STUDIES ON THE EFFECT OF CISPLATIN ON MITOCHONDRIA IN DALTON'S LYMPHOMA - BEARING MICE

By

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Declaration

I, Arpaia Kharbangar, do hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other university/Institute.

This is being submitted to the North Eastern Hill University for the degree of **Doctor of Philosophy** in Zoology.

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To Meimei & Papa

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(Arpaia Kharbangar)

CONTENTS

ABBREVIATIONS

BSA	Bovine serum albumin
DL cells	Dalton's lymphoma cells
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GST	Glutathione-s-transferase
i.p.	Intraperitoneally
LPO	Lipid peroxidation
MDH	Malate dehydrogenase
Mt	Mitochondrial
NPSH	Non-protein sulfhydryl
O.D.	Optical density
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
SDH	Succinate dehydrogenase
SOD	Superoxide dismutase
TSH	Total sulfhydryl

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INTRODUCTION

INTRODUCTION

i) CANCER

The multiplication of cells in the body is carefully regulated. In an adult, some cell types e.g. nerve cells do not divide while others like skin and progenitors of the blood cells divide throughout the life in order to replace billions of cells that die everyday. Thus, a very carefully controlled programme exists to determine the multiplication of different types of cells in the body.

Eukaryotic cell division occurs in four well defined phases: synthesis or S phase, gap 2 or G2 phase, mitosis or M Phase and gap 1 or G1 phase. After passing through mitosis and into G1, a cell either continues through another division or ceases to divide, entering a quiescent phase (Go) that may last hours, days or the lifetime of the cell. When a cell in Go begins to divide again, it re-enters the division cycle through the G1 (Hunt and Nasmyth, 1997). The cell cycle is controlled by a family of protein kinases that are the heterodimers with a regulatory subunit, cyclin and a catalytic subunit, cyclin-dependent protein kinase (CDK). The cell division is also regulated by a family of extracellular growth factors (Weinberg, 1996).

If occasionally, the exquisite control mechanisms of regulating cell multiplication break down, a cell begins to grow and divide in an uncontrolled manner. Descendants of such cells inherit the propensity to proliferate without responding to regulation and expand indefinitely to develop as a lump, which is commonly referred to as a tumor. Defects in the synthesis, regulation or recognition of growth factors may also be involved in developing a tumor (Rubin, 1985).

Tumors are strictly defined as neoplasm, although the term tumor may be applied to any swelling (Vincent, 1985). The terms neoplasm and tumor are commonly used interchangeably (Friedberg, 1986). Tumors violate the basic homeostatic principle of

the body and ideally fall into one of the two categories, the slowly growing 'benign' and the rapidly growing 'malignant' forms which are invasive, disseminating and show metastasis (Vincent, 1985). The spread of tumor from the primary organ or tissue in which neoplasm initially occurs to secondary sites is called metastasis (Fidler and Hart, 1982).

The development of malignant tumor is a multistep process characterised by a progression of genetic alterations in a single line of cells. Various cancer causing agents are called carcinogens. These carcinogens could be (i) physical agents (ultraviolet rays, γ-rays, X-rays) (ii) chemicals (Benzpyrene, aflatoxin B1, benzanthracene, methylchlolanthrene, lead, carbon tetrachloride, asbestos) and (iii) viral agents (Rous, sarcoma virus, polyoma virus, simian virus 40, adenovirus, Epstein barr virus etc) (Fearon, 1997).

In the carcinogenesis two categories of genes (tumor suppressor genes and oncogenes) may be implicated. Tumor suppressor genes (about 20 in human) normally act as cell's brakes. They encode proteins that restrain cell growth and prevent cells from becoming malignant. The transformation of a normal cell to a cancer cell is accompanied by the loss or decrease of function of one or more tumor suppressor genes. Most of the proteins encoded by tumor suppressor genes act as negative regulators of cell proliferation which may be as transcription factors (p53 and WT1), cell cycle regulators (RB and p16), components regulating signalling pathways (NF1) and components regulating RNA polymerase II elongation (VHL). Thus, their elimination contributes and promotes uncontrolled cell growth (Haber and Harlow, 1997).

In contrast to tumor suppressor genes, oncogenes (Greek; onkos, a tumor) encode proteins that promote the loss of growth control and conversion of a cell to a malignant state. Oncogenes are generally derived from proto-oncogenes which are genes that encode proteins having a function in the normal cell. These oncogenes products act in many

ways, for example, i) as growth factors or their receptors e.g. SIS oncogenes for platelet derived growth factor (PDGF), erbB oncogenes which directs the formation of EGF receptor, ii) as cytoplasmic protein kinases e.g. RAF that heads the MAP kinase cascade, the primary growth controlling signalling pathway in cells, iii) as nuclear transcription factors e.g. myc oncogene and iv) as the products that inhibits apoptosis e.g. bcl-2 oncogene (Hunter, 1997).

Malignant tumors are commonly referred to as cancers. The word cancer (Latin = crab) suggests its capacity to reach out and cling tenaciously to adjacent tissues. Cancer is considered to be a dynamic developmental disorder and a disease of cellular differentiation (Rubin, 1985). Cancer cells have unlimited life span, require less serum factors and exhibit anchorage independence for growth. Cancer cells are generally rounded/convex shape, show reduced adhesion to substratum with the loss of contact inhibition of movement and multilayering in culture. Cancer cells also acquire increased production of proteolytic enzymes, altered antigenicity, increased negative surface charge, disorganised cytoskeleton etc (Hynes, 1979).

Cancers are generally classified into three broad groups: carcinomas, sarcomas, and leukemia/lymphomas (Cairns, 1986). About 85% of cancers are carcinomas i.e. tumors that arise from endodermal or ectodermal tissues such as skin or the epithelial lining of internal organs and glands, colon, breast, prostrate, ovary, lungs etc. Sarcomas (~5%) arise less frequently and are derived from mesodermal connective tissues as such as bone marrow, fat and cartilage. The leukemias and lymphomas (~10%) are cancers of haematopoietic cells.

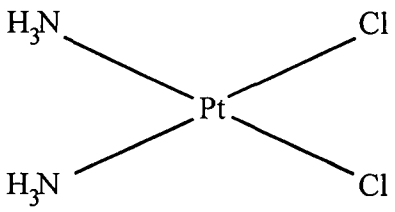
Although the idea about cancer dates back to the late 17th century (Currie and Currie, 1982) and in spite of a lot of scientific advancements, cancer is still an unpredictable and fearful disease. Considerable efforts have been directed towards improving the diagnosis and treatment of cancer. Surgery, chemotherapy and radiotherapy

are the three main ways of widely accepted treatment for cancer. In chemotherapy, cisplatin plays a pivotal role and can be used singly or in combination with radiotherapy and/or surgery in the treatment of many cancers.

ii) CISPLATIN

Rosenberg et al. (1965) while studying the effect of electric fields on bacterial (*Escherichia coli*) growth, noted that the bacterial growth continued but cell division was inhibited. The inhibition of cell division was attributed to the formation of amminechloro compounds from platinum electrodes and ammonium chloride in the growth medium and it was subsequently identified as *cis*-diamminedichloroplatinum (II) (Rosenberg et al., 1967). It is now commonly known as cisplatin.

The cell division inhibiting property of cisplatin evoked to study the antitumor activity of cisplatin and it was recognized as a potential antitumor agent (Rosenberg et al., 1969). Now cisplatin has been established to be a potent antitumor drug against a wide spectrum of experimental tumors such as leukemia L1210, DMBA mammary carcinoma, Rous sarcoma, Dunning ascites leukemia, Walker 256 carcinoma (Kociba et al., 1970; Sarna and Sodhi, 1978; Rosenberg, 1985) and also in human malignancies such as ovarian and testicular tumors, bladder carcinoma, head and neck cancer (Pil and Lippard, 1997; Lebwohl and Canetta, 1998).



Structure of Cisplatin

Cisplatin is a water soluble, square planar coordination complex containing a central platinum atom surrounded by two chloride atoms and two ammonia moieties. The antitumor activity of the complex is much greater when the chloride and ammonia moieties are in the *cis* position as compared to the *trans* position. In an aqueous solution, one or both chloride ions on cisplatin may be replaced by water to produce hydrated intermediate known as an '**aquo**' cisplatin species [$Pt(NH_3)_2Cl_2 + H_2O \rightarrow Pt(NH_3)_2Cl(H_2O)^+ + Cl^-$; $Pt(NH_3)_2Cl(H_2O)^+ + H_2O \rightarrow Pt(NH_3)_2(H_2O)_2^{++} + Cl^-$] and the monochloro monohydroxy platinum species in an alkaline environment [$Pt(NH_3)Cl_2 + OH \rightarrow Pt(NH_3)_2Cl(OH) + Cl^-$] (Rosenberg, 1985).

Isotonic and hypertonic saline solutions as well as human blood plasma have high chloride ion concentrations, which helps to maintain a greater amount of cisplatin as a $Pt(NH_3)_2Cl_2$ species in these fluids. Acidic pH tends to stabilize cisplatin's reactivity towards nucleophiles such as water, sulfate and free thiols (Hausheer et al., 1998). After passive diffusion across the plasma membrane, however, the chloride ion concentration decreases sharply, promoting the hydrolysis of the labile chloride ligands (Chu, 1994).

The major cytotoxic target of cisplatin in the cell is suggested to be DNA (Eastman, 1986), causing inhibition of DNA synthesis by acting on DNA template rather than on DNA polymerase (Howle and Gale, 1970; Pinto and Lippard, 1985). It has been suggested that the *cis* configuration of cisplatin favours the formation of intrastrand cross links in DNA (Roberts and Pascoe, 1972; Roberts, 1974; Roos and Arnold, 1977).

In intact DNA, cisplatin binds preferentially to the N-7 position of guanine and adenine (Pinto and Lippard, 1985). The cytotoxicity of cisplatin against cultured neoplastic cells correlate closely with platinum DNA interstrand cross links and to the formation of intrastrand bifunctional N-7 adducts at d(GPG) and d(APG) (Zwelling et

al., 1979; Plooy et al., 1984; Coste et al., 1999). All bifunctional adducts cause major distorsions of the local DNA structure involving both bending and unwinding of the double helix. Such structural differences between the complexes formed by cisplatin may serve as a molecular basis for their differential biological and chemotherapeutical activity. Intrastrand cross-links strongly inhibit DNA synthesis and its transcription (Heminger et al., 1997; Cullinane et al., 1999). Two classes of proteins HMG1 and HMG2 have recently been identified that bind preferentially to cisplatin-damaged DNA sites. The formation of these specific proteins or damaged DNA complexes may actually function in blocking repair by competing out the binding of the repair-related recognition proteins and hence the cytotoxity of the drug (Zamble and Lippard, 1995; Yaneva et al., 1997; Zlatanova et al., 1998).

