetus. This study provides evidence that maternal exposure to prooxidants during gestation can affect early stages of organogenesis, leading to embryonic deaths. Also, early phases of rodent organogenesis are sensitive to the prooxidant influences.

t-butyl hydroperoxide model:

Organic hydroperoxides such as t-butyl hydroperoxide have been employed as prototypic inducer of oxidative stress *in vitro* and *in vivo* systems *(Sakaida et al., 1991; Latour et al., 1995; Rajeshkumar et al., 2002)*. In our laboratory, we have employed hydroperoxides such as tbHP and cumene hydroperoxide as model prooxidants to induce oxidative stress in testis of mice and studied its genotoxic implications *(Rajeshkumar et al., 2002)*. Further in adult rats tbHP induced significant increase in lipid peroxidation and enhanced ROS generation in testis following short term exposure *(Kaur et al., 2006; Kumar & Muralidhara, 2007)*.

In the present study, administration of tbHP at sublethal dosages (1/10 and 1/5 LD_{50}/d for 3 days) induced a significant increase in LPO and enhanced generation of ROS levels sampled on GD_{13} in the maternal organs such as liver and kidney irrespective of the dosing regimen (GD_{5-7} or GD_{8-10}) clearly suggesting its potential to induce oxidative stress. These results in pregnant rats are consistent with the earlier data on tbHP induced oxidative damage in rat liver (*Younes & Wess, 1990),* testis of mice and rats *(Rajeshkumar et al., 2002; Li et al., 2006; Kumar & Muralidahra, 2007)*. In maternal liver, enhanced generation of ROS and MDA levels were evident in both cytosol and mitochondria suggesting a uniform response. A comparison of the degree of response among GD_{5-7} and GD_{8-10}, however, revealed a more robust induction with the earlier dosing regimen. Although the reasons are not clear, it may be related to the enhanced antioxidant potency in the maternal milieu which is known to be relatively higher among pregnant dams to cope up with enhanced endogenous LPO events.

Further evidence of tbHP-induced oxidative stress in maternal tissues was discernible in terms of diminished activity of antioxidant enzymes such as catalase and GST and in the levels of reduced GSH levels. The reduced GSH pool in the cytosol is indicative of the protective role of GSH and related enzymes during the metabolism of tbHP. GSH is the major sulfhydryl compound that serves as an effective reductant and a nucleophile that interacts with numerous electrophilic and oxidizing compounds *(Kaur et al., 2006)*. Depletion of GSH levels can also lead to decreased concentration of non-enzymic antioxidant molecules such as ascorbic acid and α-tocopherol. Although we have not measured the levels of these antioxidants in pregnant rats, earlier, in male rats we have found significant decrease in ascorbic acid and tocopherol levels following tbHP administration at similar dosages *(Kumar & Muralidhara, 2007)*.

Metabolism of tbHP has been elucidated by various workers employing hepatocyte models *in vitro* and two major mechanisms have been speculated. tbHP is known to be metabolized by GPX to t-butyl alcohol in isolated rat

heptocytes and this reaction consumes reduced GSH, generating oxidised glutathione, GSSG which is then reduced to GSH by Glutathione reductase, a reaction simultaneously oxidizes NADPH to NAD^+ *(Rush et al., 1985; Rush & Alberts, 1986)*. Depletion of GSH and oxidation of pyridine nucleotides are known to be associated with altered Ca^{2+} homeostasis which is thought to be critical event in the formation of blebs on the plasma membrane. Alternatively, tbHP can be metabolized to free radical intermediates by cytochrome P450 (present in hepatocytes) or hemoglobin (erythrocytes) which in turn can initiate lipid peroxidation. Since the integrity of cellular membranes is critical to normal cell function, the peroxidative decomposition of membrane lipids may have widespread implications in tbHP toxicity (*Rush et al., 1985*). It is also likely that tbHP free radicals may also form covalent bonds with cellular macromolecules resulting in cell injury as speculated earlier (*Orrenius et al., 1983*). However, the relative contribution of each of these metabolic pathways *in vivo* in the pathogenesis of tbHP-induced cell death is not clear.

A further evidence of tbHP-induced oxidative stress in the maternal milieu was evident from the elevated levels of protein carbonyls at higher doses. Among the various oxidative modifications of aminoacids in proteins, protein carbonyl formation may be an early biomarker of ROS mediated protein oxidation (*Stadtman, 1990)*. Accumulation of high amounts of protein carbonyls due to tbHP treatment reflects a high rate of protein oxidation consistent with high LPO state. Further, it also reflects a very low rate of oxidized protein degradation and or low repair activity since oxidized forms of some proteins and proteins modified by LPO products are not only resistant to proteolysis, but can also inhibit the ability of proteases to degrade the oxidized forms of other proteins. Alterations in protein conformation can lead to increased aggregation, fragmentation, distortion of secondary and tertiary structures, susceptibility to proteolysis and diminution of normal function. This data corroborates our earlier findings in rat testis in which we found elevated protein carbonyl following short term exposure to tbHP *(Kumar & Muralidhara, 2007).*

In the present model, tbHP induced oxidative damage was not confined only to the maternal milieu, as we found significant elevation in both MDA and ROS levels in the placental tissue irrespective of the dosing regimen. The protective role of placenta in part depends on the activity of its detoxifying enzyme (*Avissar et al., 1994*) and has the propensity to protect the fetus from free radical mediated damage by lowering the level of LPO in fetal blood *(Takehara et al., 1990).* Numerous workers have suggested that both placenta and the yolk sac play a critical role in the fetal development including protection from oxidative stress. GSH is shown to play an important role and glutamate-cysteine ligase, the enzyme that catalyses the rate limiting step in GSH biosynthesis is demonstrated to be widely expressed in the rodent embryos and extra membranes throughout development (*Trocino et al., 1995; Thompson, 2000; Diaz et al., 2004*). In the present study, tbHP administered during both dosing regimens resulted in marked placental oxidative damage as revealed by robust elevations in the ROS and MDA levels in both cytosol and mitochondrial fractions. However, the decline in GSH levels were moderate and were associated with reduced activities of antioxidant enzymes.

In the current study, induction of oxidative stress in maternal milieu and placental tissue was associated with significant oxidative impact on the embryos isolated on GD_{13}. While the lower dose failed to induce any significant impact, at the higher dose, embryos were subjected to significant oxidative stress as evidenced by elevated ROS and dramatic elevation of MDA levels. In general, the degree of oxidative response was relatively higher, among embryos recovered from dams administered tbHP during dosing regimen $GD_{5\text{-}7}$ compared with those of $GD_{8\text{-}10}$. The susceptibility of GD_{13} embryos to oxidative stress was also reflected in the altered redox state measured in terms of reduced GSH and total thiols. The embryos showed a decline in the GSH and thiols levels only at the higher dose which was accompanied with significant reduction in the activities of both catalase and GST levels.

Iron model

The primary aim of employing iron as a prooxidant was based on the fact that the oxidative stress and *in vivo* consequences caused by reactive iron species (mostly free iron) have been well studied in various species of experimental animals *(Casanueva & Viteri, 2003)*. Many of the deleterious effects of hemochromatosis and other chronic iron-overload conditions, some cancers, aging and neurodegerneartive diseases, are attributed to or contributed by them. ROS damage is observed in and explains many of the effects secondary to 'temporary iron excess' as in acute iron intoxication, hemolytic episodes and reperfusion injury *(Lauffer, 1991)*. The effects of iron excess can be generalised or local. Local iron excess and iron-mediated oxidative stress has been demonstrated in the intestinal mucosa, liver, spleen, bone marrow and placenta.

In the present study, administration of iron at sublethal dosages (10, 25 and 50 mg/kg bw/d for 3 days) induced a significant increase in LPO and enhanced generation of ROS sampled on GD_{13} in maternal milieu irrespective of the dosing regimen (GD_{5-7} or GD_{8-10}) at higher doses clearly suggesting induction of OS. In maternal liver, enhanced generation of ROS and MDA levels were evident in both cytosol and mitochondria suggesting a uniform response. A comparison of the degree of response among GD_{5-7} and GD_{8-10}, however, revealed a relatively higher degree of induction with the earlier regimen. This data suggests that brief exposure to prooxidants during early post-implantation period in rat can result in significant maternal OS as revealed at both sampling times (GD_{13} and GD_{20}).

Iron administration to pregnant dams induced significant OS in maternal milieu was also reflected, as hepatic tissue (sampled at GD_{13} and GD_{20}) showed diminished activity of antioxidant enzymes viz., catalase and GST and diminution in the levels of reduced glutathione and total thiols. Thiols have been found elevated in erythrocyte lysates and SOD activity in erythrocytes and

plasma thiol levels were found to be lower during pregnancy than in non pregnant women, suggesting an oxidative environment and stress *(Wisdom et al., 1991; Ilouno et al., 1996).*

It is well understood that pregnancy favors oxidative stress mostly because of the mitochondria-rich placenta. Transition metals, especially iron, which are particularly abundant in the placenta, are important in the production of free radicals. In the present model, iron administration also resulted in significant oxidative stress in the placental tissue, as we found marked elevation in both MDA and ROS levels in the placental tissue irrespective of the dosing regimen. As discussed above, placental environment is one of enhanced oxidative stress that induces protective mechanisms against free radicals as normal gestation progresses. Overall, the plasma free radical trapping and antioxidant potential are able to counteract OS in normal pregnancy through enzymatic induction and activity of various antioxidant enzymes as well as through non-enzymatic free radical scavengers (*Watson et al., 1997; Wang & Walsch, 1996; Kharb, 2000).* The fact that placental markers of oxidative damage were elevated among dams administered iron clearly indicates that the adaptation and equilibrium is easily disrupted.

In the current study, induction of oxidative stress in maternal milieu and placental tissue was associated with significant oxidative impact on the embryos isolated on GD_{13}. While the lower dose failed to induce any significant impact, at the higher dose, embryos were also subjected to significant oxidative stress as evidenced by moderately elevated ROS and dramatic elevation of MDA levels. In general, the degree of oxidative response was relatively higher, among embryos recovered from dams administered tbHP during dosing regimen $GD_{5\text{-}7}$ compared with those dosed on $GD_{8\text{-}10}$. The susceptibility of GD_{13} embryos to OS was also reflected in the altered redox state measured in terms of reduced GSH and total thiols. The embryos showed a decline in the GSH and thiol levels only at the higher dose which was accompanied with significant reduction in the activities of both catalase and GST levels.

The intrauterine environment can significantly affect embryonic and fetal development. In the current study, iron administration also significantly impacted the embryonic oxidative markers as both ROS levels and MDA levels were elevated irrespective of the dosing regimen. Embryos of iron administered dams showed diminution in the levels of reduced glutathione and total thiols which is suggestive of the ongoing oxidative stress *in utero.* These oxidative perturbations clearly indicate that iron at the higher dosages resulted in a temporary iron overload condition. Such a situation could be produced given the passive diffusion of soluble iron molecules and iron transported to the liver as non-transferrin bound iron (NTBI). It may be relevant to note that NTBI uptake by hepatocytes is independent of the iron content of the liver, and is known to be very rapid and more efficient than the liver uptake of transferring-bound uptake *(Casanueva & Viteri, 2003).*

The effect of iron administration had a negative impact on the frequency of survival of embryos (Table 2.2) as it produced a dose-dependent increase in the incidence of embryolethality. The percent dead implantations (%DI) which were less than 6% among the controls were elevated by several folds (2-5) among iron administered dams. Interestingly, the incidence was relatively higher when iron was administered during GD_{5-7} compared to GD_{8-10}. Correspondingly the fetal weights among iron administered dams were significantly reduced at higher dosages suggesting that it may be related to the enhanced induction of oxidative stress. However, we have not measured the nature of cell death among these embryos.

In conclusion, the results obtained from these two models suggest that induction of oxidative stress in the maternal milieu during early post implantation period has significant oxidative impact on placental tissue and growing embryos and fetuses. Our findings also revealed that prooxidant exposure during GD_{5-7} induced relatively higher oxidative perturbations in both embryos and fetus. Higher incidence of embryolethality among dams administered iron is suggestive of the fact that the placental as well as the embryonic defenses under the conditions of exposure were overwhelmed by iron-induced oxidative stress

culminating in death of the conceptus at an early period. We could also demonstrate that the timing and severity of the perinatal prooxidant insults are clear determinants of the pregnancy outcome interms of severity of oxidative damage and embryonic deaths.

6.0 SUMMARY

1. Administration of a model prooxidant, viz. t-butyl hydroperoxide at sublethal doses to pregnant dams during specific periods of gestation (GD_{5-7} or GD_{8-10}) induced significantly elevated oxidative stress response in the maternal tissues (viz., liver and kidney) measured on GD_{13}.

2. In liver, both cytosolic and mitochondrial fractions were subjected to significant oxidative stress as evident by the elevated MDA and ROS levels which were accompanied with depleted GSH levels, total thiols and diminished levels of antioxidant enzymes.

3. Induction of oxidative response by tbHP was relatively of higher magnitude when the exposure occurred during GD_{5-7} compared to the exposure of similar doses during GD_{8-10} suggesting the increased vulnerability of early implantation embryos to prooxidants.

4. However, tbHP exposure caused significant oxidative stress in placental tissue which was of similar degree irrespective of the dosing period.

5. Maternal exposure to tbHP resulted in markedly elevated oxidative perturbations in GD_{13} embryos measured in terms of ROS generation and MDA levels which were associated with reduced antioxidant enzyme activities, total thiols and non-protein thiols.

6. Elevated oxidative dysfunctions among embryos of tbHP exposed dams was associated with increased frequency of embryolethality (4.6 fold higher than the background incidence.

7. Administration of iron at sublethal doses to pregnant dams during early implantation period (GD_{5-7}) or late implantation period (GD_{8-10}) caused significant oxidative impairments in maternal tissues such as liver and kidney.

8. In liver, both cytosolic and mitochondrial fractions were subjected to oxidative stress as evident by elevated MDA and ROS levels were accompanied with depleted GSH levels, total thiols, and diminished levels of antioxidant enzymes.

9. Iron administered during early implantation period (GD_{5-7}) appeared to induce a higher degree of oxidative response in the maternal tissues.

10. Iron exposure also caused elevated oxidative stress in placenta irrespective of dosing period.

11. Maternal exposure to iron resulted in markedly elevated oxidative perturbations in GD_{13} embryos measured in terms of ROS generation and MDA levels which were associated with reduced antioxidant enzyme activities, total thiols and non-protein thiols.

12. Both brain and liver of fetuses (GD_{20}) obtained from diabetic mothers showed markedly enhanced ROS levels, MDA levels, protein carbonyls which were accompanied with depleted GSH content and diminished activities of antioxidant enzymes.

13. Collectively these data suggest that maternal exposure to prooxidants cause significant oxidative stress in maternal organs, placenta and increased incidence of embryonic deaths. The oxidative impact on the embryos appeared to be of higher magnitude if the prooxidants are exposed during early post-implantation period (GD_{5-7}) compared to their exposure during GD_{8-10} suggesting the enhanced vulnerability of early post implantation embryos.

1.0 INTRODUCTION

Lipid peroxidation (LPO) can result in reversible and irreversible cell and tissue damage. The process of lipid peroxidation is initiated by ROS, such as hydroxyl radicals and is terminated by antioxidants such as vitamin E. LPO is also stimulated by iron ions which catalyze the formation of the hydroxyl radical and accelerate the decomposition of lipid hydroperoxides. The degradation of lipid hydroperoxides into hydrocarbons appears to be dependent on the presence of metal ions and a number of animal studies have shown that excess iron, when given either intraperitoneally or in the diet resulted in increased exhalation of ethane and/or pentane. Increases in tissue MDA levels in rat models of dietary iron overload have also been reported.

Despite extensive literature on iron and lipid peroxidation, few studies have investigated the effects of oral iron supplements on lipid peroxidation under pregnancy conditions. Iron supplements are almost universally prescribed for pregnant women at doses ranging from 30 mg/d in the USA to as high as 240 mg/d where prevalence of anemia is high. For iron supplementation programs where anemia is high, the International Nutritional Anemia Consultative Group has recently changed its recommendations from 120 to 60 mg/d. However, if duration of iron supplementation during pregnancy is short, or if anemia is present, 120 mg/d is still recommended. Pregnant women in developing countries are commonly given daily supplements containing 120 mg of iron to prevent and correct gestational iron deficiency *(Lynch & Stoltzfus, 2003)*. This dose of iron (calculated to be 10 times the normal dietary intake) has been demonstrated to cause gastrointestinal side effects *(Hollan & Johansen, 1993)*.

With these iron doses in pregnant women, few workers have shown high rates of undesirable gastrointestinal side effects and have suggested some toxic effects, possibly involving iron-related oxidative stress. Daily high-iron supplements in rats results in an abnormal accumulation of intestinal mucosal and hepatic nonheme iron and significant increases in LPO *(Viteri et al., 1995; Knutson et al., 2000)*. Few studies have shown markedly increased LPO

suggesting that both iron deficiency and iron excess promote oxidative stress *(Shigenaga et al., 1994)*.

Iron deficiency is a significant public health concern *(Yip, 2001)* that is associated with an increased risk of poor pregnancy outcome *(Viteri, 1997)* and impaired cognitive development in young children. Iron deficiency continues to be one of the most common single-nutrient deficiencies in the world *(WHO, 2001; Viteri, 1997; Beard, 2000; Walter, et al., 2002)*. The consequences of Iron deficiency on the well being of the mother and on the growth of the fetus have been reviewed *(Allen, 1997)*. The major consequence of severe iron deficiency anemia in pregnancy is related to both impaired fetal and maternal health and associated with reduced birth weight, complications, impairments in maternal functions *(Beard, 2000)*. Many studies have reported that iron deficiency in early life is likely to have negative consequences for normal neural development and functioning *(Beard et al., 1993; Felt & Lozoff, 1996)*.

Iron deficiency has also been shown to cause increased lipid peroxidation. Recent studies *(Knutson et al., 2000; Walter et al., 2002)* showed significant increase in ethane, pentane, liver/kidney MDA levels. There are conflicting data in iron deficient rats since one group reports significantly decreased liver MDA levels in deficient rats *(Rao & Jagadeesan, 1996)* and other reports provide evidence that iron deficiency is protective against *in vivo* lipid peroxidation *(Chandler et al., 1988)* and hydroxyl radical formation *(Patt et al., 1990)*. Several factors such as elevated copper levels, accumulation of higher levels of triglycerides in liver and plasma, and increased fragility of mitochondrial membrane of iron deficient rat tissue mitochondria are speculated to contribute to elevated lipid peroxidation in iron deficient rats.

Although WHO has made its recommendations, there is no worldwide consensus concerning iron prophylaxis and therapy during pregnancy. There exists a contradiction between scientific results and common practice. Based on

animal models, the question has been raised whether previously iron-deficient pregnant women were more susceptible to therapeutic-iron-induced oxidative stress than iron replete subjects.

More and more recent articles emphasize the necessary caution considering iron therapy. High grade oxidative stress (and iron) is now widely acknowledged to have an important role in the pathogenesis of neurodegenerative diseases, atherosclerosis, diabetic complications, arthritis etc. Pregnancy itself is associated (as explained in Introduction section of chapter 2) with increase in oxidative stress.

In this context, we have addressed *two major issues* related to iron excess and iron deficiency. The **first issue** is whether oral administration of iron during specific periods of gestation causes oxidative implications in maternal milieu and subsequent oxidative impact in embryos and fetuses. For this we have employed three doses of Iron viz. 2, 4 and 8 mg/rat/d administered either on $GD_{5\text{-}12}$ or $GD_{5\text{-}19}$ in order to investigate the effects on embryos or fetuses (The criterion of dosage selection was based on recent studies of (*Walter et al., 2002)*. The ***second issue*** examined here is related to the often asked question whether iron deficiency during pregnancy causes significant oxidative stress in mother and its correlations with oxidative dysfunctions in embryos and fetuses. The results obtained from these studies are presented in two separate sections A and B.

SECTION A

IRON EXCESS: OXIDATIVE IMPLICATIONS IN MOTHER, EMBRYOS AND FETUS

2.0 OBJECTIVE

The primary focus of this study was to examine the spectrum of oxidative implications in embryos and fetus following induction of oxidative stress during the post-implantation gestation period in pregnant rats. Iron excess model was employed for this purpose and iron dextran (ID) was administered (oral) during two dosing regimens viz., GD_{5-12} and GD_{5-19} to dams which were maintained on normo-protein diet.

3.0 EXPERIMENTAL DESIGN

Two sets of experiments were conducted in pregnant rats to assess the implications of maternal iron excess on embryos and fetuses. Pregnant rats were orally administered ID at dosages of 2, 4, and 8 mg/rat/d at 9:00 am daily and were provided with a known amount of pellet diet. Dams were also provided with iron free (deionized) water *ad libitum*. Both control and treated dams were sacrificed on GD_{13} (for isolation of embryos) and GD_{20} (for isolation of fetuses) under mild ether anesthesia and uterine contents were analysed for total, live and dead implantations. Further, placenta, embryos and fetuses were excised, trimmed, rinsed in ice-cold PBS, blotted weighed and stored until further analysis.

3.1 Oxidative damage in maternal organs, embryos and fetus

3.1.1 Status of lipid peroxidation and generation of ROS

Both control and pregnant rats (n=6) administered ID were sacrificed on GD_{13} or GD_{20}. The degree of oxidative damage was determined in maternal organs such as liver and kidney in cytosol and mitochondrial fractions. Markers of oxidative stress were measured in embryonic homogenates and fetal tissues

(brain and liver). In order to examine a correlation, LPO and ROS were also determined in placenta at both sampling times.

3.1.2 Redox Status

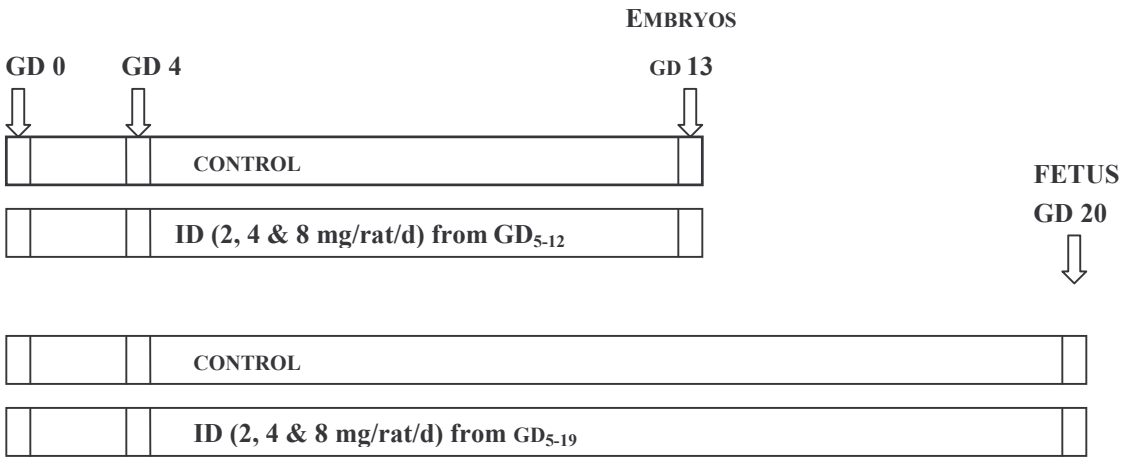
Reduced glutathione, total thiols and non-protein thiols were quantified in maternal tissues, placental tissue, embryos and fetal tissues of both control and ID administered rats.

3.1.3. Response of antioxidant enzymes

As a measure of oxidative stress, activities of selected antioxidant enzymes viz., CAT, SOD, GST, and GPX were determined in cytosolic fractions of embryos, placenta, maternal and fetal tissues of control and ID treated rats.

3.1.4 Protein carbonyls content

As a measure of protein oxidation, the protein carbonyl content was quantified in embryos and fetal tissues of control and ID treated rats. Protein carbonyls were also determined in placenta and maternal tissues.



Treatment regimen and dosages of iron employed for the study

SECTION B

IRON DEFECIENCY DURING PREGNANCY: OXIDATIVE PERTURBATIONS IN EMBRYOS AND FETUSES

2.0 OBJECTIVE

This study has two objectives: i) to establish whether iron deficiency during gestation results in significant oxidative stress in the maternal milieu and ii) to examine the oxidative impact of maternal oxidative stress on both embryos and fetus

3.0 EXPERIMENTAL DESIGN

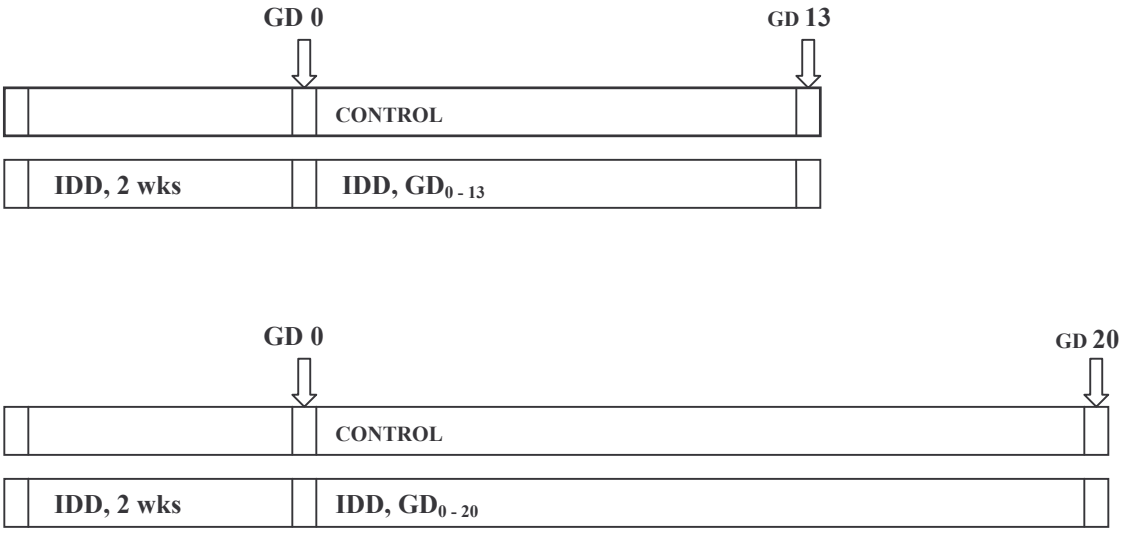
3.1 Preliminary Study - selection of dosages

Virgin female rats (10-12 wks old) were used for the investigations. With an objective of determining the percentage of dietary iron and duration of feeding which would induce consistent iron deficiency prior to pregnancy, 2 sets of preliminary studies were conducted. Initially, groups (n=6) of virgin female rats were maintained on iron deficient (100 and 50%) diet for a period of either 7 or 14 days and terminally rats were sacrificed humanely to assess the hemoglobin levels.

3.2 Determinative Study

Based on the data obtained in the preliminary study, diet regimen completely devoid of iron was fed for 14 days. Rats were then paired with proven males and pregnancy was ascertained by examination of vaginal smears for the presence of sperms. Pregnant rats were continued on their respective dietary regimen. All the rats were provided with iron free (deionized) water. Rats were provided with a known quantity of diet and daily food consumption was monitored. To examine the effect on growth, weekly body weight gain was monitored during the entire experimental period.

Two sets of experiments were conducted to assess the oxidative implications of iron deficiency during pregnancy in embryos and fetuses. In order to study the effects on embryos and fetuses, dams were sacrificed on GD_{13} and GD_{20} respectively.



Treatment regimen Scheme showing the schedule employed for the study
IDD: Iron deficient diet (2 wks prior to mating and throughout the experimental period)

3.3 Analysis of uterine contents for post implantation losses

Pregnant rats from control and IDD groups were sacrificed on GD_{13} (for isolation of embryos) and GD_{20} (for isolation of fetuses) under mild ether anesthesia, uterine horns excised and pinned on to a wax base immersed in ice-cold phosphate buffered saline. Further, uterine horns were longitudinally cut open contents were analysed for total implantation sites, number of liver embryos, resorptions and fetal deaths. Placentas, live embryos and fetuses were excised, trimmed, rinsed in ice-cold physiological saline, blotted, weighed and stored at -70^0 C for further analysis.

3.4 Oxidative damage in maternal tissues, placenta, embryos and fetus

3.4.1 Status of lipid peroxidation (LPO) and generation of ROS

Pregnant rats were sampled on GD_{13} and GD_{20} to measure the degree of oxidative damage in mitochondrial and cytosolic fractions of embryonic and fetal tissues (brain and liver). In order to obtain a comparative picture, LPO was also determined in placenta and maternal tissues (liver and kidney) at both sampling times. Cytosolic and mitochondrial fractions of embryos, placental, maternal and fetal tissues were assayed for the generation of ROS levels (employing DCF-DA as the probe) and the induction of lipid peroxidation was quantified in terms of MDA levels.

3.4.2 Perturbations in non-enzymic antioxidants

At both sampling intervals, the levels of non-enzymic antioxidants viz., GSH, total thiols and non protein thiols were determined in maternal tissues, embryos, and fetal tissues.

3.4.3 Perturbations in enzymic antioxidants

At both sampling intervals, the activities of antioxidant enzymes viz., CAT, SOD, GST, GPX and GR and levels of non-enzymic antioxidants viz., GSH, TSH and non protein thiols were determined in maternal, embryos and fetal tissues.

3.4.4 Oxidative damage to proteins

Fresh mitochondrial and cytosolic samples of embryos, placenta, fetal and maternal tissues of both control and treated groups were processed for the determination of total protein carbonyls content at both sampling intervals.

4.0 RESULTS

SECTION A

IRON EXCESS: OXIDATIVE IMPAIRMENTS IN MOTHER, EMBRYOS AND FETUS

4.1 Growth characteristics

4.1.1 Body weight gain, placenta and fetal weights

Data on the effect of oral iron administration on body weight gain and placental weights are presented in the Table 3.1a. ID at the administered dosages (2-8 mg/rat/d) failed to induce any clinical signs of toxicity or mortality. However, at the highest dose it significantly affected the maternal body weight gain (GD_{13} control, 45.2±4.6; ID, 35.7±2.6). A similar decrease was also evident among rats administered ID during $GD_{5\text{-}19}$ (control, 98.8±5.8; ID, 80.5±3.2). At the lowest dose ID did not affect the placental weights, but the weights increased at highest dose during both the dosing regimens (GD_{13} by 21%; GD_{20} by 45 %).

4.1.2 Fetal weights and embryolethality

Data on the effect of oral iron administration on fetal weights and embryolethality are presented in Table 3.1b. A dose dependent increase in the embryonic deaths was evident with both dosing regimen. At highest dose, the incidence of embryolethality was increased by 4 to 5 folds compared to controls. The fetal weights among dams administered lower doses (2 and 4 mg/rat/d) were not appreciably affected. However, at the highest dose (8mg/rat/d) the fetal weights were moderately decreased (20-25%).

4.2 Status of oxidative damage in maternal organs

4.2.1 Generation of ROS and status of LPO

The effect of ID induced oxidative damage measured as LPO and generation of ROS in cytosol and mitochondria of liver is illustrated in Fig 3.1.

Differential induction levels were evident in both cytosol and mitochondria. At higher doses, the induction of ROS levels appeared to be higher in mitochondria compared to the cytosol during dosing regimen GD_{5-12} (cytosol, 10-64%; mitochondria, 102-107%). A similar trend also occurred during the dosing regimen GD_{5-19} (cytosol, 87-88%; mitochondria, 116-144%). However, the MDA levels were higher during the dosing regimen GD_{5-12} (cytosol, 69-126%; mitochondria, 24-62%). A similar trend was also evident in the dosing regimen, GD_{5-19} (cytosol, 77-144%; mitochondria, 42-85%).

The effect of ID induced oxidative damage measured as LPO and generation of ROS in both cytosol and mitochondria of kidney is illustrated in Fig 3.2. At higher doses, the ROS levels appeared to be higher in cytosol compared to the mitochondria (GD_{5-12}: cytosol, 44-100%; mitochondria, 5-76%; GD_{5-19}: cytosol, 63-123%; mitochondria, 11-108%). However, the MDA levels were higher in ID administered mothers during the dosing regimen GD_{5-12} (cytosol, 38-96%; mitochondria, 25-86%). A similar trend also evident during the dosing regimen GD_{5-19} (cytosol, 54-109%; mitochondria, 42-99%).

4.2.2 Oxidative damage in placenta

Data on the ROS and MDA levels measured in placenta sampled at both dosing regimens are illustrated in Fig 3.3. The low dose of ID did not induce any significant response. While at higher doses, the ROS levels were markedly enhanced (GD_{5-12}: cytosol, 5-80%; mitochondria, 35-120% and GD_{5-19}: cytosol, 100-210%; mitochondria, 29-107%). In contrast, the induction levels of MDA were relatively of lesser magnitude (GD_{5-12}: cytosol, 10-66%; mitochondria, 35-117% and GD_{5-19}: cytosol, 10-37%; mitochondria, 23-86%).

4.2.3 Glutathione levels and protein carbonyls in maternal organs and placenta

Status of protein carbonyls in maternal liver, kidney and placenta of pregnant rats administered ID are shown in Fig 3.4. At the highest dose the carbonyls levels were elevated significantly during both dosing regimens (liver: 82-74%; kidney: 114-93%; placenta, 95%).

No significant alteration was evident at the GSH levels at the low dose of ID (Fig 3.4). At higher doses, the GSH levels were found to be marginally reduced in maternal organs (liver: GD_{5-12}: 12-32%; GD_{5-19}: 26-51% and kidney: GD_{5-12}: 23-46%; GD_{5-19}: 29-67%. However, there was a uniform response in the placental tissue (GD_{5-12}: 25-62%; and GD_{5-19}: 21-63%).

4.2.4 Status of total and non protein thiols in maternal liver and placenta

The thiol content and non protein thiol levels measured in maternal liver and placental tissue is shown in Fig 3.5. In maternal liver, the thiol levels were diminished uniformly at the higher doses irrespective of the dosing regimen (GD_{5-12}, 30-46%; GD_{5-19}, 27-52%) and a similar response was also evident in non-protein thiol levels. The placental tissue also showed significant decrease in both total thiol and non-protein thiol levels during both dosing regimens.

4.2.5 Antioxidant enzymes in maternal liver and placenta

Alterations in the activities of antioxidant enzymes viz., catalase, GST and GPX measured in maternal liver and placenta are illustrated in Fig 3.6. In general, the activities of enzymes were significantly decreased only at higher doses. In liver, the activities were significantly reduced (catalase, 19-21%; GST, 10-19%; and GPX, 27-43%). The activities in placenta were also significantly diminished (catalase, 25-55%; GST, 10-39%; GPX, 22-53%).

4.3 Oxidative implications in embryos (GD_{13})

Data on the oxidative makers measured in embryos of control and ID treated rats are depicted in Table 3.2. While the lower doses of ID caused no measurable effect, significant elevation in ROS levels occurred at the highest dose (ROS: cytosol, 36%, mitochondria, 54%). Similar enhancements in the MDA levels were also observed (cytosol, 52%, mitochondria, 44%).

Data on other markers of oxidative stress determined in embryos are presented in Table 3.3. In general, at the lower doses, there were no significant alterations. However, at the highest dose, ID markedly diminished the levels of GSH (40%), total thiols, (47%) and non protein thiols (36%). The protein

carbonyl content among the embryos of ID treated dams was elevated at the higher doses (19-63%).

Alterations in the activities of catalase, GST and GPX in embryos of control and ID treated dams are illustrated in the Fig 3.7. Significant reduction in the activities of enzymes was evident only at the highest dose (catalase: 52 %, GST: 30% and GPX: 34 %).

4.4 Oxidative implications in fetal tissues

Data on the status of lipid peroxidation and ROS levels measured in brain and liver tissue of fetuses obtained on GD_{20} are presented in Table 3.4. The lowest dose of ID had no significant effect on any of the parameters studied. The fetal brain among ID treated dams showed significantly elevated ROS levels (cytosol, 34-66%; mitochondria, 87-109%). Likewise, the MDA levels in brain were also elevated (cytosol, 46-92%; mitochondria, 47-81%). However, in fetal liver, the induction of LPO was less robust (ROS: cytosol, 26-51%; mitochondria, 18-41% and MDA: cytosol, 12-41%; mitochondria, 13-29%).

Data on other markers of oxidative induction in fetal tissues of control and treated dams are presented in Table 3.5. In general the low dose of ID had no significant effect. At higher doses, the significant diminutions in the levels of reduced glutathione (brain, 17-44%; liver, 22-45%) were accompanied with elevated total thiol levels (brain, 46-92%; liver, 12-41%) and non protein thiols (brain, 47- 81%; liver, 13-29%). On the other hand, the protein carbonyl levels were significantly elevated (brain, 66-96%; liver, 12-25%).

Alterations in the activities of antioxidant enzymes in brain and liver of fetuses of control and ID treated dams are represented in the Fig 3.8. The low ID dose had no effect on any of the enzyme activities. However, significant reduction in the activity was evident at higher doses in brain (catalase, 13-33%; GST, 13-37%; GPX, 27-45%). The reductions in fetal liver were less robust (catalase, 30%; GST, 20-27%; GPX, 21-35%).

4.0 RESULTS

IRON DEFICIENCY DURING PREGNANCY: OXIDATIVE IMPAIRMENTS

4.1 Hemoglobin (Hb) content

In a preliminary study, iron deficient diet (either 50 or 100 %) was fed to adult female rats for a period of 7 or 14 days. Hemoglobin levels were not significantly altered among rats fed with 50% iron deficient diet for a week, although marginal decrease was noticed among rats fed with 100% iron deficient diet. However, moderate decrease (40%) was evident among rats fed 100% iron deficient diet for 2 weeks and hence this dietary regimen was selected.

4.2 Food consumption and growth pattern

The diet intake among rats fed iron deficient diet was relatively lower (15-20%) (data not shown). The maternal body weight gain among rats fed iron deficient diet was significantly decreased at both sampling times (GD_{13}, 17%; GD_{20}, 25%) (Table 3.6).

4.3 Embryolethality and placental weights

Data on the placental weights and incidence of embryolethality among control and iron deficient diet rats are presented in Table 3.6. There was no significant change in placental weights among IDD group compared to that of controls at both sampling times. However, dams fed iron deficient diet showed increased incidence of dead implantations (GD_{13}: %DI 18.8; $GD_{20:}$ %DI 28).

4.4 Evidences of oxidative stress in maternal tissues

4.4.1 Generation of ROS in maternal brain, liver and kidney

Data on the generation of ROS in maternal tissues of both control and Iron deficient diet fed dams is presented in Fig 3.9. In general, the ROS levels

were significantly elevated among IDD group in all organs irrespective of the dosing regimen.

The brain ROS levels were uniformly elevated both in cytosol (30-45%) and mitochondria (23-41%). The elevation in ROS levels in liver cytosol were more robust (48-152%) than the mitochondria (31-51%). A similar trend of increase in ROS levels was also evident in both cytosol and mitochondria of kidney.

4.4.2 Lipid peroxidation in maternal brain, liver and kidney

The status of lipid peroxidation determined in maternal organs of both control and iron deficient diet fed dams is presented in Fig 3.10. In general, the MDA levels were significantly elevated among ID group in all organs irrespective of the dosing regimen. The brain MDA levels were elevated both in cytosol (114-118%) and mitochondria (74-76%). The elevation in MDA levels in liver cytosol were more robust (73-160%) than the mitochondria (62-83%). A similar trend of increase in MDA levels was also evident in kidney.

4.4.3 Protein carbonyls in maternal brain, liver and kidney

Data on protein carbonyls content measured among the control and Iron deficient diet fed dams is shown in Table 3.7. While the levels in brain mitochondria of rats fed IDD increased moderately (GD_{20}: 35%), there was only a marginal increase (10-18%) in protein carbonyl levels in cytosolic and mitochondrial fractions in both liver and kidney.

4.4.4 Reduced glutathione status in maternal brain, liver and kidney

The GSH content in cytosol and mitochondrial fractions of maternal tissues in both control and iron deficient diet fed rats is presented in Table 3.7. A moderate decrease (30-40%) in cytosol and mitochondria was evident in maternal brain irrespective of dosing regimen. Likewise, in liver, significant reduction in GSH levels were evident in both cytosol (GD_{13}: 26 %; GD_{20}: 50%) and mitochondria (GD_{13}: 38%; GD_{20}: 43%).

4.4.5 Response of antioxidant enzymes

Data on the activities of antioxidant enzymes in maternal organs of control and iron deficient diet fed dams is shown in Fig 3.11. In brain, the activity of SOD was significantly reduced in cytosol (15-47%) and mitochondria (26-38%).

Likewise the SOD levels in liver were also decreased significantly (cytosol, 31-23%; mitochondria, 46-60%). However, alterations in SOD levels in kidney were less robust.

In brain, the activity of GST was significantly reduced in both cytosol (14-21%) and mitochondria (31-46%). Likewise the GST levels in liver were also decreased significantly (cytosol, 21-25%; mitochondria, 31-37%). However, the decreases in GST levels in kidney were more robust in cytosol (50-64%) and mitochondria (34-40%). Further, the activity of GPX in brain was significantly reduced in both cytosol (20-28%) and mitochondria (27-39%). Likewise the GPX levels in liver were also decreased significantly (cytosol, 13-24%; mitochondria, 27-31%). A similar trend of decrease in the activity of GPX levels was evident in kidney.

4.5 Status of oxidative damage in placenta

Data on the ROS and MDA levels measured in placenta sampled at both dosing regimens are presented in Table 3.8. The ROS levels were markedly enhanced (GD_{13}: cytosol, 32%; mitochondria, 46% and GD_{20}: cytosol, 15%; mitochondria, 59%). In contrast, the induction levels of MDA were relatively of higher magnitude ($GD_{13:}$ cytosol, 63%; mitochondria, 29% and GD_{20}: cytosol, 42%; mitochondria, 23%).

Significant depletion of placental GSH levels was also evident among Iron deficient diet fed dams (Table 3.8). The GSH levels were found to be reduced

in placenta (GD_{13}*:* cytosol, 38%; mitochondria, 45% and GD_{20}: cytosol, 18%; mitochondria, 39%). The protein carbonyls content in placental tissues were significantly increased at both sampling times (GD_{13}: cytosol, 40%; mitochondria, 27% and GD_{20}: cytosol, 17%; mitochondria, 17%).

4.6 Evidences of oxidative damage in embryos (GD_{13})

Data on the oxidative makers measured in embryos of control and iron deficient diet fed dams is shown in Table 3.9. There was significant elevation in both ROS (cytosol, 83%; mitochondria, 58%) and MDA levels (cytosol, 31%; mitochondria, 50%). Among embryos of IDD group, the levels of GSH (cytosol, 32%; mitochondria, 50%), total thiols, (cytosol, 44%; mitochondria, 29%) and non protein thiols (cytosol, 20%; mitochondria, 23%) was diminished. The protein carbonyl content among the embryos of Iron deficient diet fed dams was marginally elevated (cytosol, 86%; mitochondria, 59%). Marginal alterations in the activities of catalase, GST and GPX were observed among the embryos of dams fed iron deficient diet (Fig 3.12).

4.7 Evidences of oxidative damage in fetus (GD_{20})

Data on the status of lipid peroxidation and ROS levels measured in brain and liver of fetuses obtained on GD_{20} are presented in Fig 3.13. The fetal brain of iron deficient diet fed dams showed significantly elevated ROS levels (cytosol, 129%; mitochondria, 54%). Likewise, the MDA levels in brain were also elevated (cytosol, 33%; mitochondria, 64%). Further, in fetal liver, the induction of LPO was significant (ROS: cytosol, 45%; mitochondria, 63% and MDA: cytosol, 40%; mitochondria, 49%).

Data on other markers of oxidative induction in fetal tissues of control and iron deficient diet fed dams are presented in Fig 3.14 and 3.15. Significant diminution in the levels of reduced glutathione (brain: cytosol, 61%; mitochondria, 30%) were accompanied with reduction in total thiol levels (cytosol, 27%; mitochondria, 31%) and non protein thiols (cytosol, 48%;

mitochondria, 45%). On the other hand, the protein carbonyl levels were significantly elevated (cytosol, 23%; mitochondria, 42%). A similar trend was obtained in the fetal liver of iron deficient diet fed dams.

The effect of feeding iron deficient diet on the activities of antioxidant enzymes in brain and liver of fetuses are represented in the Fig 3.16. In general, the activities of enzymes were significantly decreased (excepting for SOD). The activities of GST were significantly reduced in both brain (cytosol, 24%, mitochondria, 42%) and liver (20%). The activities of GPX were also reduced in brain (cytosol, 32%, mitochondria, 35%), while the decrease was significant only in liver mitochondria (43%). Further, the activity of SOD was marginally increased in cytosol (brain, 16% and liver, 17%). On the other hand, the activity of catalase was reduced in both organs (brain, 28%; liver, 23%).

Table 3.1a Body weight gain, placental weight and incidence of embryolethality among pregnant rats administered iron dextran

Iron dextran (mg/rat/d)	**Body wt gain** (g)	**Mean placental wt** (g)
Exposure days (GD_{5-12})		
0	45.19 ± 4.60	0.26 ± 0.015
2	46.89 ± 1.80	0.27 ± 0.018
4	42.35 ± 2.70	0.28 ± 0.020
8	35.66 ± 2.55[a]	0.32 ± 0.010[a]
Exposure days (GD_{5-19})		
0	98.81 ± 5.76	0.51 ± 0.025
2	96.25 ± 3.15	0.50 ± 0.022
4	96.62 ± 4.50	0.47 ± 0.018[a]
8	80.46 ± 3.18[a]	0.74 ± 0.026[a]

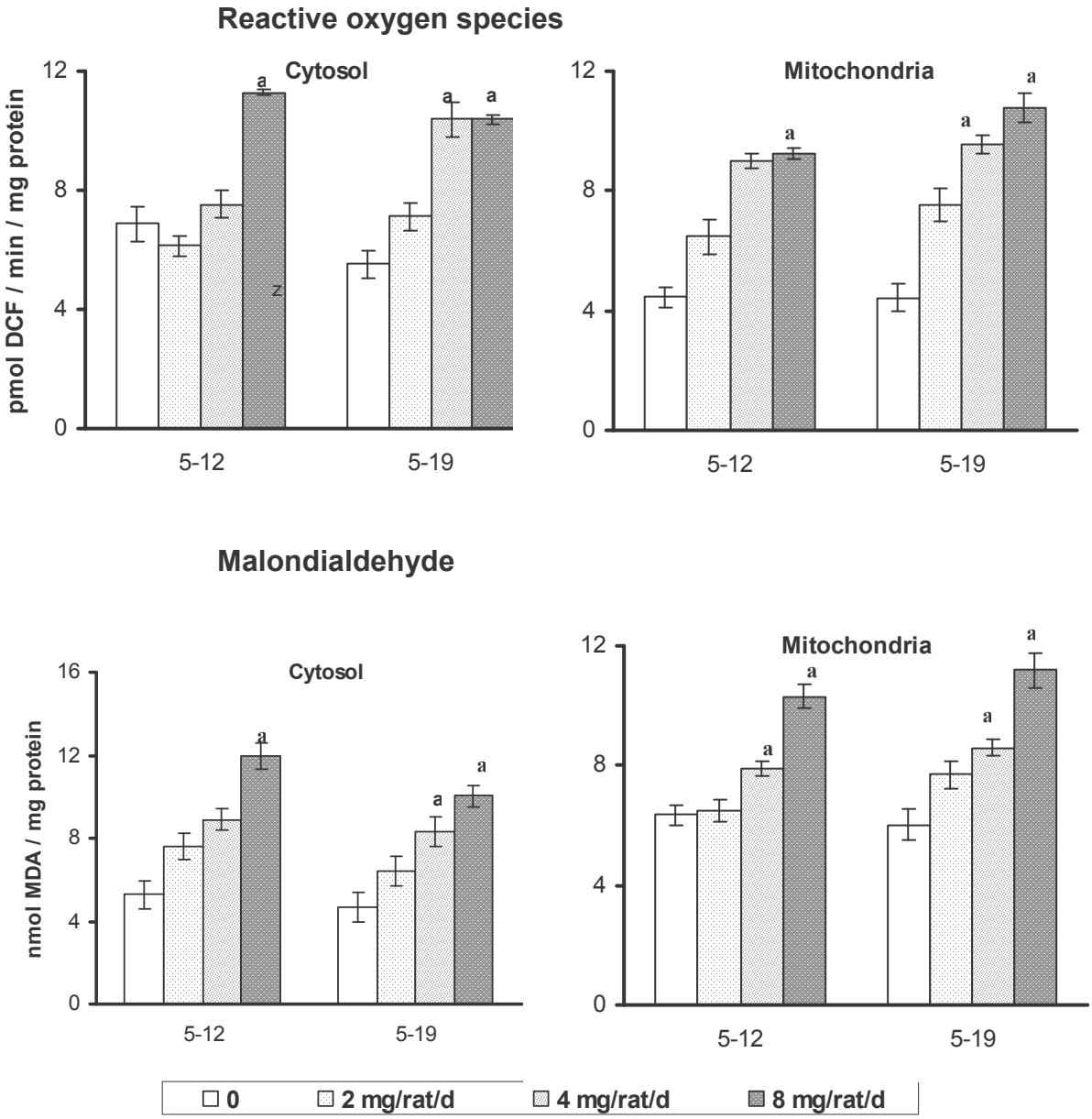
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 3.1b Fetal weights and incidence of embryolethality among pregnant rats administered iron dextran

Iron dextran (mg/rat/d)	**Fetal wt** (g)	**Embryolethality** (%DI)
Exposure days (GD_{5-12})		
0	5.36 ± 0.45	4.05 ± 0.65
2	5.25 ± 0.25	4.45 ± 0.50
4	5.18 ± 0.35	8.50 ± 0.75
8	4.05 ± 0.18[a]	18.50 ± 1.00
Exposure days (GD_{5-19})		
0	5.50 ± 0.25	3.15 ± 0.40
2	5.48 ± 0.35	5.60 + 0.80
4	5.28 ± 0.18	8.80 + 1.00
8	4.25 ± 0.29[a]	20.50 + 1.50

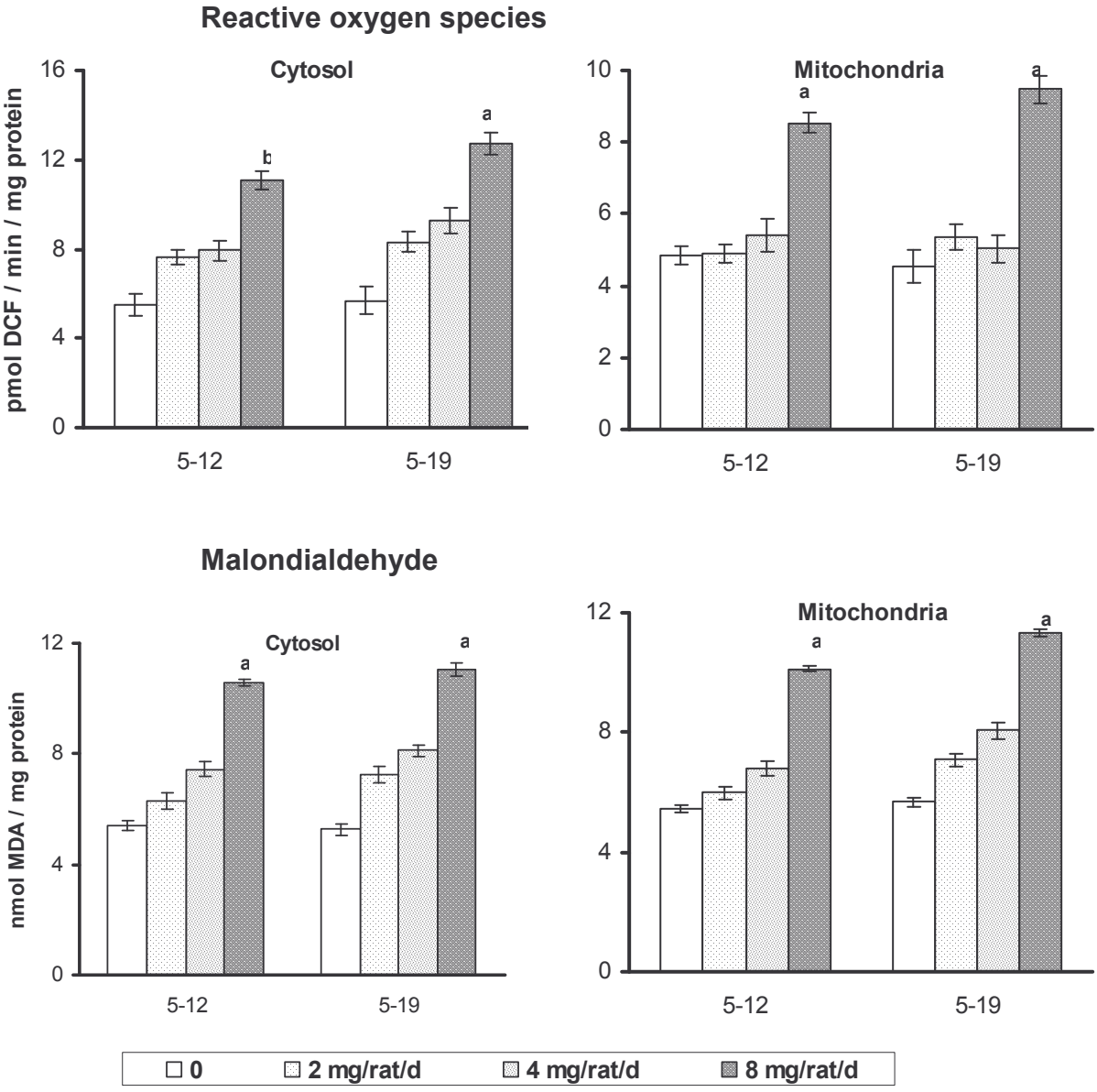
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Fig 3.1 **Induction of lipid peroxidation measured as malondialdehyde levels and generation of ROS levels in liver of pregnant rats administered (oral) with iron dextran during gestational period (GD_{5-12} and GD_{5-19})**



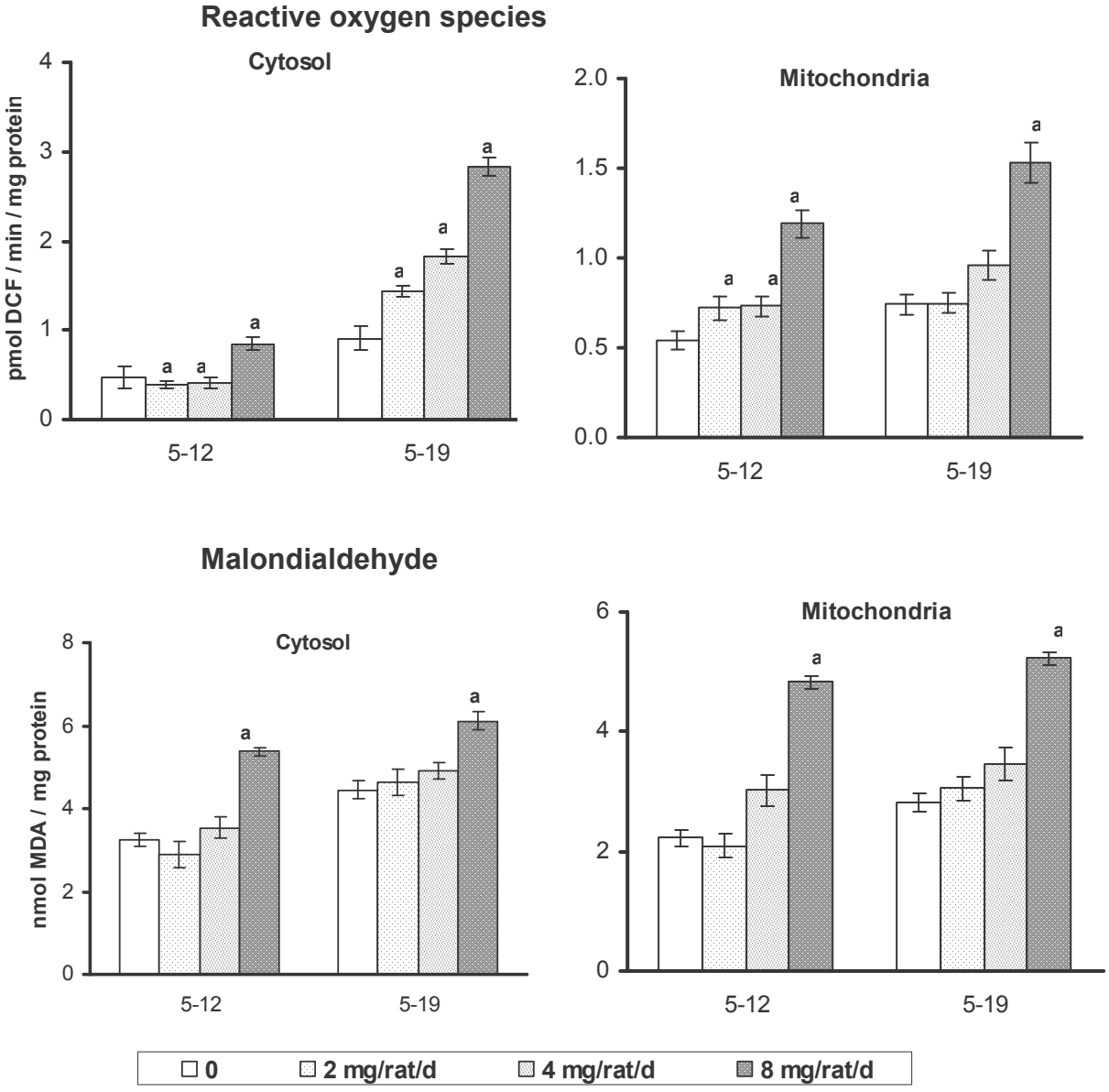
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Fig 3.2 Induction of lipid peroxidation and generation of ROS in kidney of pregnant rats administered (oral) with iron dextran during gestational period (GD_{5-12}, GD_{5-19})



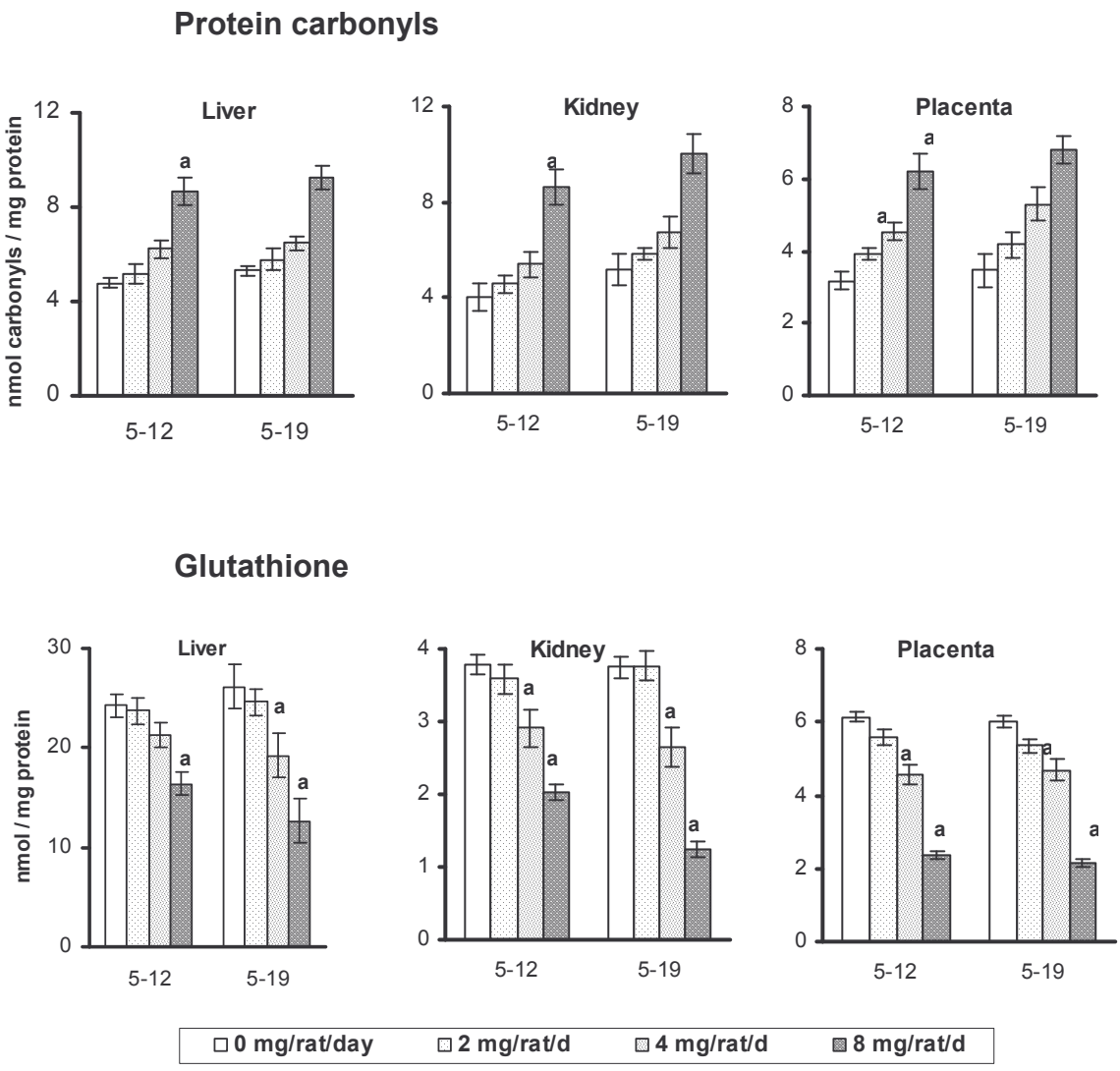
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$, $^{b}P<0.01$

Fig 3.3 Pattern of ROS generation and lipid peroxidation in placenta of pregnant rats administered with ID (oral) during gestation.



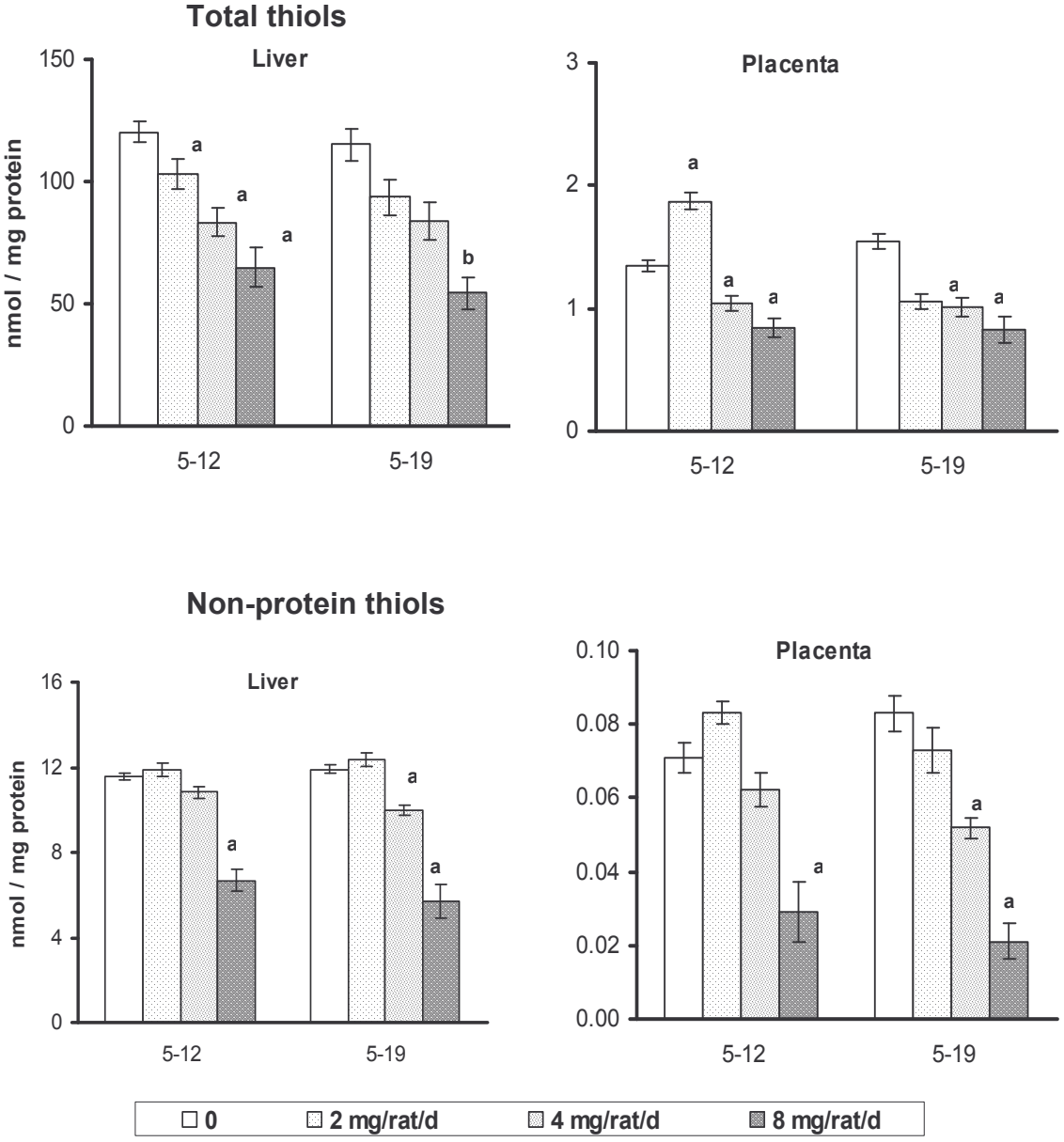
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; $^{a}P<0.001$

Fig 3.4 **Status of protein carbonyls and reduced glutathione in maternal tissues and placenta of pregnant rats administered oral iron dextran during gestation**



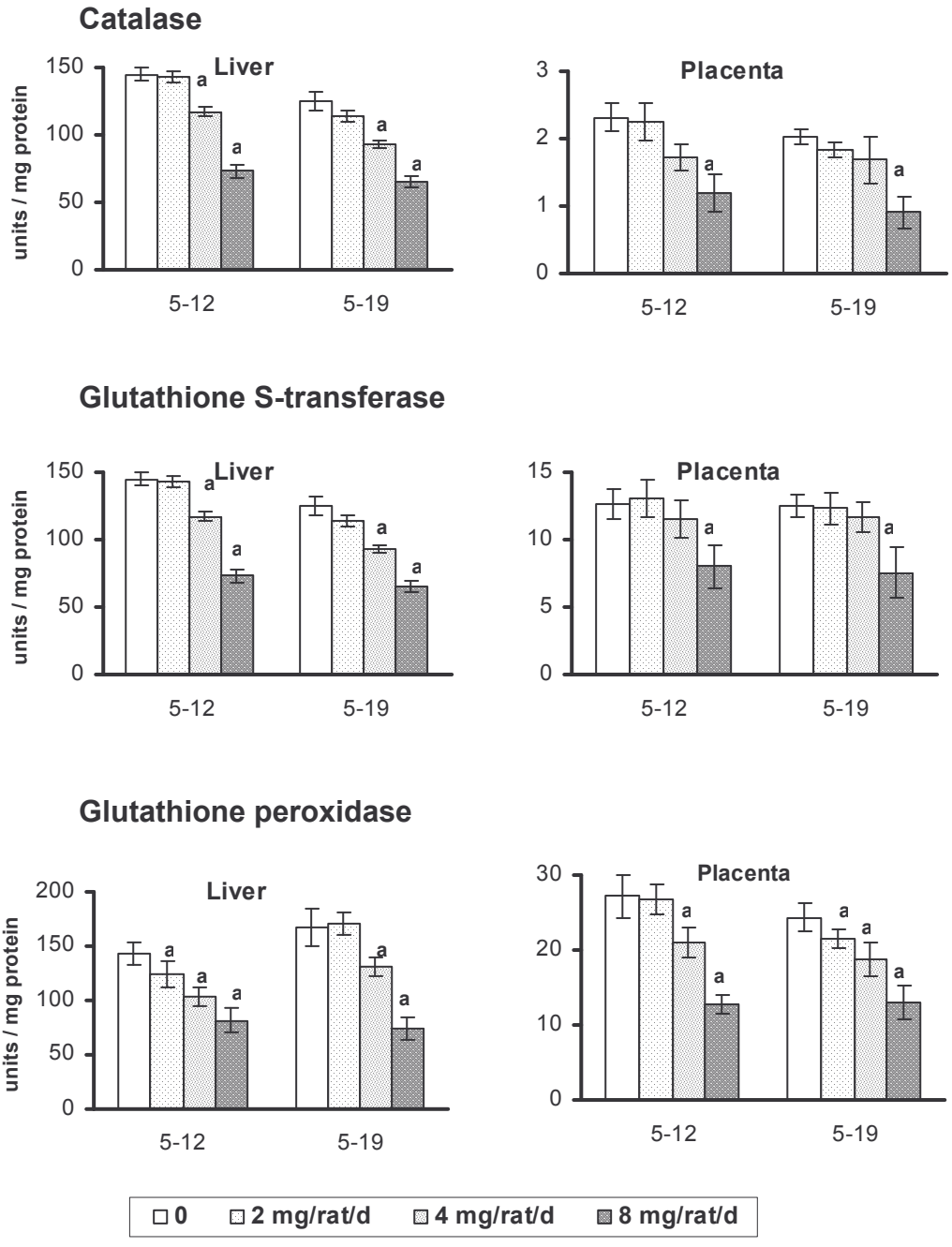
Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Fig 3.5 **Status of total thiols and non-protein thiols in liver and placenta of pregnant rats administered oral iron dextran**



Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*, [b]*P<0.01*

Fig 3.6 **Activities of catalase, glutathione transferase, glutathione peroxidase in liver and placenta of rats administered iron dextran**



Values are mean ± SD (n=6)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 3.2 Status of oxidative stress markers measured in GD_{13} embryos of rats administered (oral) with iron dextran during GD_{5-12}

		Iron dextran (mg/rat/d)			
		0	**2**	**4**	**8**
ROS[1]	Cyto	0.094 ± 0.02	0.085 ± 0.05	0.107 ± 0.07	0.128 ± 0.04
	Mito	0.127 ± 0.09	0.111± 0.04	0.139 ± 0.07	0.196 ± 0.07
MDA[2]	Cyto	2.18± 0.11	2.01 ± 0.09	2.37 ± 0.10[a]	3.33 ± 0.14[a]
	Mito	1.13 ± 0.02	1.17 ± 0.04	1.36 ± 0.05[a]	1.63 ± 0.06[a]

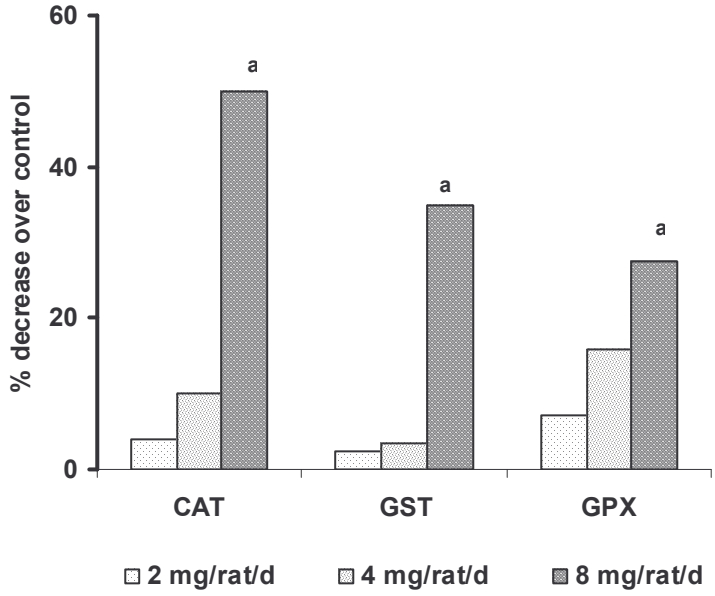
Values are mean ± SD (n=6)
[1]pmol/min/mg protein; [2]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 3.3 Status of antioxidant molecules measured in GD_{13} embryos of rats administered (oral) with iron dextran during GD_{5-12}

	Iron dextran (mg/rat/d)			
	0	**2**	**4**	**8**
Reduced Glutathione[3]	5.74 ± 0.35	5.67 ± 0.75	4.75 ± 0.29[a]	3.43 ± 0.53[a]
Protein Carbonyls[3]	3.29 ± 0.17	3.75 ± 0.55	3.93 ± 0.69[a]	5.39 ± 0.73[a]
Thiols, Total[3]	25.10 ± 1.11	23.61 ± 1.20[a]	19.89 ± 1.59[a]	13.12 ± 1.31[a]
Thiols, non protein[3]	5.85 ± 0.58	5.19 ± 0.59	4.37 ± 0.29[a]	3.71 ± 0.55[a]

Values are mean ± SD (n=6)
[3]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001*

Fig 3.7 **Activities of catalase (CAT), glutathione transferase (GST) and glutathione peroxidase (GPX) in GD_{13} embryos of rats administered (oral) ID**



Values are mean ± SD (n=12)
Data analysed by Holm-Sidak method; $^{a}P<0.001$

Table 3.4 Induction of lipid peroxidation measured as MDA levels and generation ROS in brain and liver of fetuses obtained from rats administered (oral) with iron dextran during gestation

Parameters	Iron Dextran (mg/rat/d)			
	0	2	4	8
Brain				
ROS, Cyto[1]	1.16 ± 0.20	1.22 ± 0.04	1.56 ± 0.07[a]	1.93 ± 0.05[a]
ROS, Mt[1]	0.49 ± 0.11	0.51 ± 0.10	0.92 ± 0.06[a]	1.18 ± 0.02[a]
MDA, Cyto[2]	1.53 ± 0.04	1.72 ± 0.02[a]	2.24 ± 0.09[a]	2.94 ± 0.13[a]
MDA, Mt[2]	1.74 ± 0.02	2.17 ± 0.07	2.56 ± 0.06[a]	3.15 ± 0.13[a]
Liver				
ROS, Cyto[1]	3.37 ± 0.10	3.46 ± 0.19	4.27 ± 0.47[a]	5.09 ± 0.39[a]
ROS, Mt[1]	2.25 ± 0.09	2.51 ± 0.05[a]	2.66 ± 0.03[a]	3.19 ± 0.06[a]
MDA, Cyto[2]	5.49 ± 0.12	5.63 ± 0.15	6.19 ± 0.16	7.79 ± 0.15[a]
MDA, Mt[2]	6.37 ± 0.39	6.63 ± 0.43[a]	7.24 ± 0.54[a]	8.26 ± 0.84[a]

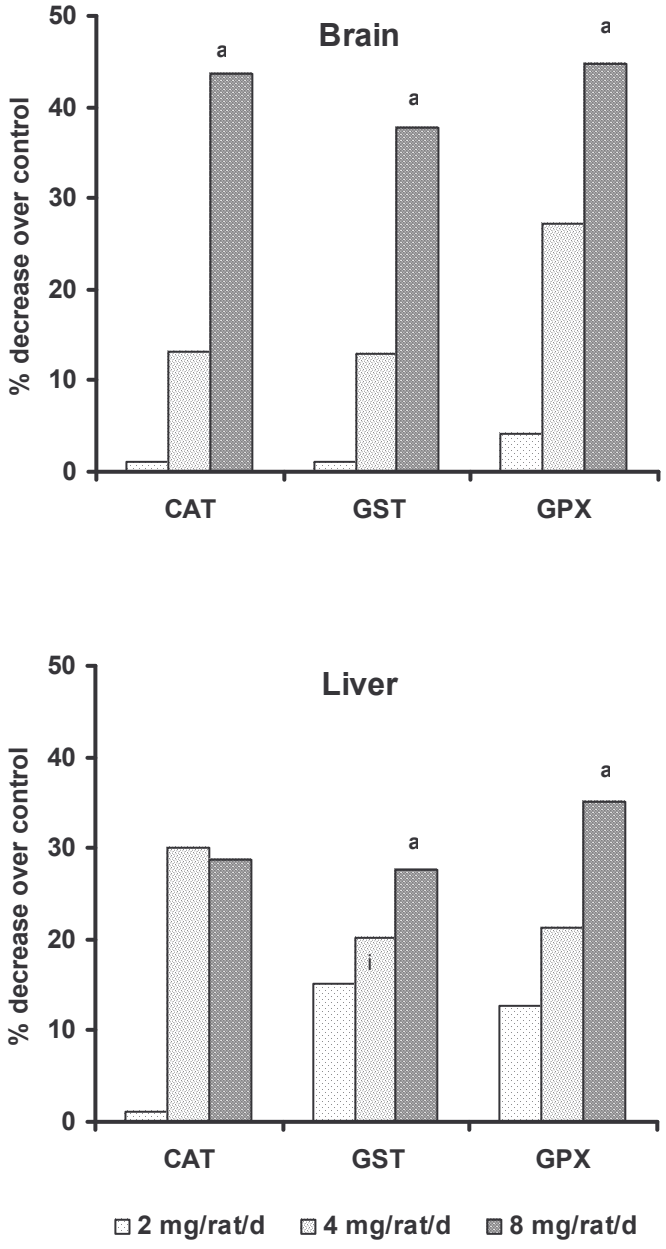
Values are mean ± SD (n=12)
[1]pmol/min/mg protein; [2]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 3.5 Protein carbonyls (PC) content and reduced glutathione (GSH) levels in fetal tissues of rats administered (oral) with iron dextran during GD_{5-19}

Parameters	Iron Dextran (mg/rat/d)			
	0	2	4	8
Brain				
PC[1]	2.57 ± 0.18	3.39 ± 0.41[a]	4.28 ± 0.16[a]	5.05 ± 0.31[a]
GSH[2]	19.75 ± 0.38	17.37 ± 1.83[a]	16.45 ± 1.55[a]	11.25 ± 1.17[a]
TSH[2]	1.53 ± 0.04	1.72 ± 0.02[a]	2.24 ± 0.09[a]	2.94 ± 0.13[a]
NPSH[2]	1.74 ± 0.02	2.17 ± 0.07[a]	2.56 ± 0.06[a]	3.15 ± 0.13[a]
Liver				
PC[1]	4.90 ± 0.47	4.89 ± 0.46	5.49 ± 0.61	6.17 ± 0.74[b]
GSH[2]	31.16 ± 2.57	30.71 ± 1.57	24.49 ± 2.33[a]	17.37 ± 0.72[a]
TSH[2]	5.49 ± 0.12	5.63 ± 0.15	6.19 ± 0.16	7.79 ± 0.15[a]
NPSH[2]	6.37 ± 0.39	6.63 ± 0.43	7.24 ± 0.54	8.26 ± 0.84[a]

Values are mean ± SD (n=12)
[1]nmol / mg protein; [2]nmol/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001*, [b]*P<0.01*

Fig 3.8 **Activities of catalase (CAT), glutathione transferase (GST) and glutathione peroxidase (GPX) in brain of fetuses of rats administered iron dextran (ID) during $GD_{5\text{-}19}$**



Values are mean ± SD (n=12)
Data analysed by Holm-Sidak method; $^{a}P<0.001$

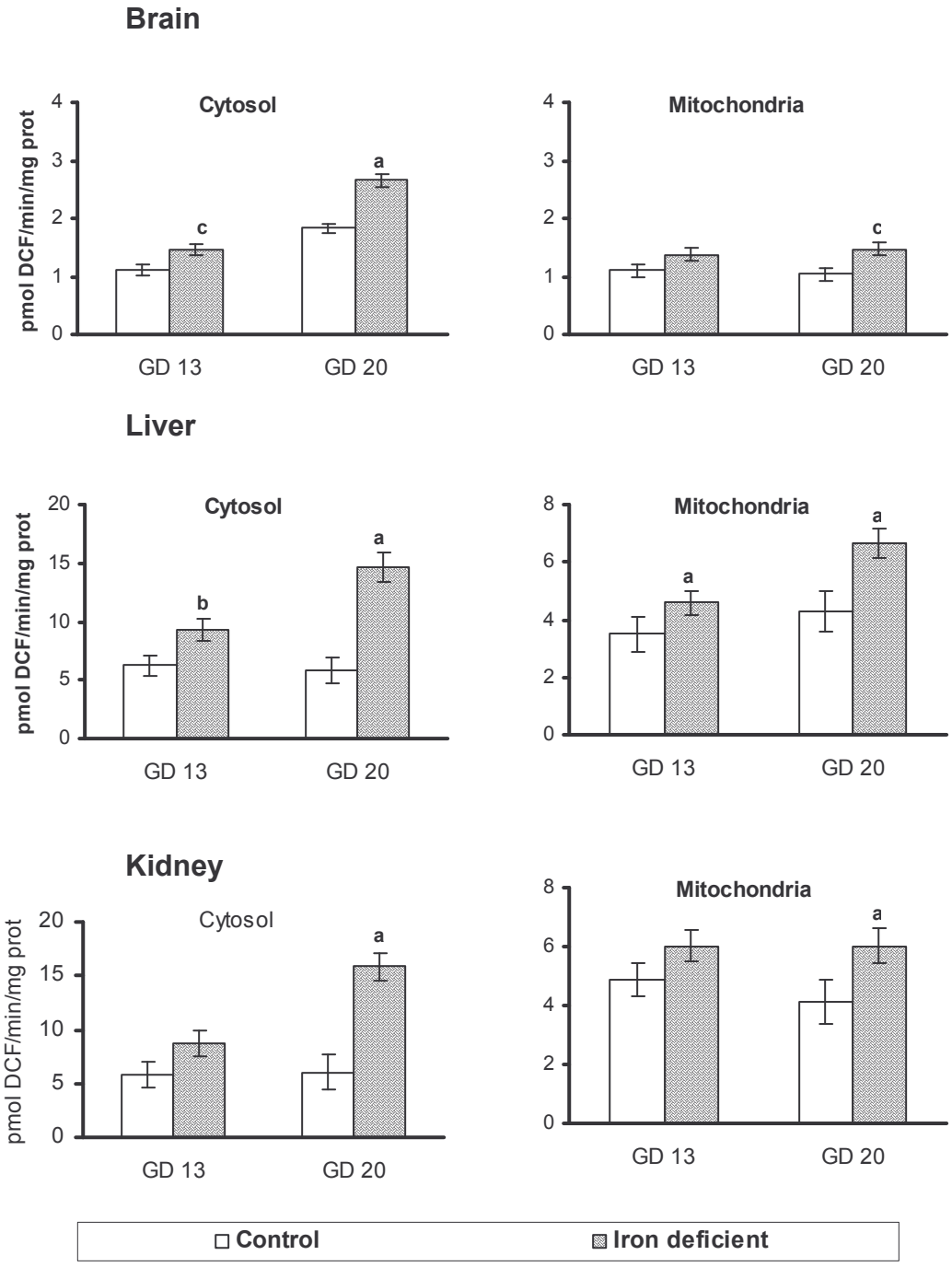
Section B

Table 3.6 Body weight gain, placental weights and embryolethality among pregnant rats fed iron deficient diet (IDD)