Cisplatin has also been reported to interact with the side chains and terminal amino and carboxylate groups of proteins, small peptides and amino acids and negatively charged phospholipids (Taylor et al., 1995; Burger et al., 1999). Prasad and Sodhi (1981, 1982) reported that cisplatin treatment of normal and tumor cells causes the removal of the sialic acid and mucopolysaccharides from the cell surface which may lead to an increase in the antigenicity of tumor cells and render them more immunogenic and accessible to the cells of the immune system. It has also been shown that treatment of tumor cells with cisplatin increases the expression of tumor cell surface associated antigens and thereby permits the immunological recognition and subsequent rejection of cisplatin treated cells (Sarna and Sodhi, 1978). Sodhi and Prasad (1985) reported differential effect of cisplatin on the lectin, concanavalin A (Con A) and wheat germ agglutinin (WGA), agglutinability of splenocytes and Dalton's lymphoma cells. The agglutinability of normal cells (splenocytes) increased and Dalton's lymphoma (tumor) cells decreases after cisplatin treatment. The depolymerization of microfilaments, formation of giant multinucleated cells after cisplatin treatment have also been reported (Sodhi, 1976)

In the antitumor studies against murine ascites Dalton's lymphoma, Prasad and Giri (1994) showed that during tumor regression ascites fluid volume decreases sharply and there is an increase in carbohydrate and decrease in protein concentration in the ascites supernatants with the infiltration of leukocytes towards the tumor cells. Membrane vesicles and vacuoles were also formed before the disintegration and lysis of tumor cells. Prasad and Giri (1999) reported that cisplatin treatment of mice results in a decrease in the lactate dehydrogenase (LDH) activity. Isozyme pattern of LDH revealed the presence of five isozymes along with the tissue specificity of different isozymes with only LDH-5 in tumor cells and appearance of some specific isozyme variant LDH-T in the serum of tumor-bearing host. Prasad et al. (1999) reported a decrease in the activities of enzymes such as Na^+K^+-ATPase, 5'-nucleotidase, arginase and lactate dehydrogenase in tumor cells and tissues of tumor-bearing mice after cisplatin treatment. Nicol and Prasad (2002) reported that cisplatin treatment of tumor-bearing mice resulted in an overall decrease of sialic acid contents in the DL cells as well as other tissues such as liver, kidney and testes which may help in tumor regression. Based on the various findings on the effect of cisplatin in the cells particularly involving the cellular components other than DNA, it has been suggested that these may play an additional significant role in the anticancer activity of cisplatin and this led to propose the involvement of multistep and multilevel effects of cisplatin in the tumor cells or host (Giri, 1995).

a) Cisplatin and side effects

Although cisplatin is one of the most widely used chemotherapeutic agents, human tumors exhibit a spectrum of response to cisplatin according to their histology, with tumors of germ cell origin the most sensitive and those arising from the breast, pancreas and gastro intestinal tract the most resistant (Kaye et al., 1992).

The therapeutic efficacy of cisplatin is limited due to its side effects which include

nephrotoxicity, neurotoxicity, gastrointestinal toxicity, ototoxicity, embryotoxicity and mutagenicity (Prestayko et al., 1979; Keller and Aggarwal, 1983; Roberts et al., 1988; Giri et al., 1998). Cisplatin-induced nephrotoxicity shows i) an increase in blood urea nitrogen, ii) a decrease in glomerular filtration rate, iii) impaired renal concentrating capacity, iv) non-specific tubular necrosis with tubule dilatation and cast formation, and v) specific tubular lesions characterised by renal potassium and magnesium wasting (Lippman et al., 1973; Gonzales-Vitale et al., 1977; Dentino et al., 1978; Schilsky and Anderson, 1979; Safirstein et al., 1981; Blachley and Hill, 1981). A variety of strategies have been proposed to protect the kidney and neurological functions following cisplatin treatment. It has been shown that the administration of glutathione (GSH) provides protection against cisplatin-induced nephrotoxicity without reducing the antitumor activity of the cytotoxic agent (Zunino et al., 1998).

In an attempt to overcome nephrotoxicity the use of cisplatin in combination with other agents have also been tried with different degrees of success (Treskes and Van der Vijgh, 1993; Giri et al., 1998). Vitamin C has been reported to be effective as a protectant against a variety of toxic chemical agents including heavy metals (Holloway and Peterson, 1984). The protective role of vitamin C on cisplatin- induced nephrotoxicity and mutagenicity have been observed (Giri and Prasad, 1996; Giri et al., 1998).

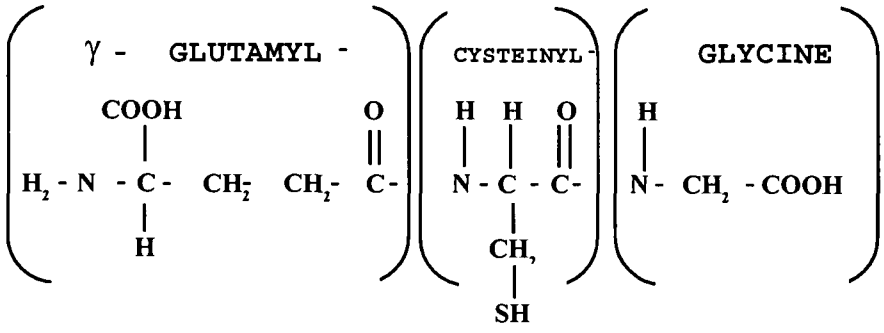
The mutagenic potential of cisplatin reported in bacteria (Cross et al., 1996) as well as in mammalian system (Khynriam, 2001) raises concern that its use in cancer chemotherapy may have carcinogenic risk with the development of secondary malignancies.

Another main limitation for the full clinical evaluation of cisplatin, is the development of drug resistance (Dabholkar and Reed, 1996) by the cancer cells and it may be multifactorial involving reduced drug uptake, enhanced DNA repair mechanisms, enhanced cellular GSH levels and impaired influx through the cell membrane or enhance efflux (Andrews and Howell, 1990).

b) Cisplatin and mitochondria

Cisplatin has been shown to bind preferentially to mitochondrial DNA (mtDNA) as compared to nuclear DNA (nDNA) (Olivero et al., 1995,1997). As compared to nDNA, cisplatin adduction in mtDNA is reported to be 4-fold to 8-fold higher (Olivero et al., 1995,1997). This preferential binding of cisplatin could be explained by the naked structure of mtDNA, which makes it highly accessible to damaging agents (Salazar et al., 1982). The increased susceptibility of mtDNA to damaging agents could be due to the existence of a non-nucleoprotein structure, the lipophilic nature of the mitochondrial membrane and prevalent oxidative environment in the mitochondria (Sawyer and Van Houten, 1999). mtDNA encodes proteins which participate in the electron transport chain and a damage to mtDNA will diminish electron transport function resulting in a fall in cell bioenergetics and subsequent cellular dysfunction. (Olivero et al., 1997). Mitochondrial dysfunction have been reported in hepatotoxicity (Pessayre et al., 1999) and cisplatin-induced nephrotoxicity (Kruidering et al., 1997).

c) Cisplatin and Glutathione



Structure of Glutathione

Glutathione, a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), is an important antioxidant. In the cells under normal physiological conditions more than 98% of glutathione exists in reduced form (GSH) (Arrick and Nathan, 1984; Wang and Ballatori, 1998). Cellular GSH is involved in a variety of important physiological and metabolic

functions including the synthesis of proteins and DNA, transport, enzyme activity and protection of cells (Meister, 1988; Deleve and Kaplowitz, 1991; Wang and Ballatori, 1998). It inhibits free radical mediated injury by eliminating toxic peroxides and protects protein sulfhydryl groups from oxidation (Meister, 1988). GSH has also become the focus of intensive interest in cancer chemotherapy and also shown to be implicated in the metabolism of cisplatin causing alterations in the rate of drug uptake. GSH may also be involved in the detoxification of cisplatin (Li et al., 1997). It has been observed that cisplatin treatment of tumor bearing mice causes a decrease of GSH levels in blood, ascites supernatants and tumor cells. Blood haemoglobin, erythrocytes, packed cell volume (PCV) and leukocytes were also decreased along with the development of various morphological abnormalities in erythrocytes after the treatment. It was suggested that blood glutathione level and development of haematological abnormalities are inversely related (Khynriam and Prasad, 2001). In the studies involving GSH-related enzymes, Khynriam and Prasad (2002) have reported that the activity of glutathione related enzymes is variable in different tissues. In liver, glutathione-S-transferase (GST), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) activity decreased while glutathione reductase (GR) increased after cisplatin treatment. In kidney, catalase and GST activity decreased while GR, GPx and SOD increased. In DL cells activities of GST, GPx, catalase and GR decreased but SOD increased. It was suggested that these changes could affect cellular antioxidant defense potential which may play an important contributory role in cisplatin-mediated toxicity particularly nephrotoxicity and anticancer activity in the host.

iii) Mitochondria

a) General features

Mitochondria (Gr, mito = thread; chondrion = granule) are considered as the direct descendants of a bacterial endosymbiont representative that became established at an early stage in a nucleus containing host cell (Gray et al., 1999). Mitochondria consist of two membranes and two compartments. The outer membrane limits the intermembranous space and inner membrane surrounds the mitochondrial matrix and itself projects as infoldings, called mitochondrial cristae (Detailed review, Fromenty and Pessayre, 1995). The outer membrane contains majority of integral protein, called porins, which form small aqueous channels so that outer membrane is freely permeable to most small molecules with the molecular weights in the range of 4000-5000. The inner membrane is highly specialized with a variety of transport proteins and contains high proportion of double phospholipid, cardiolipin (Melnick and Parker, 1971).