		Control	Iron deficient
Body wt gain (g)	GD 13	47.28 ± 0.87	39.29 ± 0.62[a]
	GD 20	98.81 ± 0.76	74.63 ± 0.30[a]
Placental wt (g)	GD 13	0.368 ± 0.016	0.387 ± 0.027
	GD 20	0.547 ± 0.013	0.602 ± 0.039[b]
Embryolethality (%Dead Implants)	GD 13	5.05 ± 0.68	18.83 ± 1.36[a]
	GD 20	3.11 ± 0.04	28.03 ± 0.70[a]

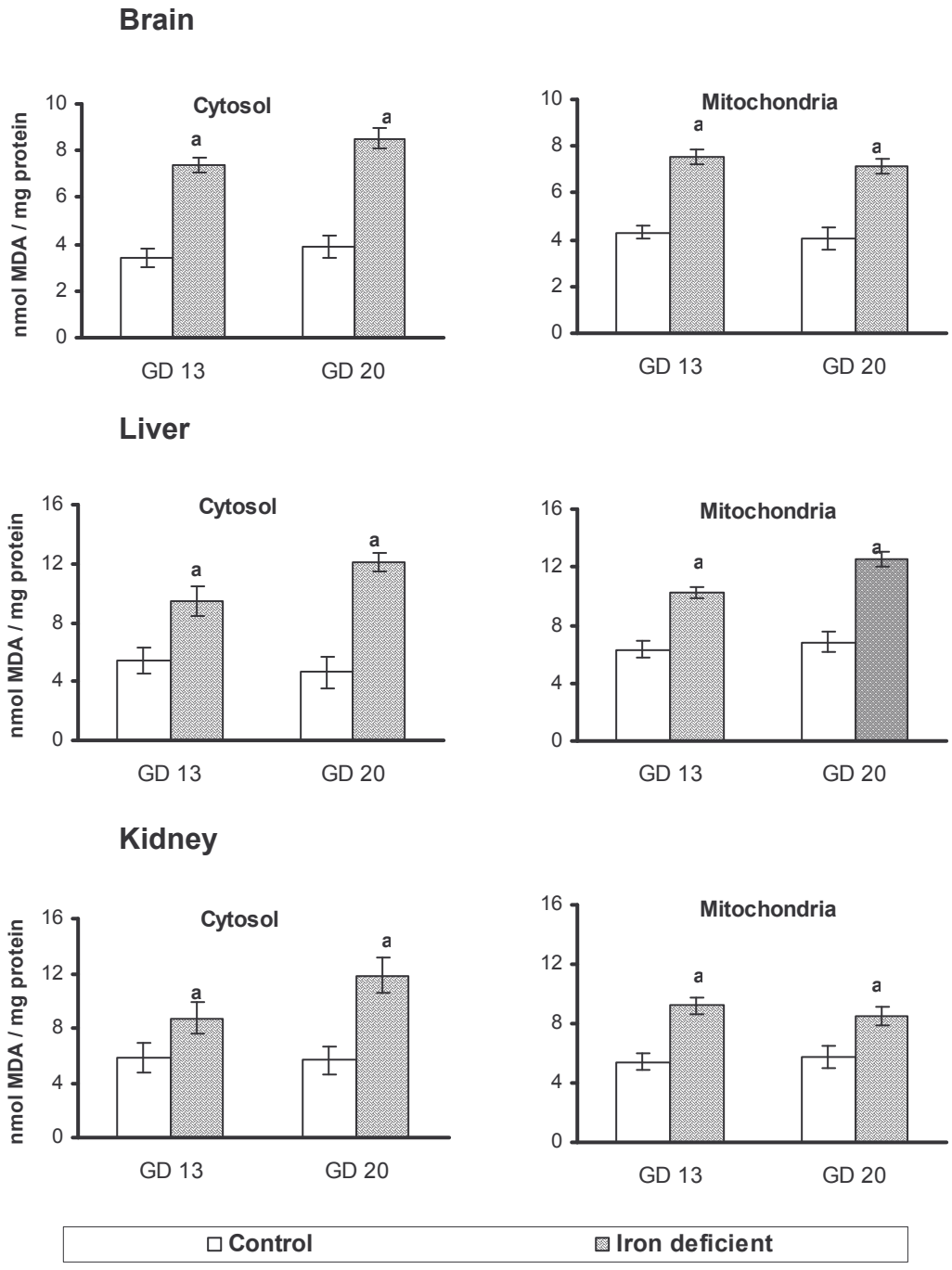
Values are mean ± SD (n=6)
Data analysed by t- test; [a]*P<0.001,* [b]*P<0.01*

Fig 3.9 ROS generation in maternal organs of pregnant rats fed iron deficient diet



Values are mean ± SD (n=6)
Data analysed by t- test; $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$

Fig 3.10 Induction of lipid peroxidation measured as malondialdehyde (MDA) levels in maternal organs of rats fed iron deficient diet



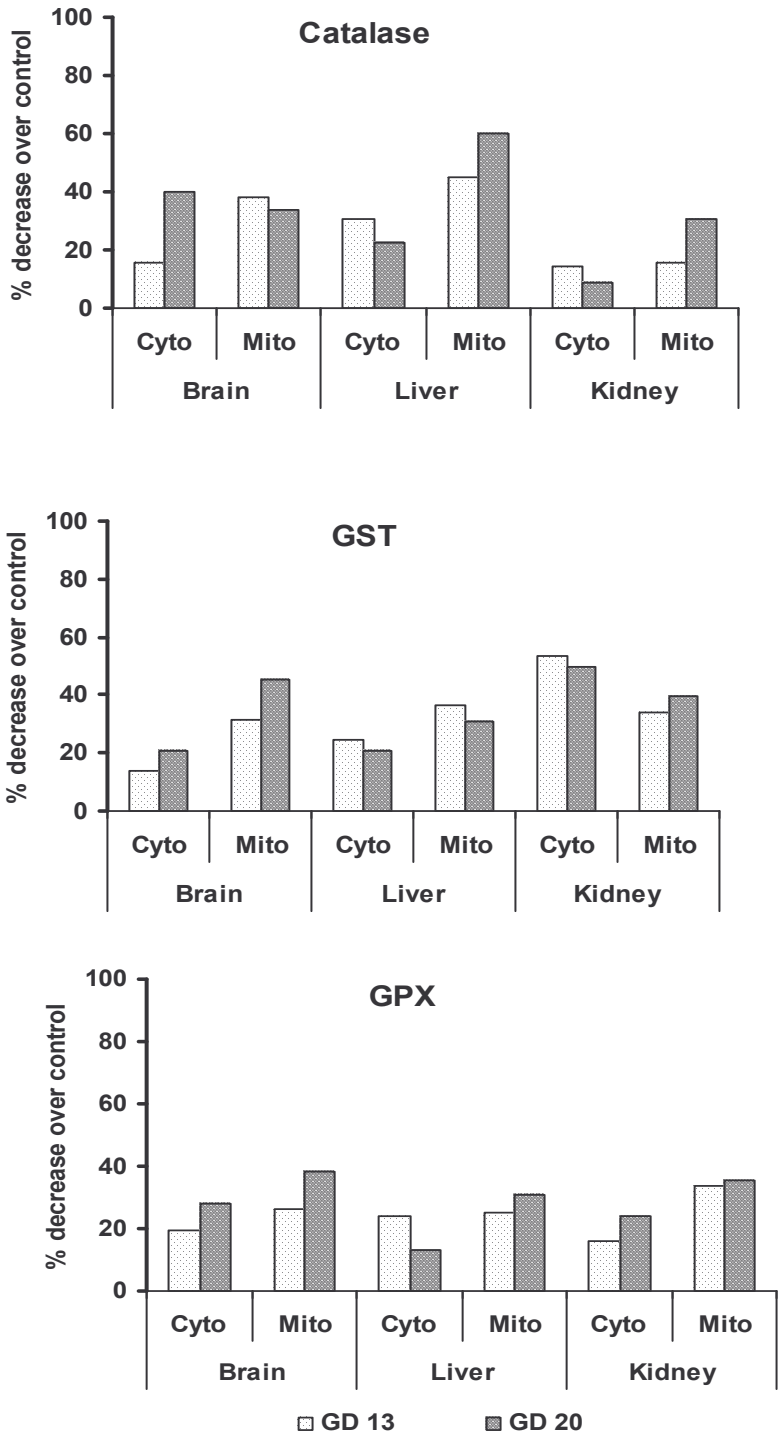
Values are mean ± SD (n=6)
Data analysed by t- test; $^{a}P<0.001$

Table 3.7 Status of protein carbonyls and reduced glutathione levels in maternal organs of rats fed iron deficient diet

		Cytosol		Mitochondria	
		Control	Iron deficient	Control	Iron deficient
PC[1]					
Brain	GD 13	5.52 ± 0.19	6.00 ± 0.36[c]	4.63 ± 0.19	4.91 ± 0.24[a]
	GD 20	5.69 ± 0.26	6.44 ± 0.02[b]	5.13 ± 0.16	6.67 ± 0.02[a]
Liver	GD 13	5.56 ± 0.35	4.81 ± 0.17[a]	6.06 ± 0.35	4.82 ± 0.17[a]
	GD 20	6.39 ± 0.14	5.63 ± 0.10[a]	6.10 ± 0.14	5.45 ± 0.10[a]
Kidney	GD 13	3.67 ± 0.35	4.31 ± 0.09[a]	6.78 ± 0.35	5.16 ± 0.09[a]
	GD 20	3.96 ± 0.54	5.52 ± 0.03[a]	7.57 ± 0.54	5.84 ± 0.03[a]
GSH[1]					
Brain	GD 13	19.59 ± 0.09	13.9 ± 0.06[a]	18.32 ± 0.09	13.52 ± 0.06[a]
	GD 20	16.58 ± 0.16	7.32 ± 0.02[a]	11.29 ± 0.16	8.92 ± 0.02[c]
Liver	GD 13	29.72 ± 0.35	33.08 ± 0.17	35.15 ± 0.35	30.9 ± 0.17[c]
	GD 20	21.98 ± 0.14	16.28 ± 0.10[b]	21.75 ± 0.14	17.5 ± 0.10[a]
Kidney	GD 13	4.73 ± 0.35	4.88 ± 0.09	4.68 ± 0.35	4.51 ± 0.09
	GD 20	2.03 ± 0.54	2.39 ± 0.03	2.42 ± 0.54	3.15 ± 0.03[c]

Values are mean ± SD (n=6)
[1]nmol/mg protein
Data analysed by t- test; *[a]P<0.001, [b]P<0.01, [c]P<0.05*

Fig 3.11 Activities of antioxidant enzymes in maternal organs of pregnant rats fed iron deficient diet



Values are mean ± SD (n=6)
Data analysed by t- test; $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$

Table 3.8 Pattern of ROS generation, status of lipid peroxidation protein carbonyl content and reduced glutathione in placenta of rats fed iron deficient diet

			Control	Iron deficient
ROS[1]	GD 13	Cyto	0.52 ± 0.09	0.69 ± 0.06[b]
		Mito	0.62 ± 0.16	0.91 ± 0.02[a]
	GD 20	Cyto	1.57 ± 0.35	1.82 ± 0.17
		Mito	0.66 ± 0.14	1.05 ± 0.10[a]
MDA[2]	GD 13	Cyto	5.87 ± 0.35	9.60 ± 0.09[a]
		Mito	3.96 ± 0.54	5.14 ± 0.03[a]
	GD 20	Cyto	5.51 ± 0.79	7.86 ± 0.13[a]
		Mito	8.72 ± 1.50	10.74 ± 1.56[c]
PC[2]	GD 13	Cyto	3.75 ± 0.26	5.28 ± 0.40[a]
		Mito	3.40 ± 0.25	4.34 ± 0.27[a]
	GD 20	Cyto	3.90 ± 0.48	4.57 ± 0.35[c]
		Mito	4.61 ± 0.33	5.39 ± 0.50[b]
GSH[2]	GD 13	Cyto	26.52 ± 1.64	16.51± 0.81[a]
		Mito	3.57 ± 0.76	1.99 ± 0.44[a]
	GD 20	Cyto	34.48 ± 0.48	28.33 ± 0.38[a]
		Mito	41.11 ± 12.07	25.33 ± 2.38[c]

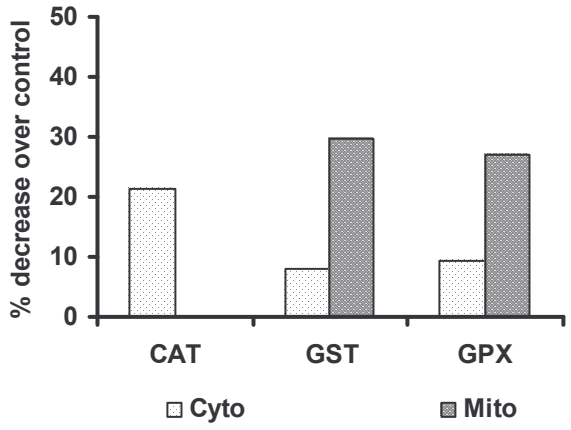
Values are mean ± SD (n=6)
[1]pmol/min/mg protein; [2]nmol/mg protein
Data analysed by t- test; [a]*P<0.001,* [b]*P<0.01,* [c]*P<0.05*

Table 3.9 Oxidative stress biomarkers in embryos of rats fed iron deficient diet

	Cytosol		Mitochondria	
	Control	Iron deficient	Control	Iron deficient
ROS[1]	0.12 ± 0.01	0.22 ± 0.06[a]	0.17 ± 0.05	0.27 ± 0.02[a]
MDA[2]	2.92 ± 0.22	3.83 ± 0.67[b]	2.56 ± 0.08	3.84 ± 0.07[a]
PC[2]	6.12 ± 1.16	3.45 ± 1.62[b]	110.9 ± 0.14	44.59 ± 0.10
GSH[2]	83.88 ± 0.54	76.39 ± 0.03[a]	2.01 ± 0.17	1.26 ± 0.09[a]
TSH[2]	20.60 ± 2.78	11.41 ± 3.32[a]	28.64 ± 4.91	20.21 ± 0.10[a]
NPSH[2]	6.16 ± 0.31	4.87 ± 0.26[a]	5.53 ± 0.03	4.21 ± 0.23[a]

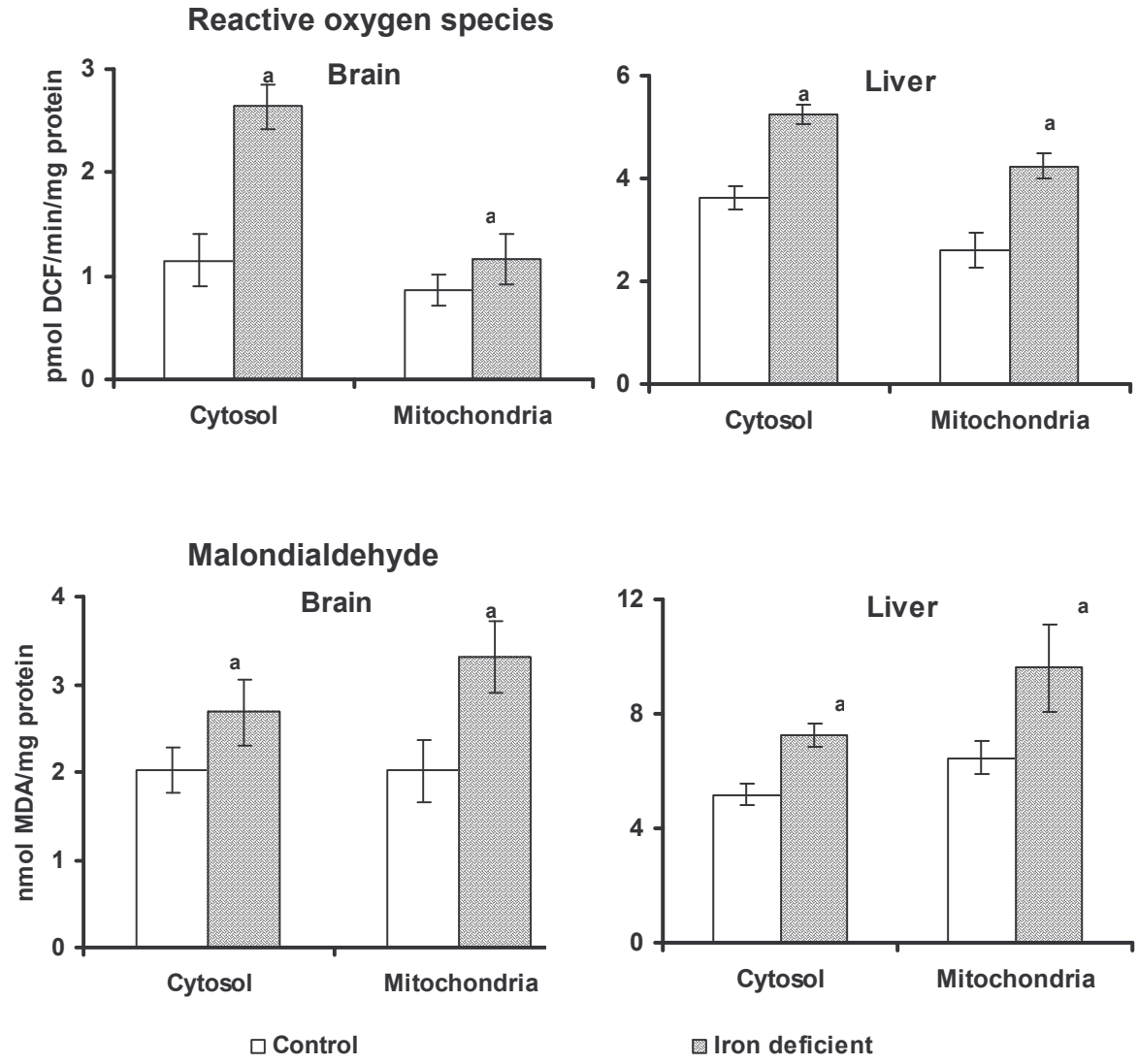
Values are mean ± SD (n=6)
[1]pmol/min/mg protein; [2]nmol/mg protein
Data analysed by t- test; [a]*P<0.001,* [b]*P<0.01*

Fig 3.12 Activities of antioxidant enzymes in embryos of rats fed iron deficient diet



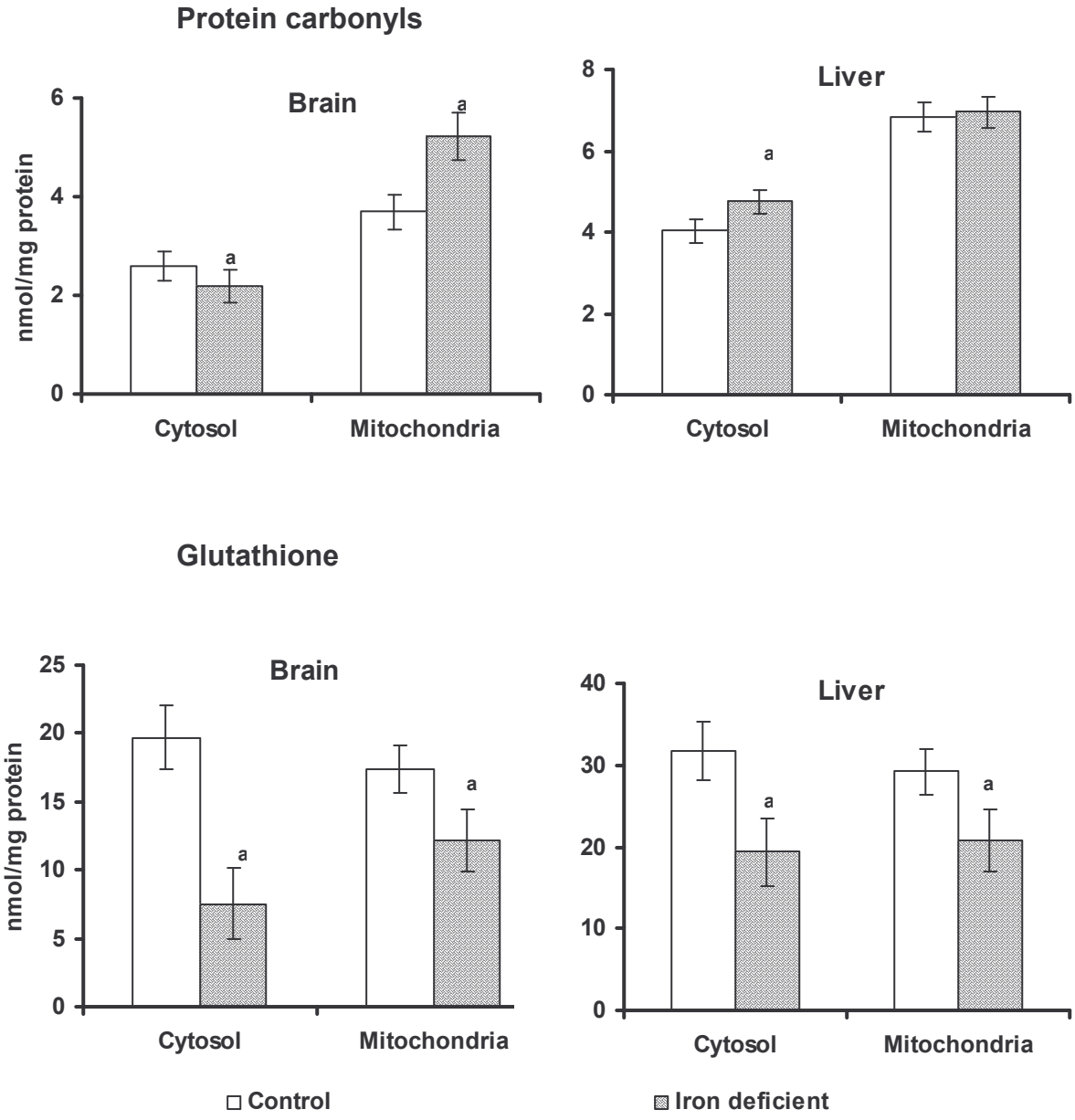
Values are mean ± SD (n=6)
Data analysed by t- test; [a]*P<0.001,* [b]*P<0.01*

Fig 3.13 Pattern of ROS generation and status of lipid peroxidation in brain and liver of fetuses of pregnant rats fed iron deficient diet



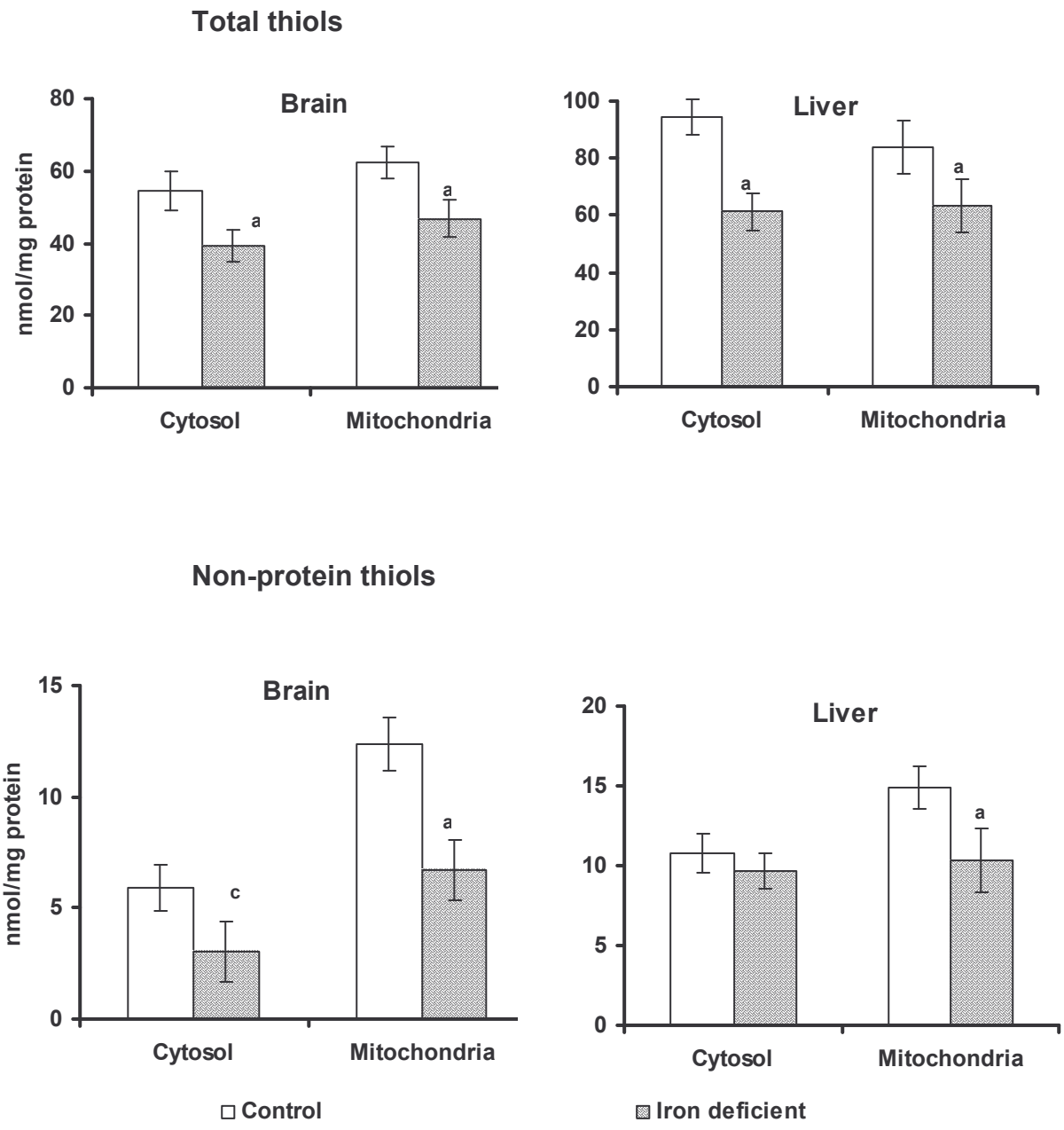
Values are mean ± SD (n=6)
Data analysed by t- test; [a]*P<0.001*

Fig 3.14 Status of protein carbonyls and reduced glutathione in brain and liver of fetuses of rats fed iron deficient diet



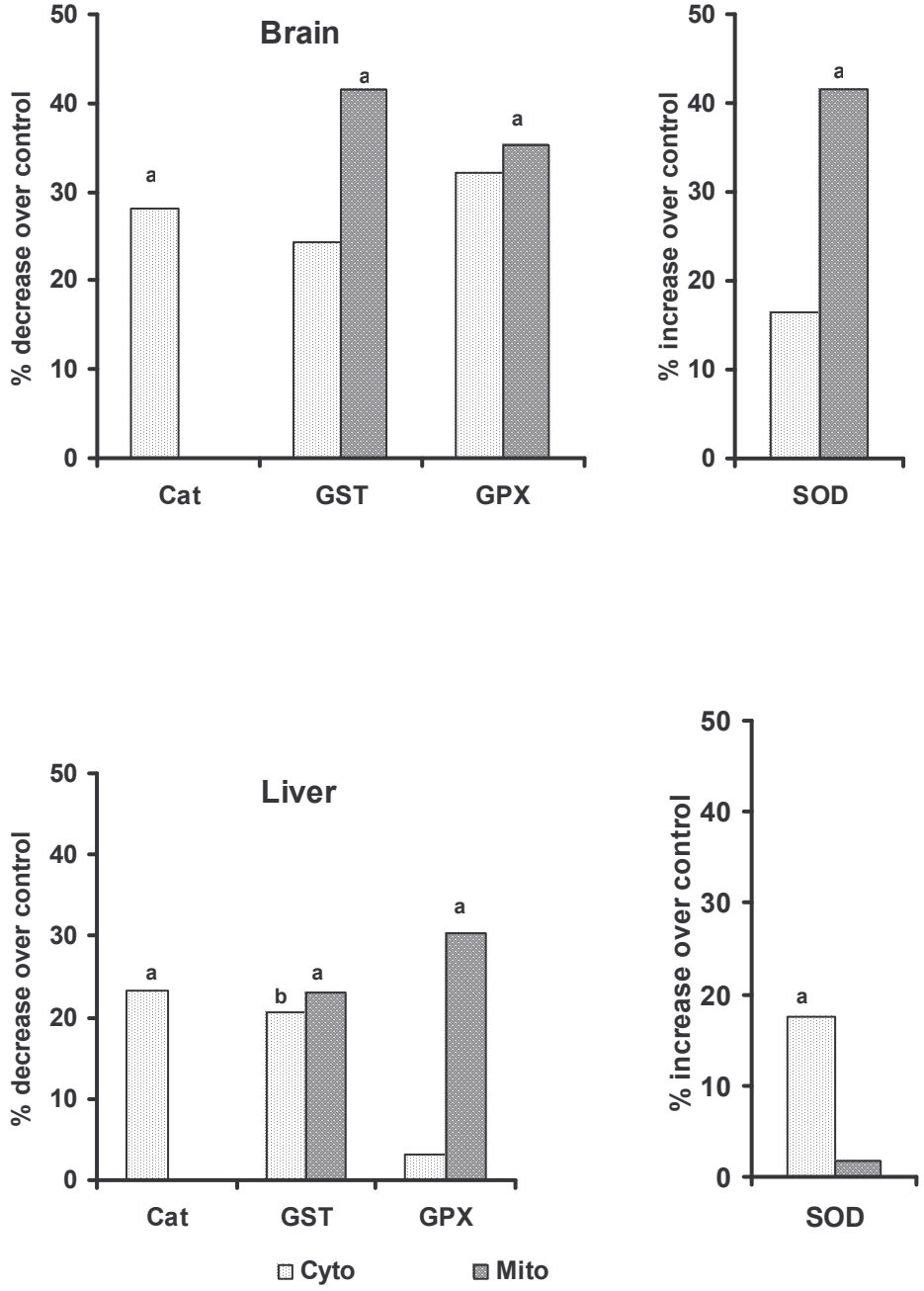
Values are mean ± SD (n=6)
Data analysed by t- test; $^{a}P<0.001$

Fig 3.15 Status of total and non protein thiols in brain and liver of fetuses of pregnant rats fed iron deficient diet



Values are mean ± SD (n=12)
Data analysed by t- test; $^{a}P<0.001$, $^{c}P<0.05$

Fig 3.16 **Activities of superoxide dismutase (SOD), catalase (Cat), glutathione transferase (GST) and glutathione peroxidase (GPX) in brain and liver of fetuses of pregnant rats fed iron deficient diet**



Values are mean ± SD (n=6)
Data analysed by t- test; [a]*P<0.001,* [b]*P<0.01*

5.0 DISCUSSION

Employing 'iron excess' and 'iron deficiency' model, we have addressed the following major questions in this study. I) whether oral supplementation of iron at dosages equivalent to those employed in human supplementation regimens induces oxidative stress in the maternal milieu and its oxidative impact on the embryos and fetuses II) whether iron deficiency prior to, and during pregnancy causes significant oxidative stress in the maternal milieu and its correlation with oxidative dysfunctions in embryos and fetuses.

Iron excess model

In order to address the first question, our selection of dosages for this study was essentially based on some recent seminal observations reported in literature. A brief mention of these findings would be relevant here and would justify our dosage selection of iron. Pregnant women in developing countries are commonly given daily supplements containing 120 mg of iron (*Lynch & Stoltzfus, 2003*) to prevent and correct gestation iron deficiency and this dose of iron is shown to be 10 times the normal daily dietary intake and can cause gastrointestinal side effects (*Hollan and Johansen, 1993*). Earlier studies (*Viteri et al., 1995; Knutson et al., 2000*) have also demonstrated that equivalent doses of daily high–iron supplements in male rats (which is equivalent to 10X normal intake or 8000 µg of iron/day) resulted in an abnormal accumulation of intestinal mucosal and non heme iron and significant increases in lipid peroxidation. Interestingly studies in iron deficient rats *(Knutson et al., 2000)* also showed markedly increased lipid peroxidation clearly suggesting that both iron deficiency and iron excess promote oxidative stress.

For the iron excess experiments, dosages of 2, 4 and 8 mg of iron/rat/day were selected. Daily supplements (***ds*)** of iron were given to dams either during $GD_{5\text{-}12}$ (8 doses) or $GD_{5\text{-}19}$ (15 doses) in order to examine the correlation between maternal oxidative impairments and oxidative impact in embryos as well as fetus. While iron induced no signs of toxicity or maternal mortality, at the

highest dose, there was a marginal reduction in weight gain among dams. Elevated oxidative stress was evident in the maternal milieu as observed in terms of enhanced levels of ROS and MDA in both cytosol and mitochondria of liver and kidney. At higher doses, iron caused moderate depletion of GSH, total thiols and non-protein thiols which were accompanied with concomitant perturbations in the activities of various antioxidant enzymes. This data is consistent with earlier findings in which excess iron given either intraperitoneally or in the diet has been demonstrated to result in increased exhalation of ethane and/or pentane (*Dillard & Tappel, 1979: Harvey & Klassen, 1983: Dresow et al., 1995; Knutson et al., 2000*). Likewise, significant increases in tissue MDA in rat models of dietary iron overload have also been reported *(Brown et al., 1997; Houglum et al., 1990).* In some of these studies, supplementation with vitamin E completely mitigated the increase d lipid peroxidation *(Brown et al., 1997; Dresow et al., 1995)*. With high iron doses in pregnant women, various undesirable gastrointestinal side effects have also been reported *(Hollan & Johansen, 1993*).

Despite extensive literature on iron and LPO, few studies have investigated the effects of daily supplements (**ds**) of iron on LPO especially in normal pregnant rats. Data on the LPO in rats provided daily iron supplementation are rather conflicting. While a recent study in weanling male rats given daily iron–supplements *(Knutson et al., 2000),* the MDA levels in liver, kidney and plasma were not elevated suggesting absence of elevated lipid peroxidation. However, an earlier study *(Srigiridhar and Madhavan Nair*, *1998*) reported that *ds* increased lipid peroxidation as well as protein oxidation in rat intestinal mucosa of *iron deficient* rats.

In the present study, ***ds*** rats showed extensive mitochondrial oxidative damage during both dosing regimens. The degree of oxidative damage was relatively higher in mitochondria when compared to the cytosol. The increased oxidative stress observed both in iron excess and ***IDD*** rats in the present study

may involve mitochondrial dysfunctions as has been found in aging and associated neurodegenerative diseases (*Shigenaga et al., 1994*). Mitochondria use 90% of inspired oxygen, produce a significant amount of cellular superoxide, and accumulate iron for heme and iron-sulfur cluster formation. Numerous studies of severe iron overload modeling hemochroamtosis have also reported increased hepatic lipid peroxidation, nuclear DNA damage and mitochondrial dysfunction (as reviewed by *Britton et al., 1994*). Interestingly, previous morphological and biochemical investigations have shown that *id* causes significant mitochondrial dysfunctions in various organs such as heart, skeletal muscle, liver and blood cells *(Dallman, 1986)* and the mitochondria are shown to be enlarged, rounded with few cristae, had decreased cytochrome concentration, respiratory control and gluconeogenesis compared to iron normal controls (*Jarvis and Jacobs, 1991: Masini et al., 1994: Klempa et al., 1989*).

Iron deficiency model

Iron deficiency is the most common nutritional deficiency and is associated with an increased risk of poor pregnancy outcomes and impaired cognitive development in young children. In the present study, feeding of an iron deficient diet (***IDD)*** also induced significant oxidative stress in the maternal milieu. This finding corroborates with observations of few recent workers who found that ***IDD*** rats had increased lipid peroxidation, ethane, pentane, liver MDA and Kidney MDA levels *(Knutson et al., 2000; Walter et al., 2002*). Prior to these reports, numerous workers have reported that under experimentally ***IDD*** conditions, liver MDA decreased as measured by TBARS (*Rao & Jagadeesan, 1996*) and other reports provide evidence that ***IDD*** is protective against *in vivo* lipid peroxidation (*Chandler et al., 1988*) and hydroxyl formation (*Patt et al., 1990*). However, increased peroxidation in ***IDD*** rats has also been reported (*Uehara et al., 1997*) who showed elevated levels of serum and liver phosphatidylcholine hydroperoxide, an reliable indicator of membrane peroxidation.

While the exact mechanism/s underlying induction of oxidative stress under ***IDD*** conditions is not clear, several mechanisms have been proposed and few evidences favour such possibility. a) First it has been demonstrated repeatedly that ***IDD*** rats rapidly accumulate liver copper *(Sherman & Moran, 1984; Sherman & Tissue, 1981*). Hence as with excess iron, excess copper can also catalyse lipid peroxidation *(Bremner, 1998*). The *id* rats in another study had liver copper levels which were 8-fold higher than normal rats (*Uehara et al., 1997*). b) Second, *id* rats have been shown to accumulate triglycerides in liver and plasma (*Masini et al., 1994; Uehara et al., 1997*) and offer more lipid substrate for lipid peroxidation which may contributed to the high levels of liver and kidney MDA levels. c) the increase in LPO may be due to increased fragility of mitochondrial membranes of ***IDD*** rat tissue mitochondria which have been described as being greatly enlarged and swollen (*Dallman and Goodman, 1970*). In the current study, hepatic tissue of ***IDD*** rats showed elevated mitochondrial ROS and MDA levels suggesting that mitochondria may be subjected to elevated peroxidation events.

Recent studies of *Walter et al., 2002* have convincingly shown that *id* induced increased levels of oxidants and LPO damages the integrity of mitochondrial membrane resulting in uncoupling of mitochondria. Further, the higher levels of oxidants could be caused by decreased heme levels and complex IV activity. Decreased cytochrome concentration of the ETC could contribute to higher superoxide levels. Other speculated mechanisms include i) Higher hepatic copper accumulation which can participate in Fenton chemistry, ii) loss of activity of some important iron–containing repair enzymes such as ribonuceleotide reductase, iii) decrease in mitochondrial aconitase activity which may prevent further mitochondrial release of oxidants by diminishing the supply of reducing equivalents to the ETC and the reduced electron flow may be one way by which the cell protects from oxidant stress under *id* conditions *(Chen et al., 1997)*.

Impact of maternal oxidative stress on Placenta, embryos and fetuses

In the **ds** study, iron administration caused significant oxidative stress in placental tissue as evident from enhanced levels of various oxidative markers measured in cytosol as well as mitochondria. Oxidative alterations were more robust when *ds* were given during GD_{5-19}. A similar trend of results were also observed with respect to diminution of GSH , thiol content, and antioxidant defenses suggesting a higher degree of oxidative induction during GD_{5-19} regimen. Placental protein carbonyls were markedly elevated suggesting protein oxidation. There was a good correlation among alterations in oxidative markers in the hepatic tissue and embryonic tissue. The embryonic ROS, and MDA levels were considerably elevated, while the levels of GSH, thiols were significantly depleted along with lowered activities of antioxidant enzymes at higher doses (4 and 8 mg/rat) suggesting a consistent induction of oxidative stress. Further, **ds** during GD_{5-19}, oxidative alterations induced in the maternal milieu significantly impacted the dysfunctions in fetal tissues as well. We chose to measure the oxidative markers in fetal brain and liver since both are known to be subjected to iron–induced oxidative damage.

Oxidation of protein may alter its structure, function and integrity. In the cell, a system of proteolytic enzymes is responsible for the recognition and selective degradation of oxidatively denatured proteins (*Carney et al., 1991*). The mechanism/s by which cellular proteinases, proteases and peptidases recognize oxidatively modified proteins are not clearly understood (*Davies et al., 1987*). Protein carbonyls are employed as biomarkers of ROS mediated protein oxidation (*Isabella et al., 2003*) and elevated levels of oxidized protein have been reported in animal tissue and cell models under various conditions of oxidative stress *(Berlett & Stadman, 1997*). Elevated ROS can lead to oxidation of amino acid residue side chains, formation of protein–protein cross linkage, and oxidation of protein backbone resulting in protein fragmentation. Protein carbonyls are introduced in to the protein by reaction with aldehydes (eg., 4-hydroxy nonenal and MDA) produced during LPO or with reactive carbonyl

derivatives generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins. Consistently higher levels of protein carbonyls in maternal tissues, embryonic homogenates, placental tissues and fetal tissues observed in the iron excess model, in the present study reflects a high rate of protein oxidation in all the compartments. Furthermore, it also reflects a very low rate of oxidized protein degeneration and/or low repair activity as oxidized forms of some proteins (for eg., cross-liked proteins) and proteins modified by LPO products are not only resistant to proteolysis, but can also inhibit the ability of proteases to degrade the oxidized forms of other proteins (*de Catalfo & Gomez Dumm, 1998*). The potential role of oxidized proteins in drug-induced teratogenesis has been demonstrated both *in vivo* (*Liu & Wells, 1995 a, b*)

Placental oxidative stress

The placenta provides an extensive and intimate interface between the maternal and fetal blood streams. A burst of oxidative stress occurs in the normal placenta as the maternal circulation is established and it is speculated that this may serve a physiological role in stimulating normal placental differentiation, but may also may be factor for the pathogenesis of pre-ecclampsia and early pregnancy failure if antioxidant defenses are depleted (*Jauniaux et al., 2000*). The protective role of the placenta depends in part on the activity of its detoxifying enzymes (*Avissar et al., 1994*). The placenta is equipped with antioxidant enzymes such as catalase, SOD, GPX, GST and GR. The placenta also expresses both enzymes necessary for synthesis of GSH and it has been suggested that the placenta could protect the developing fetus from free radicals by lowering the level of lipid peroxides in fetal blood (*Takehara et al., 1990*). In the present study, placenta of rats administered iron excess showed higher ROS and MDA levels at higher doses, which were accompanied with diminished GSH, reduced activity of antioxidant enzymes, reduced total thiols and non-protein thiols, and elevated protein carbonyl content suggesting that the ongoing oxidative dysfunctions in this protective tissue. These

significant perturbations clearly indicate that the placental antioxidant defenses were overwhelmed by the administered iron at higher doses and were unable to cope up with the oxidative implications.