The total number of different proteins making up a mitochondrion is approximated to be more than 1000 (Bauer et al., 1999). Most of these proteins are encoded by the nuclear genome and synthesized in the form of precursor proteins and imported into mitochondria in a multi-step process which is facilitated by the coordinated action of independent translocation systems in the mitochondrial outer and inner membranes. In the yeast, *sacharomyces cerevisiae*, three district translocases have been described to mediate mitochondrial protein import: one translocase complex in the outer membrane (TOM complex) and two translocase complexes in the inner membrane (TIM 23.17 and TIM 22.54). For import of preproteins into the matrix and into the inner membrane, the TOM complex cooperates with two distinct translocation machineries in the inner membrane, (Neupert, 1997; Rassow et al., 1999). The TIM 23.17 complex mediates the import of precursor proteins which carry on N-terminal presequence into mitochondrial matrix in an ATP - and membrane potential dependent manner (Bauer et al., 1996). The membrane potential $\Delta\Psi$ is required for translocation of the presequence across the

inner membrane and ATP is needed to complete translocation of the mature portion of a percursor. TIM 17 and TIM 23 are parts of a protein conducting channel across the inner membrane that can only accomodate unfolded proteins. The preproteins are not tightly bound within the translocation channel (Berthold et al., 1995). When the presequence emerges from the matrix side of the translocation channel it is bound by mt-Hsp70 which together with its co-chaperone Mge 1p and TIM 44 forms an import motor which then drives further translocation (Review, Neupert, 1997). For import and membrane insertion of a class of hydrophobic carrier proteins such as ADP/ATP carrier into the inner membrane, the TOM complex cooperates with the TIM 22.54 complex via additional soluble components located in the intermembrane space (Sirrenberg et al., 1998; Koehler et al., 1998). Upon entering the intermembrane space, the hydrophobic proteins first bind to the small proteins TIM 9 and TIM 10 and are then handed over to TIM 12. The insertion of the hydrophobic preproteins into the inner membrane is then mediated by TIM 22, which has been shown to interact with TIM 9-10-12 proteins (Sirrenberg et al., 1998). It has now been shown that TOM and TIM complexes of yeast and mammals are conserved and apparently composed of homologous components (Bauer et al., 1999).

Mitochondrial enzymes are highly compartmentalized. The outer membrane is characterised by the presence of monoamine oxidase while the inner membrane contains succinate dehydrogenase (SDH). Malate dehydrogenase (MDH) is the enzyme of the matrix and is present in two forms, cytosolic and mitochondrial (Lehninger et al., 1993).

Within all mammalian cells there are two distinct genomes, one located in the nucleus and the other in the mitochondria. mtDNA encodes for only 13 proteins of the respiratory chain, 22 tRNA and 2 rRNA species. The remaining proteins (around 80) needed in the respiratory chain are encoded by nuclear DNA. Respiratory chain

polypeptides are located in the inner membrane although cytochrome C is loosely associated with this membrane, in the intermembranous space (Fromenty and Pessayre, 1995; Saraste, 1999).

mtDNA is a circular double stranded molecule and it is extremely sensitive to oxidative damage owing to its proximity to the inner membrane, the absence of protective histones and incomplete repair mechanisms in mitochondria (Bogenhagen, 1999). The mitochondrial genome contains very few non-coding sequences, no introns and is not protected by histones and the damage to mitochondrial DNA can be expected to have a significant impact on cell functions. Therefore, as compared to nuclear DNA, mtDNA is more sensitive to be affected under different pathological conditions. Interest in mtDNA damage has risen with the discovery that defects in the mitochondrial genome are associated with several human hereditary diseases such as Kearns-Sayre syndrome, Lebers hereditary optic neuropathy, Pearson's syndrome and some cases of chronic progressive external opthalmoplegia (Wallace, 1992;Taylor, 1992). Additionally accumulations of mutations and deletions in mtDNA with their associated defects in oxidative phosphorylation have been implicated in diabetes, ischemic heart disease, Parkinson's disease, demyelinating polyneuropathy, cancer and aging (Ikebe et al., 1990; Wallace, 1992; Taylor, 1992; Ballinger et al., 1992).

b) Mitochondria and apoptosis

Apoptosis or programmed cell death is an active physiological cell death that controls cell populations during embryogenesis, immune response, hormone regulation and normal tissue homeostasis. Changes in the mechanism of apoptosis are also associated with the pathophysiology of cancer, AIDS or neurodegenerative diseases. The process of apoptosis involves a cascade of biochemical events which in the late stages of the process, leads to activation of specific cysteine proteases called caspases, and finally to characteristic changes in nuclear morphology and DNA fragmentation (Green, 1998).

Although apoptosis is a well-defined morphological process, the biochemical mechanisms involved in it, remain under investigation. It is well known that cellular redox status modulates various aspects of cellular function. Kane et al. (1993) suggested that proto-oncogene Bcl-2; an inhibitor of apoptosis exerts its action by reducing the production of reactive oxygen intermediates (ROI), thus, working as an antioxidant in neurons. Recent reports have emphasised the role of oxidative stress and nuclear DNA damage in apoptosis (Polyak et al., 1997; Vanden Dobbelsteen et al., 1996). In addition, antioxidants have been shown to protect against apoptosis in different experimental models. However, normal apoptosis occurs in very low oxygen environments (Jacobson and Raff, 1995). It has been shown that apoptosis is closely related to mitochondrial impairment also (Zamzami et al., 1996). Although, the possible effect of apoptosis on mtDNA is not known, it has been reported that during the apoptotic process, the following events may take place in mitochondria: mtDNA oxidation, increased mitochondrial peroxide production and cytosolic peroxide levels, early oxidation of the mitochondrial and cytosolic GSSG/GSH couple and decreased mitochondrial membrane potential (Esteve et al., 1999).

c) Mitochondria and aging

Mitochondria has been an adversely affected organelle during aging. The electron transport chain is known to be the major intracellular site for the generation of 0_2 and H_2O_2 and subsequently other potentially deleterious reactive oxygen species (ROS) as well as for ATP production via oxidative phosphorylation (Chance et al., 1979). There is a large body of evidence indicating an age-related increase in the rate of mitochondrial O_2 and H_2O_2 generation and the amounts of oxidative damage to mitochondrial proteins and DNA (Sohal, 1997; Martinez et al., 1996; Shigenega et al., 1994). Oxidative damage to mitochondria has been experimentally demonstrated to cause both an elevation in the rate of mitochondrial ROS, H_2O_2 generation and a derangement of

mitochondrial respiratory activity (Sohal and Dubey, 1994). Furthermore, the rate of mtDNA transcription, as indicated by the levels of cytochrome C oxidase RNA relative to mt DNA also seems to decline in human skeletal muscle during aging (Barrientos et al., 1997) which indicates that mitochondria become progressively more damaged during senescence (Yan et al., 1997). Nevertheless, whether activities of mitochondrial electron transport complexes uniformly decline during aging remains controversial. In human skeletal muscles, Cooper et al. (1992) have reported an age-associated decline in the activities of complex I and IV, whereas Barrientos et al. (1996) found no correlation between specific activities of electron transport complexes and age. Furthermore, the pattern of age related changes in the enzymatic activities of the complexes, observed in the liver mitochondria (Miquel et al., 1995), was dissimilar from that observed in mitochondria isolated from skeletal muscle, heart and brain (Desai et al., 1996; Guerrieri et al., 1993, Bowling et al., 1993; Fernandiz et al., 1994).The mitochondrial theory of aging postulates that organisms age due to the accumulation of DNA damage and mutations in the multiple mitochondrial genomes, leading to mitochondrial dysfunctions. Among the many types of DNA damage, 8-oxodG has received the most attention due to its mutagenicity and because of the possible correlation between its accumulation and pathological processes like cancer, degenerative diseases and aging (Croteau et al., 1999).

d) Mitochondria and calcium regulation

Since calcium ions (Ca^{++}) function as important cellular signals regulating many physiological processes, the cytosolic concentration of Ca^{++} is maintained at very low levels (approximately 10^{-7}M) with the concentration gradient between extra and intracellular environments of the order of 10,000. Cells use multiple mechanisms to tran-

siently increase the Ca^{++} concentration in the cytoplasm, in response to appropriate stimuli (Clapham, 1995; Berridge, 1997). Mitochondria, in addition to their function as cellular power plants, have been recognised to play a central role in Ca^{++} homeostasis and cellular Ca^{++} signalling (Thomas et al., 1996; Simpson and Russell, 1998).

The cell achieves low cytosolic Ca^{++} concentration by actively expelling Ca^{++} ions to the exterior and by judicious compartmentalization of Ca^{++} into specialised cellular stores (Clapham, 1995; Berridge, 1997). Plasma membrane Na^{+}-Ca^{++} exchanger (PMNCE) and a Ca^{++} ATPase pump (PMCA) move Ca^{++} across the plasma membrane to the cell exterior and the sarcoendoplasmic reticulum Ca^{++} pump known as SERCA which moves Ca^{++} ions into ER Ca^{++} stores. In addition, mitochondria have long been recognised to be important in removing cytoplasmic Ca^{++} particularly when the Ca^{++} levels reach above tens of micromolar in concentration in the space around mitochondria (Rizzuto et al., 1993). Thus, mitochondria can act as low-affinity, but high capacity cytosolic Ca^{++} sponges. Furthermore, a number of Ca^{++} binding proteins present in the cytoplasm act as local buffers of Ca^{++} and help to remove signalling Ca^{++} away from its targets. These proteins together with the mitochondria and ER Ca^{++} transport systems function to restrict rapid diffusion of Ca^{++} in the cytosol and contribute to discrete local signals (Simpson and Russell, 1998).

e) Mitochondria and Lipid peroxidation

Lipid peroxidation is a complex process known to occur in both plants and animals. It involves the formation and propagation of lipid radicals, the uptake of oxygen, a rearrangement of the double bonds in unsaturated lipids and the eventual destruction of membrane lipids producing a variety of breakdown products. Lipid peroxidation usually begins with the obstruction of a hydrogen atom from an unsatur-

ated fatty acid resulting in the formation of a lipid radical. An attack by molecular oxygen produces a lipid peroxy radical, which can abstract a hydrogen atom from an adjacent lipid to form a lipid endoperoxide, which can lead to the formation of malondialdehyde as a breakdown product. Malondialdehyde formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for determining the extent of the peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid, which in total is referred to as thiobarbituric acid reacting substance (TBARS) (Buege and Aust, 1978).

The polyunsaturated fatty acids located in the mitochondrial membranes are excellent targets for lipid peroxidations. Conversely, it is known that the lipid environment can affect membrane function (Pamplona et al., 1996), including mitochondrial electron transport which could influence ROS production. Microsomes (Catala et al., 1994) as well as mitochondria are susceptible to lipid peroxidation (Wright et al., 1979 ; Vladimirov et al., 1980) and the measurement of lipid peroxidation has become one of the most commonly used parameter for radical-induced damage (Svingen et al., 1979; Sevanian and Hochstein, 1985).

f) Mitochondria and tumorigenicity

Various studies have suggested a role of cytoplasmic components, including mitochondria, in maintaining the tumorigenic phenotype (Cavalli and Liang, 1998). Because the mitochondrion has the distinctive property of harbouring its own DNA, studies have indicated that the mitochondria may play a direct role in the tumorigenic phenotype. The studies of cytoplasmic fusion of cancer and normal cells have suggested extra-nuclear cellular components can maintain the tumorigenic phenotype (Welter et al., 1989). Creation of mtDNA-less yeast cells can mimic cell surface changes similar to vertebrate malignancy, and in mtDNA-depleted hamster cells, membrane glycopep-

tide profiles have been found to be similar to transformed tumorigenic cells (Wilkie et al., 1983). Other studies have suggested that mutations in the nuclear p53 and *bcl-2* gene are involved in the induction of anchorage-independent growth *in vitro* (Nikiforov et al., 1996; Sierra et al., 1995). However, some evidence has also been provided that mtDNA-less tumor cells withour p53 mutation and *bcl-2* overexpression can loose the anchorage-independent phenotype (Cavalli and Liang, 1998).

The biochemical and ultrastructural events in mitochondria that may eventuate cisplatin-induced effects in the tumor cells/ host remains to be investigated in detail. In the present piece of work involving the effect of cisplatin in the cells/host, mitochondria have been chosen as cellular targets because i) their highly specialized membrane-linked functions are very sensitive to drugs, ii) mitochondria play a central role in cellular homeostasis and the study of the effects of cisplatin might shed light on the importance of mitochondria in cellular toxic effects, and iii) platinum (II) complexes interact with energy-dependent functions in the cells (Beltrame et al., 1984). Thus, in this study investigations were undertaken to look into the changes in mitochondrial protein, glutathione, lipid peroxidation, succinate dehydrogenase activity, malate dehydrogenase activity, oxygen consumption and ultrastructure in relation to tumorous condition and cisplatin treatment. This may help to understand further the significance of mitochondria in the effectiveness of cisplatin in toxicity/cytotoxicity in tumor-bearing mice.

MATERIALS AND METHODS

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MATERIALS AND METHODS

i) Chemicals

Most of the biochemicals were purchased from the Sigma chemical company, St Louis, Mo, USA. However, some specific chemicals obtained from India or other countries has been mentioned accordingly. All other chemicals used in the experiments were of analytical grade. Cisplatin solution (1 mg/ml of 0.89 % NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai, India. Glass double distilled water was always used to prepare various solutions.

ii) Animals and tumor maintenance

Inbred Swiss albino mice were maintained in the laboratory under conventional conditions at room temperature of 20 ± 4^0C with free access to food pellets and water *ad libitum*. Ascites Dalton's lymphoma tumor was maintained *in vivo* in 10-12 weeks old mice of both sexes by serial intraperitoneal (i.p.) transplantations of approximately $1x10^7$ tumor cells per animal (0.25 ml in phosphate-buffered saline (PBS), pH 7.4. Tumor transplanted hosts usually survived for 19-21 days.

iii) Cisplatin treatment

The therapeutic dose of cisplatin against malignant tumors has been established to be 8-10 mg/kg body weight (Rosenberg, 1985). A single dose of cisplatin (8 mg/kg body weight, i.p) was administered to tumor-bearing mice on the 10^{th} day following tumor transplantation which is the approximately mid phase of tumor growth. With the regression of Dalton's lymphoma (DL) tumor, very little ascites is recoverable from the hosts after 6-7 days of cisplatin treatment (Giri, 1995). Thus, the cisplatin treatment schedule for only 4 days i.e., 24, 48, 72 and 96 h was followed. Liver, kidney, testes and spleen were dissected out from the normal, tumor-bearing and cisplatin-treated tumorous mice. Ascites Dalton's lymphoma collected from the mice was centrifuged (2000xg , 4° C, 10 min) and the pellet was used as the DL cells.

iv) Mitochondrial isolation

The particular tissue was homogenized in ice cold 0.25 mol /L sucrose in a teflon pestle tissue homogenizer (Remi instruments, Mumbai, India). The homogenate was then centrifuged (800xg, 4^{0}C, 10 min). The supernatant was decanted and centrifuged (14,000xg, 4^{0}C, 30 min). The sedimented mitochondrial fraction was collected and washed in cold sucrose solution. The mitochondrial pellet was weighed, resuspended in sucrose solution, sonicated (Soniprep-150, MSE, UK) and used for various determinations. Biochemical studies were conducted in the mitochondrial fraction as well as in the corresponding tissue to get a comparative idea about the changes in the cell as a whole and its mitochondrial fraction after cisplatin treatment.

v) Protein Estimation

Protein was estimated following the method of Lowry et al. (1951). Tissues and the mitochondrial fractions were homogenized in 0.25 mol/L sucrose solution. To 1 ml of the homogenate sample in duplicate (after appropriate dilutions), 5 ml of the alkaline solution [50 ml of 20 g/L Na_2CO_3 dissolved in 0.1 mol/L NaOH, + 1 ml of copper sulphate-sodium potassium tartarate solution (5g/L $CuSO_4$-5 H_2O in 10 g/L Na,K tartarate) prepared immediately before use] was added, mixed thoroughly and allowed to stand at room temperature for 15 min. Then 0.5 ml diluted (1:1,v/v) Folin-Ciocalteu reagent was added with immediate mixing. The final solutions were kept at room temperature for 30 min for the completion of the reaction and the optical density was measured at 750 nm against the blank which contained all the reagents except homogenate sample. The protein concentrations were determined from a standard curve prepared in the same way using bovine serum albumin (BSA) as the standard.

vi) Glutathione estimation

Glutathione was determined as non-protein (NPSH) and total (TSH) sulfhydryl contents using the method of Sedlak and Lindsay (1968). Briefly, 5% homogenates of tissues and mitochondria were made in 0.02 mol/L EDTA, pH 4.7. For determination of NPSH, the homogenate (500 µl) was precipitated with 10% trichloroacetic acid (500 µl) and centrifuged (2000xg, 4 °C, 10 min). To the supernatant (800 µl), 1.6 ml of Tris-EDTA buffer (0.4 mol/L, pH 8.9) was added before the addition of 25 µl of Ellman's reagent (10 mmol/L, 5,5'-dithio-bis-2-nitrobenzoic acid in methanol). For the determination of TSH, to the homogenate (100 µl), 1 ml of tris-EDTA buffer (0.2 mol/L, pH 8.2) and 0.9 ml of EDTA solution (0.02 mol/L, pH 4.7) was added which is followed by the addition of 20 µl of Ellman's reagent. After 30 min of incubation at room temperature, the absorbance was read at 412 nm in a Beckman DU- 640 spectrophotometer. Samples for TSH determinations were centrifuged before absorbances of supernatants were measured. To prepare the standard curve pure reduced form of glutathione was taken keeping the similar volume as that of sample.

vii) Assay of lipid peroxidation

The concentration of thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde, was determined using the method of Buege and Aust (1978). Tissues and mitochondria were homogenized (5%) in 0.15 mol/L NaCl. To 1 ml of the homogenate, 2 ml of trichloroacetic acid-thiobarbituric acid-HCl reagent (15 % trichloroacetic acid and 0.375 % thiobarbituric acid dissolved in 0.25 mol/L HCl) was added and mixed thoroughly. The solution was heated in a boiling water bath for 15 min. After cooling at room temperature, the precipitate was removed by centrifugation (1000x g, 4°C, 10 min). The absorbance of the supernatant was read at 535 mm. The malondialdehyde concentration in the samples was calculated using an extinction coefficient of 1.56×10^5 L/mol and has been expressed as nmol/mg protein.

viii) Determination of O_2 consumption

Mitochondrial O_2 consumption was determined polarographically using clark-type oxygen electrodes. The temperature of the system was maintained using an isotemp circulator water bath. The oxygen electrodes and monitor were calibrated with distilled water at the desired temperature (25^0 C). ADP was added to a respiration medium to a final concentration of 100 μM. Necessary substrate (1 mM succinate) was added to an aliquot of the mitochondrial preparation to a final volume of 3 ml. Rates of O_2 consumption was normalized on a per mg motochondrial protein basis. The amount of mitochondrial protein used for the assay was determined by the Lowry (1951) method described in detail above (section-v of methodology). Data are reported as nmol O_2/mg protein/min.

ix) Assay of succinate dehydrogenase (SDH)

SDH activity was assayed in the mitochondrial fraction using the method described by King (1967). Mitochondrial samples were sonicated on ice to prepare a 5 % homogenate in 0.25 mol/L sucrose solution. The reaction mixture contained 0.2 mol/L phosphate buffer, pH 7.8 (0.375 ml), 0.045 mol/L KCN (0.05 ml), 0.6 mol/L succinate (0.1 ml), 0.0015 mol/L dichlorophenolindophenol (DCIP, 0.05 ml), 0.009 mol/L phenazine methosulfate (PMS , 0.15 ml) in a 3 ml spectrophotometer glass cuvette. The volume was made up to 2.95 ml with distilled water. The reaction was started by the addition of 0.05 ml of mitochondrial homogenate. The change in absorbance at 600 nm (Δ600 nm) was recorded at one minute intervals in a Beckman DU-640 spectrophotometer. The Δ600 nm was converted to μmols succinate oxidized by multiplying Δ600 nm by 0.0476.

The unit of enzyme activity was expressed as nmol succinate oxidized/ min and the specific activity as units / mg mitochondrial protein.

x) Assay of malate dehydrogenase (MDH)

MDH activity was assayed in the mitochondrial fractions using the method described by Kitto (1967). Mitochondrial homogenate (5 %) was prepared in 0.25 mol/L sucrose solution by sonication. The reaction mixture contained 0.03 ml of NADH (14.3 mmol/L), 0.05 ml of oxaloacetate (20 mmol/L), enzyme and buffer (potassium phosphate buffer, 0.1 M, pH 7.5) to a final volume of 3.0 ml. The reaction was started by the addition of either oxaloacetate or enzyme. The optical density was read at 340 nm against a blank containing all components of the assay mixture except NADH at one minute intervals. Enzyme activity was calculated from the initial rate of oxidation of NADH. The amount of enzyme used was adjusted to give a decrease in the optical density of approximately 0.04 per min.

The unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 nmol of oxaloacetate per minute and the specific activity as units/mg mitochondrial protein.

xi) Transmission electron microscopy (TEM)

Tissues (liver, kidney, testes) and DL cells pellet collected from animals under varying experimental conditions were used for transmission electron microscopy. The tumor ascites was collected from the peritoneal cavity using a glass syringe with a disposable needle and it was centrifuged (1000xg, 4^0C, 8-10 min). The cells were washed with PBS and collected by centrifugation at 500xg for 5 min at 4^0C. The cell pellet was resuspended in PBS (1 : 4 , w/v).