In the iron excess model, embryos which were sampled on GD_{13} showed significant increases in the biomarkers of oxidative stress as compared with the controls. Both cytosol and mitochondrial fractions exhibited elevated levels of ROS and MDA levels clearly suggesting increased lipid peroxidation occurring *in vivo*. Further, our findings such as depleted GSH levels, reduced activities of antioxidant enzymes, total thiols and non–protein thiols among embryos exposed to high maternal iron levels also suggested that the embryos were indeed subjected to elevated oxidative stress situation. Interestingly, the protein carbonyl content of embryos were also elevated significantly indicating the occurrence of protein oxidation.

Fetal oxidative stress

The intrauterine environment influences the development of the fetus by modifying gene expression in both pluripotential and terminally differentiated, poorly replicating cells. The fetus adapts to an inadequate supply of substrates (such as glucose, aminoacids, fatty acids, and oxygen) by metabolic changes, redistribution of blood flow, and changes in the production of fetal and placental hormones which control growth *(Simmons, 2006)*.

Fetal growth and development are dependent upon the adequate provision of oxygen and substrates from the maternal circulation. Intrauterine growth restriction is a condition sometimes associated with placental insufficiency caused by shallow trophoblast invasion during the early stages of gestation *(Cetin, 2003)*. In the present study, we observed significant increase in placental weights only at the highest dose (8 mg/d) in the dosing regimen GD_{5-12}, where as significant increase was evident at higher doses (4 and 8 mg/d) in the dosing regimen 5-19. Increased placental weights, placental size and placental ratios (placental weight to birth weigh ratio)/have been documented in pregnancies complicated by gestational diabetes *(Naeye, 1987; Desoye & Shafir, 1996; Lao et al., 1997)*.

Pregnancy outcome

Iron nutrition and hematological status are known to be critical throughout pregnancy for mother, fetus and beyond *(Casanueva & Viteri, 2003*). Epidemiological evidence has shown an association between anemia, premature delivery and low birth weight *(Scholl & Reilly, 2000*). Prolonged Iron deficiency conditions are known to have pronounced negative effect on the course and product of pregnancy. In the present study, iron administered at higher doses induced significant elevation in the incidence of embryonic deaths (2-5 fold higher over the background incidence) during both dosing regimens.

Although we have not examined the nature of cell death mechanisms under iron excess or iron deficiency models, in the present study, the incidence of early embryonic deaths were higher in both models. However, a small percentage (approximately 10% deaths) of fetal deaths occurred in the dosing regimen $GD_{5\text{-}19}$ clearly suggesting the vulnerability of early embryos to oxidative stress. The nature of cell death involved in the embryonic deaths was not examined in the present study. However, earlier numerous studies with teratogens, such as hyperthermia, cyclophosphamide, sodium arsenite, cadmium and deoxyadenosine have demonstrated induction of apoptosis in early post-implantation mouse embryos *(Mirkes & Little, 1998; Ghatnekar et al., 2004)*.

In conclusion, the present investigations have clearly demonstrated that both iron excess (daily supplements) and iron deficiency during gestation causes not only elevated oxidative impairments in the maternal milieu, but has significant impact on the embryonic development. The embryos are subjected to oxidative stress as revealed by the various oxidative dysfunctions which may significantly contribute towards the higher frequency of early embryonic deaths. Further the fetal tissues are also subjected to significant oxidative stress as revealed in the elevated oxidative dysfunction in the fetal organs which is likely to be responsible for the altered development of fetus and subsequent development of adult diseases.

6.0 SUMMARY

1. Oral administration (2-8 mg/rat/d) of iron failed to induce any clinical signs of toxicity or mortality, while at the highest dose it significantly affected the maternal body weight gain. Placental weights were found increased (21-45%) at the highest dose during both the dosing regimens.

2. In maternal organs, differential pattern of induction of ROS levels was evident in cytosol and mitochondria. At higher doses, the induction of ROS levels appeared to be higher in mitochondria during both regimens.

3. Marginal reduction in GSH levels in maternal organs were accompanied by uniformly diminished total thiol/non-protein thiols levels at higher doses irrespective of the dosing regimen; Protein carbonyls levels were elevated significantly only at higher doses. In general, the activities of antioxidant enzymes were significantly decreased only at higher doses.

4. Placental tissue showed marked elevation in ROS levels only at the highest dose, while the increase in MDA levels was relatively of lesser magnitude. The placental tissue also showed significant decrease in total thiol and non-protein thiol levels during both dosing regimens.

5. Significant oxidative impact was evident among GD_{13} embryos following maternal exposure of iron at the highest dose as evidenced by elevated ROS and MDA levels in both cytosol and mitochondria. That the embryos are subjected to OS was also reflected in the diminished activities of antioxidant enzymes, marked diminution of GSH, total thiols, and non protein thiols, while the carbonyl content were found significantly elevated.

6. Maternal iron exposure resulted in a significant oxidative impact in the fetal tissues. While brain tissue showed significant elevations in MDA and ROS levels, the liver tissue showed less robust induction.

7. In fetal tissues, maternal Iron exposure caused significant reduction in the activity of antioxidant enzymes, reduced levels of GSH and was accompanied with elevated total thiol levels, while the protein carbonyl levels were higher.

8. Among iron deficient dams, the incidence of embryolethality was significantly higher compared to the controls during both GD_{13} and GD_{20}.

9. Maternal tissues of iron deficient dams were subjected to significant OS as evident from the elevated ROS levels and lipid peroxidation irrespective of the dosing regimen. In brain, the ROS levels were uniformly elevated both in cytosol and mitochondria, while in liver, the cytosol showed more robust increase than mitochondria.

10. The GSH content of maternal tissues (brain, liver and kidney) showed moderate decrease in cytosol/mitochondria which were associated with significantly reduced activities of antioxidant enzymes.

11. The placenta of iron deficient dams showed moderate elevations in ROS levels, marked induction of MDA levels, depleted GSH levels and elevated protein carbonyls content suggesting the ongoing oxidative stress *in vivo.*

12. Embryos of iron deficient dams were indeed subjected to oxidative impact as evident by significant elevation in both ROS and MDA levels, diminished levels of GSH, total thiols/non protein thiols and marginal alterations in the activities of antioxidant enzymes.

13. Embryos were also subjected to protein oxidative damage since the protein carbonyl content among the embryos of Iron deficient diet fed dams was marginally were observed among the embryos of dams fed iron deficient diet

14. The fetal organs (brain and liver) of iron deficient diet fed dams showed significantly elevated ROS/MDA levels. Significant diminution in the levels of reduced glutathione accompanied with reduction in total thiol levels and non protein thiols, while the protein carbonyl levels were significantly elevated.

15. The results obtained in the iron excess and iron deficiency models clearly suggest the occurrence of elevated oxidative impairments in the maternal milieu, and significant oxidative impact on the growing embryos, which may contribute towards the higher frequency of early embryonic deaths. Further, elevated oxidative dysfunction in the fetal organs may to be responsible for the altered development of fetus and subsequent development of adult diseases.

1.0 INTRODUCTION

The steadily increasing trend in the prevalence of diabetes mellitus (DM) is of global concern. Women are reported to be more prone to type 1 DM than men. DM is often diagnosed in women during pregnancy and about 5 % of all pregnancies occur among diabetic women. Maternal diabetes during pregnancy is associated with increased risk for growth disturbances and congenital malformations. There is 2-3 fold increased incidence of major congenital malformations in the offspring of diabetic women compared to that of the general population. The malformations which occur during the period of embryonic organogenesis and the nature observed in type-1, type-2 and gestational diabetes are similar. Although the cellular mechanisms of diabetic embryopathy are not completely understood, a number of possible teratological processes have been suggested mainly on experimental studies. Both disturbed metabolism of arachidonic acid and excess of radical oxygen species (ROS) have been implicated in diabetes-induced embryonic dysmorphogenesis *(Eriksson et al., 1996; Eriksson et al., 2003)*.

Several findings have suggested a role for oxidative stress in the development of diabetic complications *(Oberley, 1988; Baynes & Thorpe, 1999; Gurler et al., 2000)*. Mitochondrial overproduction of the oxygen radical superoxide has been suggested recently as a critical element in diabetic complications *(Brownlee, 2001)*. Earlier studies have demonstrated the involvement of oxidative stress in the pathogenesis of diabetic embryopathy *(Eriksson & Borg, 1991)*. Some evidence also suggests that disturbances in the arachidonic acid cascade *(Goldman et al., 1985)* including prostaglandin E2 metabolism cause diabetic embryopathy *(Goto et al., 1992; Piddington et al., 1996; Wentzel et al., 1999)* since this pathway plays a vital role in neural tube development.

Uncontrolled diabetes has been implicated as an etiological factor for recurrent pregnancy loss and there is an estimated incidence of 17% spontaneous abortion in DM pregnancies. Prevention of fetal complications in maternal DM requires a thorough understanding of the underlying pathophysiology of pregnancy in diabetes. Poor maternal metabolic control during pregnancy is attributed to be the major cause for the augmented malformation rate. Major organ system anomalies arise in embryos of diabetic women during the period of organogenesis and are known to affect various systems rather nonspecifically. Experimental studies on maternal diabetes in laboratory animals have demonstrated a consistent intrauterine growth restriction (IUGR) and fetoplacental abnormalities *(Padmanabhan et al., 2006)*. However, the mechanisms of diabetic embryopathy are not clearly understood so far, although there is some strong evidence that there is oxidant-antioxidant disequilibrium in DM.

Employing animal models, numerous workers have demonstrated that exposure of embryos to diabetes *in vivo* or high glucose concentration *in vitro (Eriksson & Borg, 1981)* increases malformation rate in the offspring. More importantly, in experimental diabetic pregnancy, various antioxidative treatments have been demonstrated to be beneficial by improving fetal outcome, thereby implicating oxidative stress as an important etiological factor for diabetic embryopathy *(Wentzel & Eriksson, 2005; Cederberg & Eriksson, 2005)*.

In the field of obstetrics, diabetes during pregnancy is a cause for great concern due to a number of reasons. Firstly, diabetic women are more susceptible to spontaneous abortion than non-diabetic pregnant women. The increased risk may be eliminated if glycemic control is obtained at the time of conception or during the first 3 months of pregnancy. In addition, congenital anomalies occur in 6-8% of the fetuses of diabetic women (Type 1) compared to 2 % in non-diabetics.

Several studies have reported the positive effects of antioxidant treatment in experimental diabetic pregnancy. The antioxidants viz., ascorbic acid and alpha tocopherol have been demonstrated to improve fetal outcome and diminish embryonic dysmorphogenesis *(Cederberg & Eriksson, 2005)* in rodents. However, administering antioxidants in extremely high doses also constitutes a high teratological risk and hence finding the optimal dosage level of antioxidant is critical in clinical studies with dietary supplementation to pregnant diabetic women.

Accordingly, in the present investigation, it was aimed to establish the nature and degree of oxidative impairments in CFT-Wistar rats, rendered diabetic by an acute injection of STZ, a well-known diabetogen. Following preliminary studies, an optimum dosage of 45 mg/kg bw and the gestation day (GD) 4 was selected for these investigations. In the first series of investigation, we have quantified the oxidative implications in maternal tissues at GD_{13} and GD_{20} in order to examine the progression and pattern of oxidative stress. Induction of hyperglycemia and the effects of diabetes on body growth and urine output were monitored. More importantly, we chose to examine the impact of maternal hyperglycemia on embryonic and fetal development, oxidative implications in GD_{13} embryos and fetal organs (GD_{20}). The degree to which the placental tissue is subjected to oxidative stress has also been assessed at both sampling times. In the second series of investigation, the protective propensity of garlic (a widely consumed spice) and selected medicinal plants were assessed. These studies focused on their ability to modulate diabetic associated oxidative impairments in maternal embryonic and fetal milieu. The results have been presented under two sections, A and B.

SECTION A

OXIDATIVE PERTURBTIONS IN PREGNANT DIABETIC RATS

2.0 OBJECTIVE

The primary objective of this study was to arrive at the optimum dosage of STZ required to induce consistent hyperglycemia, moderate embryopathy and assessment of embryonic and fetal growth characteristics in CFT-Wistar strain of rats. Further, it was aimed to establish the degree of oxidative damage in maternal milieu and its correlation with oxidative implications in the embryos (GD_{13}) and fetal (GD_{20}) tissues. These data were a primary requisite for our subsequent ameliorative studies.

3.0 EXPERIMENTAL DESIGN

3.1 Preparation of STZ

Streptozotocin was dissolved (75 mg/ml) in freshly prepared citrate buffer (0.05 M, pH4.5) and immediately used for administration.

3.2 Preliminary studies and dose selection

With an objective of determining the optimum dosage of STZ that would induce a consistent hyperglycemia and moderate embryolethality, preliminary studies were conducted. Initially, groups of pregnant rats (n=4) were administered (i.p) STZ at acute dosages - 30, 45 and 60 mg/kb bw on GD_4. Animals were provided with glucose water (5% w/v) for 48 h following STZ treatment to prevent initial drug-induced hyperglycemic mortality. Random plasma glucose was measured by drawing blood by cardiac puncture from both control and STZ-injected rats. Based on the incidence of embryolethality and hyperglycemic response, a dosage 45 mg/kg bw was selected as the optimum dosage in our strain for our further experimentation.

3.3 Determinative study

In order to assess the diabetes associated oxidative impairments in embryos and fetuses, pregnant rats were rendered diabetic by administration of an acute dose of STZ (i.p, 45 mg/kg bw) on GD_4. Rats of both control and STZ groups were provided with a known amount of diet and water *ad libitum*. Daily food consumption was recorded by weighing the residual diet. Weekly body weights were monitored during the experimental period of 20 days. Dams (n=6) from both groups were sacrificed on GD_{13} or GD_{20} under light ether anesthesia. Maternal serum glucose levels were determined at sacrifice. Embryos, placenta and fetuses were excised, trimmed, rinsed in ice-cold physiological saline, blotted and weighed.

3.4 Oxidative impairments in mother, placenta, embryos and fetus

3.4.1 Status of lipid peroxidation and generation of ROS

Status of oxidative damage in embryonic tissue and fetal organs (brain and liver) was measured in terms of MDA levels. In order to obtain a comparative data, MDA levels were also determined in placenta and selected maternal organs such as liver and kidney at both sampling times (GD_{13} and GD_{20}). Cytosolic and mitochondrial fractions of embryos, placenta, maternal and fetal tissues from both control and treated groups were subjected to biochemical analysis.

3.4.2 Perturbations in enzymic and non-enzymic antioxidants

At both sampling intervals, the activities of antioxidant enzymes viz., CAT, SOD, GST, GPX and GR and levels of non-enzymic antioxidants viz., GSH, total thiols and non protein thiols were determined in embryos, placenta, maternal and fetal tissues.

3.4.3 Oxidative damage to proteins: protein carbonyls formation

Fresh mitochondrial and cytosolic samples of embryos, placental, fetal and maternal tissues of both control and treated groups were processed for the determination of total protein carbonyls content at both sampling intervals.

SECTION B

ABROGATION OF DIABETES ASSOCIATED OXIDATIVE IMPAIRMENTS

2.0 OBJECTIVE

The primary objective of these studies was to assess the ameliorative propensity of selected medicinal plants viz., *Gymnema sylvestre* (GS), *Ipomea aquatica* (IA), *Tinospora cordifolia* (TC) and *Withania somnifera* (WS) in pregnant rats rendered diabetic by an acute dose of STZ on GD_4. Dried powder of leaves (GS and IA) and roots (TC and WS) were used as dietary supplements. A separate study was also conducted to assess the beneficial effects of garlic supplements (freeze dried garlic powder) in the diabetic model.

3.0 EXPERIMENTAL DESIGN

3.1 Ameliorative effects of garlic powder on diabetes associated oxidative damage in maternal and fetal tissues

Pregnant rats were randomly distributed into four groups (n=6) as follows: Group I: Control; Group II: Garlic (G); Group III: Streptozotocin (STZ) and Group IV: STZ + Garlic. While Group II and IV rats were fed with powdered diet supplemented with garlic (2%) from $GD_{0\text{-}19}$ throughout the gestation (total 20 days), other groups were maintained on powdered commercial pellet diet (M/s Amruth feeds Ltd., India). STZ (45 mg/kg bw) was administered to pregnant rats of Group III and IV on GD_4.

Terminally, all rats were sacrificed under mild diethyl ether anesthesia. Blood was drawn by cardiac puncture for separation of serum. Maternal organs (liver and kidney) were excised, rinsed in ice-cold PBS, blotted, and processed. Placenta and fetuses were weighed after rinsing in ice-cold PBS and blotted. Further, fetuses were dissected, brain and liver excised, rinsed in ice-cold PBS,

blotted and processed. The following biochemical indices of oxidative damage were determined in placenta, maternal and fetal tissues (brain and liver): TBARS and ROS levels, GSH and total thiols, protein carbonyls, selected enzymic antioxidants.

3.2 Ameliorative effects of medicinal plants on diabetes induced oxidative impairments in maternal and fetal tissues

Rats were randomly grouped in to ten groups (n=6) as follows:

Group I – non-diabetic control (CTR); Group II: GS control; Group III: IA control; Group IV: TC control; Group V: WS control; Group VI: STZ; Group VII: STZ + GS; Group VIII: STZ + IA; Group IX: STZ + TC; Group X: STZ + WS.

Weighed amounts of diet (supplemented with 2% of GS/IA/TC/WS) were fed to the rats throughout the gestation starting from GD_0. On GD_4 pregnant rats of Group VI, VII, VIII, IX and X) were rendered diabetic by STZ. While body weight gain was monitored regularly, urine excretion was measured from GD_{17-20} after housing them in individual metabolic cages.

Rats were sacrificed on GD_{20} by mild diethyl ether anesthesia. Blood was drawn by cardiac puncture for measuring serum glucose levels. Maternal organs (liver and kidney) were excised, rinsed in ice-cold phosphate buffered saline, blotted, weighed and stored in cryovials at -80^0C until further analysis. Uterine horns were excised and pinned to wax base immersed in ice cold PBS. Using surgical procedures, fetuses and placenta were excised and weighed. Further, fetal liver and brain were excised, rinsed in ice-cold PBS, blotted and at -80^0C until further analysis. The modulatory effects of selected medicinal plants were assessed employing the following selected markers: TBARS and ROS levels, reduced GSH and total thiols, enzymic antioxidants (CAT and GST).

4.0 RESULTS

SECTION A

OXIDATIVE PERTURBATIONS IN PREGNANT DIABETIC RATS

4.1 Hyperglycemic response

Blood glucose measured following administration of STZ at the dosages of 30, 45 and 60 mg/kg bw, has been presented in Fig 4.1. The data showed a consistent and progressive hyperglycemic response following STZ injection. While the glucose levels in control rats were in the range of 82-104 mg%, those of STZ rats ranged from 223-636 mg%. At the highest dosage (60 mg/kg bw), plasma glucose increased by 6-fold whereas, the lowest dose (30 mg/kg bw) induced only a 2-fold increase and a 4-fold increase in the glucose levels was evident at the dosage of 45 mg/kg bw.

4.2 Incidence of embryo lethality: fetal/placental weight

STZ at the administered dosages (30, 45 and 60 mg/kg bw) failed to induce any maternal mortality. However, STZ administration resulted in a higher incidence of embryolethality as evident by significant increase in the incidence of dead implantation (DI), the percent dead implants expressed as a percentage of total implantations. While the highest dosage induced complete resorptions (100% DI), the lowest dosage (30mg/kg bw) induced only a marginal increase (12%) (Table 4.1). However, at a dosage of 45 mg /kg bw STZ induced consistent degree of DI (30-40% DI). Hence, based on the incidence of embryolethality and hyperglycemic response, 45 mg/kg bw was considered as the optimum dosage which was deployed for all further investigations.

Data on the fetal and placental weights among control and diabetic rats determined on GD_{20} is presented in Fig 4.2. Among diabetic dams the fetal weights were markedly reduced (38%), while the mean placental weights were highly increased (70%).

4.3 Food consumption and growth pattern

A significant decrease (50%) in food intake was evident in the STZ treated rats during the first four days of post STZ administration. Further, the food intake was significantly increased (30-40%) in diabetic rats compared to the controls during the rest of experimental period (data not shown). In general, a decrease in the body weight gain was evident among the STZ treated rats throughout gestation. Body weight gain in the diabetic rats (19$\pm$1.5g) was significantly reduced than that of controls (31$\pm$2.6g) at GD_{13}. Similarly, on GD_{20}, there was a 43% decrease in the body weight gain (CTR 86 $\pm$ 5.6g v/s STZ: 49 $\pm$ 6.5g)

4.4 Evidences of oxidative damage in maternal tissues (GD_{13})

4.4.1 Lipid peroxidation (LPO) *in maternal liver and kidney*

The lipid peroxidation (LPO) status measured as MDA levels in maternal liver of both control and STZ treated rats on GD_{13} is presented in Fig 4.3. Diabetes induced significant elevation (30%) in MDA levels in mitochondria as well as cytosolic fractions of maternal liver. However, the extent of elevation in MDA levels in cytosol of kidney was marginal (15%). There was a marked elevation (60%) in LPO in serum of diabetic rats (data not shown).

4.4.2 Generation of ROS in maternal liver and kidney

STZ treatment resulted in a significant elevation in ROS levels in cytosolic and mitochondrial fractions of both liver and kidney (Fig 4.3). Interestingly the pattern of elevation was similar in both fractions. While the hepatic cytosolic and mitochondrial fractions showed marked (90%) elevation, the percent increase in renal ROS levels was relatively less (70%) in both fractions.

4.4.3 Perturbations in antioxidant enzymes and molecules

Data on the activity of selected antioxidant enzymes in hepatic and renal tissue is presented in Fig 4.4. In general, the activities of antioxidant enzymes were reduced marginally in the cytosolic fractions of liver and kidney of diabetic rats. The extent of reduction was more in kidney compared to liver. While there was no significant difference in hepatic GPX, activities of other enzymes were decreased in both liver (CAT: 23%; GST: 17%) and kidney (GST: 47%; GPX: 17%; CAT: 30%). Data on reduced GSH and thiol status are depicted in Fig 4.4. While, hepatic GSH and non protein thiol levels were moderately decreased (35%), total thiol levels decreased by 25% in pregnant rats administered STZ on GD_4. Similar pattern of decrease in levels of antioxidant molecules was also evident in kidneys of diabetic rats.

4.5 Status of oxidative damage in placenta (GD_{13})

4.5.1 Status of lipid peroxidation

The status of lipid peroxidation determined in placenta sampled on GD_{13} is presented in Fig 4.5. There was a marked elevation in the MDA levels in both cytosolic (64%) and mitochondrial (70%) fractions of placenta. A similar trend was also evident in ROS levels (cytosol, 50%; mitochondria, 70%).

4.5.2 Alterations in antioxidant defenses

The levels of reduced GSH, total thiols, non protein thiols and activities of antioxidant enzymes in placenta of control and STZ treated rats are shown in Fig 4.5. In general, a significant decrease in CAT, GPX and GST activities were evident (30-40%) in cytosol of diabetic rats sampled on GD_{13}. Further, a significant reduction was observed with reduced GSH (45%) and NPSH (36%) while on the other hand, there was only a marginal (10%) increase of total thiols.

4.6 Status of oxidative impairments in embryos (GD_{13})

4.6.1 MDA and ROS levels in embryos

The effect of diabetic pregnancy on LPO and ROS levels in embryos, measured on GD_{13} is shown in Fig 4.6. MDA levels in embryos of diabetic rats showed a significant elevation in both cytosolic (63%) and mitochondrial (53%) fractions compared to controls. Likewise, there was a marked increase (70-90%) in the ROS levels in both the fractions of embryos.

4.6.2 Response of antioxidant defenses

Data on the levels of antioxidant molecules (reduced glutathione and total thiols) (Fig 4.7) showed a significant (30%) decrease. However, there was a higher degree of reduction in non-protein thiols (60%) in embryos of STZ rats compared to their corresponding controls. While the activities of GPX and CAT decreased moderately (30%), the reduction in the activity of GST was marginal (18%) (Fig 4.7).

4.7 Oxidative damage in maternal tissues (GD_{20})

4.7.1 Status of lipid peroxidation and ROS

The lipid peroxidation status measured as MDA levels in maternal serum, liver and kidney in both control and STZ treated rats on GD_{20} is presented in Table 4.2. There was a marked elevation (50%) in LPO in serum of diabetic rats. Hepatic and renal MDA levels showed a significant elevation in both mitochondrial and cytosolic fractions, which ranged from 35-40%.

STZ administration induced significant increase in the ROS levels in both liver and kidney and the ROS levels were enhanced (45%) in both the compartments of liver. While, in kidney, the ROS levels measured were relatively less elevated (35%) in cytosol than mitochondrial fractions (81%).

4.7.2 Perturbations in antioxidant molecules and enzymes

A marked decrease (liver: 40% and kidney: 25%) in the endogenous GSH levels was evident in maternal tissues on GD_{20} (Table 4.2). However, TSH levels showed only a moderate decrease (kidney: 35% and liver: 15%) due to STZ treatment. On the contrary, NPSH levels were greatly reduced (70%) in maternal liver, whereas in kidney, the decrease was marginal (26%). Alterations in activities of antioxidant enzymes showed similar levels in maternal hepatic and renal tissues on GD_{13}. While, hepatic antioxidant enzymes showed marginal reduction in their activities (CAT and GST: 22% and GPX: 16%), that of kidney showed moderate reduction (CAT: 30%; GST: 35% and GPX: 20%).

4.7.3 Protein carbonyls content

Marginal increases of 19 and 24% in protein carbonyl levels were found in the maternal kidney and liver of diabetic rats on GD_{20} (Table 4.2).

4.8 Evidences of oxidative damage in fetal tissues

4.8.1 Status of lipid peroxidation and generation of ROS

Data on the ROS and MDA levels in brain and liver of fetuses in non-diabetic and diabetic rats is presented in Tables 4.3 and 4.4. The hepatic cytosol showed enhanced ROS levels (by 53%) over the controls, while it was relatively higher in mitochondrial fractions (by 66%). The fetal brain cytosol and mitochondria of STZ rats also showed enhanced ROS levels (65-80%). However, the increase in MDA levels was less robust (20-30%).

4.8.2 Effect on reduced GSH, thiols and antioxidant enzymes

Changes in reduced GSH, thiols and antioxidant enzymes in fetal brain and liver are presented in Table 4.4. While NPSH showed marked decrease (50%) in fetal liver, it showed marginal decrease in fetal brain (23%). However, there was marginal to moderate decrease in the levels of other antioxidant molecules (GSH and TSH) in both the fetal tissues which ranged between 30-40%. While diabetic pregnancy induced marginal change (10-18%) in the activities of antioxidant enzymes in fetal liver, it induced moderate change (15-35%) in fetal brain (Table 4.4).

4.0 RESULTS

SECTION B

ABROGATION OF DIABETES ASSOCIATED OXIDATIVE IMPAIRMENTS.

(i) Ameliorative effects of dietary garlic

4.1 Effect on diet intake and body weight gain

Data on serum glucose levels, body weight gain and urine output among control and treatment groups are presented in Table 4.5. There was a marginal decrease (20%) in diet intake of diabetic rats, which was normalized with garlic diet (data not shown). Terminally, the average body weight gain among diabetic dams was significantly (45%) lower compared to non-diabetic controls. However, weight gain was restored partially among garlic supplemented diabetic dams, although it was still lower (25%) in comparison with non-diabetic dams. Urine output among diabetic dams measured on gestation days 14-19 showed a marked polyuria (STZ: 98 ml v/s CTR: 11 ml/rat/d), while diabetic dams fed garlic diet showed relatively lower degree of polyuria (STZ+G: 73 ml v/s STZ: 98ml /rat/d).

4.2 Incidence of embryolethality and fetal weights

Data on the incidence of total, live and dead implantations (DI) among control and treatment groups is presented in Table 4.6. The DI calculated as percentage of total implantations showed marked increase in diabetic rats (STZ: 40% v/s control: 4%). While garlic *per se* had no measurable effect on the incidence of DI, garlic supplements in diabetic dams significantly reduced the incidence of embryolethality (12% v/s 40%). Further, the mean fetal weights in diabetic dams were markedly (37%) decreased in comparison with non-diabetic controls. However, garlic supplements in diabetic dams considerably normalized the fetal weights. Interestingly, there was significant (50%) restoration of fetal weights among garlic supplemented diabetic dams

4.3 Oxidative impairments in maternal organs

4.3.1 Markers of oxidative stress

In general, garlic supplements had no measurable effect on various biochemical markers of oxidative damage determined in maternal liver and kidney of non-diabetic dams (Table 4.7). However, induction of diabetes caused a significant elevation in ROS levels (liver-29%; kidney-55%) as well as MDA levels in maternal tissues (liver-45%; kidney-43%). With garlic supplements, these markers were significantly restored to normalcy among diabetic dams. Likewise, the reduced GSH and total thiols in maternal liver of diabetic dams which were reduced by 40% and 30% respectively in diabetic dams were also restored to normalcy by garlic supplements among diabetic dams. However, the protein carbonyl levels in liver of diabetic dams were protected only marginally by garlic. A similar trend of result was also evident in maternal kidney with respect to total thiols and protein carbonyls (Table 4.7).

4.4 Placental weights and markers of oxidative damage in placenta

In general, garlic supplements had no measurable effect on either placental weights or biochemical markers of oxidative damage in placenta of non-diabetic dams (Table 4.8). While induction of diabetes caused a marked (75%) elevation in the placental weights, garlic supplements significantly (42%) reduced the weights among diabetic dams. In diabetic dams, the elevated placental MDA (by 64%) and ROS (by 49%) levels were restored significantly (50% restoration) by garlic supplementation. A similar degree of protection was also evident with respect to other markers viz., reduced GSH, total thiols and protein carbonyls in diabetic dams provided with garlic supplements.

4.5 Oxidative stress markers in fetal brain and liver

4.5.1 Status of lipid peroxidation

Status of lipid peroxidation (quantified as MDA levels) in the mitochondrial and cytosolic fraction of fetal liver and brain are shown in Fig 4.8. The MDA levels were elevated significantly (27%) in fetal brain and liver obtained from diabetic dams. While garlic supplements did affect the basal levels in non-diabetic controls, it restored partially the augmented MDA levels in the fetal organs of diabetic dams. The fetal liver of diabetic dams showed relatively higher degree of oxidative damage and the garlic diet afforded a lower degree of protection.

4.5.2 ROS generation in fetal brain and liver

Levels of ROS generated in the mitochondrial and cytosolic fractions of fetal brain and liver are shown in Figs 4.8 and 4.9. The MDA levels were elevated significantly (27%) in fetal brain and liver obtained from diabetic dams. While garlic supplementation did not affect the basal levels in non-diabetic controls, it restored partially the augmented levels in the fetus of diabetic dams. The fetal liver of diabetic dams showed relatively higher degree of oxidative damage and the garlic supplements afforded a lower degree of protection.

4.5.3 Antioxidant enzyme activity

Data on the activity of antioxidant enzymes viz., catalase (CAT) and glutathione transferase (GST) measured in maternal and fetal tissues are presented in Table 4.9. Among diabetic dams, the activity of catalase was significantly lower in maternal tissues (liver, 30%; kidney, 44%). Likewise GST activity was also lower (liver, 25%; kidney, 50%). However, the enzyme activities were restored to varying degrees of normalcy by garlic diet among diabetic dams. In fetus obtained from diabetic dams, significant decrease in CAT activity was observed in brain (40%) and liver (28%). Likewise, GST activity was also reduced in fetal brain (49%) and liver (25%). However, diabetic dams fed garlic showed relatively higher levels of enzyme activities.

(ii) Ameliorative effects of medicinal plants

4.6 Serum glucose levels, diet intake, urine output and body weight gain

In general, supplementation of selected medicinal plants to non-diabetic rats had no significant effect on diet intake, body weight gain, serum glucose and urine output (Table 4.10). There was a significant increase (3.5 fold) in the serum glucose levels of diabetic rats. However, dietary supplements lowered the increased serum glucose levels in diabetic rats to varying extent (GS: 40%, TC: 35%, IA and WS: 20%). There was a marginal decrease (20%) in diet intake of diabetic rats, which was normalized with GS, IA and WS diet. TC did not alter the diet intake in diabetic rats. Terminally, the average body weight gain among diabetic dams was significantly lower (43%) compared to non-diabetic dams. Dietary supplements offered varying degree of protection among diabetic rats (GS: 65%, IA: 25%, TC: 42% and WS: 40%). The urine output in diabetic rats was dramatically enhanced (8-folds higher) compared to control. The elevated urine output was brought down marginally by the dietary supplements (GS and TC: 35%; IA and WS: 20%).

4.7 Incidence of embryolethality, fetal and placental weights

Data on the incidence of embryolethality among control, STZ and various treatments groups is presented in Table 4.11. Diabetic rats showed high incidence of (40%) embryolethality compared to non-diabetic controls. Dietary supplements offered significant protection against STZ induced embryolethality. While GS and TC offered maximum protection (65%), IA and WS brought down the elevated incidence of dead implants by 36 and 47%. Fetal weights in diabetic rats which were decreased significantly (intra uterine growth retardation) were restored by dietary supplements.

4.8 Oxidative impairments in maternal organs

In general, dietary supplements fed to non diabetic rats had no appreciable effect on various biochemical markers of oxidative damage determined in maternal liver and kidney (Table 4.12) except for GS and TC supplements which reduced MDA levels by 25% in maternal liver. However, STZ administration on elevated the MDA levels in maternal tissues (M_L: 45% and M_K: 32%) as well as ROS levels (M_L: 72% and M_K: 55%). GS supplementation offered maximum protection against elevated MDA levels (M_L: 90% and M_K: 71%) followed by TC (M_L: 65% and M_K: 70%), IA (M_L: 58% and M_K: 62%) and WS (M_L and M_K: 35%). Similar patterns of protection were also evident in reduction of ROS levels.

Diabetic rats showed marked decline in GSH levels in maternal tissues (M_L: 53 % and M_K: 40%) (Table 4.13). Dietary supplement in diabetic rats significantly offset the decrease in GSH levels. Further, the protection offered by various supplements in maternal liver was between 40-60%. In maternal kidney, GS and TC offered moderate but significant protection (30-40%) while on the contrary IA and WS treatments offered no appreciable protection. STZ administration induced a moderate (25-45%) reduction in TSH levels of maternal tissues. While dietary supplements offered 30-50% protection in maternal liver, they offered 30-70% protection in kidney.

In diabetic rats, the activities of antioxidant enzymes were significantly diminished (Cat, 35-40%; GST, 35%) in both liver and kidney (Table 4.14). The percent protection offered against STZ induced reduction in CAT activity by GS and TC varied from 50-75 in maternal liver. However, IA and WS supplements restored the decreased CAT activity by 30-60%. Similar pattern of protection by dietary supplements was evident in restoring the reduced GST activity.

4.9 Oxidative stress markers in fetal tissues

4.9.1 Modulation of MDA and ROS levels in fetus

Data on fetal tissues (brain and liver) MDA levels in mitochondria and cytosolic fractions are shown in Fig 4.10. Dietary supplements *per se* had no effect on the basal MDA levels in non-diabetic rats except in fetal liver. Significant elevation (cytosol, 45% and mitochondria, 65%) in the fetal brain MDA levels was evident in diabetic rats while dietary supplementation offered varying degree of protection in both cytosol (TC and WS: >70%; GS and IA: >45%) and mitochondrial fractions (GS: 65%; IA and TC: ≅ 50%; WS: 30%).

In fetus, the ROS levels were markedly enhanced in brain (mitochondria, 40%; cytosol, 36%) and liver (mitochondria, 55%; cytosol, 44%) of STZ administered rats (Fig 4.11). Both GS and TC supplements resulted in a marked (70%) protection in fetal brain cytosol and mitochondria (73 and 60%). However, IA and WS treatment offered marginal (20-35%) protection in both cytosolic and mitochondrial fractions of fetal brain. A similar pattern of protection was also evident in fetal liver. While GS and TC offered 60-70 % protection, IA and WS offered only 25-50% protection respectively.

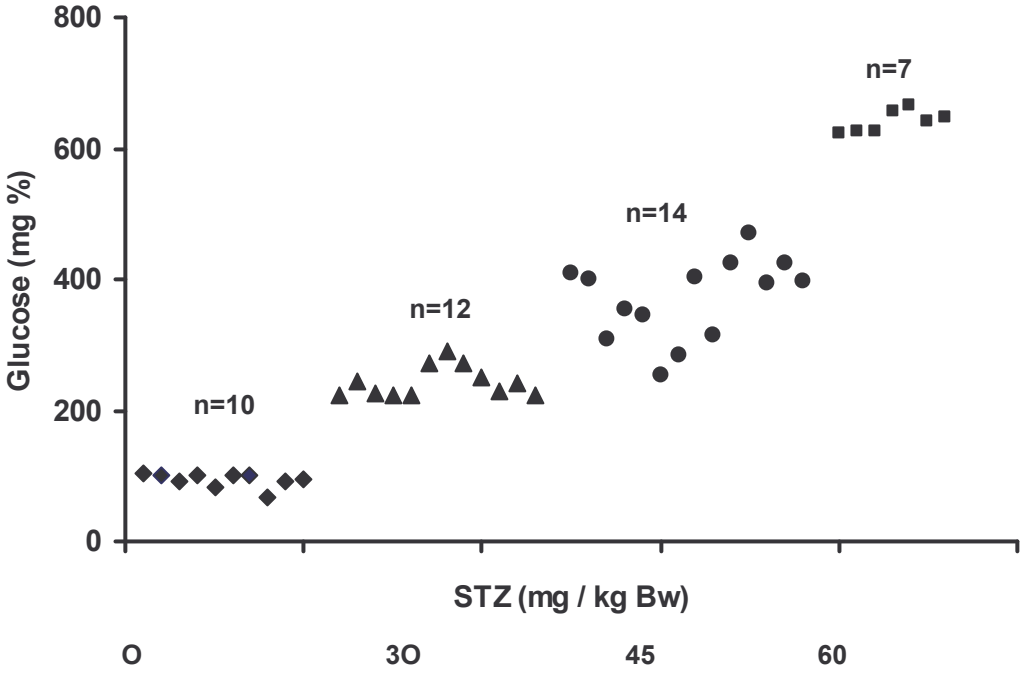
4.9.2 Status of Reduced Glutathione and Total Thiols (TSH)

Dietary supplements *per se* had no measurable effect on the levels of GSH and TSH in fetal tissues, except for the marginal increase (12%) in F_B GSH by GS and TC (Fig 4.12). Diabetic rats showed marked decline in GSH levels in both the fetal tissues (F_B: 64% and F_L: 26%). Dietary treatment in diabetic rats significantly offset the decrease in GSH levels. While GS and TC supplementation restored the GSH levels in fetal brain by ≅ 80%, IA and WS supplementation restored it by 60%. In fetal liver, GS and TC offered moderate but significant protection (30-40%). On contrary, IA and WS treatments had no appreciable protection in fetal liver. STZ induced a moderate (45%) reduction in TSH levels of fetal tissues which were restored to varying degree by the plant supplements.

4.9.3 Antioxidant enzyme activity

Data on the activities of antioxidant enzymes catalase (CAT) and glutathione transferase (GST) are presented in Fig 4.13. In non-diabetic rats, GS/IA/TC/WS supplements did not appreciably alter the GST and CAT activity in and fetal tissues, since the activities were on par with those of controls. In diabetic rats, CAT activity decreased by 30-40%, whereas GST activity decreased by 25-35% in fetal tissues. The percent protection offered by GS and TC against STZ induced reduction in CAT activity varied from 50-75% in fetal tissues. However, IA and WS supplements restored the decreased CAT activity by 30-60%. Similar pattern of protection by dietary supplements was evident in restoring the reduced GST activity of fetal tissues.