Tissues and DL cells were fixed in 3% glutaraldehyde (prepared in 0.1 M cacodylate buffer) for 2 h at 4^0C. After fixation, the cells were washed and rinsed in 0.1 M cacodylate buffer. Then the tissues and cells were cut into small pieces and post-fixed in 1% osmium tetroxide for15 min at 4^0C. The post-fixed samples were then dehydrated with an ascending grade of acetone (30-50-70-80-90-95%, twice at each

concentration for 15 min each) at room temperature and finally kept in dry acetone (prepared by adding $CuS0_4$ crystals in excess to absolute acetone and filtered). These were then kept in propylene oxide, twice for 1 hr at room temperature and at different ratio of propylene oxide and embedding medium [(3 : 1, overnight), (1 : 1 , 1 h), (1 : 3, 1 h) and in pure embedding medium twice for 2h at 50^0 C. The embedding medium (araldite) was prepared by thoroughly mixing 10ml of araldite Cy212, 10ml of dodecenyl succinic anhydride (DDSA), 0.4ml of tridimethylamino methyl phenol (DMP 30) and 1.0ml of dibutylphdyhalate. Ultrathin sections (60-80 nm) were cut in an ultramicrotome (ultratome-RMC, MTX, USA) and collected on copper grids. These were stained with lead citrate (5%) and uranyl acetate (5%) (1:1v/v). Then viewing was done in the electron microscope (Jeol electron microscope) operated at a voltage of 80 KV. The grids/sections were scanned and photomicrographs were taken after observing thoroughly different portions of the sections collected on at least four grids.

xiii) Statistical analysis

The significance of various changes at a particular time point of treatment as compared to respective control was determined by Student's *t*-test. The whole group of treatment at various time points was compared using ANOVA. The P value $P \leq 0.05$, was considered as significant.

RESULTS

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i) Protein Determination

a) Tissue protein

Protein contents in the different tissues of the normal mice revealed that kidney has the highest protein concentration (232.19 mg/g) followed by liver (228.56 mg/g), testes (133.7 mg/g) and spleen (116.42 mg/g). As compared to those in normal mice, protein contents of liver and kidney decreased to about 16% and 38% respectively while in testes and spleen an increase (~14% and ~ 45% respectively) was noted in tumor-bearing mice (Table 1).

b) Mitochondrial protein

The highest protein concentration was noted in liver mitochondrial fraction (138.02 mg/g) and it is followed by the mitochondrial fraction of kidney (77.86 mg/g), spleen (48.52 mg/g) and testes (42.48 mg/g) (Table 1). As compared to the corresponding normal tissue, the mitochondrial protein concentration decreased in liver and testes (~22% and ~4% respectively) while in kidney and spleen an increase (6% and 28% respectively) was noted in tumor-bearing mice.

c) Tissue protein in cisplatin-treated mice

Protein concentration in liver did not change significantly after cisplatin treatment. In kidney, the concentration of proteins decreased (~15%) after cisplatin treatment. However, average protein decrease was much greater in DL cells (about 30%) than that in liver (~ 7%) and kidney (about 15%) after 24–96 h of cisplatin treatment (Table 1). In testes an increase (~7%) was seen at 48h of treatment while in spleen it decreased (~13%) at 24h after which it recovers slowly.

d) Mitochondrial protein in cisplatin-treated mice

Cisplatin treatment of tumor-bearing mice resulted in a significant decrease of protein concentration in the mitochondria of all the tissues. The decrease is more significant in liver mitochondria (~31%) at 96h, kidney mitochondria (~38%) at 96h and testes mitochondria (~49%) at 72h of cisplatin treatment. In DL cells mitochondria a very significant decrease (~45%) was observed at 96h of the treatment while in spleen, a decrease (~25%) was seen at 48h of treatment (Table 1). However as compared to that in the corresponding tissues, the decrease was considerably larger in the respective mitochondrial fraction and the maximum decrease was noted in DL cells (Fig.2). However in the testes mitochondria it was found to increased to about 14% after 24h of treatment but decreased during the later period of cisplatin treatment.

ii) GSH Determination

a) Tissue GSH

In the normal mice, liver showed the highest total GSH (TSH) and non-protein thiols (NPSH) concentrations (11.72 and 4.20μmoles/g) followed by testes (10.16 and 4.14μmoles/g), kidney (8.03 and 2.70μmoles/g) and spleen (7.86 and 1.82μmoles/g). As compared to those in normal mice, GSH concentration in liver and kidney of tumor-bearing mice did not change significantly. In spleen, TSH and NPSH concentrations increased significantly to about 27% and 59% while in testes a decrease in NPSH level (~17%) was noted in tumor-bearing mice as compared to the respectives tissue of the normal mice (Table 2).

b) Mitochondrial GSH

The highest mitochondrial-TSH concentration was noted in spleen, liver (~2.65 μmoles/g), followed by kidney (2.28 μmoles/g) and testes (0.82 μmoles/g). Mitochon-

drial-NPSH content was found to be highest in spleen (0.304 μmoles/g) followed by kidney (0.210 μmoles/g), testes (0.090 μmoles/g), and liver (0.071 μmoles/g) (Table 2). As compared to the corresponding normal tissue, mitochondrial-TSH of tumor-bearing mice increased significantly in liver (43%), testes (36%) and spleen (12%) while in kidney a slight decrease was noted (4%). In comparison to the normal tissues, mitochondrial-NPSH in the respective tissue of tumor-bearing mice increased significantly in liver (125%) and testes (44%) while it decreased in kidney (39%) and spleen (21%).

c) Tissue GSH in cisplatin-treated mice

The total GSH concentration of liver and testes showed no significant change after cisplatin treatment for 24-96h. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease in total GSH concentration in kidney (~29%) at 24h, DL cells (~21%) at 24h and in spleen (~19%) at 48 h. The concentration of NPSH decreased in liver (~15%) and testes (~ 20%) at 24h, kidney (~27%) at 24h and spleen (~34%) after 48h of cisplatin treatment (Table 2).

d) Mitochondrial GSH in cisplatin-treated mice

Cisplatin treatment of tumor-bearing mice resulted in a significant decrease of mitochondrial-TSH in liver (~60%), kidney (~35 %) after 48h and spleen (~42%) at 24 h of treatment. Contrary to the changes in other tissues, a significant increase in mitochondrial-TSH was seen in DL cells (~32%) and testes (~58%) at 24h of cisplatin treatment (Table 2). The concentration of mitochondrial-NPSH decreased significantly in liver (~69%) and kidney (29%) at 24h of cisplatin treatment. Mitochondrial-NPSH concentration in kidney was found to recover slowly after 48h of treatment. In spleen there is no significant change in mitochondrial-NPSH after treatment while a significant increase was observed in DL cells (~66%) and testes (~21%) at 24h of treatment.

Comparison of the tissue and its mitochondrial fractions revealed that cisplatin treatment resulted in a very significant decrease of GSH contents in liver mitochondria but not in liver tissue (Fig.3a). TSH contents decreased in kidney as well as kidney mitochondria after the treatment (Fig.3b). However, a sharp increase in kidney mitochondrial-NPSH was observed at 72h of cisplatin treatment (Fig.3b). In DL cells (Fig.3c) and testes (Fig.3d) the treatment resulted in an increase in mitochondrial-GSH. In spleen, mitochondrial-TSH level decreased initially at 24-48h and it recovered later at 72-96h of cisplatin treatment (Fig.3e).

iii) Lipid Peroxidation (LPO)

a) Tissue LPO in tumor-bearing mice

LPO measured in terms of malondialdehyde levels in the different tissues of the normal mice revealed that spleen has the highest LPO concentration (0.87 nmol/mg protein) followed by testes (0.61 nmol/mg protein), kidney (0.35 nmol/mg protein) and liver (0.29 nmol/mg protein). As compared to those in normal mice, LPO concentration in liver and kidney of tumor-bearing mice increased to about 17% and 25% respectively while in testes and spleen it decreased to about 42% and 50% respectively (Table 3).

b) Mitochondrial LPO in normal and tumor-bearing mice

The highest concentration of mitochondrial LPO (1.64 nmol/mg protein) was noted in testes and it is followed by the spleen (1.61 nmol/mgptn), kidney (1.31 nmol/mg protein) and liver (0.67 nmol/mg protein). As compared to the corresponding normal tissues, the mitochondrial LPO concentration of tumor-bearing mice, increased significantly in liver and testes (78% and 37% respectively) while a decrease was noted in kidney and spleen (8% and 38% respectively) (Table 3).

c) Tissue LPO in cisplatin-treated mice

Cisplatin treatment of tumor-bearing mice resulted in a significant increase in LPO concentration in kidney (~25%) at 48h, DL cells (~66%) at 24h, testes (~77%) at 72h and spleen (~50%) at 24-48 h of cisplatin treatment. However in the liver, no significant change could be observed (Table 3).

d) Mitochondrial LPO in cisplatin –treated mice

Cisplatin treatment of tumor-bearing mice resulted in a significant increase in mitochondrial-LPO concentration in liver (~41%) at 48h of treatment, kidney (~40%) at 48h of treatment, DL cells (~91%) at 48h, testes (~23%) at 72h and spleen (~30%) at 24-96h of cisplatin treatment (Table 3). As compared to respective tissue, the more prominent increase in mitochondrial-LPO is seen after cisplatin treatment in liver and kidney (Fig.4a and b). In DL cells there is an overall increase in mitochondrial-LPO although there is a sharp increase in tissue-LPO at 48h of treatment (Fig.4c). In testes and spleen, there is a decrease in mitochondrial-LPO as compared to that in the respective tissue (Fig.4d and e).

iv)Protein, GSH and LPO in ascites supernatant.

To understand if any changes are noticed in the ascites supernatants along with mitochondria and tissue, biochemical determination was done in supernatants also i.e protein, lipid peroxidation and thiol contents.

Cisplatin treatment of tumor-bearing mice resulted in a significant decrease in protein concentration in the ascites supernatant (~16%) at 48h although an overall decrease was observed. Lipid peroxidation was noted to decrease during the initial periods of cisplatin treatment but increased (~25%) at 72h of treatment (Table 4). The total GSH content showed an overall decrease after cisplatin treatment although it is significant at 24h (~46%), 48h (~34%) and 72h (~26%). The content of NPSH decreased significantly after cisplatin treatment at 24h (~60%), 48h (~70%), 72h (~60%) and 96h (~40%) (Table 4, Fig.5).

v) Oxygen consumption

a) O_2 consumption in DL cells

Cisplatin treatment of tumor-bearing mice resulted in a significant increase in O_2 consumption in DL cells (~65%) at 24h of cisplatin treatment. However, at later periods of treatment i.e. at 48-96h, oxygen consumption decreased consistently in a time dependent manner (Table 5, Fig.6).

b) O_2 consumption in the mitochondria of DL cells

After cisplatin treatment, O_2 consumption in mitochondrial fractions of DL cells also showed a similar pattern as that of whole cells. A significant increase in O_2 consumption was observed in the mitochondria of DL cells (~74%) at 24h, after which a consistent decrease can be seen during 48-96h of treatment (Table 5, Fig.6).

vi) Mitochondrial Enzymes

a) Succinate Dehydrogenase (SDH)

Among the tissues of normal mice, testes showed the more SDH activity (10.15 units) followed by kidney (9.28 units), liver (8.93 units) and spleen (3.70 units) (Table 6). As compared to the respective tissue of normal mice, the unit of SDH activity in the tissue of tumor- bearing mice, increased in kidney and testes to about 21% and 23% respectively while it decreased in liver and spleen to about 20% and 15% respectively. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease of SDH activity in kidney (~38%) at 24h, DL cells (~30%) at 24-48h, testes (~41%) at 72h and spleen (~32%) at 48-72h of cisplatin treatment. In liver, an increase in the units of SDH activity (~30%) was observed after 48-72h of cisplatin treatment (Fig.7).

b) Malate Dehydrogenase (MDH)

In the normal mice, liver was found to have higher MDH activity (2.52 units) than other tissues, kidney (1.84 units), testes (1.32 units) and spleen (1.10 units) (Table 7). As compared to the corresponding normal tissue, the units of MDH activity in the tumor-bearing mice increased in kidney to about 34% while it decreased in liver, testes and spleen to about 13%, 7% and 27% respectively. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease in the units of MDH activity in liver (~42%) at 24h, kidney (~43%) at 24-48h and DL cells (~20%) at 24h. However, the units of MDH activity increased significantly in testes (~20%) at 24h and in spleen (~ 62%) at 48h of cisplatin treatment (Fig.8).

vii) Transmission Electron Microscopy (TEM)

a) Liver

In control, the normal mitochondrial structure showing elongated shape with regular pattern of cristae was observed (Fig.9a). After cisplatin treatment of mice for 24-94h irregularities in the arrangement of mitochondrial cristae developed along with its thickening (Fig.9c) and disruptions in some parts of the mitochondrial membrane (Fig.9e).

b) Kidney

In control, the normal, regular pattern of mitochondrial cristae with elongated shapes were observed (Fig.10a). Cisplatin treatment of tumor-bearing mice resulted in the appearance of the more roundish mitochondria (Fig.10b and c) with thickened membranes and reduction in the number of cristae along with the formation of some vacuoles (Fig. 10d and e).

c) Testes

Different morphological variations of the mitochondria with more roundish appearance was seen in the control (Fig.11a). As compared to other tissues, early disruption or disappearance of cristae was observed after 24h and 48h of cisplatin treatment (Fig.11b and c). Formation of vacuoles along with the thickening of cristae (Fig.11d) and deformations in the elongated structure of the mitochondria was also noticed (Fig.11e).

d) DL Cells

Different morphological forms of the mitochondria was observed in the control (Fig.12a). After cisplatin treatment, irregularities in cristae shapes and disruptions in some parts of the mitochondrial membrane was observed (Fig.12b and c). Thickening of cristae and formation of vacuoles were also prominent during later periods of treatment (Fig.12d and e).

FIGURES AND TABLES
FIGURES (1 - 12)
TABLES (1 -7)

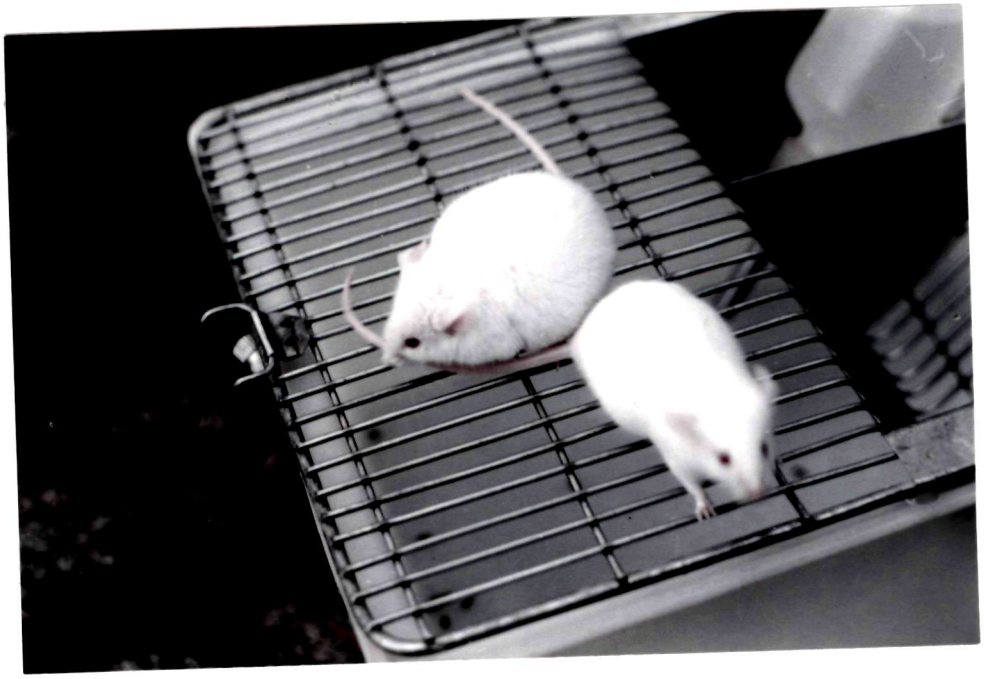
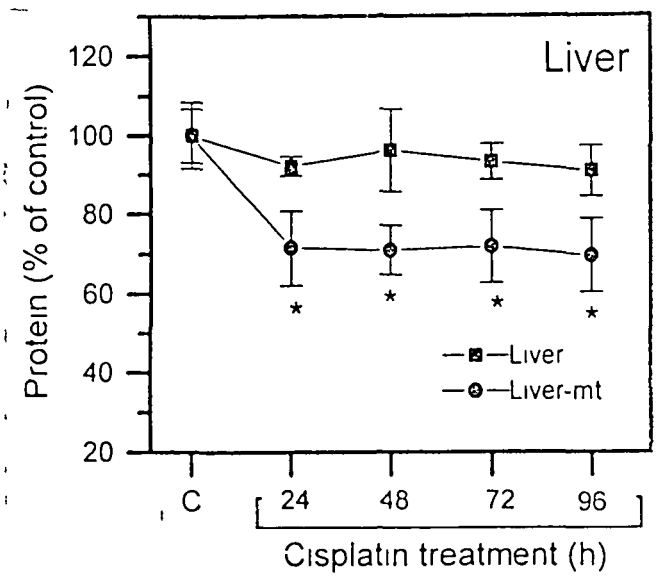


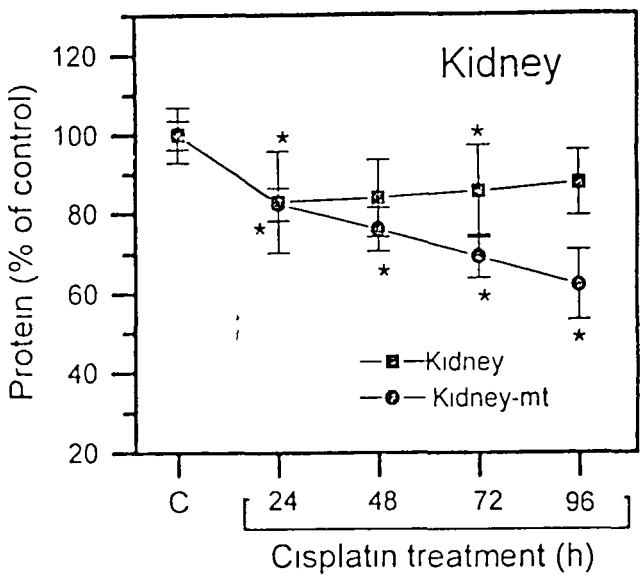
Table 1: Total protein content (mg/g wet weight) in the tissues and their mitochondrial fractions (mean ± SD)