Fig 4.1 Serum glucose levels in rats administered STZ on GD_4



Values are mean ± SD (n=7 to 14)
Data analysed by t-test; [a]*P<0.001*

Table 4.1 Serum glucose levels, urine output and embryolethality in pregnant rats rendered diabetic by an acute dose of STZ on GD_4

	Serum Glucose	Urine Output **	Implantations			
	(mg %)	ml/rat/d	TI	LI	DI	% DI *
CTR	113 ± 9.64	10.50 ± 02.05	180	173	7	3.89
STZ	514.7 ± 20.6[a]	98.0 ± 26.91[a]	158	102	53	33.54

Values are mean ± SD (n=15)
TI: Total implants; LI: Live implants; DI: Dead implants
*Dead implants expressed as a percentage of total implantations
** Urine output was measured on last 5 days
Data analysed by t-test; [a]*P<0.001*

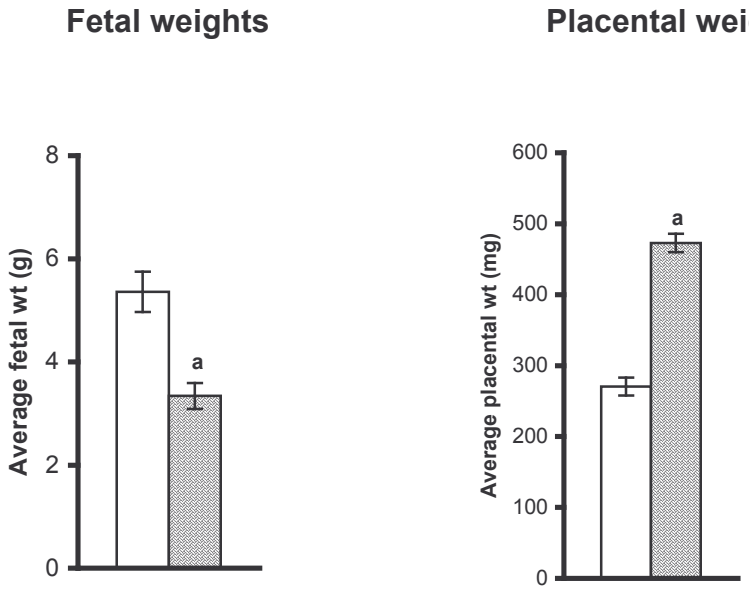
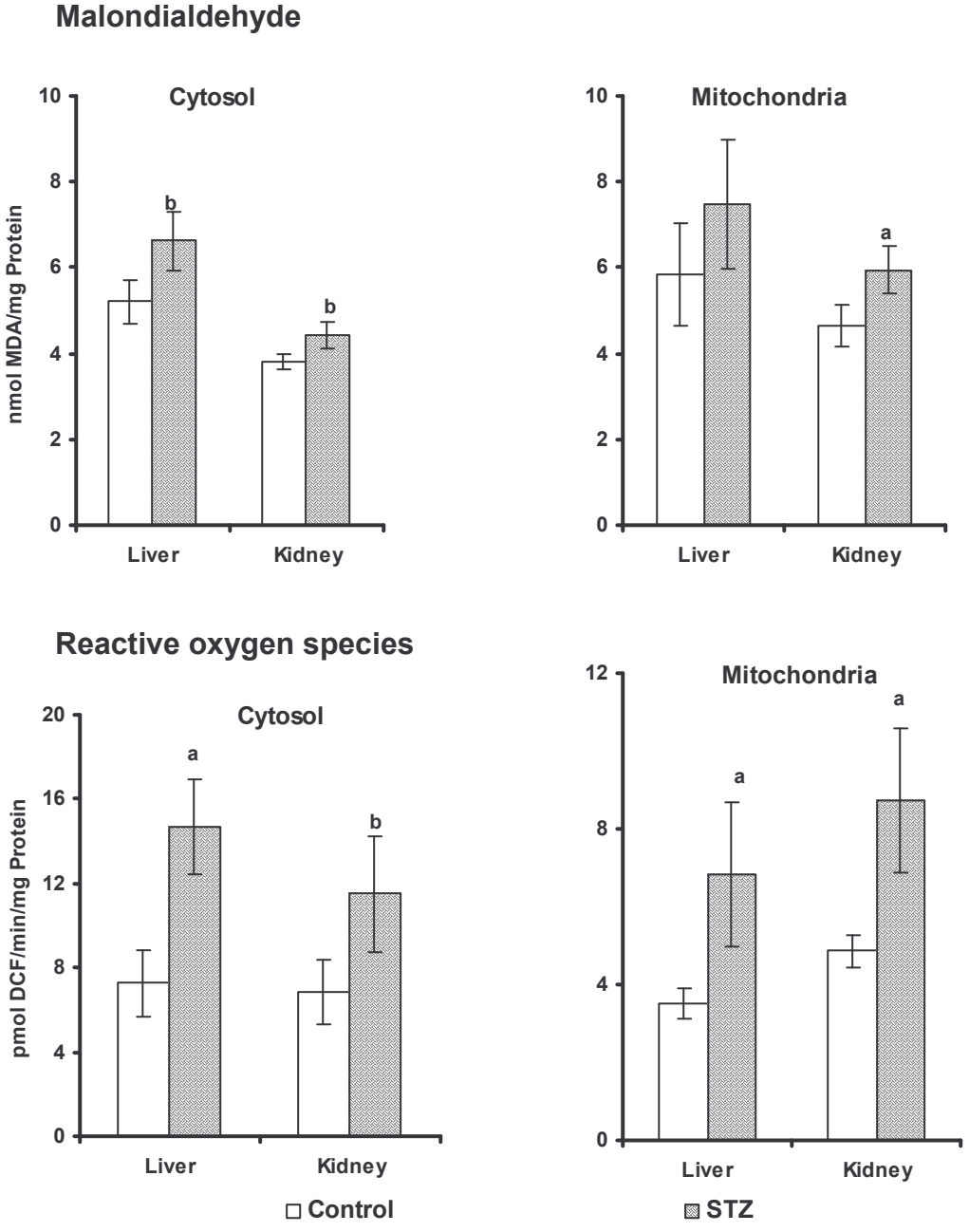


Fig 4.2 Mean fetal and placental weights of pregnant rats rendered diabetic by an acute dose of STZ on GD_4 (measured on GD_{20})

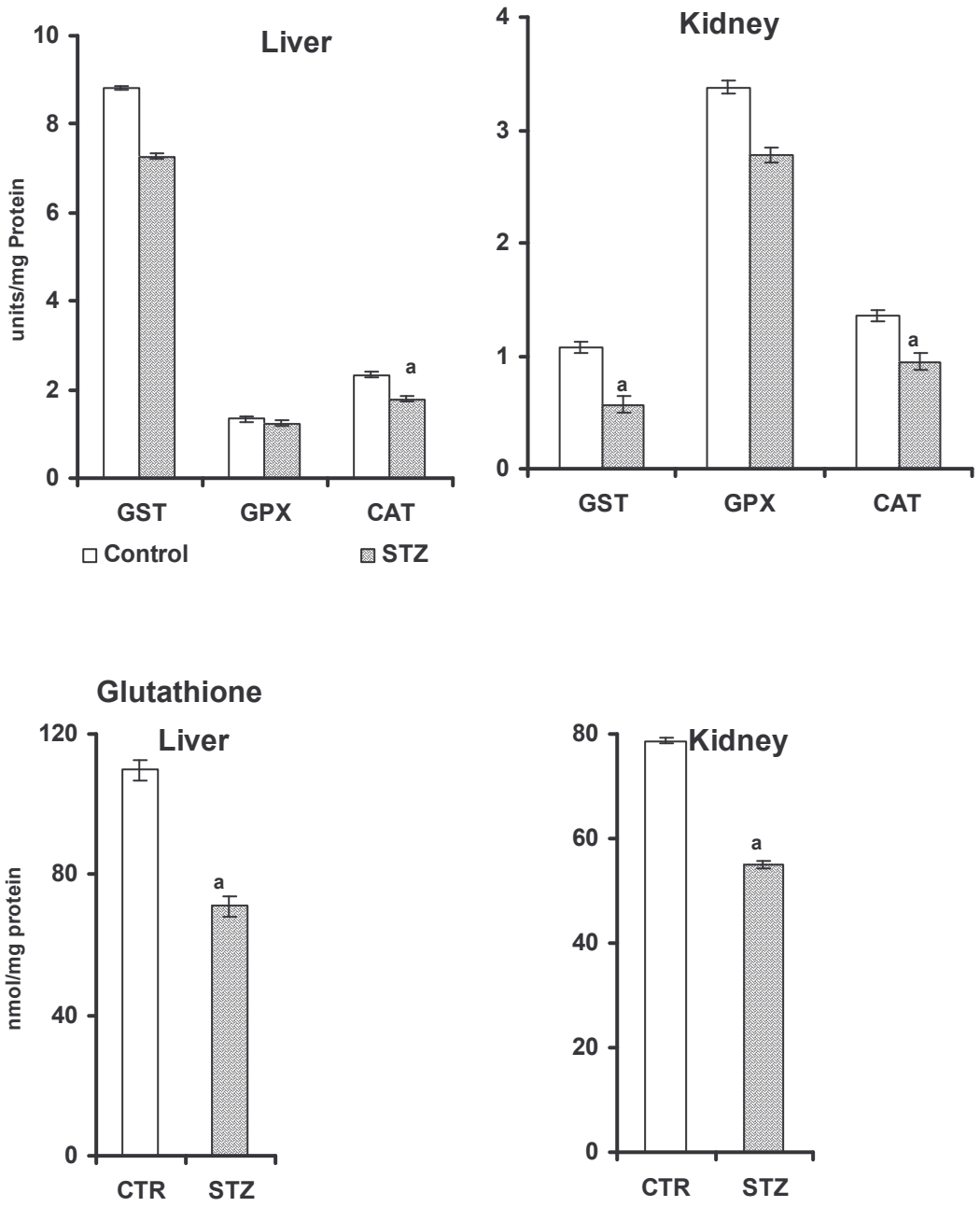
Values are mean ± SD (n=15)
Data analysed by t-test; [a]*P<0.001*

Fig 4.3 Malondialdehyde and ROS levels in maternal tissues of STZ-diabetic rat measured on GD_{13}



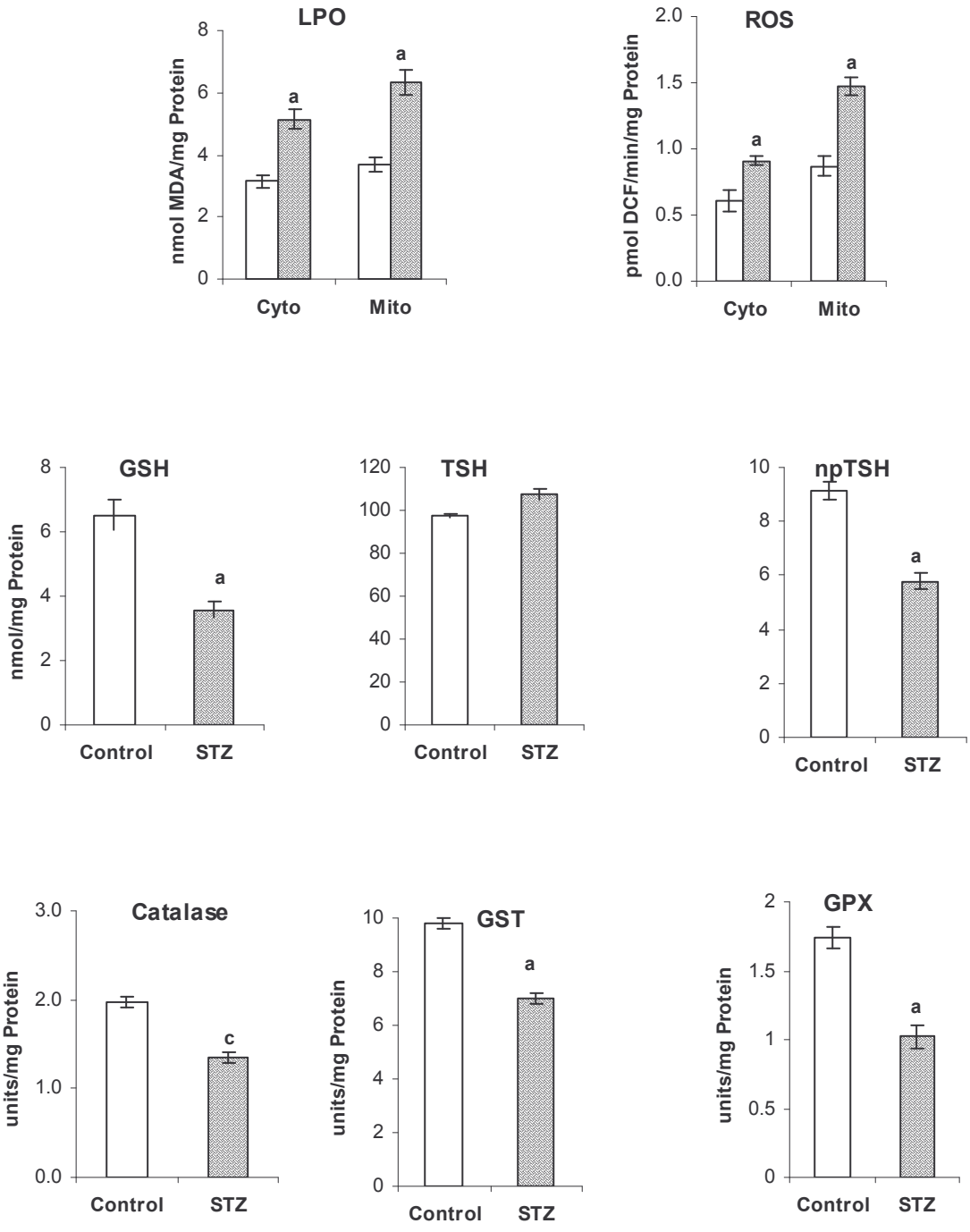
Values are mean ± SD (n=6)
Data analysed by t-test; [a]*P<0.001*, [b]*P<0.01*

Fig 4.4 Activities of antioxidant enzymes in maternal tissues of STZ-diabetic rat measured on GD_{13}



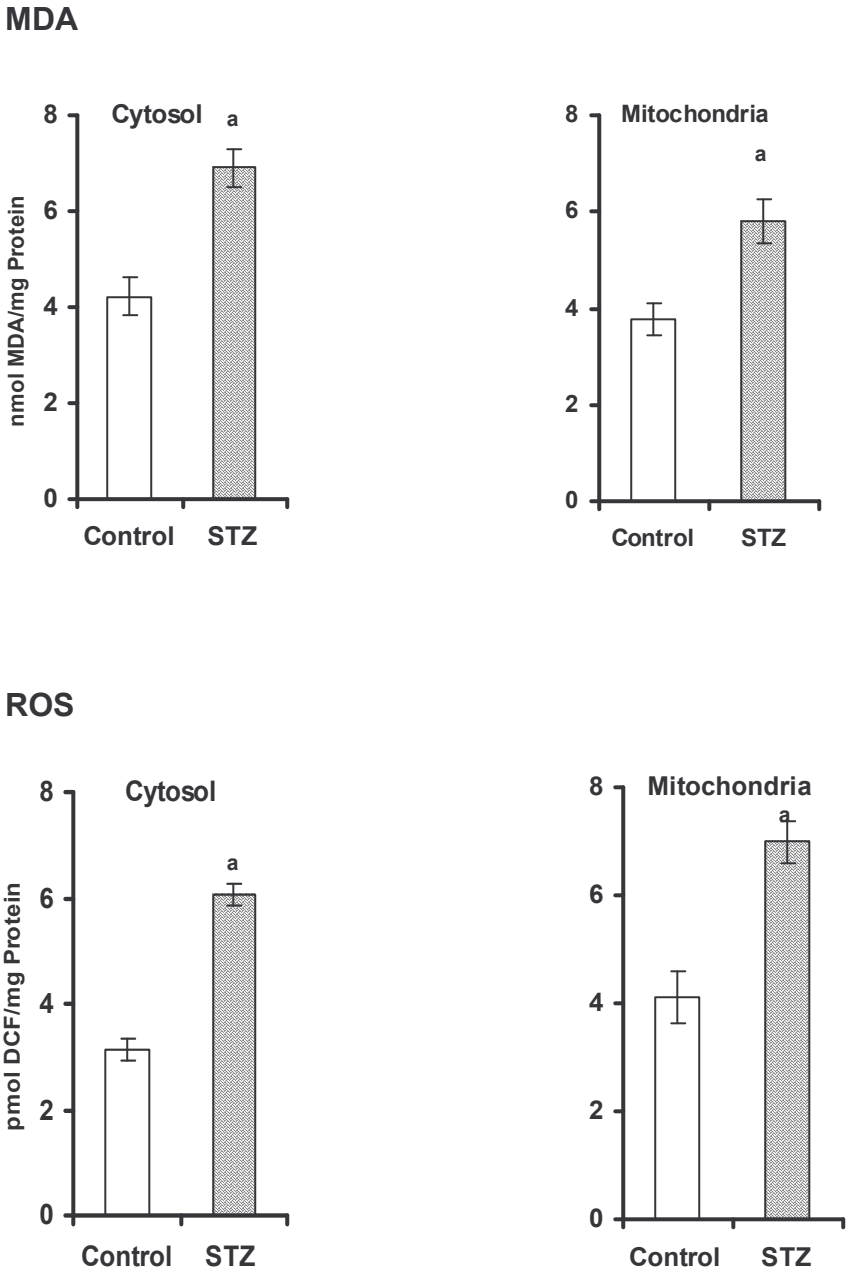
Values are mean ± SD (n=6)
Data analysed by t-test; $^{a}P<0.001$

Fig 4.5 Placental oxidative stress in STZ-diabetic rat measured on GD_{13}



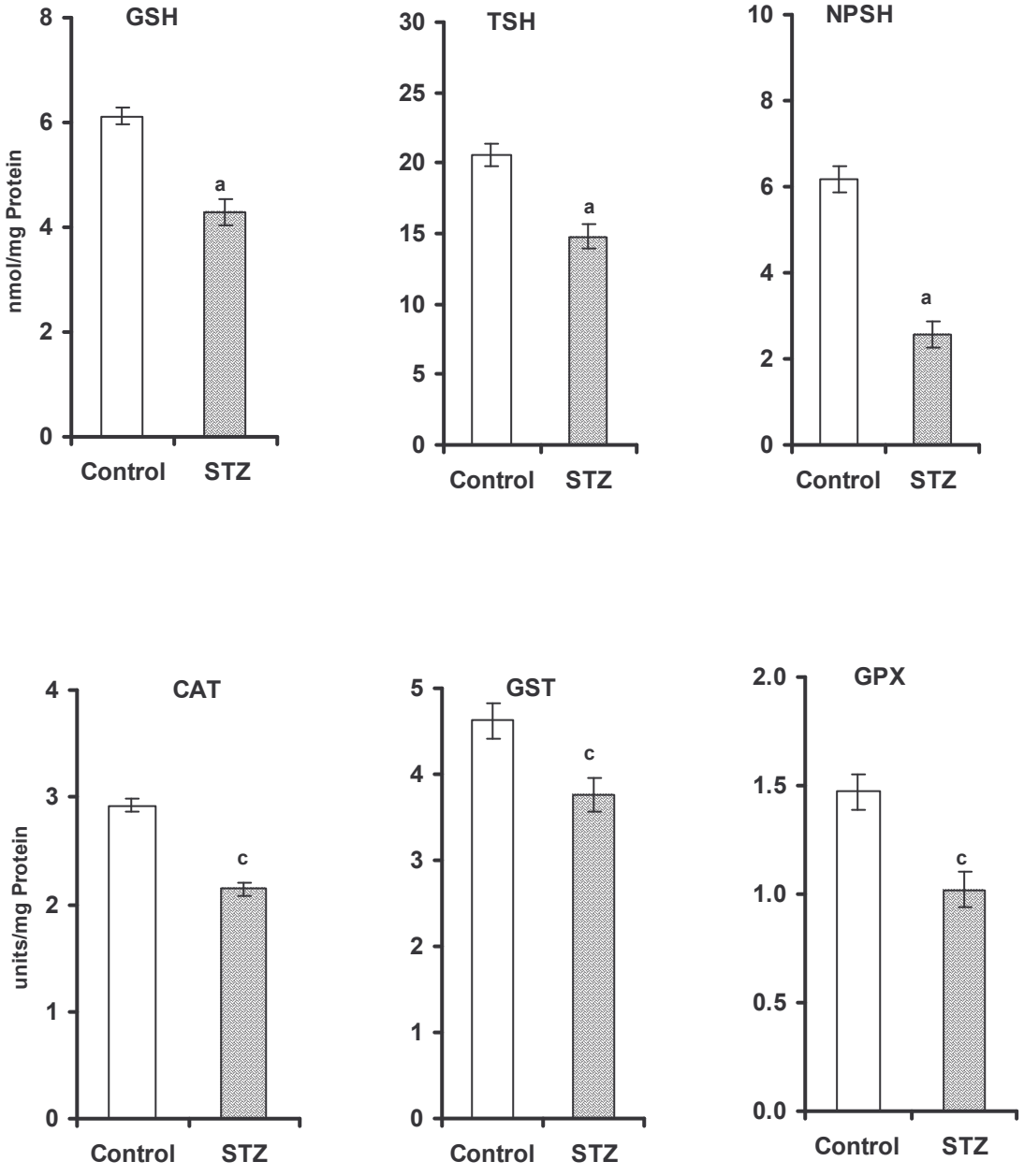
Values are mean ± SD (n=6)
Data analysed by t-test; $^{a}P<0.001$; $^{c}P<0.05$

Fig 4.6 Malondialdehyde (MDA) and ROS levels in cytosolic and mitochondrial fractions of GD_{13} embryos of STZ-diabetic rats



Values are mean ± SD (n=6)
Data analysed by t-test; $^{a}P<0.001$

Fig 4.7 Levels of antioxidant molecules and activities of antioxidant enzymes in GD_{13} embryos of STZ-diabetic rats



Values are mean ± SD (n=6)
Data analysed by t-test; $^{a}P<0.001$; $^{c}P<0.05$

Table 4.2 Biochemical markers of oxidative stress in maternal tissues of STZ-diabetic rat measured on GD_{20}

Tissue	Parameter	Control	STZ
Serum	MDA[2]	48.77 ± 0.37	74.96 ± 1.40[a]
Liver	ROS[1] (cyto)	8.81 ± 0.76	12.69 ± 0.86[a]
	ROS (mito)	4.31 ± 0.60	6.35 ± 0.95[a]
	MDA[2] (cyto)	6.53 ± 0.72	9.23 ± 1.33[a]
	MDA (mito)	5.77 ± 0.63	7.96 ± 0.80[a]
	PC[2]	4.09 ± 0.22	5.08 ± 0.23[b]
	GSH[2]	31.02 ± 5.98	18.45 ± 6.69[b]
	TSH[2], Total	101.54 ± 8.74	86.12 ± 5.23[b]
Kidney	ROS (cyto)	6.02 ± 0.68	8.14 ± 1.25[b]
	ROS (mito)	3.11 ± 0.04	5.63 ± 0.68[a]
	MDA (cyto)	7.875 ± 0.28	11.07 ± 0.65[a]
	MDA (mito)	4.63 ± 0.37	6.34 ± 0.44[a]
	PC	6.12 ± 0.03	7.27 ± 1.45
	GSH	8.38 ± 1.88	6.32 ± 1.24[c]
	TSH	71.68 ± 6.41	46.16 ± 3.22[a]

Values are mean ± SD (n=6)
[1]pmol DCF/min/mg protein; [2]nmol/mg protein
Data analysed by t-test; [a]*P<0.001,* [b]*P<0.01,* [c]*P<0.05*

Table 4.3 Biochemical markers of oxidative stress in fetal brain of STZ-diabetic rat measured on GD_{20}

	CTR	STZ
ROS[1] (Cyto)	3.10 ± 0.29	5.18 ± 0.56[a]
ROS (Mito)	1.72 ± 0.17	3.18 ± 0.62[a]
MDA [2](Cyto)	6.95 ± 0. 81	9.76 ± 1.12[a]
MDA (Mito)	5.16 ± 0.48	6.63 ± 0.65[a]
GSH[2]	19.84 ± 3.93	13.19 ± 2.56[b]
TSH[2]	54.50 ± 9.62	36.60 ± 6.39[b]
NPSH[2]	6.59 ± 0.87	5.02 ± 1.18[c]
CAT[3]	1.68 ± 0.19	1.09 ± 0.03[a]
GST[4]	2.20 ± 0.06	1.74 ± 0.02[a]
GPX[5]	2.10 ± 0.15	1.79 ± 0.03[b]

Values are mean ± SD (n=6)
[1]pmol DCF/min/mg protein; [2]nmol/mg protein
[3]µmol H_2O_2 oxidized/min/mg protein
[4]nmol complex/min/mg protein;
[5]nmol NADPH oxidized/min/mg protein
Data analysed by t-test; [a]*P<0.001,* [b]*P<0.01,* [c]*P<0.05*

Table 4.4 Biochemical markers of oxidative stress in fetal liver of STZ diabetic rat measured on GD_{20}

	CTRL	STZ
ROS[1] (Cyto)	3.62 ± 0.45	5.55 ± 0.65[a]
ROS (Mito)	1.59 ± 0.34	2.65 ± 0.33[a]
MDA [2](Cyto)	8.47 ± 0.69	10.30 ± 0.84[a]
MDA (Mito)	5.53 ± 0.35	7.73 ± 0.61[a]
GSH[2]	27.84 ± 6.19	16.43 ± 3.71[b]
TSH[2]	84.56 ± 10.73	56.48 ± 3.93[a]
NPSH[2]	9.84 ± 0.84	6.12 ± 1.25[a]
CAT[3]	217. 13 ± 16.87	192.47 ± 20.47[c]
GST[4]	90.51 ± 7.61	73.54 ± 9.15[b]
GPX[5]	99.66 ± 10.85	85.63 ± 9.95[c]

Values are mean ± SD
[1]pmol/min/mg protein
[2]nmol/mg protein,
[3]µmol H_2O_2 oxidized/min/mg protein
[4]nmol complex/min/mg protein
[5]nmol NADPH oxidized/min/mg protein
Data analysed by t-test: [a]*P<0.001,* [b]*P<0.01,* [c]*P<0.05*

Table 4.5 Effect of garlic supplements on plasma glucose, body weight, urine output and fetal weight of pregnant rats rendered diabetic by an acute dose of STZ

	Plasma Glucose (mg %)	Body wt gain (g/rat)	Urine output (ml/rat/d)	Fetal Weight (g/fetus)
CTR	113.0 ± 11.0	86.0 ± 5.65	10.50 ± 02.69	5.36 ± 0.45
Garlic (2%)	123.5 ± 05.5	94.5 ± 5.45[a]	13.25 ± 04.08	4.96 ± 0.25[a]
STZ	514.7 ± 25.0[a]	49.0 ± 6.50[a]	98.00 ± 29.48[a]	3.34 ± 0.75[a]
STZ + Garlic (2%)	419.6 ± 11.4[a]	66.0 ± 4.00[a]	73.01 ± 25.00[a]	4.24 ± 0.50[a]

Values are mean ± SD (n=10)
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 4.6 Effect of garlic supplements on total implantation (TI), live Implants (LI), dead implants (DI) % dead implants (%DI) of STZ-diabetic rat

	IMPLANTATIONS			
	Total	Live	Dead	% DI
CTR	96	92	4	4.16
Garlic (2%)	93	88	5	5.37
STZ	80	48	32	40.0
STZ + Garlic (2%)	90	89	11	12.2

Values are mean ± SD (n=10)

Table 4.7 Abrogation of oxidative stress markers in maternal tissues of STZ- diabetic rat by fresh garlic powder during gestation

		CTR	Garlic	STZ	STZ + Garlic
Liver	MDA[1]	5.31 ± 0.12	4.18 ± 0.72[a]	7.72 ± 0.59[a]	5.58 ± 0.70
	ROS[2]	4.47 ± 0.29	3.28 ± 0.23[a]	5.76 ± 0.91[a]	7.56 ± 0.32[a]
	GSH[1]	20.38 ± 1.07	25.22 ± 1.17[a]	12.14 ± 0.70[a]	18.11 ± 1.41[a]
	TSH[1]	110.85 ± 7.23	107.94 ± 7.10	74.45 ± 2.96[a]	100.55 ± 5.55[a]
	PC[1]	3.55 ± 0.24	3.68 ± 0.31	5.07 ± 0.16[a]	4.45 ± 0.32[a]
Kidney	MDA	4.56 ± 1.04	4.58 ± 0.62	6.55 ± 0.48[a]	5.27 ± 0.83[a]
	ROS	3.28 ± 0.32	3.17 ± 0.78	5.11 ± 0.34[a]	3.96 ± 0.32[a]
	GSH	3.18 ± 0.06	3.82 ± 0.14[a]	1.28 ± 0.02[a]	2.75 ± 0.13[a]
	TSH	73.86 ± 6.57	80.30 ± 12.22	39.82 ± 8.6[a]	64.66 ± 5.37[a]
	PC	2.91 ± 0.29	2.86 ± 0.70	4.81 ± 0.91[a]	3.43 ± 0.39

Values are mean ± SD (n=6)
Data Pooled from 2 independent experiments
[1]nmol/mg protein; [2]pmol/min/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001*

Table 4.8 Modulatory effect of garlic powder on the status of placental oxidative stress markers in STZ-diabetic rat

	CTR	Garlic	STZ	STZ + Garlic
Weight	270 ± 12.49	290 ± 11.31	473 ± 13.0[a]	386 ± 27.40[a]
MDA[1]	1.04 ± 0.18	1.25 ± 0.21	1.71 ± 0.31[c]	1.4 ± 0.55
ROS[2]	0.61 ± 0.08	0.59 ± 0.07	0.91 ± 0.06[a]	0.72 ± 0.08[a]
GSH[1]	6.52 ± 0.48	6.65 ± 0.09	3.57 ± 0.24[a]	5.24 ± 0.31[a]
TSH[1]	9.75 ± 1.84	10.75 ± 2.66	6.78 ± 2.6	8.56 ± 2.54
PC[1]	2.48 ± 0.19	2.69 ± 0.16	3.85 ± 0.19[a]	2.95 ± 0.28[a]
CAT[3]	1.98 ± 0.2	1.86 ± 0.11	1.34 ± 0.12[a]	1.58 ± 0.20[a]
GST[4]	9.79 ± 0.68	8.64 ± 0.44[a]	6.99 ± 0.41[a]	7.93 ± 0.60[a]

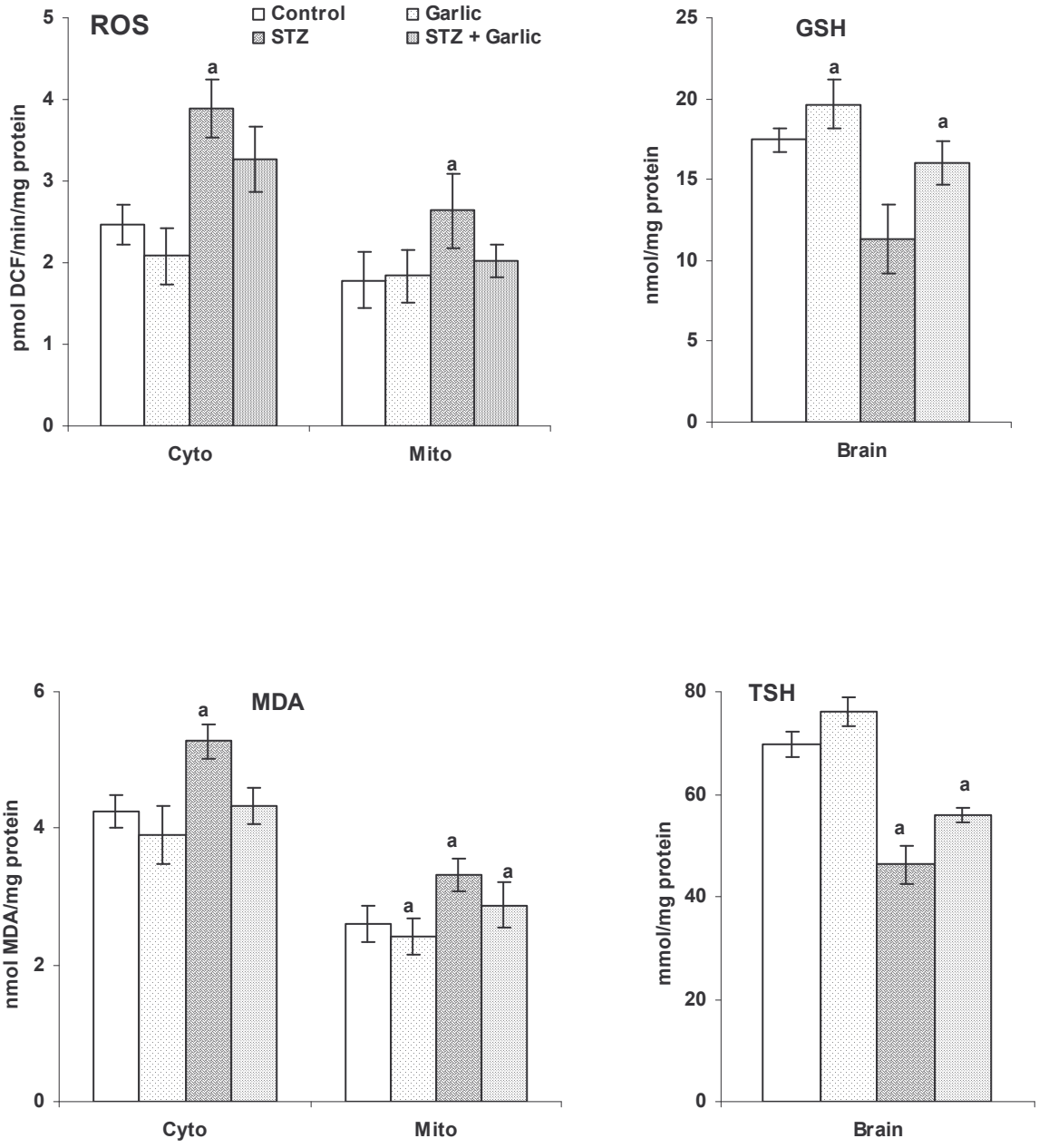
Values are mean ± SD (n=12)
[1]nmol/mg protein; [2]pmol/min/mg protein
[3]µmol H_2O_2 oxidized/min/mg protein; [4]nmol complex/min/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001,* [c]*P<0.05*

Table 4.9 Effect of garlic supplements on the activities of antioxidant enzymes in maternal and fetal tissues of STZ diabetic rat

		CTR	Garlic	STZ	STZ + Garlic
Maternal Liver	CAT[1]	380.5 ± 8.60	385.0 ± 16.8	264.20 ± 38.0[a]	305.8 ± 18.2[a]
	GST[2]	100.31 ± 8.39	116.30 ± 12.76[a]	72.41 ± 2.62[a]	104.71 ± 3.76
Kidney	CAT[1]	119.8 ± 8.6	112.0 ± 6.3	67.5 ± 3.2[a]	89.0 ± 6.7[a]
	GST[2]	16.41 ± 1.0	21.16 ± 2.38[a]	8.41 ± 0.95[a]	15.41 ± 1.34
Fetal Brain	CAT[1]	3.42 ± 0.15	3.38 ± 0.12	2.06 ± 0.11[a]	2.93 ± 0.11[a]
	GST[2]	19.98 ± 1.96	22.02 ± 3.60	13.69 ± 1.41[a]	21.33 ± 2.89
Liver	CAT[1]	244.1 ± 27.4	231.53 ± 36.2	175.88 ± 34.6[b]	215.68 ± 35.9
	GST[2]	71.23 ± 3.2	72.58 ± 4.09	53.31 ± 8.54[a]	60.44 ± 6.17[a]

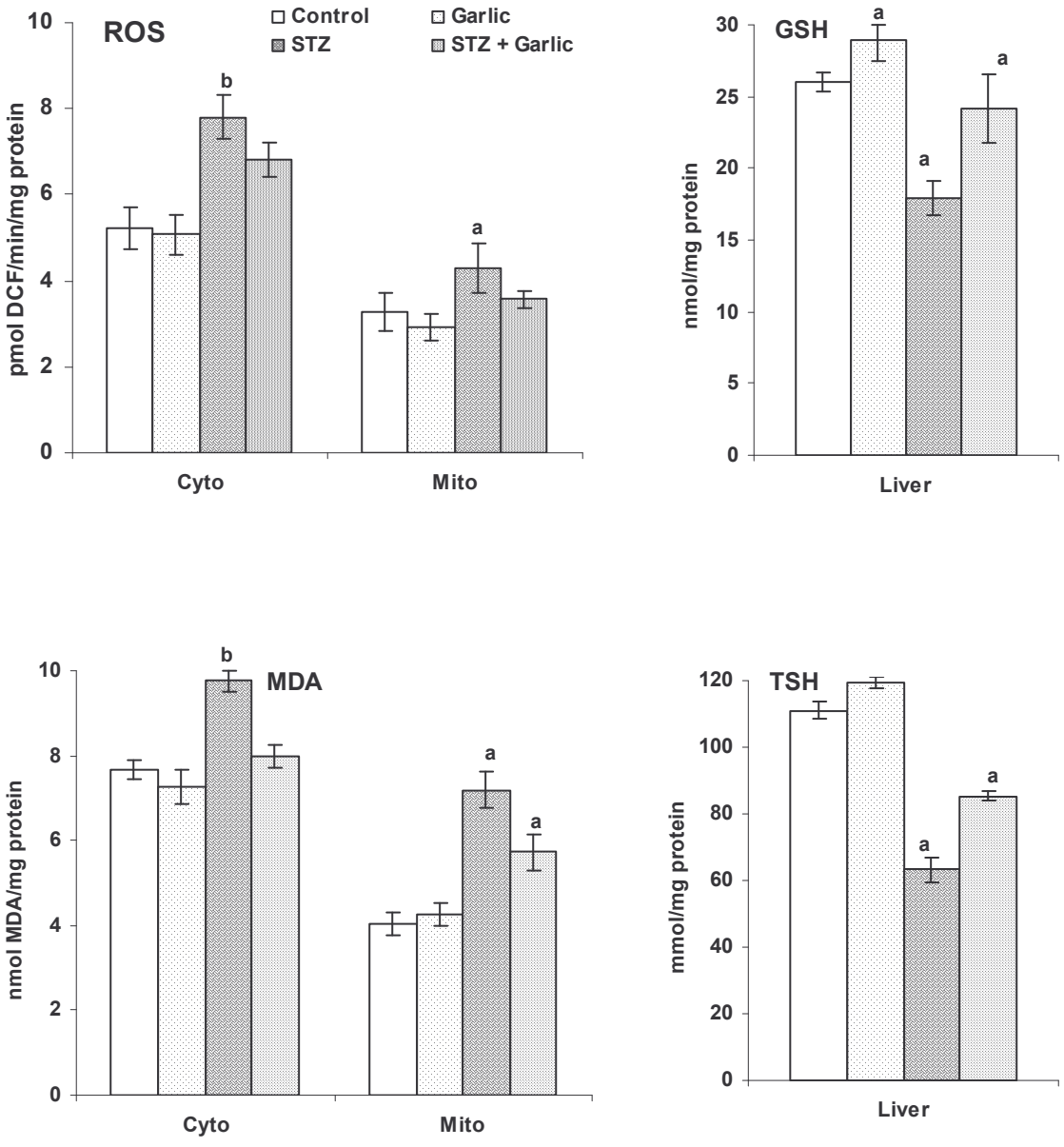
Values are mean ± SD; n=6 (dams) and n=12 fetuses
[1]μmol H_2O_2 oxidized/min/mg protein
[2]nmol complex/min/mg protein
Data analysed by Holm-Sidak method; [a]*P<0.001,* [b]*P<0.01*

Fig 4.8 Ameliorative effect of dietary garlic on oxidative stress markers in fetal brain of STZ diabetic rat



Values are mean ± SD; n=6 (dams) and n=12 (fetuses)
Data analysed by Holm-Sidak method; [a]P<0.001

Fig 4.9 Ameliorative effect of dietary garlic on oxidative stress markers in fetal liver of STZ diabetic rat



Values are mean ± SD; n=6 (dams) and n=12 (fetuses)
Data analysed by Holm-Sidak method; $^{a}P<0.001$, $^{b}P<0.01$

Table 4.10 Effect of medicinal plant supplements on body weight gain, serum glucose levels, urine output among STZ diabetic rat

Group	Serum Glucose (mg%)	Urine output (ml/rat/d)	Body wt gain (g/rat)
CTR	113.0 ± 11.0	10.50 ± 02.69	86.0 ± 5.65
GS	106.0 ± 03.0	12.50 ± 01.46	92.5 ± 3.25
IA	115.0 ± 05.0	14.00 ± 02.26	91.0 ± 3.50
TC	121.0 ± 08.0	12.00 ± 03.22	88.5 ± 2.50
WS	103.0 ± 03.0	11.90 ± 02.34	90.5 ± 2.50
STZ	514.7 ± 25.0[a]	98.00 ± 29.48[a]	49.0 ± 6.50[a]
STZ + GS	353.2 ± 11.7[ab]	65.42 ± 29.60[ab]	73.0 ± 6.00[ab]
STZ + IA	468.6 ± 12.4[ab]	80.50 ± 08.90[a]	58.5 ± 5.50[ab]
STZ + TC	377.4 ± 13.5[ab]	67.50 ± 24.00[ab]	65.0 ± 4.00[ab]
STZ + WS	432.6 ± 08.2[ab]	79.40 ± 18.48[a]	63.5 ± 2.50[ab]

Values are mean ± SD (n=6)
Urine output was measured on $GD_{17\text{-}19}$
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with non-diabetic control)*
[b]*P<0.001(compared with STZ)*

Table 4.11 Modulatory effect of medicinal plants on fetal/placental weights and incidence of embryolethality among STZ diabetic rat

Group	Fetal wt (g)	Placental wt (g)	Implantations			
			Total	Live	Dead	% DI
CTR	5.36 ± 0.93	0.308 ± 0.037	96	92	4	4.16
GS	5.39 ± 0.57	0.283 ± 0.038	94	89	5	5.32
IA	5.13 ± 0.72	0.315 ± 0.041	92	88	4	4.35
TC	5.54 ± 0.74	0.311 ± 0.022	93	90	3	3.22
WS	5.28 ± 0.78	0.284 ± 0.044	95	89	6	6.31
STZ	3.36 ± 0.55[a]	0.548 ± 0.028[a]	80	48	32	40.0
STZ + GS	4.72 ± 0.57[b]	0.362 ± 0.053[b]	90	78	12	13.33
STZ + IA	4.81 ± 0.53[b]	0.355 ± 0.041[b]	85	67	18	21.18
STZ + TC	5.02 ± 0.60[b]	0.362 ± 0.035[b]	88	75	13	14.77
STZ + WS	4.96 ± 0.65[b]	0.352 ± 0.052[b]	86	64	22	25.58

Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera ; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with non-diabetic control)*
[b]*P<0.001(compared with STZ)*

Table 4.12 Attenuation of oxidative stress markers in maternal tissues of STZ-diabetic rat by medicinal plant supplements

Groups	Reactive oxygen species (pmol DCF/min/mg protein)		Malondialdehyde (nmol/mg protein)	
	Liver	Kidney	Liver	Kidney
CTR	5.96 ± 1.33	4.38 ± 1.20	8.35 ± 1.21	6.08 ± 0.94
GS	5.06 ± 1.63	4.54 ± 0.23	6.50 ± 1.17	5.83 ± 1.59
IA	5.50 ± 1.99	4.47 ± 0.72	8.29 ± 1.23	5.59 ± 1.65
TC	5.17 ± 1.15	4.37 ± 0.47	6.25 ± 1.29	5.98 ± 1.43
WS	5.19 ± 1.22	4.74 ± 0.63	7.14 ± 1.53	6.30 ± 1.52
STZ	10.25 ± 1.07[a]	6.81 ± 1.17[b]	12.09 ± 2.70[a]	8.07 ± 1.41
STZ + GS	7.09 ± 1.14[b]	5.64 ± 0.82	8.77 ± 1.49	6.62 ± 1.02
STZ + IA	9.07 ± 1.89[a]	5.09 ± 1.41	9.98 ± 1.17	6.84 ± 2.24
STZ + TC	8.43 ± 1.25	5.15 ± 0.96	9.75 ± 2.50	6.66 ± 1.04
STZ + WS	9.12 ± 2.43[a]	5.70 ± 2.02	10.76 ± 2.25	7.32 ± 0.55

Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with non-diabetic control)*
[b]*P<0.001(compared with STZ)*

Table 4.13 Attenuation of oxidative stress markers in maternal tissues of STZ-diabetic rat by medicinal plant supplements

Groups	Glutathione (nmol/mg protein)		Total thiols (nmol/mg protein)	
	Liver	Kidney	Liver	Kidney
CTR	30.24 ± 1.61	7.86 ± 1.69	91.74 ± 6.59	63.73 ± 12.80
GS	34.02 ± 2.27	9.09 ± 1.48	103.22 ± 15.11	60.42 ± 5.95
IA	30.39 ± 3.65	7.41 ± 1.55	93.92 ± 11.59	64.53 ± 6.59
TC	34.33 ± 3.59	8.51 ± 1.49	89.96 ± 13.36	65.36 ± 7.39
WS	28.33 ± 3.41	7.48 ± 1.74	86.55 ± 11.89	61.87 ± 8.42
STZ	14.16 ± 11.57[a]	3.17 ± 2.05[a]	51.63 ± 12.32[a]	42.65 ± 5.73[a]
STZ + GS	23.38 ± 4.57	6.06 ± 1.43	71.12 ± 15.68	55.41 ± 7.96
STZ + IA	20.5 ± 2.60	5.08 ± 2.51	62.51 ± 20.07[a]	55.01 ± 7.52
STZ + TC	20.56 ± 9.19	5.60 ± 2.66	64.36 ± 18.38[a]	51.90 ± 6.64
STZ + WS	22.47 ± 13.35	4.94 ± 1.61	66.86 ± 18.82	48.38 ± 12.28

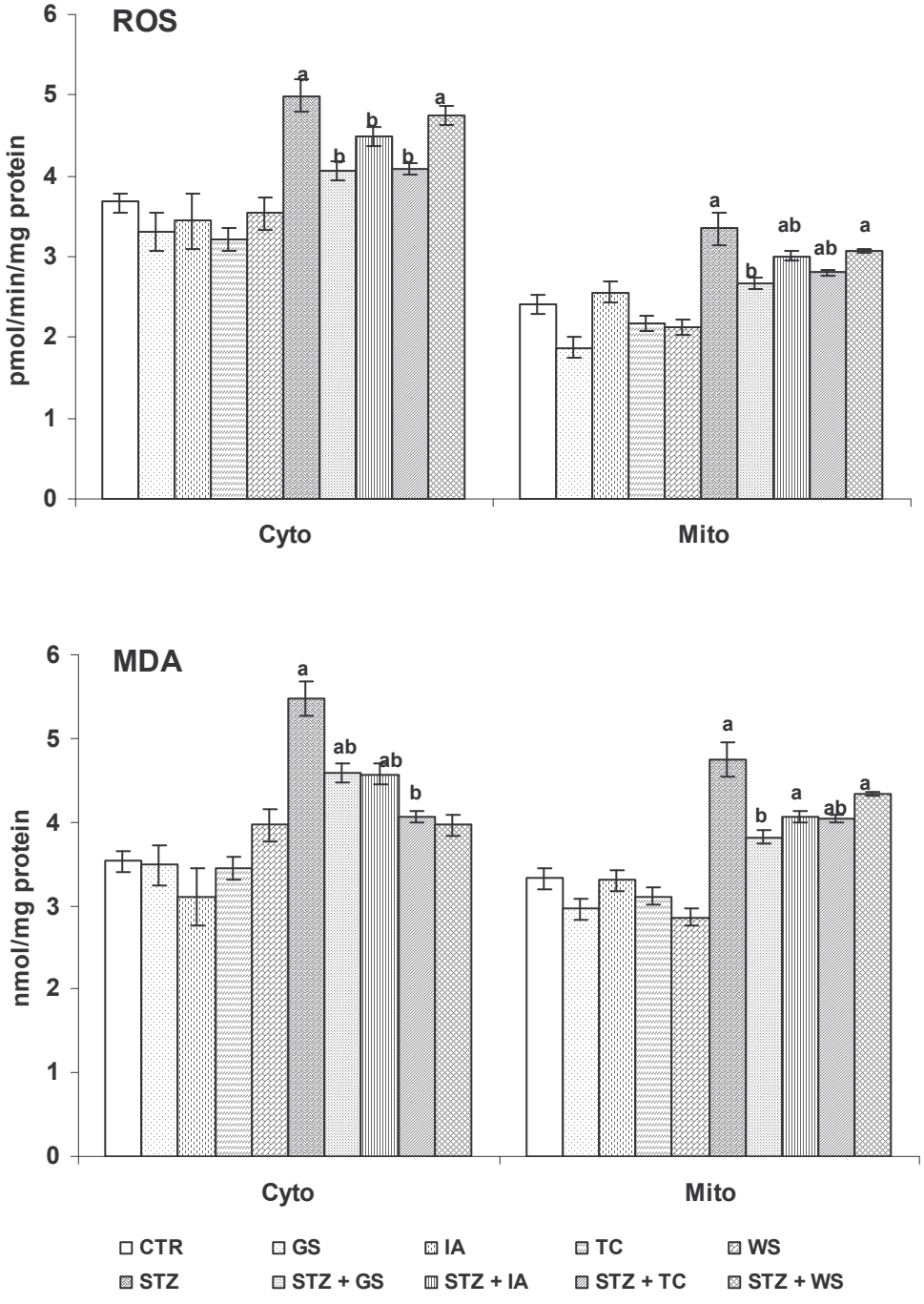
Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with non-diabetic control)*

Table 4.14 Effect of medicinal plants supplements on the activities of antioxidant enzyme in maternal tissues of STZ-diabetic rat

Groups	CAT (units / mg protein)		GST (units / mg protein)	
	Liver	Kidney	Liver	Kidney
CTR	385. 71± 23.49	11.98 ± 1.53	100.31 ± 14.63	16.41 ± 1.10
GS	412.57 ± 15.83	10.92 ± 1.31	98.42 ± 13.67	14.68 ± 1.08
IA	353.35 ± 17.83	10.87 ± 1.47	105.27 ± 12.91	18.02 ± 1.55
TC	395.78 ± 17.46	12.04 ± 1.23	96.34 ± 12.11	13.68 ± 1.16
WS	334.95 ± 32.29a	11.53 ± 0.97	89.67 ± 12.05	15.36 ± 1.35
STZ	264.23 ± 22.29[a]	7.74 ± 1.12[a]	72.41 ± 9.70[a]	8.41 ± 1.52[a]
STZ + GS	347.38 ± 23.90[b]	9.88 ± 1.18	90.60 ± 9.27	14.14 ± 1.95[b]
STZ + IA	303.62 ± 21.91[a]	10.16 ± 1.17	86.24 ± 8.32	14.08 ± 1.71[b]
STZ + TC	348.95 ± 27.51[b]	12.21 ± 1.25[b]	90.96 ± 9.11	12.22 ± 1.46[ab]
STZ + WS	298.62 ± 25.17[a]	10.16 ± 1.54	79.75 ± 8.33	14.13 ± 1.64

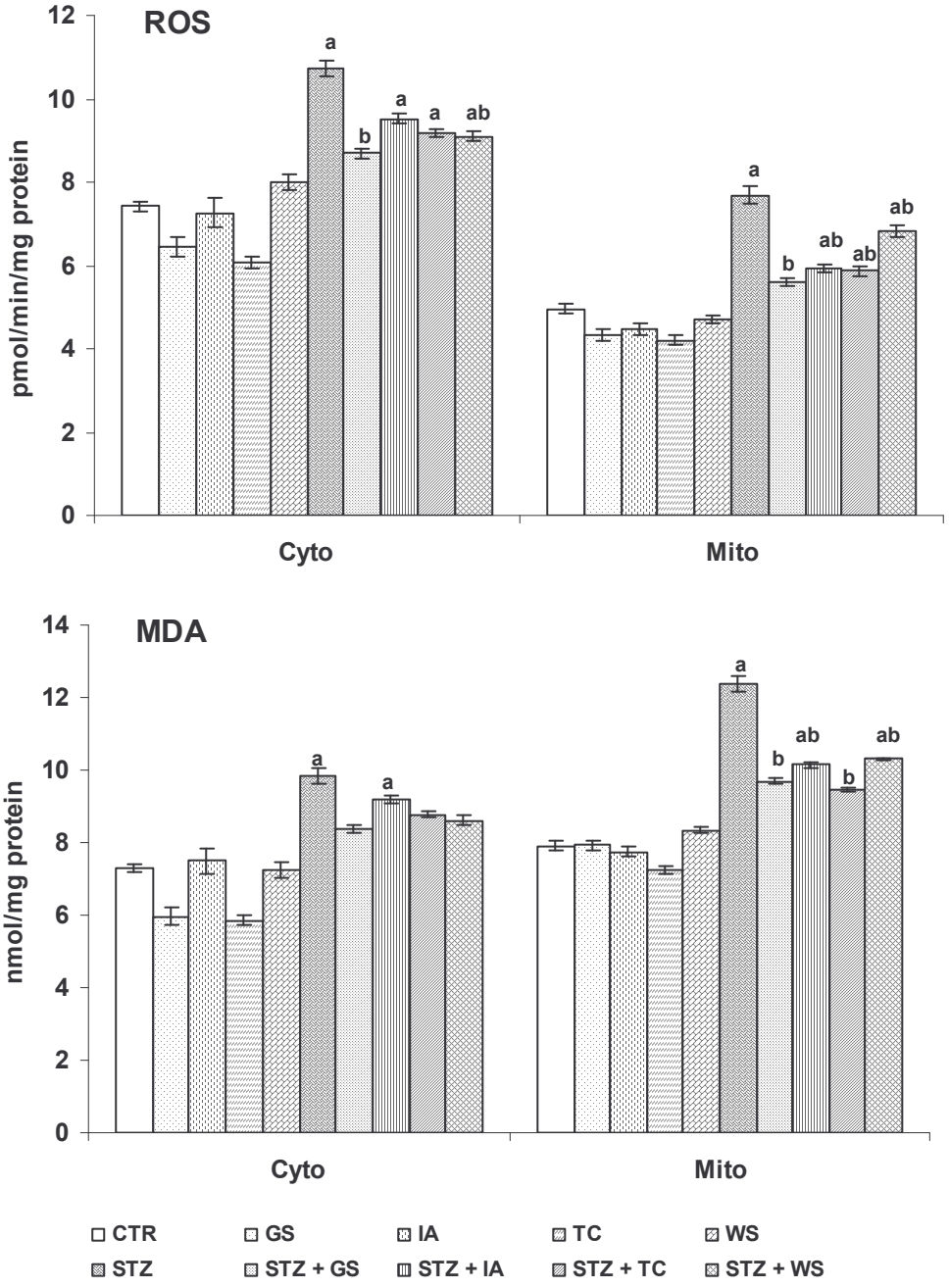
Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with non-diabetic control)*
[b]*P<0.001(compared with STZ)*

Fig 4.10 Effect of medicinal plant supplements on reactive oxygen Species (ROS) generation and malondialdehyde (MDA) levels in fetal brain of STZ-diabetic rats



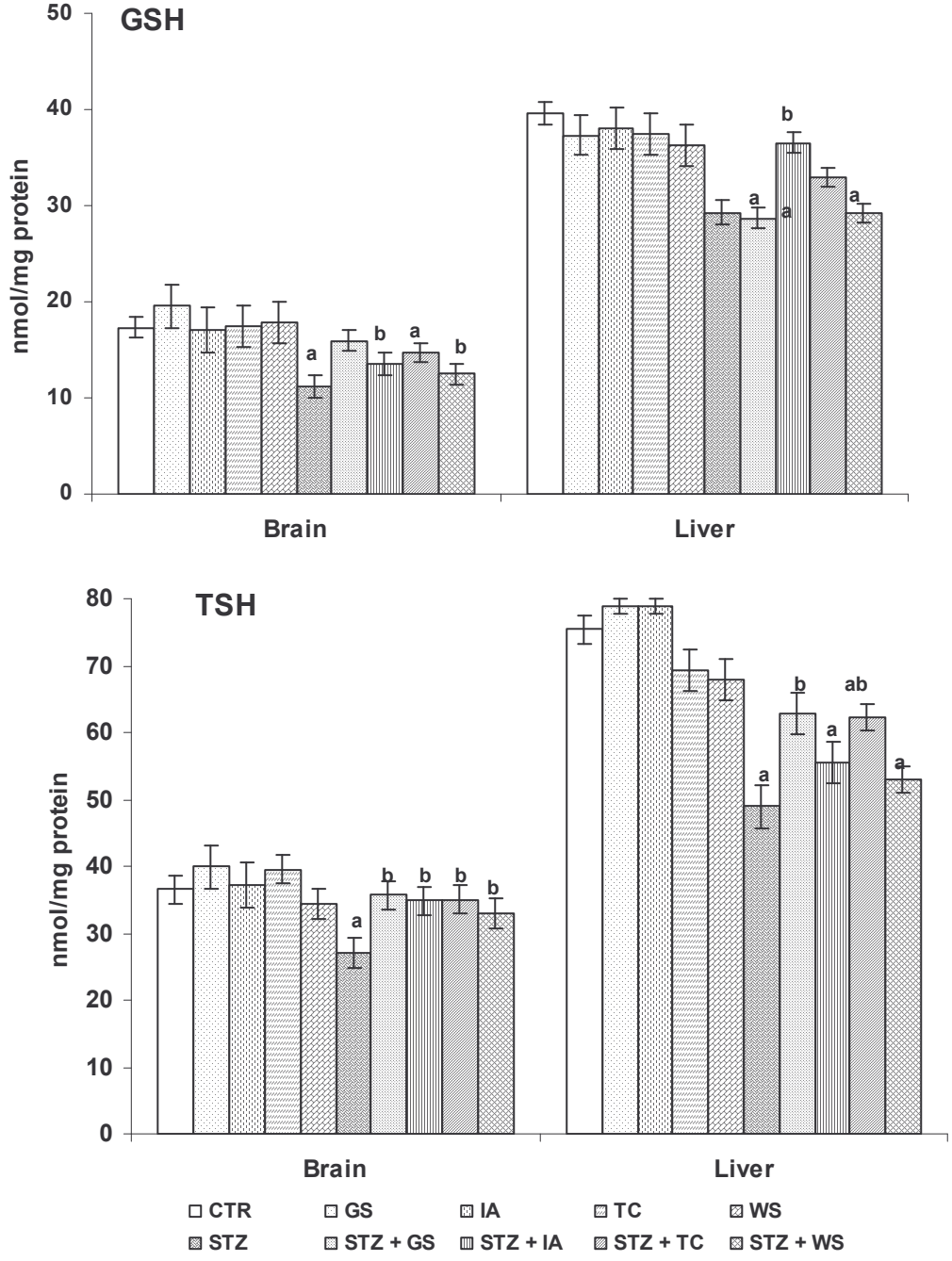
Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with control)*
[b]*P<0.001(compared with STZ)*

Fig 4.11 Effect of medicinal plants supplements on the pattern of reactive oxygen species (ROS) and levels of malondialdehyde (MDA) in fetal liver of STZ diabetic rat



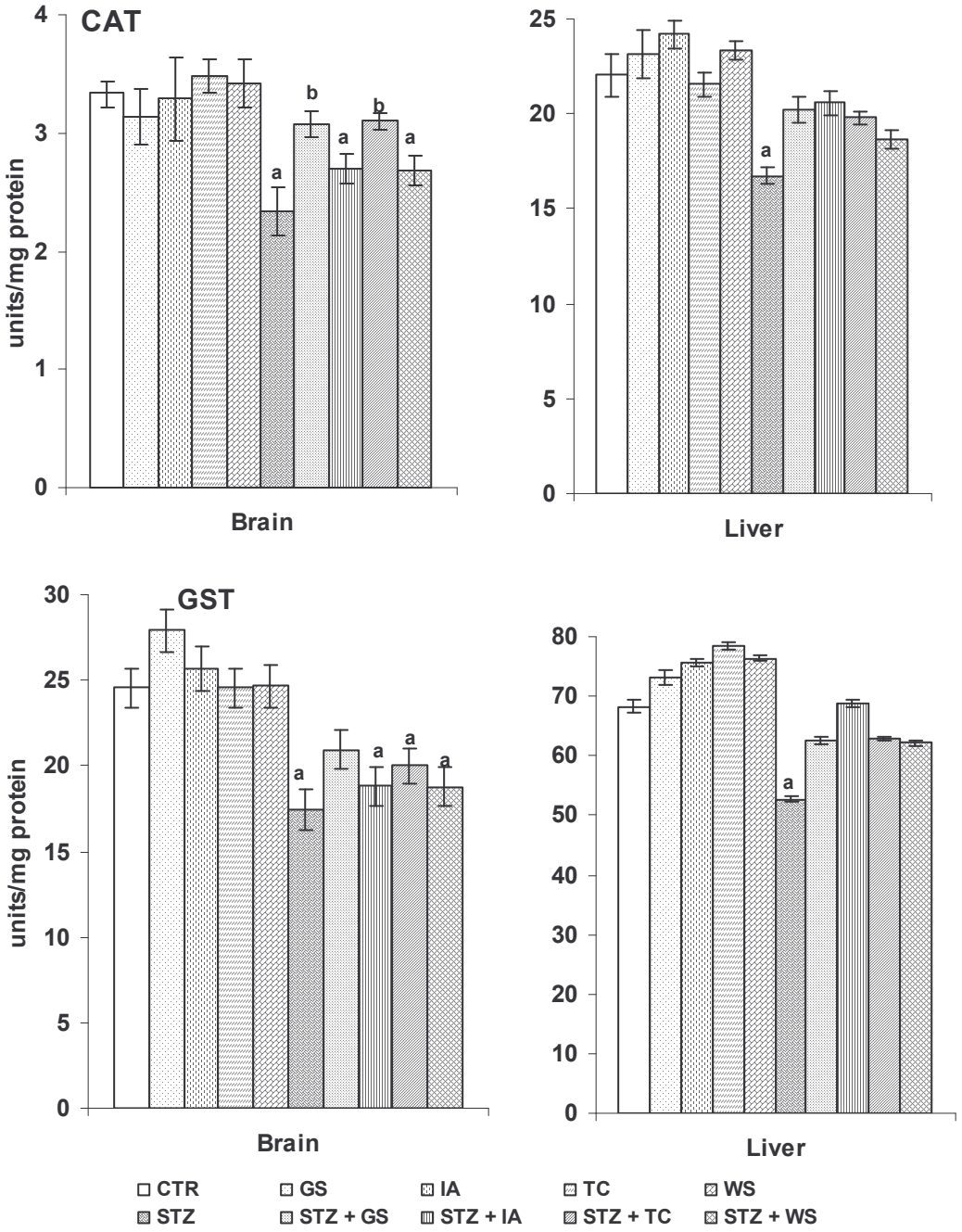
Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with control)*
[b]*P<0.001(compared with STZ)*

Fig 4.12 Effect of medicinal plants supplements on the reduced glutathione (GSH) and total thiol (TSH) levels in fetal brain and liver of STZ-diabetic rats



Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with control)*
[b]*P<0.001(compared with STZ)*

Fig 4.13 Effect of medicinal plants supplements on the activities of catalase (CAT) and glutathione s-transferase (GST) in fetal brain and liver of STZ-diabetic rat



Values are mean ± SD (n=6)
IA: Ipomea aquatica; TC: Tinospora cordfolia
WS: Withania somnifera; GS: Gymnema sylvestre
Data analyzed by Holm-Sidak method; [a]*P<0.001(compared with control)*
[b]P<0.001(compared with STZ)

5.0 DISCUSSION

The purpose of the present study was to determine the perturbations in markers of oxidative stress in maternal milieu of pregnant rats rendered diabetic by an acute STZ dose on gestation day 4 and more importantly its implications on embryonic/fetal growth and oxidative consequences in placenta, embryos and fetal organs. Once these were established, the possibilities of ameliorating diabetes induced oxidative impairments during pregnancy by oral supplements of garlic powder and selected medicinal plants were investigated.

Oxidative perturbations in pregnant diabetic rats

In general**,** in animal models, two strategies have been employed to investigate the implications of chemical diabetogens during pregnancy. In the first, diabetes is induced by diabetogens (such as alloxan, STZ) in normal female rats, and the animals mated. In the second strategy, diabetes is induced after mating *(Kalter, 1996)*. In the present study, we chose to administer STZ on GD_4 and the basis for selection of GD_4 consisted of the following a) administration of STZ prior to mating may result in failure of pregnancy b) administration during preimplantation days is likely to cause preimplantation deaths of embryos. In order to circumvent these problems, we chose to administer STZ on GD_4. Further, our observations in preliminary experiments suggested GD_4 to be ideal, since the degree of preimplantation losses were minimal in the diabetic group and were comparable to those of non-diabetic pregnant rats. This regimen has been adopted by earlier workers *(Palomar-Morales et al., 1998)* and it has been suggested that this regimen may cause a state similar to that of gestational diabetes mellitus in humans.

In the present study, administration of STZ to pregnant rats on GD_4 induced typical symptoms of diabetes such as hyperglycemia, lesser body weight gain, hyperphagia and polydipsia after one week. These observations are consistent with earlier findings in STZ diabetic pregnant rats *(Viana et al., 2000)*. Further, there was a marked increase in blood glucose in STZ-induced diabetic pregnant rats. Significant increases in the incidence of post-

implantation embryonic deaths were observed among diabetic dams as reported by earlier workers *(Eriksson et al., 2000; Wentzel et al., 1999; Cederberg et al., 2003; Cederberg et al., 2001)*. In the present study, maternal diabetes in the rat increased both resorption rates. Similar large-sized resorptions have been reported in diabetic litters by earlier workers *(Eriksson et al., 2000)*. Both malformations and resorptions are speculated to result from the same developmental abnormality, i.e., a fetus with a severe malformation will die prematurely and then be counted as a resorption and not as a malformation.

In the present study, maternal tissues of diabetic mothers on GD_{13} showed elevated lipid peroxidation and ROS levels and perturbations in antioxidant defenses clearly suggesting that a state of oxidative stress exists *in vivo*. The maternal organs showed more robust elevations in markers of oxidative stress sampled on GD_{20} suggesting a clear progression of oxidative impairments. Interestingly, these dysfunctions were associated with significantly enhanced ROS and MDA levels in embryos. Among diabetic rats, the fetal tissues (GD_{20}) also exhibited marked oxidative damage as evident by increased ROS and MDA levels in the brain and liver cytosol and mitochondria. Our findings in maternal tissues of diabetic rats are consistent with earlier reports *(Oberley, 1988; Baynes & Thorpe, 1999; Gurler et al., 2000)*.

Mitochondrial overproduction of the oxygen radical superoxide has been suggested recently as a critical element in the blood vessel-associated diabetic complications *(Brownlee, 2001; Rolo & Palmeira, 2006)*. Increased production of ROS *(Trocino et al., 1995; Sakamaki et al., 1999)* and enhanced lipid peroxidation *(Wentzel et al., 1999)* have been demonstrated both *in vitro* and *in vivo* in embryos of diabetic rats *(Jenkinson et al., 1986)*. Further evidence implicating oxidative stress as an important etiological factor for diabetic embryopathy stems from experimental diabetic pregnancy wherein several different types of antioxidative treatments have been shown to be beneficial in terms of improving fetal outcome *(Eriksson et al., 2000; Cederberg et al., 2001; Cederberg & Eriksson, 2005).* Apart from increased production of ROS in

diabetic pregnancy, the putative increase in oxidative stress can be attributed to impaired embryonic defense in response to an oxidative environment *(Ornoy et al., 1999; Zaken et al., 2001)*. This hypothesis is supported by findings of decreased activity and impaired mRNA response to maternal diabetes of the radical scavenging enzyme catalase in embryos of a malformation-prone rat strain. Interestingly, the fetal maldevelopment in diabetic litters is shown to be blocked by vitamin E/C, supporting a teratogenic role for free-oxygen radicals. Collectively these data provide support for the notion of oxidative stress involvement in both diabetic embryopathy and teratogenicity.

In the present study, the GSH concentration was diminished in the diabetic group compared with the non-diabetic group. The glutathione antioxidant system has a fundamental role in cellular defense against reactive free radicals and other oxidant species *(Mak et al., 1996)*. Glutathione (GSH) is present in most mammalian cells and plays an important role in cellular defense against oxidative stress by reducing protein disulfides and other cellular molecules. It also acts as a scavenger of free radicals of ROS. GSH is synthesized intracellularly by two GSH-synthesizing enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase. γ-GCS catalyzes the rate-limiting step of GSH synthesis. In many cells, the GSH redox cycle is catalyzed by both glutathione peroxidase and glutathione reductase. During oxidative stress, the reduced form of GSH is converted by glutathione peroxidase to oxidized glutathione (GSSG). Earlier studies have shown the presence of low GSH and high GSSG concentrations in erythrocytes of diabetic patients *(Nwose et al., 2006)* and in endothelial cells of diabetic rabbits *(Tagami et al., 1992)*. Further the activity of γ-GCS is reduced specifically in human erythrocytes of diabetic patients and in endothelial cells cultured under hyperglycemic conditions *(Pieper et al., 1995)*. Earlier, few workers *(Menegola et al. 1995; Sakamaki et al. 1999)* have shown a dramatic reduction in embryonic GSH content and impaired responsiveness of GSH-synthesizing enzyme to OS during organogenesis and speculated that these may have

important roles in the development of embryonic malformations in diabetes since they impair the cellular protection mechanism in the oxidative stress condition related to diabetic pathology.

Inconsistent results exist in the literature regarding the relationship between enzymes and antioxidant products. The reasons for these discrepancies have not been delineated, but it is quite possible that variations in enzyme activity are dependent upon each enzyme and the types of tissue under consideration. Furthermore, various investigators have suggested the possibility that there may be temporal changes in enzyme activity that are both transitory and biphasic in nature. For instance, after prolonged hyperglycemia in severe diabetes, the induction of certain antioxidant enzymes or a return to normal values from previously decreased values may occur as a compensatory mechanism to respond to the constant exposure to increased oxidative stress *(Pieper et al., 1995)*. In view of this, we measured the activities of selected antioxidant enzymes in maternal, embryonic and fetal tissues.

Modulation of oxidative dysfunctions in diabetic pregnancy by garlic

Recently much emphasis has been laid on understanding the protective biochemical function of natural antioxidants contained in dietary plants, which are good candidates for prevention of oxidative damage caused by free radical species *(Stavirc, 1994)*. Despite the widespread traditional use of garlic as an abortifacient, there are no epidemiologic studies suggesting an association between garlic intake and miscarriage. Hypoglycemic effect of garlic in human is not well studied. Chronic feeding of garlic oil and garlic powder *(Jain & Vyas, 1975; Augusti, 1996)* showed significant decrease in blood glucose level whereas few studies *(Liu et al., 2007)* showed no change in blood glucose level. All human studies, except one or two, have been shown the effect of garlic on blood glucose level in normal healthy individuals but not in diabetic patients. Thus the role of garlic in diabetic condition is yet to be confirmed.

In the present study, dietary garlic (2%) did not have any adverse effect on the pregnancy outcome or on any other determinants among non-diabetic rats. However, various protective effects were demonstrable among diabetic

mothers. The most notable effect was the significant reduction in incidence of embryolethality among diabetic mothers. Garlic did not provide total protection, but offered 70% protection. The second protective effect was the restoration of fetal weights among diabetic litters, where in nearly 50% protection was evident. The third protective effect was the reduction in the placental weights among diabetic dams. These protective effects were also reflected in normalization of various oxidative stress markers in tissues of diabetic mothers, and fetuses. Garlic has been used in herbal medicine for centuries for various ailments such as cardiovascular risk factors and diabetes *(Jain et al., 1973)*. To date, many favorable experimental and clinical effects of garlic preparations, including garlic extract, have been reported. These biological responses have been largely attributed to : i) reduction of risk factors for cardiovascular diseases and cancer, ii) stimulation of immune function, iii) enhanced detoxification of foreign compound, iv) hepatoprotection v) antimicrobial effect and vi) antioxidant effect.

Garlic is a complex mixture of phytochemicals and it is likely that they all interact synergistically to contribute towards its beneficial effects, especially its antioxidant capacity. Garlic has been shown to reduce blood glucose level in diabetic mice, rats *(Augusti & Sheela, 1996; Demerdash et al., 2005; Ozturk et al., 1994)*, and rabbits *(Jain & Vyas, 1975; Durak et al., 2002)*.

Ingestion of garlic juice has been shown to result in better utilization of glucose by glucose tolerance test performed in rabbits *(Jain & Vyas, 1975; Durak et l., 2002)*. Ethyl alcohol, petroleum ether and ethyl ether extracts of garlic are also reported to significantly reduce blood sugar levels in rabbits. Allicin (250mg/kg) is 60% as effective as tolbutamide in alloxan-induced diabetic rabbit. S-allyl cysteine sulfoxide (alliin), a sulfur containing amino acid in garlic (200 mg/kg bw.) has a potential to reduce diabetic condition in rat almost to the same extent as glibenclamide and insulin *(Augusti & Sheela, 1996)*. Aged garlic extract is also effective in preventing adrenal hypertrophy, hyperglycemia and elevation of corticosterone in hyperglycemic mice induced by immobilization stress. Recently, garlic oil was also shown to reduce oxidative stress in STZ-induced diabetes. Treatment of diabetic rats with garlic oil decreased serum

acid/ alkaline phosphatase, serum alanine and asparate transferases, as well as serum amylase in diabetic rats. However, few reports have shown contradictory results. Garlic intake (6.25% in diet, for 12 d) reduced hyperphagia and polydipsia but did not alter hyperglycemia and hypoinsulinaemia in STZ -induced diabetic mice. Though the exact mechanism/s of garlic as antidiabetic agent is still not clear, evidences suggest that garlic acts as an insulin secretagogue in diabetic rats. Inactivation of insulin by sulphydryl groups is a well known phenomenon, and allicin in garlic can effectively combine with compounds such as cysteine and enhance serum insulin levels. Further, it is proposed that garlic can act as an antidiabetic agent by increasing either the pancreatic secretion of insulin from the beta cells or its release from bound insulin *(Jain & Vyas, 1975)*. The antioxidant nature of S-allyl cysteine sulfoxide (isolated product from garlic) is also speculated to contribute towards the beneficial effect in diabetes (*Augusti & Sheela, 1996)*.

In the present study, the weights of the mother and fetuses, were significantly decreased by maternal diabetes. Earlier studies have reported similar findings in STZ-diabetic pregnancy and more importantly, the reversal of these effects by the antioxidant, vitamin E *(Sivan et al., 1996)*. This indicates that dietary additions aiming to reduce diabetic embryopathy should be composed of an antioxidant mixed with other protective agents. The most important findings were that antioxidative agents such as vitamin E *in vivo (Sivan et al., 1996; Siman and Eriksson, 1997; Mihalick et al., 2003)* and folic acid *in vitro (Cano et al., 2001; Joshi et al., 2001; Chiarello et al., 2003)* normalized embryonic isoprostane and PGE2 concentrations in a diabetic/hyperglycemic environment, concomitant with diminishing embryonic dysmorphogenesis. The mechanism behind these findings may be related, at least in part, to an intracellular arachidonic acid deficiency due to a diabetes/ hyperglycemia-induced overproduction of oxygen-derived radicals, leading to increased formation of isoprostanes from arachidonic acid *(Morrow et al., 1992)*.

Recent studies have shown that garlic and onion juices exert antioxidant and antihyperglycemic effects and consequently may alleviate liver and renal damage caused by alloxan-induced diabetes *(Demerdash et al. 2005)*. Several mechanisms have been suggested to explain the protective effect of garlic extract on the functional abnormalities observed in the diabetic rat aorta. Garlic extract has been shown to improve impairment in endothelium-dependent relaxation *in vitro* evoked by ACh *(Ozturk et al., 1994)* and to reduce increased lipid peroxidation caused by oxygen-free radicals *(Ide and Lau, 1997)*. Therefore, a possible mechanism by which garlic extract administration can improve the vascular reactivity in diabetes may depend on inhibition of oxidative stress. Consistent with this idea, it has been shown that *in vivo* treatment with garlic extract reduces the lipid peroxidation products *(Balasenthil et al., 2000)*. In addition, it has been shown that garlic extract activates the antioxidant system and decreases peroxidation in aortic tissue *(Durak et al., 2002)*. However, whether the lipid peroxidation-lowering effect of garlic extract results from its direct superoxide scavenging properties or increasing NO synthesis indirectly is controversial because NO also has antioxidant activity *per se* in diabetic arteries *(Chang et al., 1993)*. Further, it has been demonstrated that garlic can increase NO in endothelial cells and suppress the production of hydroxyl radical, confirming its antioxidant activity *(Kim et al., 2001).* On the other hand, advanced stage glycosylation end products (AGEs) which accumulate in diabetes *(Monnier et al., 1984)* can inactivate NO *(Bucala et al., 1991)*. Therefore, it is possible that garlic extract directly inhibits AGEs production *in vivo* through preventing NO quenching induced by AGEs.

Modulatory effects of medicinal plants in STZ model

WHO study groups have emphasized strongly the optimal and rational use of traditional and natural indigenous medicines. In Ayurvedic or indigenous folk medicines, the hypoglycemic plants have been in use generally in their natural forms (fresh juice, paste or powder). Hence in the present study, we have employed four plants which have been earlier shown to have appreciable hypoglycemic properties*. Gymnema sylvestre (GS)*, a commonly used herb in

ayurveda is demonstrated to significantly reduce blood glucose in animals with residual pancreatic function, but with no effect in pancreatectomized animals. Earlier studies with ethanol leaf extract, in diabetic rat and rabbit models have reported regeneration of islets of Langerhans, decrease in blood glucose, and increases in serum insulin *(Shanmugasundaram et al., 1990; Prakash et al., 1986; Chattopadhyay, 1998)*. Anti-hyperglycemic effects of dried leaf powder of GS are also reported in alloxanized rabbits *(Shanmugasundaram et al., 1983)*.

In the present study, dietary incorporation of GS leaf powder significantly offset diabetes induced oxidative stress in maternal organs. Earlier workers have reported the efficacy of GS leaf powder (500 mg/rat/d, 10 d) to prevent beryllium nitrate induced hyperglycemia in rats. However, no significant hypoglycemia was evident in normal rats fed with GS leaves for 25 days *(Prakash et al., 1986)*. In another study, oral administration of aqueous extracts of GS leaves (20 mg/day) for 20-60 days normalized the blood sugar levels in STZ diabetic rats *(Shanmugasundaram et al., 1990)*. Further, single/chronic administration of aqueous extract (1 g/kg) to 18-h fasted non-diabetic and STZ diabetic (mild) rats showed significant reduction in blood glucose on OGTT (1 g/kg) without any significant effect on immuno-reactive insulin levels *(Okabayashi et al., 1990)*. Oral doses GS aqueous extract (50,100,200 and 500 mg/kg) to normal and STZ diabetic rats showed significant dose-dependent hypoglycemic activity *(Chattopadhyay, 1999)*. Other workers have demonstrated hypolipidemic effect in spontaneously hypertensive rats *(Preuss et al., 1998)*.

Various hypoglycemic principles of GS referred to as gymnemosides and gymnemic acid were isolated from the saponin fraction of the plant *(Murakami et al., 1996; Yoshikawa et al., 1997)*. Its triterpene glycosides are known to inhibit glucose utilzation in muscles, inhibit glucose uptake in the intestine *(Shimizu et al., 1997)*. In humans, oral treatment of GS leaves extract (400 mg) for 18-/20 months plus conventional treatment showed beneficial effects in 22 NIDDM patients. Salient findings such as significant reduction in blood glucose, glycosylated haemoglobin and plasma proteins and lowering of conventional

drug requirement. In addition, serum insulin levels were raised suggesting insulin-releasing effect *(Baskaran et al., 1990)*. Oral administration of a water-soluble leaves extract of G. sylvestre (400 mg/day) to 27 IDDM patients on insulin therapy lowered fasting blood glucose, glycosylated haemoglobin (HbA1c), glycosylated plasma protein and insulin requirements *(Shanmugasundaram et al., 1990)*.

In the present study, another plant used, *Tinospora cordfolia* (TC) is widely used in Ayurveda as a tonic, vitalizer and as a remedy for DM and other metabolic disorders. Earlier both anti-oxidant and hypolipidemic activity of this plant have been described *(Prince & Menon, 1999; Stanely et al., 2000)*. In our study dietary supplemens of TC at 2% level significantly mitigated the oxidative stress associated with diabetes in maternal as well as fetal tissues clearly suggesting its efficacy. These findings are consistent with earlier reports in which considerable hypoglycemic effect of aqueous extract of TC was demonstrated following oral intake (400 mg/kg,15 weeks) *(Grover et al., 2000)*. In another study, aqueous extract of TC root (2.5, 5 and 7.5 mg/kg) also caused a significant reduction in blood glucose, brain lipid level, hepatic glucose-6-phosphatase, serum acid phosphatase, alkaline and lactate dehydrogenase and increase in body weight, total hemoglobin and hepatic hexokinase in alloxan-diabetic rats *(Stanely et al., 2003)*.

Withania somnifera (WS), commonly known as 'Indian cheese' has been reported to possess a variety of biological activities. A compound isolated from aqueous extract of fruit of *Withania coagulans* has been shown to exert hepatoprotective and anti-inflammatory activity *(Budhiraja et al., 1986; Rajurkar et al., 2001)*. Antifungal and antibacterial properties have also been demonstrated in the ethanolic extract of the whole plant and leaves *(Choudhary et al., 1995; Khan et al., 2006)*. Administration of aqueous extract of *W. coagulans* (1 g/kg) to non-diabetic and STZ-diabetic rats exhibited significant lowering of blood glucose levels at the end of 7 days suggesting that possibly its action may be similar to that of insulin. Aqueous extract of *Withania* to diabetic rats significantly lowered the liver/ serum LPO and decreased the STZ induced

enhanced serum cholesterol levels. Earlier, studies have shown that WS possesses anti-inflammatory *(Senthilnathan et al., 2006)*, antitumour *(Christina et al., 2004)*, cardioprotective *(Gupta et al., 2004)* and antioxidant *(Bhatnagar et al., 2005)* properties. It also appears to exert a positive influence on the endocrine *(Hemalatha et al., 2004)*, urogenital and central nervous systems *(Prasad & Malhotra, 1968)*. The biologically active chemical constituents of WS are alkaloids, steroidal lactones (including withanolides), and terpenoids with a tetracyclic skeleton like cortisol. Furthermore, treatment with WS produced an enhancement in the circulating antibody titre in the mouse spleen *(Bhattacharya et al., 2000)*.

According to indigenous medicine in Sri Lanka, *Ipomea aquatica* extracts possess an insulin-like principle *(Malalavidhane et al., 2000)*. The hypoglycemic and antihyperglycemic effects of *I. aquatica* have been reported in diabetic rats and patients (Sokeng et al., 2007; *Malalavidhane et al., 2003*). However, the mechanisms involved in the action of this plant extract remains unclear. An antidiabetic agent could exert a beneficial effect in the diabetic situation by different mechanisms including the inhibition of gastrointestinal digestion and absorption of sugars, the enhancement of insulin secretion and/or insulin action *(Malalavidhane et al., 2001)*. Consumption of the shredded, fresh, edible portion of *I. aquatica* for one week effectively reduced the fasting blood sugar level of STZ -induced diabetic rats. When subjected to a glucose challenge, the Type II diabetic subjects showed a significant reduction in the serum glucose concentration 2h after the glucose load. However, it was not significantly reduced at 1h post glucose load. There was a 29.4% decrease in the serum glucose concentration of the Type II diabetic patients *(Sokeng, 2007; Malalavidhane, 2000; 2001)*.

6.0 SUMMARY

1. Administration of STZ (45 mg/kg bw, i.p.) to pregnant rats on gestation day 4 induced significant hyperglycemia. Further, induction of diabetes consistently resulted in significant (40%) embryolethality and reduction in fetal weights.

2. Diabetes induction caused significant oxidative stress in maternal organs such as liver and kidney measured on GD_{13} in terms of, increased generation of ROS levels, elevated lipid peroxidation (MDA levels), perturbations in antioxidant defenses and significant elevation in placental lipid peroxidation and alteration in antioxidant defenses.

3. Embryos (GD_{13}) of diabetic dams were subjected to significant oxidative stress as evident from markedly elevated MDA/ROS levels in cytosolic/mitochondrial fractions, diminished GSH/total thiols and reduced activities of antioxidant enzymes.

4. Oxidative impairments in maternal organs (liver, kidney) obtained on GD_{20} showed more robust oxidative impairments as evident by marked elevation in LPO, ROS levels, depleted GSH and total thiols antioxidant enzymes and higher protein carbonyls.