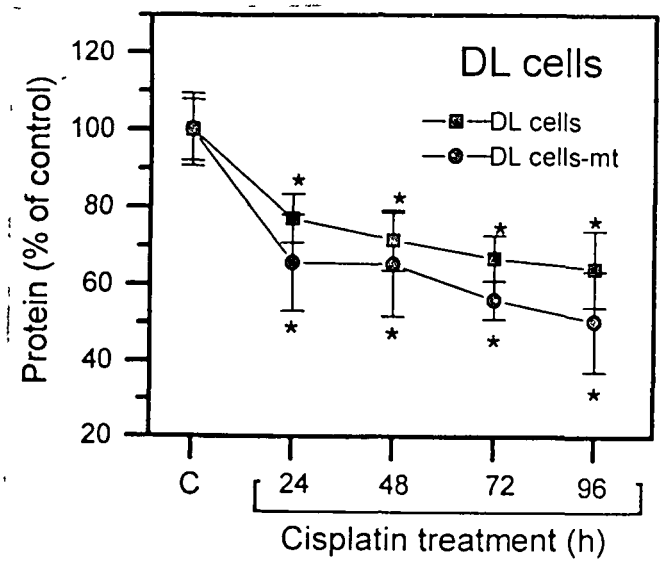
Tissues	Treatment	Tissue protein	Mt-protein
Liver	Normal	228.56 ± 33.04	138.02 ± 7.73
	Tumor-bearing(control)	197.69 ± 13.57	107.42 ± 9.13
	Cisplatin (24h)	182.10 ± 4.45	76.73 ± 7.20[a]
	Cisplatin (48h)	190.06 ± 19.95	76.06 ± 4.72[a]
	Cisplatin (72h)	184.40 ± 8.50	77.12 ± 7.00[a]
	Cisplatin (96h)	179.80 ± 11.50	74.60 ± 6.87[a]
Kidney	Normal	232.19 ± 29.53	77.86 ± 1.16
	Tumor-bearing(control)	168.33 ± 6.09	82.73 ± 5.84
	Cisplatin (24h)	139.67 ± 17.86[a]	68.16 ± 2.80[a]
	Cisplatin (48h)	141.34 ± 13.78[a]	62.90 ± 3.42[a]
	Cisplatin (72h)	144.15 ± 16.83	57.26 ± 3.11[a]
	Cisplatin (96h)	147.60 ± 12.34	51.34 ± 4.57[a]
DL cells	Tumor-bearing(control)	153.84 ± 12.06	59.26 ± 5.53
	Cisplatin (24h)	118.55 ± 7.51[a]	38.84 ± 4.87[a]
	Cisplatin (48h)	109.90 ± 8.65[a]	38.62 ± 5.20[a]
	Cisplatin (72h)	102.76 ± 6.04[a]	33.14 ± 1.64[a]
	Cisplatin (96h)	98.65 ± 9.75[a]	29.86 ± 3.93[a]
Testes	Normal	133.70 ± 15.77	42.48 ± 4.35
	Tumor-bearing(control)	152.40 ± 14.55	40.80 ± 4.09
	Cisplatin (24h)	117.10 ± 12.93[a]	46.53 ± 3.65
	Cisplatin (48h)	163.90 ± 15.05	34.79 ± 2.05[a]
	Cisplatin (72h)	126.85 ± 13.54[a]	29.07 ± 1.62[a]
	Cisplatin (96h)	120.98 ± 12.57[a]	33.46 ± 4.32[a]
Spleen	Normal	116.42 ± 6.30	48.52 ± 4.97
	Tumor-bearing(control)	168.97 ± 8.34	62.19 ± 4.81
	Cisplatin (24h)	147.31 ± 14.68[a]	53.34 ± 6.67[a]
	Cisplatin (48h)	174.50 ± 9.50	46.77 ± 7.71[a]
	Cisplatin (72h)	191.85 ± 5.46[a]	54.51 ± 2.23[a]
	Cisplatin (96h)	187.23 ± 8.59[a]	49.82 ± 5.03[a]

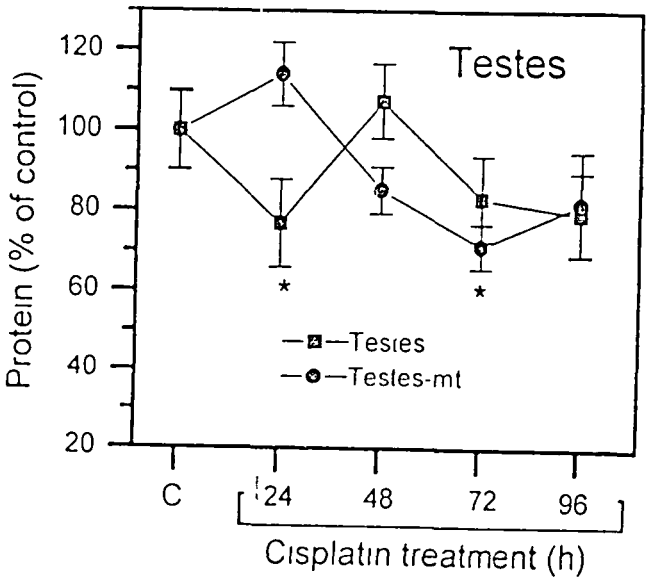
Cisplatin (8mg/kg body wt) was administered to tumor-bearing mice. Student's t-test, n = 6, as compared to the respective control, [a]$P \leq 0.05$. The significance of total changes between the control and cisplatin-treated groups was tested by ANOVA and it showed that protein contents decreased significantly ($P \leq 0.05$) in kidney, DL cells, testes and the mitochondrial fractions of liver, kidney, DL cells and spleen after cisplatin treatment.

Fig. 2. Graph showing the comparative percent changes in protein in the tissues (Liver, Kidney, Dalton's lymphoma cells, Testes and Spleen) and its mitochondrial fractions of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control, tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). *$P \leq 0.05$. It may be observed that mitochondrial protein increased significantly in liver, kidney and testes although an overall increase in mitochondrial protein was observed in all the tissues.


Liver
Protein (% of control)
120
100
80
60
40
20
C
24
48
72
96
Cisplatin treatment (h)
Liver
Liver-mt


Kidney
Protein (% of control)
120
100
80
60
40
20
C
24
48
72
96
Cisplatin treatment (h)
Kidney
Kidney-mt


DL cells
Protein (% of control)
120
100
80
60
40
20
C
24
48
72
96
Cisplatin treatment (h)
DL cells
DL cells-mt


Testes
Protein (% of control)
120
100
80
60
40
20
C
24
48
72
96
Cisplatin treatment (h)
Testes
Testes-mt

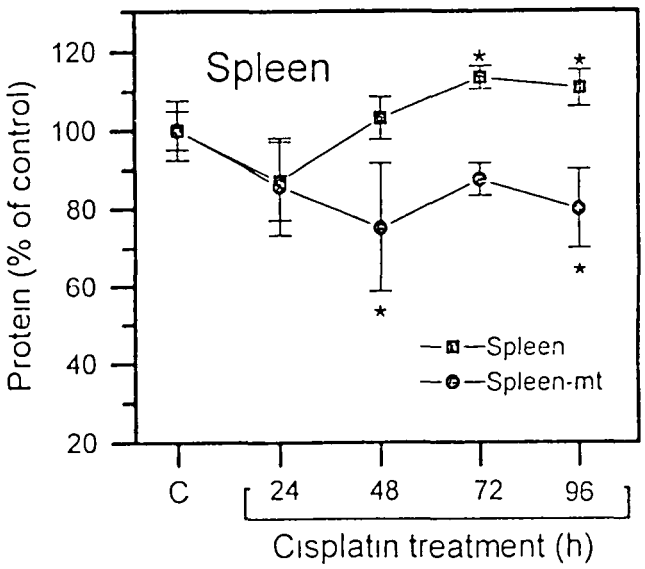
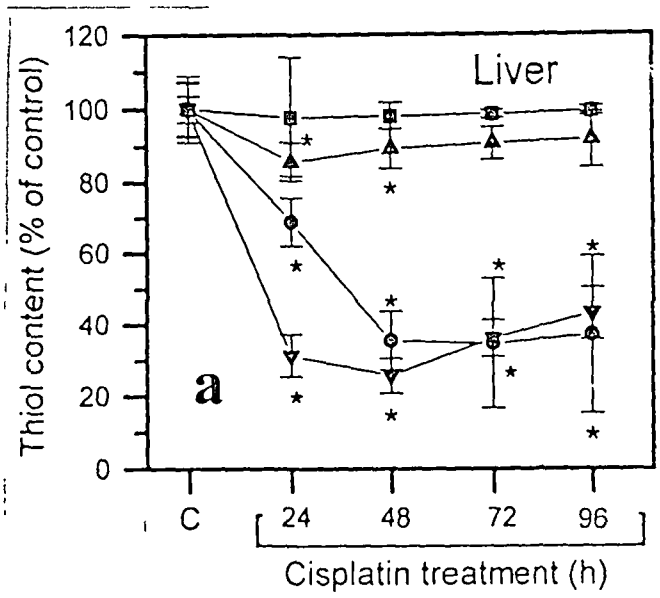

Spleen
Protein (% of control)
120
100
80
60
40
20
C
24
48
72
96
Cisplatin treatment (h)
Spleen
Spleen-mt

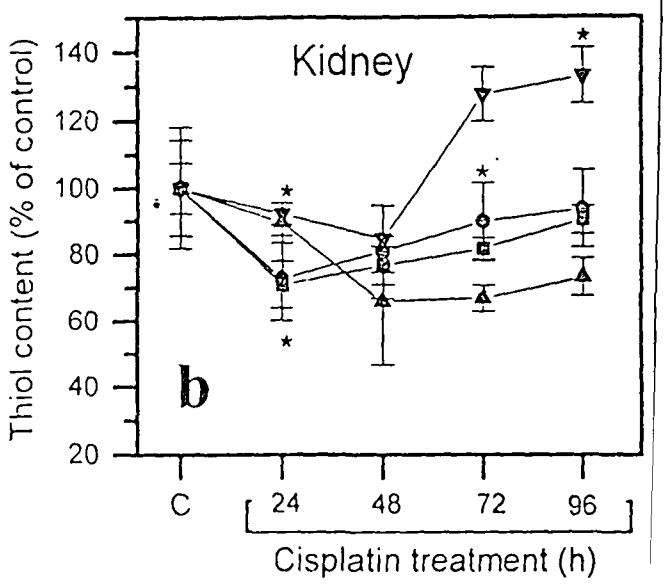
Table 2: Total (TSH) and nonprotein (NPSH) glutathione concentration (μmol/g wet weight)in the mitochondrial fractions and tissues of mice, (mean ± SD).