5. Fetal (GD_{20}) tissues of diabetic dams (brain and liver) were also subjected to marked oxidative stress as measured by increased ROS/MDA levels and alterations in antioxidant defenses.

6. Garlic supplements (2% in diet) to STZ-diabetic pregnant rats marginally reduced the blood glucose levels, partially restored the body weight loss, and reduced the magnitude of polyuria. However, garlic supplements significantly reduced the incidence of embryolethality and markedly restored the fetal weights (50% protection).

7. However, garlic supplements markedly offset diabetes associated oxidative impairments in maternal organs (liver and kidney) as evident

by the restoration of various oxidative markers such as MDA/ROS levels, GSH content and enzymic antioxidants.

8. Garlic supplementation appeared to significantly protect against placental oxidative stress and placental weights.

9. Garlic supplements among diabetic rats significantly restored the ROS generation and MDA levels, markedly restored the diminished GSH levels, perturbations in antioxidant defenses and thiol content.

10. Oral supplements of medicinal plants to non-diabetic pregnant rats had no appreciable effect. While dietary supplements to STZ-diabetic rats marginally reduced the glucose levels, significant beneficial effects were demonstrable among Diabetic rats in terms of abrogation of oxidative stress biomarkers.

11. The incidence of embryolethality was reduced significantly by all the dietary supplements (GS>TC>IA>WS). A similar protective effect was also evident in terms of reduction in intra uterine growth retardation (IUGR). Dietary supplements also provided considerable protection against diabetes associated oxidative impairments among both maternal and fetal tissues.

12. Collectively, these findings suggest that dietary supplementation of Garlic powder, GS, TC, IA and WS during pregnancy can substantially mitigate diabetes associated oxidative stress in maternal organs and fetal tissues thus rendering protection to the mother and the developing embryos and fetus.

CONCLUSIONS

1. Pregnant dams exposed to a model prooxidant, *viz.,* t-butyl hydroperoxide at sublethal doses during specific periods of gestation (GD_{5-7} or GD_{8-10}) showed elevated oxidative induction response in the maternal tissues as evident by elevated MDA/ROS levels, depleted GSH levels, total thiols and diminished levels of antioxidant enzymes.

2. Induction of oxidative response was of higher magnitude when tbHP was administered during GD_{5-7} compared to the response during GD_{8-10} suggesting the enhanced vulnerability of embryos to prooxidants during early gestation.

3. Maternal exposure to tbHP resulted in significant oxidative implications among embryos (GD_{13}) as measured by elevated ROS/MDA levels, reduced antioxidant enzyme activities, total thiols and non-protein thiols.

4. The fetal tissues obtained from tbHP exposed dams were also showed enhanced ROS/MDA levels, protein carbonyls, depleted GSH and diminished activities of antioxidant enzymes.

5. Iron administered at sublethal doses to pregnant dams during early implantation period (GD_{5-7}) or late implantation period (GD_{8-10}) induced significant oxidative impairments in maternal tissues (eg., liver and kidney). Iron administered during early implantation period (GD_{5-7}) appeared to induce a higher degree of oxidative response in the maternal and placental milieu.

6. Maternal exposure to iron caused marked oxidative perturbations among GD_{13} embryos as evident from elevated ROS and MDA levels associated with reduced antioxidant enzyme activities, total thiols and non-protein thiols.

7. Both brain and liver of fetuses obtained from iron exposed dams showed marked enhancement of ROS/MDA levels, protein carbonyls, depleted GSH content and diminished activities of antioxidant enzymes.

8. Taken together, the above data suggest that maternal exposure to prooxidants during early phases of development induces significant oxidative stress in maternal milieu, placenta and higher oxidative impact among embryos during early post-implantation period (GD_{5-7}) compared to GD_{8-10}.

9. Pregnant dams administered with iron (oral, 2-8 mg/rat/d) during gestation failed to show any clinical signs of toxicity, while the placental weights were increased (21-45%) at the highest dose.

10. Differential pattern of ROS induction was evident in cytosol and mitochondria of maternal organs which appeared to be higher in mitochondria during both regimens, while the MDA levels were higher during the dosing regimen, GD_{5-12}. Marginal reduction in GSH levels in maternal organs were accompanied by diminished total thiol/non-protein thiols levels at higher doses irrespective of the dosing regimen, while PC levels were elevated.

11. Placental tissue showed marked elevation in ROS levels (at the highest dose), marginal elevation in MDA levels, and significant decrease in total thiol and non-protein thiol levels during both dosing regimens suggesting the vulnerability to iron induced oxidative stress especially during the early phase of development.

12. Maternal exposure to iron resulted in significant oxidative impact among embryos as evidenced by elevated ROS and MDA levels in both cytosol and mitochondria. A considerable reduction in the activities of antioxidant enzymes, marked diminution of GSH, total thiols/non protein thiols were the other salient findings.

13. Significant oxidative impact in the fetal tissues was observed following maternal iron exposure. While brain showed significant elevations in MDA and ROS levels, the liver tissue showed less robust induction. Significant reduction in the activity of antioxidant enzymes, reduced levels of GSH was accompanied with elevated total thiol levels, while the protein carbonyl levels were significantly elevated.

14. Among iron deficient (Idd) diet fed dams, the incidence of embryolethality was significantly higher compared to the controls. Maternal tissues of Id dams were subjected to significant oxidative stress since both ROS levels and lipid peroxidation were significantly elevated. In brain the ROS levels were uniformly elevated in cytosol and mitochondria, while liver cytosol showed robust increase. The GSH content of maternal tissues showed moderate decrease in cytosol and mitochondria and was associated with reduced activities of antioxidant enzymes.

15. The placental tissue of Idd dams, showed moderate elevations in GSH levels and elevated protein carbonyls content suggesting that the placental tissues is subjected to oxidative stress .

16. Embryos of Idd dams were also subjected to oxidative impact as evident by significant elevation in both ROS/MDA levels, diminished levels of GSH, total thiols/non protein thiols and marginal alterations in the activities of antioxidant enzymes. Embryos were also subjected to protein oxidative damage since the protein carbonyl content among the embryos of Id diet fed dams was marginally were observed among the embryos of dams fed Idd.

17. The fetal organs (brain and liver) of Idd diet fed dams showed significantly elevated ROS/MDA levels. Significant diminution in the levels of GSH accompanied with reduction in total thiol levels and non protein thiols, while the PC levels were significantly elevated; the activities of antioxidant enzymes in fetal tissues were decreased excepting for the SOD activities.

18. Administration of an acute dose of streptozotocin (STZ, 45 mg/kg bw, i.p.) to pregnant dams during GD_4 induced significant hyperglycemia and higher incidence of embryolethality (30- 40%) and reduction in fetal weights.

19. Diabetes induction caused significant oxidative stress in maternal organs (eg. liver, kidney) measured on either GD_{13} or GD_{20} (increased generation of ROS levels, elevated lipid peroxidation, perturbations in antioxidant defenses) and elevated placental lipid peroxidation.

20. The embryos (GD_{13}) and fetal (GD_{20}) organs of diabetic dams were subjected to significant oxidative stress as evident from elevated MDA/ ROS levels in cytosolic/mitochondrial fractions, diminished GSH/total thiols and reduced activities of antioxidant enzymes.

21. Garlic supplements to diabetic dams marginally reduced the blood glucose levels, partially restored the body weight loss, and reduced the magnitude of polyuria. Interestingly, the incidence of embryolethality was markedly reduced and fetal weights (50% protection) were restored among diabetic dams.

22. Garlic supplements markedly offset diabetes associated oxidative impairments in maternal organs (liver and kidney) as evident by the restoration of various oxidative markers such as MDA/ROS levels, GSH content and enzymic antioxidants.

23. Both placental weights and oxidative stress among diabetic rats was also significantly attenuated by the garlic supplements (50% protection). Garlic supplements among diabetic rats significantly restored the ROS generation and MDA levels, markedly restored the diminished GSH levels, perturbations in antioxidant defenses and thiol content.

24. Oral supplements of medicinal plants to non-diabetic pregnant rats had no effect. While the dietary supplements to STZ-diabetic rats marginally reduced the glucose levels, significant beneficial effects were demonstrable among diabetic rats in terms of abrogation of oxidative stress biomarkers.

25. The incidence of embryolethality was reduced significantly by all the dietary supplements (GS>TC>IA>WS) and protective effect was discernible in terms of reduction in intra uterine growth retardation. Dietary supplements also provided considerable protection against diabetes associated oxidative impairments among both maternal and fetal tissues.

26. In conclusion, our findings in the diabetic model suggest that dietary supplements of garlic powder, GS, TC, IA and WS during pregnancy can significantly abrogate diabetes associated oxidative stress in maternal and fetal tissues thus rendering protection to the mother and the developing embryos/fetus.

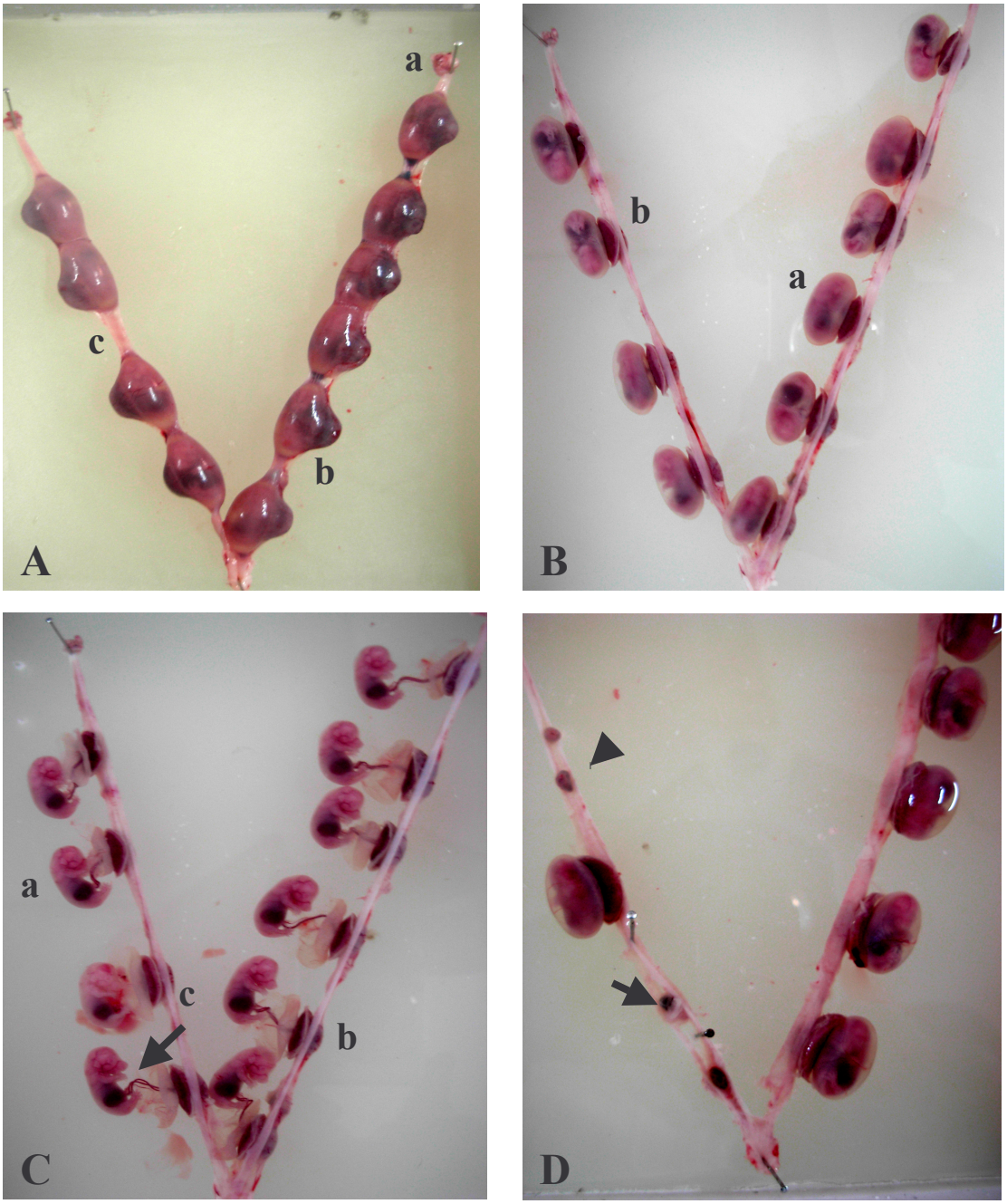


Plate I : **A)** Typical normal rat gravid uterus (GD 17) showing (a) ovary, (b) right uterine horn and (c) left uterine horn; **B)** Normal rat gravid uterus (GD 17) cut open with fetuses attached (a) fetus (b) placenta; **C)** Normal rat gravid uterus (GD 17) cut open with fetuses attached (a) fetus (b) placenta (c) umbilical cord;
D) Gravid uterus from a treated rat showing early resorption sites

Abraham SC, Yardley JH, Wu TT, 1999. Erosive injury to the upper gastrointestinal tract in patients receiving iron medication: an under recognized entity. Am J Surg Pathol. 23, 1241-1247.

Adams C, Cannell S, 2001. Women's beliefs about ''natural'' hormones and natural hormone replacement therapy. Menopause. 8(6), 433-440.

Aebi H, 1984. Catalase. Methods Enzymol. 105, 121-125.

Aisen P, Enns C, Wessling-Resnick M, 2001. Chemistry and biology of eukaryotic iron metabolism. Int.J.Biochem. Cell Biol. 33 (10), 940-959.

Akazawa M, Akazawa s, Hashimoto M, et al, 1989. Effects of brief exposure to insulin-induced hypoglycaemic serum during organogenesis in rat embryo culture. Diabetes. 38, 1573-1578.

Al Ghafli MH, Padmanabhan R, Kataya HH, Berg B, 2004. Effects of alpha-lipoic acid supplementation on maternal diabetes-induced growth retardation and congenital anomalies in rat fetuses. Mol Cell Biochem. 261(1-2), 123-35.

Allen RG, Balin AK, 1989. Oxidative influence on development and differentiation: an overview of a free radical theory of development. Free Radic Biol Med. 6, 631-661.

Allen RG, Keogh BP, Tresini M, Gerhard GS, Volker C, Pignolo RJ, Horton J, Cristofalo VJ, 1997. Development and age-Radical oxygen species during gestation associated differences in electron transport potential and consequences for oxidant generation. J Biol Chem. 272, 24805-24812.

Arrigo AP, 1999. Gene expression and the thiol redox state. Free Radic Biol Med. 27, 936-944.

Ashworth CJ, Antipatis C, 2001. Micronutrient programming of development throughout gestation. Reproduction. 122, 527-535.

Augusti KT, 1996. Therapeutic values of onion (Allium cepa L.) and garlic (Allium sativum L.). Br J Nutr. 85, S67-S74.

Augusti KT, Sheela CG, 1996. Antiperoxide effect of S-allyl cysteine sulfoxide, an insulin secretagogue, in diabetic rats. Experientia. 52(2), 115-120.

Aurousseau B, Gruffat D, Durand D, 2006. Gestation linked radical oxygen species fluxes and vitamins and trace mineral deficiencies in the ruminant. Reprod Nutr Dev. 46(6), 601-620.

Avissar N, Eisenmann C, Breen JG, Horowitz S, Miller RK, Cohen HJ, 1994. Human placenta makes extracellular glutathione peroxidase and secretes it into maternal circulation. Am J Physiol. 267 (1 Pt 1), E68-E76.

Babior BM, 1999. NADPH oxidase: an update. Blood. 93, 1464-1476.

Balasenthil S, Arivazhagan S, Nagini S, 2000. Garlic enhances circulatory antioxidants during 7, 12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis. J Ethnopharmacol. 72(3), 429-433.

Bassett ML, 2001. Haemochromatosis: iron still matters. Int Med J. 31 (4), 237-242.

Batsis JA, Nieto-Martinez RE, Lopez-Jimenez F, 2007. Metabolic syndrome: from global epidemiology to individualized medicine. Clin Pharmacol Ther. 82(5), 509-524.

Bauerova K, Bezek A, 1999. Role of reactive oxygen and nitrogen species in etiopathogenesis of rheumatoid arthritis. Gen Physiol Biophys. 18, 15-20.

Baynes JW, Thorpe SR, 1999. Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. Diabetes. 48(1), 1-9.

Beard JL, 2000. Effectiveness and strategies of iron supplementation during pregnancy. Am J Clin Nutr. 71(5 Suppl), 1288S-1294S.

Beard JL, Connor JR, Jones BC, 1993. Iron in the brain. Nutr Rev. 51(6), 157-170.

Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA, 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci. U. S. A. 87, 1620-1624.

Bentley-Lewis R, Koruda K, Seely EW, 2007. The metabolic syndrome in women. Nat Clin Pract Endocrinol Metab. 3(10), 696-704.

Berlett BS, Stadtman ER, 1997. Protein oxidation in aging, disease, and oxidative stress.J Biol Chem. 272(33), 20313-20316.

Bhatnagar M, Sisodia SS, Bhatnagar R, 2005. Antiulcer and antioxidant activity of *Asparagus racemosus* Willd and *Withania somnifera* Dunal in rats. Ann N Y Acad Sci. 1056, 261-278.

Bhattacharya SK, Bhattacharya A, Sairam K, Ghosal S, 2000. Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. Phytomedicine. 7(6), 463-469.

Bilodeau JF, Hubel CA, 2003. Current concepts in the use of antioxidants for the treatment of preeclampsia. J Obstet Gynaecol Can. 25, 742-750.

Boveris A, Oshino N, Chance B, 1972. The cellular production of hydrogen peroxide. Biochem J. 128, 617-630.

Bremner I, 1998. Manifestations of copper excess. Am J Clin Nutr. 67(5 Suppl), 1069S-1073S.

Breuer W, Ronson A, Slotki IN, Abramov A, Hershko C, Cabantchik I, 2000. The assessment of serum nontransferrin-bound iron in chelation therapy and iron supplementation. Blood. 95, 2975-2982.

Britton RS, Ramm GA, Olynyk J, Singh R, O'Neill R, Bacon BR, 1994. Pathophysiology of iron toxicity. Adv Exp Med Biol. 356, 239-253.

Brown KE, Poulos JE, Li L, Soweid AM, Ramm GA, O'Neill R, Britton RS, Bacon BR, 1997. Effect of vitamin E supplementation on hepatic fibrogenesis in chronic dietary iron overload. Am J Physiol. 272(1 Pt 1), G116-123.

Brownlee M, 2001. Biochemistry and molecular cell biology of diabetic complications. Nature. 13; 414(6865), 813-820.

Bucala R, Tracey KJ, Cerami A, 1991. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. J Clin Invest. 87(2), 432-438.

Buchanan TA, Denno KM, Sipos GF, Sadler TW, 1994. Diabetic teratogenesis. *In vitro* evidence for a multifactorial etiology with little contribution from glucose per se. Diabetes. 43, 656-660.

Budhiraja RD, Garg KN, Sudhir S, Arora B, 1986. Protective effect of 3-beta-hydroxy-2,3-dihydrowithanolide F against CCl_4-induced hepatotoxicity. Planta Med. 1, 28-29.

Buonocore G, Zani S, Perrone S, Caciotti B, Bracci R, 1998. Intraerythrocyte nonprotein-bound iron and plasma malondialdehyde in the hypoxic newborn. Free Radic Biol Med. 25, 766-770.

Burke W, Imperatore G, Reyes M, 2001. Iron deficiency and iron overload: effects of diet and genes. Proc Nutr Soc. 60 (1), 73-80.

Callapina M, Zhou J, Schmid T, Kohl R, Brune B, 2005. NO restores HIF-1 alpha hydroxylation during hypoxia: Role of reactive oxygen species. Free Radic Biol Med. 39, 925–936.

Caniggia I, Winter JL, 2002. Adriana and Luisa Castelluci award lecture 2001, 2002. Hypoxia Inducible Factor-1: oxygen regulation of trophoblast differentiation in normal and pre-eclamptic pregnancies – A review. Placenta. 23 (Suppl A): S47–S57.

Cano MJ, Ayala A, Murillo ML, Carreras O, 2001. Protective effect of folic acid against oxidative stress produced in 21-day postpartum rats by maternal-ethanol chronic consumption during pregnancy and lactation period. Free Radic Res. 34(1), 1-8.

Caragay AB, 1992. Cancer-preventive foods and ingredients. Food-Technol. Chicago, Ill: Institute of Food Technologists. 46 (4), 65-68.

Carlberg I, Mannervik B, 1985. Glutathione reductase. Methods Enzymol. 113, 507-510.

Carney JM, Starke-Reed PE, Oliver CN, Landum RW *et al.,* 1991. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-tert-butyl-alpha-phenylnitrone. Proc Natl Acad Sci U S A. 88(9), 3633-6.

Casanueva E, Mares-Galindo M, Meza C, Schnaas L, Gutierrez- Valenzuela V, Viteri FE, 2002. Iron supplementation in non-anaemic pregnant women. SCN News. Geneva. 25, 37-38.

Casanueva E, Viteri FE, 2003. Iron and oxidative stress in pregnancy. J Nutr. 133, 1700S-1708S.

CDC/DHS, 2002. Dietary intake of macronutrients, micronutrients and other constituents: United States 1988-94. Vital Health Statistics, Series 11, No 245.

Cederberg J, Eriksson UJ, 1997. Decreased catalase activity in malformation-prone embryos of diabetic rats. Teratology. 56(6), 350-357.

Cederberg J, Eriksson UJ, 2005. Antioxidative treatment of pregnant diabetic rats diminishes embryonic dysmorphogenesis. Birth Defects Res A Clin Mol Teratol. 73(7), 498-505.

Cederberg J, Picard JJ, Eriksson UJ, 2003. Maternal diabetes in the rat impairs the formation of neural-crest derived cranial nerve ganglia in the offspring. Diabetologia. 46(9), 1245-1251.

Cederberg J, Simán CM, Eriksson UJ, 2001. Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. Pediatr Res. 49(6), 755-762.

Centers for Disease Control. 1989. Criteria for anemia in children and childbearing-aged women. MMWR 38, 400-404.

Cetin I, 2003. Placental transport of amino acids in normal and growth-restricted pregnancies. Eur J Obstet Gynecol Reprod Biol. 110 Suppl 1, S50-S54.

Chandel NS, McClintock DS, Feliciana CE, Wood TM, Melandy JA, Rodriguez AM, Schinacker PT, 2000. Reactive oxygen species generated at mitochondria complex III stabilize hypoxia-inducible factor-1α during Radical oxygen species during gestation hypoxia. J Biol Chem. 275, 25130-25138.

Chandler DB, Barton JC, Briggs DD 3rd, Butler TW, Kennedy JI, Grizzle WE, Fulmer JD, 1988. Effect of iron deficiency on bleomycin-induced lung fibrosis in the hamster. Am Rev Respir Dis. 137(1), 85-89.

Chang KC, Chong WS, Park BW, Seung BW, Chun GW, Lee IJ, Park PS, 1993. NO- and NO_2-carrying molecules potentiate photorelaxation in rat trachea and aorta. Biochem Biophys Res Commun. 191(2), 509-514.

Chappell LC, Seed PT, Briley AL, Kelly FJ, Lee R, Hunt BJ, Parmar K, Bewley SJ, Shennan AH, Steer PJ, Poston L, 1999. Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial. Lancet. 354(9181), 810-816.

Chattopadhyay RR, 1998. Possible mechanism of antihyperglycemic effect of *Gymnema sylvestre* leaf extract, part I. Gen Pharmacol. 31(3), 495-496.

Chattopadhyay RR, 1999. A comparative evaluation of some blood sugar lowering agents of plant origin. J Ethnopharmacol. 67(3), 367-372.

Chen OS, Schalinske KL, Eisenstein RS, 1997. Dietary iron intake modulates the activity of iron regulatory proteins and the abundance of ferritin and mitochondrial aconitase in rat liver. J Nutr. 127(2), 238-248.

Chiang YH, Jen LN, Su HY, Lii CK, Sheen LY, Liu CT, 2006. Effects of garlic oil and two of its major organosulfur compounds, diallyl disulfide and diallyl trisulfide, on intestinal damage in rats injected with endotoxin. Toxicol Appl Pharmacol. 213(1), 46-54.

Chiarello PG, Vannucchi MT, Moyses Neto M, Vannucchi H, 2003. Hyperhomocysteinemia and oxidative stress in hemodialysis: effects of supplementation with folic acid. Int J Vitam Nutr Res. 73(6), 431-438.

Choudhary MI, Dur-e-Shahwar, Parveen Z, Jabbar A, Ali I, Atta-ur-Rahman, 1995. Antifungal steroidal lactones from Withania coagulance. Phytochemistry. 40(4), 1243-1246.

Christina AJ, Joseph DG, Packialakshmi M, Kothai R, Robert SJ, Chidambaranathan N, Ramasamy M, 2004. Anticarcinogenic activity of *Withania somnifera* Dunal against Dalton's ascitic lymphoma. J Ethnopharmacol. 93(2-3), 359-361.

Craig WJ, 1999. Health-promoting properties of common herbs. Am J Clin Nutr. 70, 491S–9S.

Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A, 2003. Protein carbonylation in human diseases. Trends Mol Med. 9(4), 169-176.

Dallman PR, 1986. Biochemical basis for the manifestations of iron deficiency. Ann Rev Nutr. 6, 13-40.

Dallman PR, Goodman JR, 1970. Enlargement of mitochondrial compartment in iron and copper deficiency. Blood. 35(4), 496-505.

Damasceno DC, Volpato GT, Calderon Ide M *et al.,* 2004. Effect of Bauhinia forficata extract in diabetic pregnant rats: maternal repercussions. Phytomedicine. 11(2-3), 196-201.

Davies KJ, 1987. Protein damage and degradation by oxygen radicals. I. general aspects. J Biol Chem. 262(20), 9895-901.

De Pasquale A, 1984. Pharmacognosy: the oldest modern science. J of Ethnopharmacol. 11, 1-16.

Dean RT, Fu S, Stocker R, Davies MJ, 1997. Biochemistry and pathology of radical-mediated protein oxidation. Biochem J. 324, 1-18.

Dean RT, Gieseg S, Davies MJ, 1993. Reactive species and their accumulation on radical damaged protein. Trends Biochem. Sci. 18, 437-441.

Demerdash FM, Yousef MI, El-Naga NI, 2005. Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. Food Chem Toxicol. 43(1), 57-63.

Desoye G, Shafrir E, 1996. The human placenta in diabetic pregnancy. Diabetes Rev.

Díaz D, Krejsa CM, White CC, Charleston JS, Kavanagh TJ, 2004. Effect of methylmercury on glutamate-cysteine ligase expression in the placenta and yolk sac during mouse development. Reprod Toxicol. 19(1), 117-129.

Dillard CJ, Tappel AL, 1979. Volatile hydrocarbon and carbonyl products of lipid peroxidation: a comparison of pentane, ethane, hexanal, and acetone as *in vivo* indices. Lipids. 14(12), 989-995.

Draper H, Hadley M, 1990. A review on recent studies on the metabolism of exogenous and endogenous malondialdehyde. Xenobiotics. 20, 901-910.

Dresow B, Albert C, Zimmermann I, Nielsen P, 1995. Ethane exhalation and vitamin E/ubiquinol status as markers of lipid peroxidation in ferrocene iron-loaded rats. Hepatology. 21(4), 1099-1105.

Durak I, Ozturk HS, Olcay E, Guven C, 2002. Effects of garlic extract supplementation on blood lipid and antioxidant parameters and atherosclerotic plaque formation process in cholesterol-fed rabbits. J Herb Pharmacother. 2(2), 19-32.

El-Demerdash FM, Yousef MI, El-Naga NI, 2005. Biochemical study on the hypoglycemic effects of onion and garlic in alloxan-induced diabetic rats. Food Chem Toxicol. 43(1), 57-63.

El-Hage S, Singh SM, 1990. Temporal expression of genes encoding free radical-metabolizing enzymes is associated with higher mRNA levels during in utero development in mice. Dev Genet. 11, 149-159.

Ellis WG, Semple JL, Hoogenboom ER, Kavlock RJ, Zeman FJ, 1987. Benomyl-induced craniocerebral anomalies in fetuses of adequately nourished and protein-deprived rats. Teratol Carcinog Mutagen. 7, 357-375.

Emerit J, Beaumont C, Trivin F, 2001. Iron metabolism, free radicals, and oxidative injury. Biomed Pharmacother.55 (6), 333-339.

Eriksson UJ, Borg LA, Cederberg J, Nordstrand H, Simán CM, Wentzel C, Wentzel P, 2000. Pathogenesis of diabetes-induced congenital malformations. Ups J Med Sci. 105(2), 53-84.

Eriksson UJ, Borg LA, 1993. Diabetes and embryonic malformations. Role of substrate-induced free-oxygen radical production for dysmorphogenesis in cultured rat embryos. Diabetes. 42, 411-419.

Eriksson UJ, Borg LAH, 1991. Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformations. *In vitro*. Diabetologia. 34, 325-331.

Eriksson UJ, Brolin SE, Naeser P, 1989. Influence of sorbitol accumulation on growth and development of embryos cultured in elevated levels of glucose and fructose. Diabetes Res. 11, 27-32.

Eriksson UJ, Siman CM, 1996. Pregnant diabetic rats fed the antioxidant butylated hydroxytoluene show decreased occurrence of malformations in the offspring. Diabetes. 45, 1497-1502.

Ernst E, 2002. The risk-benefit profile of commonly used herbal therapies: Ginkgo, St. John's Wort, Ginseng, Echinacea, Saw Palmetto,and Kava. Ann. Intern. Med. 136, 42-53

Esterbauer H, Dieber-Rotheneder M, Waeg G, Puhl H, Tatzber F, 1990. Endogenous antioxidants and lipoprotein oxidation. Biochem Soc Trans. 18(6), 1059-1061.

Fantel AG, Mackler B, Stamps LD, Tran TT, Person RE, 1998. Reactive oxygen species and DNA oxidation in fetal rat tissues. Free Radic Biol Med. 25, 95–103.

Fantel AG, 1996. Reactive oxygen species in developmental toxicity: review and hypothesis. Teratology. 53, 196-217.

Felt BT, Lozoff B, 1996. Brain iron and behavior of rats are not normalized by treatment of iron deficiency anemia during early development. J Nutr. 126(3), 693-701.

Fernandes AP, Holmgren A, 2004. Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Antioxid Redox Signal. 6(1), 63-74.

Fernando MR, Nanri H, Yoshitake S, Nagata-Kuno K, Minakami S, 1992. Thioredoxin regenerates proteins inactivated by oxidative stress in endothelial cells. Eur J Biochem. 209, 917-922.

Fine EL, Horal M, Chang TI, Fortin G, Loeken MR, 1999. Evidence that elevated glucose causes altered gene expression, apoptosis and neural tube defects in a mouse model of diabetic pregnancy. Diabetes 48, 2454-2462.

Finkel T, 2003. Oxidant signals and oxidative stress. Current Opinion Cell Biol. 15, 247-254.

Flohe L, Gunzler WA, 1984. Methods Enzymol. 105, 114-121.

Flohe L, Otting A, 1984. Superoxide dismutase assays. Methods Enzymol. 105, 93-96.

Forsberg H, Borg LAH, Cagliero E, Eriksson UJ, 1996. Altered levels of scavenging enzymes in embryos subjected to a diabetic environment. Free Rad Res. 24, 451-459.

Freeman BA, Crapo JD, 1982. Biology of disease: free radicals and tissue injury. Lab Invest. 47(5), 412-26

Frei B, England L, Ames BN, 1989. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci USA. 86, 6377-6381.

Freidovich I, 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen?. Ann NY Acad Sci. 13, 893.

Gabbay KH, 1973. The sorbitol pathway and the complications of diabetes. N Engl J Med. 288, 831-836.

Gäreskog M, Eriksson UJ, Wentzel P, 2006. Combined supplementation of folic acid and vitamin E diminishes diabetes-induced embryotoxicity in rats. Birth Defects Res A Clin Mol Teratol. 76(6), 483-490.

Gate L, Paul J, Nguyen Ba, Tew KD, Tapiero H, 1999. Oxidative stress induced in pathologies:the role of antioxidants. Biomed and Pharmacother. 53, 169-80.

Gerdin E, Tyden O, Eriksson UJ, 1985. The development of antioxidant enzymatic defense in the perinatal rat lung: Activities of superoxide dismutase, glutathione peroxidase and catalase. Pediatr. Res. 19, 687-691.

Ghatnekar GS, Barnes JA, Dow JL, Smoak IW, 2004. Hypoglycemia induced changes in cell death and cell proliferation in the organogenesis stage embryonic mouse heart. Birth Defects Res A Clin Mol Teratol. 70(3), 121-131.

Gillery P, Monboisse JC, Maquart FZ, Borel JP, 1988. Glycation of proteins as a source of superoxide. Diabete Metab. 14, 25-30.

Goldfrank L, Lewin N, Flomenbaum N, Howland MA, 1982. The pernicious panacea: herbal medicine. Hosp Physician. 10, 64-9, 73-8.

Goldman As, Baker L, Piddington R, Marx B, Herold R, Egler J, 1985. Hyperglycemia-induced teratogenesis is mediated by a functional deficiency of arachidonic acid. Proc Natl Acad Sci U S A. 82, 8227-8231.

Goto Y, Noda Y, Narimoto K, Umaoka Y, Mori T, 1992. Oxidative stress on mouse embryo development *in vitro*. Free Radic Biol Med. 13, 47–53.

Grover JK, Yadav S, Vats V, 2002. Medicinal plants of India with anti-diabetic potential. J Ethnopharmacol. 81(1), 81-100

Grover JK, Vats V, Rathi SS, 2000. Anti-hyperglycemic effect of *Eugenia jambolana* and *Tinospora cordifolia* in experimental diabetes and their effects on key metabolic enzymes involved in carbohydrate metabolism. J Ethnopharmacol. 73(3), 461-470.

Gruden N, Munic S, 1987. Effect of iron upon cadmium-manganese and cadmium-Iron interaction. Bull Environ Contam Toxicol. 38 (6), 969-974.

Guijarroa MV, Indarta OI, Aruomab, Vianaa M, Bonet B, 2002. Effects of ergothioneine on diabetic embryopathy in pregnant rats. Food and Chem Toxicol. 40, 1751-1755

Gurib-Fakim A, 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. Mol Aspects Med. 27(1), 1-93.

Guleria RS, Pan J, Dipette D, Singh US, 2006. Hyperglycemia inhibits retinoic acid-induced activation of Rac1, prevents differentiation of cortical neurons, and causes oxidative stress in a rat model of diabetic pregnancy. Diabetes. 55(12), 3326-3334.

Gupta SK, Mohanty I, Talwar KK, Dinda A, Joshi S, Bansal P, Saxena A, Arya DS, 2004. Cardioprotection from ischemia and reperfusion injury by *Withania somnifera*: a hemodynamic, biochemical and histopathological assessment. Mol Cell Biochem. 260(1-2), 39-47

Gurler B, Vural H, Yilmaz N, Oguz H, Satici A, Aksoy N, 2000. The role of oxidative stress in diabetic retinopathy. Eye. 14 Pt 5,730-735.

Gurzau E, Neagu C, Gurzau AE, 2003. Essential metals-case study on iron.Ecotoxicol and Environmental Safety. 56, 190-200

Guthenberg C, Alin P, Mannervik B, 1985. Glutathione transferase from rat testis. Methods Enzymol. 113, 507-510.

Gutteridge JM, Mumby S, Koizumi M, Taniguchi N, 1996. Free'' iron in neonatal plasma activates aconitase: evidence for biologically reactive iron. Biochem Biophys Res Commun. 229, 806-809.

Hackman RM, Hurley LS, 1983. Interaction of dietary zinc, genetic strain, and acetazolamide in teratogenesis in mice. Teratology 28, 355-368.

Haddad JJ, 2002. Antioxidant and prooxidant mechanisms in the regulation of redox-sensitive transcription factors. Cell Signal. 14, 879-897.

Hallberg L, 1992. Iron balance in pregnancy and lactation. In: Nutritional Anemias, (Fomon SJ & Zlotkin S, Eds.). Nestle Nutrition Workshop Series. 30, 13-28. Raven Press, New York, NY.

Halliwell B, 2007. Dietary polyphenols: good, bad, or indifferent for your health? Cardiovasc Res. 73(2), 341-347.

Halliwell B, Aruoma OI, 1991. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 9, 281, 9-19.

Halliwell B, Dizdaroglu M, 1992. The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. Free Radic Res Commun. 16(2), 75-87.

Halliwell B, Gutteridege JMC, 1990. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. 186, 1- 85.

Halliwell B, Gutteridge JMC, 1999. Free Radical Biology and Medecine, 3rd ed, Oxford, Oxford University Press.

Hamburger M, Hostettmann K, 1991. Bioactivity in plants: the link between phytochemistry and medicine. Phytochemistry 30 (12), 3864-3874.

Harvey MJ, Klaassen CD, 1983. Interaction of metals and carbon tetrachloride on lipid peroxidation and hepatotoxicity. Toxicol Appl Pharmacol. 71, 316-322.

Hash RB, 2001. Hereditary hemochromatosis. J Am Board Fam. Pract.14 (4), 266-273.

Hemalatha S, Wahi AK, Singh PN, Chansouria JP, 2004. Hypoglycemic activity of *Withania coagulans* Dunal in streptozotocin induced diabetic rats. J Ethnopharmacol. 93(2-3), 261-214.

Hollan S, Johansen KS, 1993. Adequate iron stores and the 'Nil nocere' principle. Haematologia (Budap). 25(2), 69-84.

Holmgren A, Aslund F, 1995. Glutaredoxin. Methods Enzymol. 252, 283-292.

Horton WE, Sadler TW, 1985. Mitochondrial alterations in embryos exposed to β-hydroxybutyrate in whole embryo culture. Anat Rec. 213,

Houglum K, Filip M, Witztum JL, Chojkier M, 1990. Malondialdehyde and 4-hydroxynonenal protein adducts in plasma and liver of rats with iron overload. J Clin Invest. 86(6), 1991-1998.

Hubel CA, Kozlov AV, Kagan VE, Evans RW, Davidge ST, McLaughlin MK, Roberts JM, 1996. Decreased transferrin and increased transferrin saturation in sera of women with reeclampsia: implications for oxidative stress. Am J Obstet Gynecol. 175, 692-700.

Hubel CA, Roberts JM, Taylor RN, Musci TJ, Rogers GM *et al.,* 1989. Lipid peroxidation in pregnancy: new perspectives on preeclampsia. Am J Obstet Gynecol. 161(4), 1025-1034.

Hurtado de Catalfo GE, De Gómez Dumm IN, 1998. Lipid dismetabolism in Leydig and Sertoli cells isolated from streptozotocin-diabetic rats. Int J Biochem Cell Biol. 30(9), 1001-1010.

Ide N, Lau BH, 1997. Garlic compounds protect vascular endothelial cells from oxidized low density lipoprotein-induced injury. J Pharm Pharmacol. 49(9), 908-911.

Idjradinata P, Pollitt E, 1993. Reversal of developmental delays in iron-deficient anaemic infants treated with iron. Lancet. 2; 341(8836), 1-4.

Ilouno LE, Shu EN, Igbokwe GE, 1996. An improved technique for the assay of red blood cell superoxide dismutase (SOD) activity. Clin Chim Acta. 247(1-2), 1-6.

Ishibashi M, Akazawa S, Sakamaki H, Matsumoto K, Yamasaki H, Yamaguchi Y *et al.,* 1997. Oxygen-induced embryopathy and the significance of glutathione-dependent antioxidant system in the rat embryo during early organogenesis. Free Radic Biol Med. 22, 447-454.

Ishida K, Tsukimori K, Nagata H, Koyanagi T, Akazawa K, Nakano H, 1995. Is there a critical gestational-age in neutrophil superoxide production activity? 85, 1331–1333.

Ishihara M, 1978. Studies on lipoperoxide of normal pregnant women and of patients with toxemia of pregnancy. Clin Chim Acta. 1; 84 (1-2), 1-9.

Jain RC, Vyas CR, 1975. Garlic in alloxan-induced diabetic rabbits. Am J Clin Nutr. 28(7), 684-685.

Jain RC, Vyas CR, Mahatma OP, 1973. Letter: Hypoglycaemic action of onion and garlic. Lancet. 2(7844), 1491.

Jarvis JH, Jacobs A, 1974. Morphological abnormalities in lymphocyte mitochondria associated with iron-deficiency anaemia. J Clin Pathol. 27(12), 973-979.

Jauniaux E, Watson A, Burton G, 2001. Evaluation of respiratory gases and acid-base gradients in human fetal fluids and uteroplacental tissue between 7 and 16 weeks' gestation. Am J Obstet Gynecol. 184, 998–1003.

Jauniaux E, Pahal G, Gervy C, Gulbis B, 2000. Blood biochemistry and endocrinology in the human fetus between 11 and 17 weeks of gestation. Reprod Biomed Online. 1(2), 38-44.

Jenkinson PC, Anderson D, Gangolli SD, 1986. Malformations induced in cultured rat embryos by enzymically generated active oxygen species. Teratog Carcinog Mutagen. 6(6), 547-554.

Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM, 2000. Fate of the mammalian cardiac neural crest. Development. 127, 1607-1616.

Johri RK, Dasgupta PR, 1980. Hydrogen peroxide formation in the uterus under hormone-induced conditions. J Endocrinol. 180, 477-481.

Joshi R, Adhikari S, Patro BS, Chattopadhyay S, Mukherjee T, 2001. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. Free Radic Biol Med. 30(12), 1390-1399.

Juchau MR, Boutelet-Bochan H, Huang Y, 1998. Cytochrome P450-dependent biotransformation of xenobiotics in human and rodent embryonic tissues. Drug Metab. Rev. 30, 541-568.

Kalter H, 1996. Reproductive toxicology in animals with induced and spontaneous diabetes. Reprod Toxicol. 10(6), 417-438.

Karakaya S, El SN, Taş AA, 2001. Antioxidant activity of some foods containing phenolic compounds. Int J Food Sci Nutr. 52(6), 501-8.

Kaur P, Kaur G, Bansal MP, Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: role of transcription factor NF-kappaB and testicular antioxidant enzymes. Reprod Toxicol. 22(3), 479-484.

Keen CL, 1992. Maternal factors affecting teratogenic response: a need for assessment. Teratology 46, 15-21.

Keen CL, Zidenberg-Cherr S, 1994. Should vitamin-mineral supplements be recommended for all women with childbearing potential?. Am. J. Clin. Nutr. 59, 532S–539S.

Khan B, Ahmad SF, Bani S, Kaul A, Suri KA, Satti NK, Athar M, Qazi GN, 2006. Augmentation and proliferation of T lymphocytes and Th-1 cytokines by *Withania somnifera* in stressed mice. Int Immunopharmacol. 6(9), 1394-1403.

Kharb S, 2000. Lipid peroxidation in pregnancy with preeclampsia and diabetes.
Gynecol Obstet Invest. 50(2), 113-116.

Kim A, Murphy MP, Oberley TD, 2005. Mitochondrial redox regulates transcription of the nuclear-encoded mitochondrial protein manganese superoxide dismutase: a proposed adaptative response to mitochondrial redox imbalance. Free Radic Biol Med. 38, 644–654.

Kim KM, Chun SB, Koo MS, Choi WJ, Kim TW, Kwon YG, Chung HT, Billiar TR, Kim YM, 2001. Differential regulation of NO availability from macrophages and endothelial cells by the garlic component S-allyl cysteine. Free Radic Biol Med. 30(7), 747-756.

Klempa KL, Willis WT, Chengson R, Dallman PR, Brooks GA, 1989. Iron deficiency decreases gluconeogenesis in isolated rat hepatocytes. J Appl Physiol. 67(5), 1868-1872.

Knutson MD, Walter PB, Ames BN, Viteri FE, 2000. Both iron deficiency and daily iron supplements increase lipid peroxidation in rats. J Nutr. 130(3), 621-628.

Kubow S, Yaylayn V, Mandeville S, 1993. Protection by acetylsalicylic acid against hyperglycemia induced glycatiion and neural tube defects in cultured early somite mouse embryos. Diabetes Res. 22, 145-158.

Kucera J, 1971. Rate and type of congenital anomalies among offspring of diabetic women. J Reprod Med. 7(2), 73-82.

Kukiela E, Cederbaum AI, 1990. NADPH- and NADH-dependent oxygen radical generation by rat liver nuclei in the presence of redox cycling agents and iron. Arch Biochem Biophys. 283, 326–333.

Kumar TR, Muralidhara, 1999. Male-mediated dominant lethal mutations in mice following prooxidant treatment. Mutat Res. 444(1), 145-149.

Kumar TR, Muralidhara, 2007. Induction of oxidative stress by organic hydroperoxides in testis and epididymal sperm of rats in vivo. J Androl. 28(1),77-85.

Laloraya M, Kumar GP, Laloraya MM, 1989. A possible role of superoxide anion radical in the process of blastocyst implantation in Mus musculus. Biochem Biophys Res Commun. 161, 762-770.

Lao TT, Chan PL,Tam KF, 2001. Gestational diabetes mellitus in the last trimester-a feature of maternal iron excess? Diabet Med. 18, 218-223.

Lao TT, Lee CP, Wong WM, 1997. Placental weight to birthweight ratio is increased in mild gestational glucose intolerance. Placenta. 18(2-3), 227-230.

Latour I, Demoulin JB, Buc-Calderon P, 1995. Oxidative DNA damage by t-butyl hydroperoxide causes DNA single strand breaks which is not linked to cell lysis. A mechanistic study in freshly isolated rat hepatocytes. FEBS Lett. 373(3), 299-302.

Lauffer RB, 1991. Iron stores and the international variation in mortality from coronary artery disease. Med Hypotheses. 35(2), 96-102.

Lebel CP, Ali SF, Mckee M, Bondy SC, 1990. Organo metal – induced increases in oxygen radical species: the potential of 2', 7'-dichloro-fluorescin diacetate as an index of neuto toxic damage. Toxicol Appl Pharmacol. 104, 17-24.

Lee AT, Eriksson UJ, 1995. Embryos exposed to high glucose *in vitro* show congenital malformations and decreased frequency of DNA mutations. Diabetologia. 38, A281 (Abstract).

Lee AT, Reis D, Eriksson UJ, 1999. Hyperglucemia-induced embryonic dysmorphogenesis correlates with genomic DNA mutation frequency *in vitro* and *in vivo*. Diabetes. 48, 371-376.

Lee AY, Chung SS, 1999. Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J. 13, 23-30.

Leonard SS, Harris GK, Shi X, 2004. Metal- induced oxidative stress and signal transduction. Free Radic Biol Med. 37, 1921-1942.

Levavasseur F, Miyadera H, Sirois J, Tremblay ML, Kita K, Shoubridge E, Hekimi S, 2001. Ubiquinone is necessary for mouse embryonic development but is not essential for mitochondrial respiration. J Biol Chem. 276, 46160-46164.

Levine RL, Garland D, Oliver C, Amici A, Climent I, Lenz A, Ahn B, Shaltiel S, Stadtman E, 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464-478.

Li HL, Brynes RW, 1999. Association of redoxactive iron bound to high molecular weight structures in nuclei with inhibition of cell growth by H_2O_2. Free Radic Biol Med. 26, 49-60.

Lillig CH, Holmgren A, 2007. Thioredoxin and related molecules--from biology to health and disease. Antioxid Redox Signal. 9(1), 25-47.

Little RE, Gladen BC, 1999. Levels of lipid peroxides in uncomplicated pregnancy: a review of the literature. Reprod Toxicol. 13(5), 347-352.

Liu CT, Sheen LY, Lii CK, 2007. Does garlic have a role as an antidiabetic agent? Mol Nutr Food Res. 51(11), 1353-1364.

Liu R, Buettner GR, Oberley LW, 2000. Oxygen free radicals mediate the induction of manganese superoxide dismutase gene expression by TNF-α. Free Radic Biol Med. 28, 1197–1205.

Liu L, Wells PG, 1995. Potential molecular targets mediating chemical teratogenesis: in vitro peroxidase-catalyzed phenytoin metabolism and oxidative damage to proteins and lipids in murine maternal hepatic microsomes and embryonic 9000g supernatant. Toxicol Appl Pharmacol. 134(1), 71-80.

Lloyd RV, Hanna PM, Mason RP, 1997. The origin of the hydroxyl radical oxygen in the Fenton reaction. Free Radic Biol Med.22, 885-888.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement using folin phenol reagent. J Biol Chem. 193, 265-275.

Lund EK, Fairweather-Tait SJ, Warf SG, Johnson IT, 2001. Chronic exposure to high levels of dietary iron fortification increases lipid peroxidation in the mucosa of the rat large intestine. J Nutr. 131, 2928-2931.

Lynch SR, Stoltzfus RJ, 2003. Iron and ascorbic Acid: proposed fortification levels and recommended iron compounds. J Nutr. 133(9), 2978S-2984S.

Maiese K, Chong ZZ, Shang YC, 2007. Mechanistic insights into diabetes mellitus and oxidative stress. Curr Med Chem. 14(16), 1729-1738.

Mak RH, 1996. Effect of recombinant human erythropoietin on insulin, amino acid, and lipid metabolism in uremia. J Pediatr. 129(1), 97-104.

Malalavidhane S, Wickramasinghe SM, Jansz ER, 2001. An aqueous extract of the green leafy vegetable *Ipomoea aquatica* is as effective as the oral hypoglycaemic drug tolbutamide in reducing the blood sugar levels of Wistar rats. Phytother Res. 15(7), 635-637.

Malalavidhane TS, Wickramasinghe SM, Jansz ER, 2000. Oral hypoglycaemic activity of *Ipomoea aquatica*. J Ethnopharmacol. 72(1-2), 293-298.

Malalavidhane TS, Wickramasinghe SM, Perera MS, Jansz ER, 2003. Oral hypoglycaemic activity of *Ipomoea aquatica* in streptozotocin-induced, diabetic wistar rats and Type II diabetics. Phytother Res. 17(9), 1098-1000.

Mancuso C, Scapagini G, Curro D, Giuffrida Stella AM *et al.,* 2007. Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders. Front Biosci.12, 1107-1123.

Maritim A, Dene BA, Sanders RA, Watkin JB III, 2002. J Biochem Mol Toxicol. 16, 203-208.

Marnett LJ, Riggins JN, West JD, 2003. Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. J. Clin. Invest. 111, 583-593.

Masella R, Di Benedetto R, Vari R, Filesi C, Giovannini C, 2005. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. J Nutr Biochem. 16(10), 577-86.

Masini A, Ceccarelli D, Gallesi D, Giovannini F, Trenti T. 1994. Lipid hydroperoxide induced mitochondrial dysfunction following acute ethanol intoxication in rats. The critical role for mitochondrial reduced glutathione. Biochem Pharmacol. 47(2), 217-224.

Mates JM, Perez-Gomez C, De Castro IN, 1999. Antioxidant Enzymes and Human Diseases. Clin Biochem. 32 (8), 595-603.

McCord JM, Ormar BA, 1993. Sources of free radicals. Toxicol Ind Health. 9, 23-37.

McKelvey TG, Hollwarth ME, Granger DN, Engerson TD, Landler U, and Jones HP, 1988. Mechanisms of conversion of xanthine dehydrogenase to xanthine oxidase in ischemic rat liver and kidney. Am J Physiol Gastrointest Liver Physiol. 254, G753-G760.

McLaren CE, Li KT, Gordeuk VR, Hasselblad V, McLaren GD, 2001. Relationship between transferrin saturation and iron stores in the African American and US Caucasian populations: analysis of data from the third National Health and Nutrition Examination Survey. Blood. 98, 2345-2351.

Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, Habermehl GG, 1989. Human fibroblast release oxygen species in response to interleukin-1 or tumor necrosis factor-α. Biochem J. 263, 539–545.

Meister A, Anderson ME, 1983. Glutathione. Annual Review of Biochemistry. 52 711-760.

Menegola E, Prati M, Broccia ML, Ricolfi R, Giavini E, 1995. *In vitro* development of rat embryos obtained from diabetic mothers. Experientia. 51(4), 394-397.

Mihalick SM, Ortiz D, Kumar R, Rogers E, Shea TB, 2003. Folate and vitamin E deficiency impair cognitive performance in mice subjected to oxidative stress: differential impact on normal mice and mice lacking apolipoprotein E. Neuromolecular Med. 4(3), 197-202.

Miki A, Fujimoto E, Ohasaki T, Mizoguti H, 1988. Effects of oxygen concentration on embryonic development in rats: a light and electron microscopic study using whole-embryo culture techniques. Anat Embryol. 178, 337-343.

Milczarek R, Klimek J, Zelewski L, 2000. The effects of ascorbate and alpha-tocopherol on the NADPH-dependent lipid peroxidation in human placental mitochondria. Mol Cell Biochem. 210, 65-73.

Miller MJS, Voelker CA, Olester S, Thompson JH *et al.,* 1996. Fetal growth retardation in rats may result from apoptosis: rate of peroxinitrite. Free Radic Biol Med. 21, 619–629.

Mirkes PE, Little SA, 1998. Teratogen-induced cell death in postimplantation mouse embryos: differential tissue sensitivity and hallmarks of apoptosis. Cell Death Differ. 5(7), 592-600.

Mokrasch LC, Teschke EJ, 1984. Glutathione content of cultured cells and rodent brain regions: a specific fluorometric assay. Anal Biochem. 140(2), 506-509.

Monnier VM, Kohn RR, Cerami A, 1984. Accelerated age-related browning of human collagen in diabetes mellitus. Proc Natl Acad Sci U S A. 81(2), 583-587.

Morris JM, Gopaul NK, Endresen MJ, Knight M, Linton EA, Dhir S, Anggård EE, Redman CW, 1998. Circulating markers of oxidative stress are raised in normal pregnancy and pre-eclampsia. Br J Obstet Gynaecol. 105(11), 1195-1199.

Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ 2nd, 1992. Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids. Proc Natl Acad Sci U S A. 89(22), 10721-10725.

Mover H, Ar A, 1997. Antioxidant enzymatic activity in embryos and placenta of rat chronically exposed to hypoxia and hyperoxia. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol. 117, 151-157.

Murakami N, Murakami T, Kadoya M, Matsuda H, Yamahara J, Yoshikawa M, 1996. New hypoglycemic constituents in "gymnemic acid" from *Gymnema sylvestre*. Chem Pharm Bull (Tokyo). 44(2), 469-471.

Nagataki S, 1995. Significance of glutathione depletion and oxidative stress in early embryogenesis in glucose-induced rat embryo culture. Diabetes. 44(8), 992-998.

Nash P, Eriksson UJ, 2007. Suramin-restricted blood volume in the placenta of normal and diabetic rats is normalized by vitamin E treatment. Placenta. 28(5-6), 505-515.

Neye RL, 1987. Functionally important disorders of the placenta, umbilical cord, and fetal membranes. Hum Pathol. 18(7), 680-691.

Niki E, 1991. Vitamin C as an antioxidant. World Rev Nutr Diet. 64, 1-30.

Niki E, Noguchi N, Gotoh N, 1993. Dynamics of lipid peroxidation and its inhibition by antioxidants. Biochem Soc Trans. 21(2), 313-317.

Nishikawa T, Edelstein D, Du XL, et al, 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature. 404, 787-790.

Nwose EU, Jelinek HF, Richards RS, Kerr PG, 2006. Changes in the erythrocyte glutathione concentration in the course of diabetes mellitus. Redox Rep. 11(3), 99-104.

Oberley LW, 1988. Free radicals and diabetes. Free Radic Biol Med. 5(2), 113-124.

Ohkawa H, Ohishi N, Yagi K, 1978. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 95(2), 351-358.

Okabayashi Y, Tani S, Fujisawa T, Koide M, Hasegawa H, Nakamura T, Fujii M, Otsuki M, 1990. Effect of *Gymnema sylvestre*, R.Br. on glucose homeostasis in rats. Diabetes Res Clin Pract. 9(2):143-148.

Ornoy A, Zaken V, Kohen R, 1999. Role of reactive oxygen species (ROS) in the diabetes-induced anomalies in rat embryos *in vitro*: reduction in antioxidant enzymes and low-molecular-weight antioxidants (LMWA) may be the causative factor for increased anomalies. Teratology. 60(6), 376-386.

Orrenius S, Ormstad K, Thor H, Jewell SA, 1983. Turnover and functions of glutathione studied with isolated hepatic and renal cells. Fed Proc. 42(15), 3177-3188.

Osawa T, 1999. Protective role of dietary polyphenols in oxidative stress. Mech Ageing Dev. 111(2-3), 133-9.

Osawa T, 1997. Biochemical and physiological importance as plant polyphenols. In: Ohigashi H, Osawa T, Terao J, Watanabe S, Yoshikawa T(Eds.), Food Factors for Cancer Prevention. Springer, Tokyo, 39-46.

Oubré AY, Carlson TJ, King SR, Reaven GM, 1997. From plant to patient: an ethnomedical approach to the identification of new drugs for the treatment of NIDDM. Diabetologia. 40(5),614-617.

Ozturk Y, Aydin S, Kosar M, Baser KH, 1994. Endothelium-dependent and independent effects of garlic on rat aorta. J Ethnopharmacol. 44(2), 109-16.

Packer L, Witt EH, Trischler HJ, 1995. Alpha-lipoic acid as a biological antioxidant. Free Rad Biol Med. 19, 227-250.

Packer, L.E, 1994. Oxygen radicals in biological systems: Part C. Methods in Enzymology. Academic Press, New York, 233.

Padmanabhan R, Mohamed S, Singh S, 2006. Beneficial effect of supplemental lipoic acid on diabetes-induced pregnancy loss in the mouse. Ann N Y Acad Sci. 1084, 118-131.

Page KR, 1993. The physiology of human placenta. UCL Press Limited, London. 164.

Palomar-Morales M, Baiza LA, Verdin-Teran L, Roman-Ramos R *et al.,* 1998. Fetal development in alloxan-treated rats. Reprod Toxicol. 12(6), 659-65.

Patt A, Horesh IR, Berger EM, Harken AH, Repine JE, 1990. Iron depletion or chelation reduces ischemia/reperfusion-induced edema in gerbil brains. J Pediatr Surg. 25(2), 224-227.

Pedersen JF, Molsted-Pedersen L, 1979. Early growth retardation in diabetic pregnancy. Br Med J. 4, 1-5.

Pedersen JF, Molsted-Pedersen L, 1982. Early growth delay predisposes the fetus in diabetic pregnancy to congenital malformation (letter). Pediatr Res. 49(6), 755-762.

Pershadsingh HA, 2007. Alpha-lipoic acid: physiologic mechanisms and indications for the treatment of metabolic syndrome. Expert Opin Investig Drugs. 16(3), 291-302.

Piddington R, Joyce J, Dhanasekaran P, Baker L, 1996. Diabetes mellitus affects prostaglandin E2 levels in mouse embryos during neurulation. Diabetologia. 39(8),915-920.

Pieper GM, Meier DA, Hager SR, 1995. Endothelial dysfunction in a model of hyperglycemia and hyperinsulinemia. Am J Physiol. 269(3 Pt 2), H845-850.

Pinn G, Pallett L, 2002. Herbal medicine in pregnancy. Complement Ther Nurs Midwifery. 8 (2), 77-80.

Porter NA, Caldwell SE, Mills KA, 1995. Mechanisms of free radical oxidation of unsaturated lipids. Lipids. 30, 277-290.

Prakash AO, Mathur S, Mathur R, 1986. Effect of feeding *Gymnema sylvestre* leaves on blood glucose in beryllium nitrate treated rats. J Ethnopharmacol. 18(2), 143-146.

Prasad S, Malhotra CL, 1968. Studies on Withania ashwagandha Kaul. VI. The effect of the alkaloidal fractions (acetone, alcohol and water soluble) on the central nervous system. Indian J Physiol Pharmacol. 12(4), 175-181.

Preuss HG, Jarrell ST, Scheckenbach R, Lieberman S, Anderson RA, 1998. Comparative effects of chromium, vanadium and *Gymnema sylvestre* on sugar-induced blood pressure elevations in SHR. J Am Coll Nutr. 17(2), 116-123.

Prince PS, Kamalakkannan N, Menon VP, 2004. Restoration of antioxidants by ethanolic *Tinospora cordifolia* in alloxan-induced diabetic Wistar rats. Acta Pol Pharm. 61(4), 283-287.

Prince PS, Menon VP, 1999. Antioxidant activity of *Tinospora cordifolia* roots in experimental diabetes. J Ethnopharmacol. 65(3), 277-281.

Prince PS, Padmanabhan M, Menon VP, 2004. Restoration of antioxidant defence by ethanolic *Tinospora cordifolia* root extract in alloxan-induced diabetic liver and kidney. Phytother Res. 18(9):785-787.

Pryor WA, 1986. Oxy-radicals and related species: their formation, lifetimes, and reactions. Ann Rev Physiol. 48, 657-667.

Qanungo S, Mukherjea M, 2000. Ontogenic profile of some antioxidants and lipid peroxidation in human placental and fetal tissues. Mol Cell Biochem. 215, 11–19.

Raijmakers MT, Dechend R, Poston L, 2004. Oxidative stress and preeclampsia: rationale for antioxidant clinical. Hypertension. 44, 374-380.

Rajesh Kumar T, Muralidhara, 2002. Oxidative stress response of rat testis to model prooxidants *in vitro* and its modulation. Toxicol *In vitro*. 16(6), 675-682.

Rajurkar NS, Pardeshi BM, 1997. Analysis of some herbal plants from India used in the control of diabetes mellitus by NAA and AAS techniques. Appl Radiat Isot. 48(8), 1059-1062.

Ranzini A, Allen A, Lai Y, 2001. Use of complementary medicines and alternative therapies among obstetric patients. Obstet Gynecol. 97(4 Suppl 1), S46.

Rao AV, Rao LG, 2007. Carotenoids and human health. Pharmacol Res. 55(3), 207-216.

Rao J, Jagadeesan V, 1996. Lipid peroxidation and activities of antioxidant enzymes in iron deficiency and effect of carcinogen feeding. Free Radic Biol Med. 21(1), 103-108.

Rashbass P, Ellington SK, 1988. Development of rat embryos cultured in serum prepared from rats with streptozotocin-induced diabetes. Teratology. 37, 51-61.

Rates SM, 2001. Plants as source of drugs. Toxicon. 39(5), 603-613.

Rauscher PM, Saders RA, Watkins JB, 2000. J Biochem Mol Tox. 14, 189-194.

Rayburn WF, Stanley JR, Garrett ME, 1996. Periconceptional folate intake and neural tube defects. J. Am. Coll. Nutr. 15, 121-125.

Rebelo I, Carvalho–Guerra F, Pereira–Leite L, Quintanilha A, 1995. Lactoferrin as a sensitive blood marker of neutrophil activation in normal pregnancies. Eur J Obstet Gynecol Reprod Biol. 62, 189-194.

Reece EA, 1999. Maternal fuels, diabetic embryopathy: pathomechanisms and prevention. Semin Reprod Endocrinol. 17(2), 183-194.

Reece EA, Khandelwal M, Wu YK, Borenstein M, 1997. Dietary intake of myo-inositol and neural tube defects in offspring of diabetic rats. Am J Obstet Gynecol. 176, 536.539.

Reeves PG, Nielsen FH, Fahey GC Jr, 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 123: 1939-1951.

Rice D, Barone S Jr, 2000. Critical periods of vulnerability for the developing nervous system: evidences from human and animal models. Environ Health Perspect. 108 suppl 3, 511-533.

Roberts JM, Redman CW, 1993. Pre-eclampsia: more than pregnancy-induced hypertension. Lancet. 341(8858), 1447-1451.

Rodriguez AM, Carrico PM, Mazurkiewicz JE, Melendez JA, 2000. Mitochondrial or cytosolic catalase reverses the MnSOD dependent inhibition of proliferation by enhancing respiratory chain activity, net ATP production, and decreasing the steady state level of H_2O_2. Free Radic Biol Med. 29, 801–813.

Rolo AP, Palmeira CM, 2006. Diabetes and mitochondrial function: role of hyperglycemia and oxidative stress. Toxicol Appl Pharmacol. 212(2), 167-178.

Rush GF, Alberts D, 1986. tert.-butyl hydroperoxide metabolism and stimulation of the pentose phosphate pathway in isolated rat hepatocytes. Toxicol Appl Pharmacol. 85(3):324-331.

Rush GF, Gorski JR, Ripple MG, Sowinski J, Bugelski P, Hewitt WR, 1985. Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. Toxicol Appl Pharmacol. 78(3), 473-483.

Sadler TW, 1980. Effects of maternal diabetes on early embryogenesis: I. The teratogenic potential of diabetec serum. Teratology. 21, 339-347.

Sadler TW, Horton WEJ, 1983. Effects of maternal diabetes on early embryogenesis: the role of insulin and insulin therapy. Diabetes. 32, 1070-1074.

Sailaja Devi MM, Suresh Y, Das, 2000. Preservation of the antioxidant status in chemically-induced diabetes mellitus by melatonin. J Pineal Res. 29(2), 108-115.

Sakaida I, Thomas AP, Farber JL, 1991. Increases in cytosolic calcium ion concentration can be dissociated from the killing of cultured hepatocytes by tert-butyl hydroperoxide.J Biol Chem. 266(2), 717-722.

Sakamaki H, Akazawa S, Ishibashi M, et al, 1999. Significance of glutathione-dependent antioxidant system in diabetes-induced embryonic malformations. Diabetes. 48, 1138-1144.

Sakanashi TM, Rogers JM, Fu SS, Connelly LE, Keen CL, 1996. Influence of maternal folate status on the developmental toxicity of methanol in the CD-1 mouse. Teratology 54, 198-206.

Sakurai T, Tsuchiya S, 1988. Superoxide production from nonenzymatically glycated protein. FEBS Lett. 236, 406-410.

Scholl TO, Hediger ML, Bendich A, Schall JI, Smith WK, Krueger PM, 1997. Use of multivitamin/mineral prenatal supplements: influence on the outcome of pregnancy. Am J Epidemiol. 146, 134-141.

Scholl TO, Reilly T, 2000. Anaemia, iron and pregnancy outcome. J Nutr. 130 (Suppl.), 443S-447S.

Schrader M, Fahimi HD, 2006. Peroxisomes and oxidative stress. Biochim Biophys Acta. 1763(12), 1755-1766.

Schumann K, 2001. Safety aspects of iron in food. Ann Nutr Metab. 45 (3), 91-101.

Schwartz R, Teramo KA, 2000. Effects of diabetic pregnancy on the fetus and newborn. Semin Perinatol. 24(2), 120-135.

Segal AW and Shatwell KP, 1997. The NADPH oxidase of phagocytic leukocytes. *Ann NY Acad Sci* 832, 215-222.

Seldak J, Lindsay RH, 1968. Estimation of total, protein bound and non-protein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem. 25, 192-205.

Senthilnathan P, Padmavathi R, Banu SM, Sakthisekaran D, 2006. Enhancement of antitumor effect of paclitaxel in combination with immunomodulatory *Withania somnifera* on benzo(a)pyrene induced experimental lung cancer. Chem Biol Interact. 159(3), 180-185.

Seshadri S, Gopaldas T, 1989. Impact of iron supplementation on cognitive functions in preschool and school-aged children: the Indian experience. Am J Clin Nutr. 50, 675-84.

Shaarawy M, Aref A, Salem ME, Sheiba M, 1998. Radical-scavenging antioxidants in pre-eclampsia and eclampsia. Int J Gynaecol Obstet. 60(2), 123-128.

Shanmugasundaram ER, Gopinath KL, Radha Shanmugasundaram K, Rajendran VM, 1990. Possible regeneration of the islets of Langerhans in streptozotocin-diabetic rats given *Gymnema sylvestre* leaf extracts. J Ethnopharmacol. 30(3), 265-279.

Shanmugasundaram ER, Rajeswari G, Baskaran K, Rajesh Kumar BR *et al.,* 1990. Use of *Gymnema sylvestre* leaf extract in the control of blood glucose in insulin-dependent diabetes mellitus. J Ethnopharmacol. 30(3), 281-294.

Shanmugasundaram KR, Panneerselvam C, Samudram P, Shanmugasundaram ER, 1983. Enzyme changes and glucose utilisation in diabetic rabbits: the effect of *Gymnema sylvestre*. J Ethnopharmacol. 7(2), 205-234.

Sherman AR, Moran PE, 1984. Copper metabolism in iron-deficient maternal and neonatal rats. J Nutr. 114(2), 298-306.

Shigenaga MK, Hagen TM, Ames BN, 1994. Oxidative damage and mitochondrial decay in aging. Proc. Natl. Acad. Sci. USA 91, 10771-10778.

Shimizu K, Ozeki M, Tanaka K, Itoh K, Nakajyo S, Urakawa N, Atsuchi M, 1997. Suppression of glucose absorption by extracts from the leaves of Gymnema inodorum. J Vet Med Sci. 59(9), 753-757.

Sies H, 1991. Oxidative stress: Introduction. In: Sies H, editor. Oxidative stress: Oxidants and antioxidants. San Diego, CA: Academic Press. 15-22.

Siman CM, Gittenberger-de Groot AC, Wisse B, Eriksson UJ, 2000. Neural crest related malformations in offspring of diabetic rats decrease with vitamin E treatment. Teratology. 61, 355-367.

Simmons RA, 2006. Developmental origins of diabetes: the role of oxidative stress. Free Radic Biol Med. 40(6), 917-922.

Simşek M, Naziroğlu M, Erdinç A, 2005. Moderate exercise with a dietary vitamin C and e combination protects against streptozotocin-induced oxidative damage to the kidney and lens in pregnant rats. Exp Clin Endocrinol Diabetes. 113(1), 53-59.

Singh J, Hood RD, 1985. Maternal protein deprivation enhances the teratogenicity of ochratoxin A in mice. Teratology. 32, 381-388.

Sivan E, Reece EA, Wu YK, Homko CJ, Polansky M, Borenstein M, 1996. Dietary vitamin E prophylaxis and diabetic embryopathy: morphologic and biochemical analysis. Am J Obstet Gynecol. 175, 793-799.

Slater TF, 1984. Free-radical mechanisms in tissue injury. Biochem J. 222(1), 1-15.

Soewondo S, Husaini M, Pollitt E, 1989. Effects of iron deficiency on attention and learning processes in preschool children: Bandung, Indonesia. Am J Clin Nutr. 50, 667-73

Sokeng SD, Rokeya B, Hannan JM, Junaida K, Zitech P, Ali L, Ngounou G, Lontsi D, Kamtchouing P, 2007. Inhibitory effect of *Ipomoea aquatica* extracts on glucose absorption using a perfused rat intestinal preparation. Fitoterapia.

Spätling L, Fallenstein F, Huch A, Huch R, Rooth G, 1992. The variability of cardiopulmonary adaptation to pregnancy at rest and during exercise. Br J Obstet Gynaecol. 99 Suppl 8, 1-40.

Srigiridhar K, Nair KM, Subramanian R Singotamu L, 2001. Oral repletion of iron induces free radical mediated alterations in the gastrointestinal tract of rat. Mol Cell Biochem. 219, 91-98.

Srigiridhar K, Nair KM, 1997. Protective effects of antioxidant enzymes and GSH *in vivo* on iron mediated lipid peroxidation in gastrointestinal tract of rat. Ind J Biochim Biophys. 34, 402-405.

Srigiridhar K, Nair KM, 1998. Iron-deficient intestine is more susceptible to peroxidative damage during iron supplementation in rats. Free Radic Biol Med. 25, 660-665.

Srigiridhar K, Nair KM, 2000. Supplementation with alpha-tocopherol or a combination of alpha-tocopherol and ascorbic acid protects the gastrointestinal tract of iron-deficient rats against iron-induced oxidative damage during iron repletion. Br J Nutr. 84, 165-173.

Stadtman ER, 1990. Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences. Free Radic Biol Med. 9(4), 315-325.

Stadtman ER, Levine RL, 2003. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids. 25(3-4), 207-218.

Stanely Mainzen Prince P, Menon VP, 2003. Hypoglycaemic and hypolipidaemic action of alcohol extract of *Tinospora cordifolia* roots in chemical induced diabetes in rats. Phytother Res. 17(4), 410-413.

Stanely P, Prince M, Menon VP, 2000. Hypoglycaemic and other related actions of *Tinospora cordifolia* roots in alloxan-induced diabetic rats. J Ethnopharmacol. 70(1), 9-15.

Stavric B, 1994. Role of chemopreventers in human diet. Clin Biochem. 27(5), 319-332.

Steer P, Alam MA, Wadsworth J, Welch A, 1995. Relation between maternal haemoglobin concentration and birth weight in different ethnic groups. BMJ 310, 489-491.

Stolzfus RJ, Dreyfuss M, 1998. Guidelines for the use of iron supplementation to prevent and treat iron deficiency anaemia. International Nutritional Anaemia Consultative Group (INACG), ILSI Press, Washington, DC.

Sugino N, Nakamura Y, Takeda O, Ishimatsu M, Kato H, 1993. Changes in activities of superoxide dismutase and lipid peroxide in corpus luteum during pregnancy in rats. J Reprod Fertil. 97(2), 347-351.

Sussman I, Matschinsky FM, 1988. Diabetes affects sorbitol and myo-inositol levels of neuroectodermal tissue during embryogenesis in rat. Diabetes. 37, 974-981.

Suzuki N, Svensson K, Eriksson UJ, 1996. High glucose concentration inhibits migraton of rat cranial neural crest cells *in vitro*. Diabetologia. 39, 401-411.

Svensson AM, Borg LA, Eriksson UJ, 1992. Glucose metabolism in embryos of normal and diabetic rats during organogenesis. Acta Endocrinol (Copenh). 127, 252-257.

Tagami S, Kondo T, Yoshida K, Hirokawa J, Ohtsuka Y, Kawakami Y, 1992. Effect of insulin on impaired antioxidant activities in aortic endothelial cells from diabetic rabbits. Metabolism. 41(10), 1053-1058.

Takehara Y, Yoshioka T, Sasaki J, 1990. Changes in the levels of lipoperoxide and antioxidant factors in human placenta during gestation. Acta Med Okayama. 44(2):103-111.

Tappel AL, 1973. Lipid peroxidation damage to cell components. Fed Proc. 32(8), 1870-1874.

Thibodeau PA, Koksis-Bedard M, Courteau J, Niyonsenga T, Paquette B, 2001. Thiols can either enhance or suppress DNA damage induction by catecholoestrogens. Free Radic Biol Med. 30, 62-73.

Thompson JG, 2000. *In vitro* culture and embryo metabolism of cattle and sheep embryos - a decade of achievement. Anim Reprod Sci. 60-61, 263-275.

Tiran D, 2003. The use of herbs by pregnant and childbearing women: a risk–benefit assessment. Complementary Therapies in Nursing & Midwifery. 9, 176-181.

Trocino R, Akazawa S, Takino H, et al, 1994. Cellualr-tissue localization and regulation of the GLUT-1 protein in both the embryo and the visceral yolk sac from normal and experimental diabetic rats during the early postimplantation period. Endocrinol. 134, 869-878.

Trocino RA, Aakazawa S, Ishibashi M, et al, 1995. Significance of glutathione depletion and oxidative stress in early embryogenesis in glucose-induced rat embryo culture. Diabetes. 44, 992-998.

Trounce IA, Kim YL, Jun AS, Wallace DC, 1996. Methods Enzymol. 264, 484-509.

Uehara M, Tateishi S, Chiba H, Suzuki K, Goto S, 1998. Effects of iron and copper supplementation on the formation of thiobarbituric acid-reactive substances and phosphatidylcholine hydroperoxide in the livers of iron- and copper-deficient rats. J Nutr Sci Vitaminol (Tokyo). 44(5):705-14.

Unterman TG, Buchanan TA, Freinkel N, 1989. Access of maternal insulin to the rat conceptus prior to allantoic placentation. Diabetes Res. 10, 115-120.

van Rensburg SJ, Potocnik FC, Kiss T, Hugo F, van Zijl P, Mansvelt E *et al.,* 2001. Serum concentrations of some metals and steroids in patients with chronic fatigue syndrome with reference to neurological and cognitive abnormalities. Brain Res Bull. 55(2), 319-325.

Viana M, Aruoma OI, Herrera E, Bonet B, 2000. Oxidative damage in pregnant diabetic rats and their embryos. Free Radic Biol Med. 29(11), 1115-1121.

Visavadiya NP, Narasimhacharya AV, 2007. Hypocholesteremic and antioxidant effects of *Withania somnifera* (Dunal) in hypercholesteremic rats. Phytomedicine. 14(2-3), 136-142.

Viteri FE, 1997. Iron supplementation for the control of iron deficiency in populations at risk. Nutr Rev. 55, 195-209.

Viteri FE, 1998. A new concept in the control of iron deficiency (ID): community-based preventive supplementation (PS) of at-risk groups by weekly intake of iron supplements. Biomed Environ Sci. 11, 46-60.

Viteri FE, 1998. Prevention of iron deficiency. In: Micronutrient Deficiencies: A Toolkit for Policymakers and Public Health Workers (Howson CP, Kennedy E, Horwitz A, eds.), 45-102.

Viteri FE, Ali F, Tujague J, 1999. Long-term weekly iron supplementation improves and sustains nonpregnant women's iron status as well or better than currently recommended short-term daily supplementation. J Nutr. 129, 2013-2020.

Viteri FE, Liu XN, Martin A, Tolomei K, 1995. True absorption and retention of supplemental iron is more efficient when administered every-three days rather than daily to iron-normal and iron-deficient rats. J Nutr 125, 82-91.

Viteri FE, Mendoza C, Guiro A, Hercberg S, Galan P, 1999. Daily and weekly and reference-dose iron (Fe) absorption in Berkeley, Ca. and Dakar, Senegal. FASEB J. 13, A536.4.

Vulto AG, Smet PAGM, 1988. In: Dukes, M.M.G. (Ed.). Meyler's Side Effects of Drugs. 11th Ed. Elsevier, Amsterdam, 999-1005.

Walsh SW, Wang Y, 1993a. Deficient glutathione peroxidase activity in preeclampsia is associated with increased placental production of thromboxane and lipid peroxides. Am J Obstet Gynecol. 169(6), 1456-1461.

Walsh SW, Wang Y, 1993b. Secretion of lipid peroxides by the human placenta. Am J Obstet Gynecol. 169, 1462-1466.

Walter PB, Knutson MD, Paler-Martinez A, Lee S, Xu Y, Viteri FE, Ames BN, 2002. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. Proc Natl Acad Sci. USA 99, 2264-2269.

Wang Y, Walsh SW, 1996. Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. J Soc Gynecol Investig. 3(4), 179-184.

Watson AL, Palmer ME, Jauniaux E, Burton GJ, 1997. Variations in expression of copper/zinc superoxide dismutase in villous trophoblast of the human placenta with gestational age. Placenta. 18(4), 295-299.

Wefers H, Sies H, 1988. The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. Eur J Biochem. 2, 353-357.

Wegger I, Palladin B, 1994. Vitamin C deficiency causes hematological and skeletal abnormalities during fetal development in swine. J Nutr. 124, 241-248.

Wells PG, Bhuller Y, Chen CS, Jeng W, Kasapinovic S, Kennedy JC, Kim PM *et al.,* 2005. Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species. Toxicol Appl Pharmacol. 1;207(2 Suppl), 354-66.

Wells PG, Kim PM, Nicol CJ, Parman T, Winn LM, 1997. Reactive intermediates. In: Kavlock RJ, Daston, GP. (Eds.), Drug Toxicity in Embryonic Development. Springer-Verlag, Heidelberg, 453-518.

Wells PG, Winn LM, 1996. Biochemical toxicology of chemical teratogenesis. Crit. Rev. Biochem. Mol. Biol. 31, 1-40.

Wentzel P, Eriksson UJ, 1996. Insulin treatment fails to abolish the teratogenic potential of serum from diabetic rats. Eur J Endocrinol. 134, 459-464.

Wentzel P, Eriksson UJ, 1998. Antioxidants diminish developmental damage induced by high glucose and cyclooxygenase inhibitors in rat embryos *in vitro*. Diabetes. 47, 677-684.

Wentzel P, Welsh N, Eriksson UJ, 1999. Developmental damage, increased lipid peroxidation, diminished cyclooxygenase-2 gene expression, and lowered prostaglandin E2 levels in rat embryos exposed to a diabetic environment. Diabetes. 48(4), 813-820.

Wessling-Resnick M, 2000. Iron transport. Annu Rev Nutr. 20, 129-151.

WHO, 2001. Iron deficiency anaemia: assessment, prevention, and control. WHO/NHD/01.3, Geneva.

Willett WC, 2000. Will high-carbohydrate/low-fat diets reduce the risk of coronary heart disease? Proc Soc Exp Biol Med. 225(3), 187-90.

Winitzer A, Ayalon N, Hershkovitz R, et al, 1999. Lipoic acid prevention of neural tube defects in offspring of rats with streptozocin-induced diabetes. Am J Obstet Gynecol. 180, 188-193.

Wisdom SJ, Wilson R, McKillop JH, Walker JJ, 1991. Antioxidant systems in normal pregnancy and in pregnancy-induced hypertension. Am J Obstet Gynecol. 165(6 Pt 1), 1701-1704.

Wolff SP, Dean RT, 1987. Glucose autoxidation and protein modification. The potential role of ‘autooxidative glycosylation’ in diabetes. Biochem J. 245, 243-250.

Wood ZA, Poole LB, Karplus PA, 2003. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. Science. 300, 650-653.

Yan SD, Schmidt AM, Anderson GM, et al, 1994. Enhanced cellualar oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. J Biol Chem. 269, 9889-9897.

Yang X, Borg LA, Eriksson UJ, 1995. Altered mitochondrial morphology of rat embryos in diabetic pregnancy. Anat Rec. 241, 255-267

Yip R, 2001. Better use of data to define nutrition priority and action. Biomed Environ Sci. 14(1-2), 61-65.

Yoshikawa M, Murakami T, Kadoya M, Li Y, Murakami N, Yamahara J, Matsuda H, 1997. Medicinal foodstuffs. IX. The inhibitors of glucose absorption from the leaves of *Gymnema sylvestre* R. BR. (Asclepiadaceae): structures of gymnemosides a and b. Chem Pharm Bull (Tokyo). 45(10), 1671-1676.

Younes M, Wess A, 1990. The role of iron in t-butyl hydroperoxide-induced lipid peroxidation and hepatotoxicity in rats. J Appl Toxicol. 10(5), 313-317.

Zabihi S, Eriksson UJ, Wentzel P, 2007. Folic acid supplementation affects ROS scavenging enzymes, enhances Vegf-A, and diminishes apoptotic state in yolk sacs of embryos of diabetic rats. Reprod Toxicol. 23(4), 486-98.

Zaken V, Kohen R, Ornoy A, 2001. Vitamins C and E improve rat embryonic antioxidant defense mechanism in diabetic culture medium. Teratology. 64(1), 33-44.

Zanninelli G, Loreal O, Brissot P, Konijn AM, Slotki IN, Hider RC Ioav Cabantchik Z, 2002. The labile iron pool of hepatocytes in chronic and acute iron overload and chelator-induced iron deprivation. J Hepatol. 36 (Suppl.), 39-46.