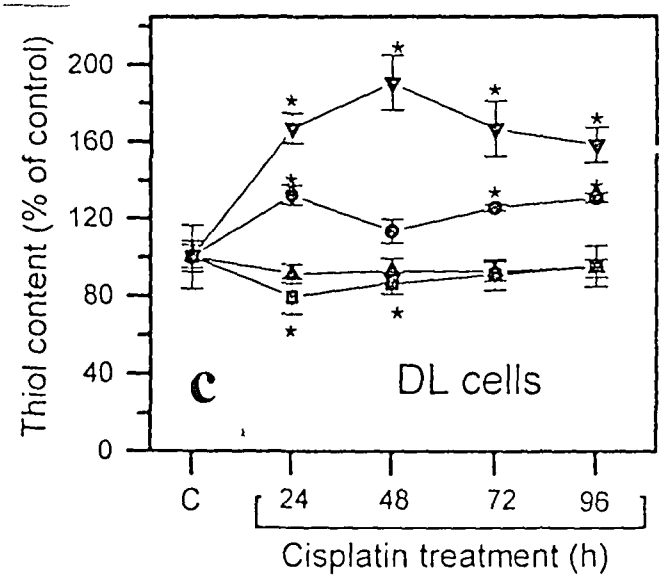
Tissues	Treatment	TSH	NPSH	mt-TSH	mt-NPSH
Liver	Normal	11.72 ± 1.50	4.20 ± 0.80	2.65 ± 0.26	0.071 ± 0.002
	Tumor-bearing (control)	11.91 ± 1.06	4.50 ± 0.16	3.80 ± 0.27	0.160 ± 0.012
	Cisplatin (24h)	11.64 ± 1.88	3.85 ± 0.20[a]	2.61 ± 0.18[a]	0.050 ± 0.003[a]
	Cisplatin (48h)	11.68 ± 0.42	4.01 ± 0.22[a]	1.35 ± 0.11[a]	0.041 ± 0.002[a]
	Cisplatin (72h)	11.75 ± 0.13	4.08 ± 0.18[a]	1.32 ± 0.24[a]	0.058 ± 0.003[a]
	Cisplatin (96h)	11.84 ± 0.15	4.12 ± 0.31	1.41 ± 0.31[a]	0.069 ± 0.005[a]
Kidney	Normal	8.03 ± 1.58	2.70 ± 0.37	2.28 ± 0.37	0.210 ± 0.031
	Tumor-bearing (control)	8.50 ± 1.22	3.02 ± 0.55	2.20 ± 0.40	0.130 ± 0.010
	Cisplatin (24h)	6.04 ± 0.42[a]	2.20 ± 0.28	1.97 ± 0.12	0.120 ± 0.004
	Cisplatin (48h)	6.52 ± 0.37	2.44 ± 0.34	1.45 ± 0.28	0.110 ± 0.011
	Cisplatin (72h)	6.94 ± 0.22	2.71 ± 0.32	1.47 ± 0.06[a]	0.166 ± 0.013
	Cisplatin (96h)	7.68 ± 0.33	2.83 ± 0.33	1.61 ± 0.09	0.173 ± 0.014[a]
DL cells	Tumor-bearing (control)	4.43 ± 0.26	0.67 ± 0.11	2.27 ± 0.18	0.084 ± 0.005
	Cisplatin (24h)	3.51 ± 0.32[a]	0.61 ± 0.03	3.00 ± 0.15[a]	0.140 ± 0.011[a]
	Cisplatin (48h)	3.82 ± 0.21[a]	0.62 ± 0.04	2.57 ± 0.16	0.160 ± 0.023[a]
	Cisplatin (72h)	4.02 ± 0.32	0.62 ± 0.03	2.85 ± 0.05[a]	0.140 ± 0.020[a]
	Cisplatin (96h)	4.21 ± 0.45	0.63 ± 0.03	2.97 ± 0.07[a]	0.133 ± 0.012[a]
Testes	Normal	10.16 ± 0.28	4.14 ± 0.16	0.82 ± 0.08	0.090 ± 0.010
	Tumor-bearing (control)	9.28 ± 0.15	3.46 ± 0.18	1.12 ± 0.02	0.130 ± 0.002
	Cisplatin (24h)	9.03 ± 0.29	2.81 ± 0.20[a]	1.77 ± 0.10[a]	0.158 ± 0.010[a]
	Cisplatin (48h)	9.08 ± 0.23	2.89 ± 0.42[a]	2.37 ± 0.17[a]	0.140 ± 0.001[a]
	Cisplatin (72h)	9.10 ± 0.33	3.04 ± 0.36	2.28 ± 0.10[a]	0.157 ± 0.006[a]
	Cisplatin (96h)	9.11 ± 0.39	3.10 ± 0.22	1.98 ± 0.15[a]	0.147 ± 0.007[a]
Spleen	Normal	7.86 ± 1.64	1.82 ± 0.47	2.68 ± 0.09	0.304 ± 0.020
	Tumor-bearing (control)	9.95 ± 0.14	2.91 ± 0.83	3.00 ± 0.12	0.240 ± 0.150
	Cisplatin (24h)	9.80 ± 0.14	2.10 ± 0.19[a]	1.73 ± 0.14[a]	0.228 ± 0.130[a]
	Cisplatin (48h)	8.13 ± 0.12[a]	2.02 ± 0.07[a]	2.14 ± 0.05[a]	0.247 ± 0.007[a]
	Cisplatin (72h)	9.10 ± 0.11	1.95 ± 0.11[a]	3.04 ± 0.07	0.248 ± 0.011[a]
	Cisplatin (96h)	8.88 ± 0.17[a]	1.89 ± 0.14[a]	2.83 ± 0.08	0.232 ± 0.009[a]

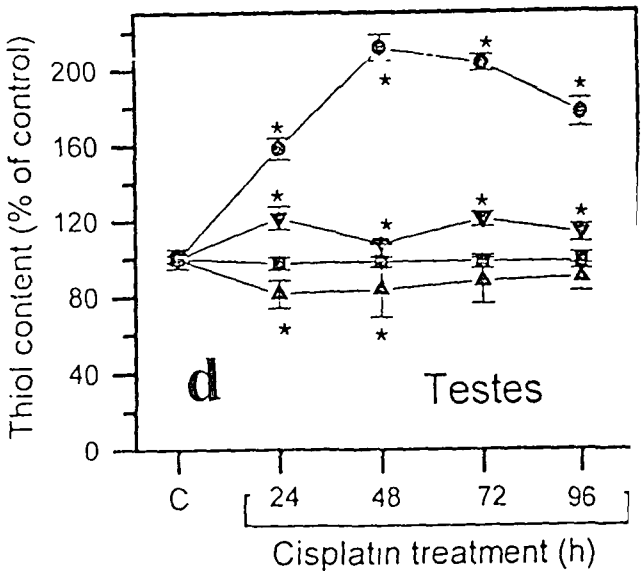
Cisplatin treatment same as in Table 1. Student's t-test, n = 6, as compared to the respective control, [a]P ≤ 0.05. The significance of variation between the control and cisplatin-treated groups was tested by ANOVA and it showed that thiol contents decreased significantly (P≤0.05) in the mitochondrial fractions of liver and kidney after cisplatin treatment.

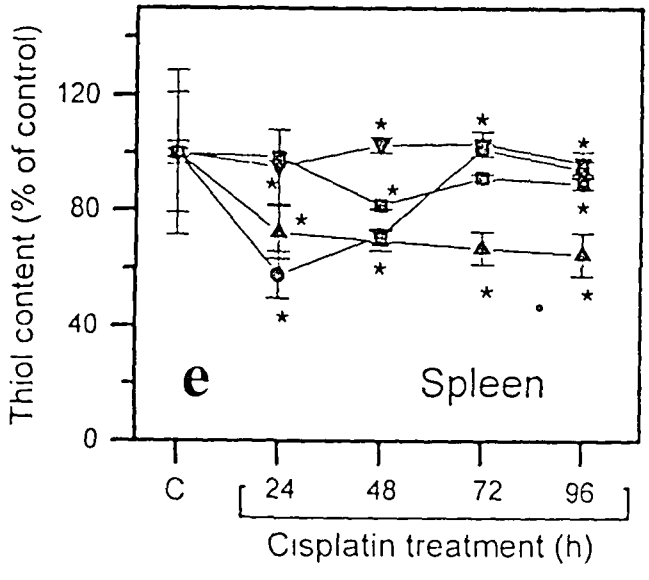
Fig. 3. Graph showing the comparative percent changes in total GSH and non-protein thiol (NPSH) content in the tissues (Liver, Kidney, Dalton's lymphoma cells, Testes and Spleen) and its mitochondrial fractions of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control, tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). *$P \leq 0.05$. It may be observed that glutathione content decreased in the mitochondria of liver and kidney while it increased in DL cells and testes.


Liver
a
Thiol content (% of control)
120
100
80
60
40
20
0
C
24
48
72
96
Cisplatin treatment (h)


Kidney
b
Thiol content (% of control)
140
120
100
80
60
40
20
C
24
48
72
96
Cisplatin treatment (h)


DL cells
c
Thiol content (% of control)
200
160
120
80
40
0
C
24
48
72
96
Cisplatin treatment (h)


Testes
d
Thiol content (% of control)
200
160
120
80
40
0
C
24
48
72
96
Cisplatin treatment (h)


Spleen
e
Thiol content (% of control)
120
80
40
0
C
24
48
72
96
Cisplatin treatment (h)

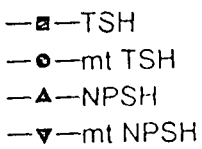
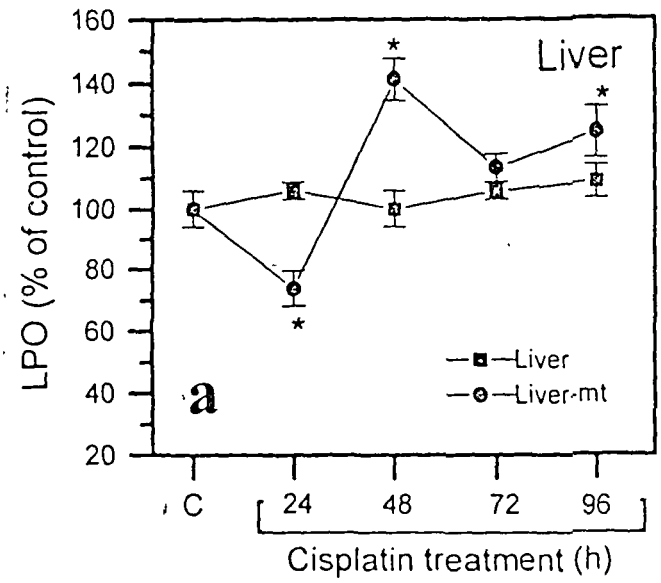

TSH
mt TSH
NPSH
mt NPSH

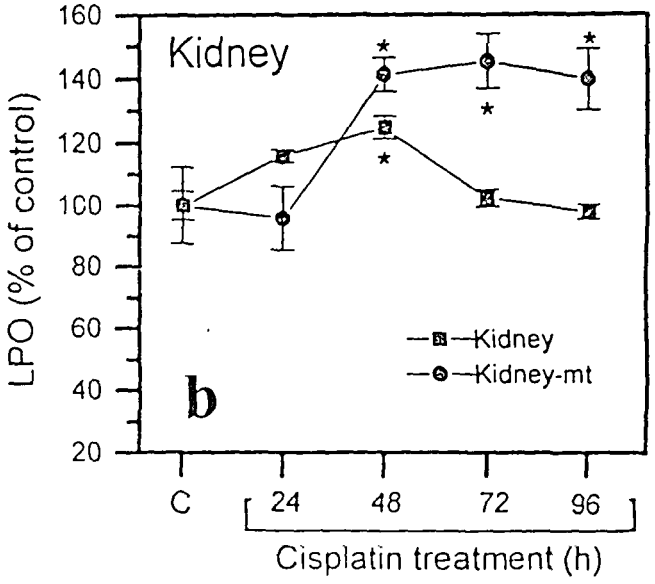
Table 3: Total lipid peroxide (LPO) content (nmol/mg protein) in the tissues and their mitochondrial fractions (mean±SD).

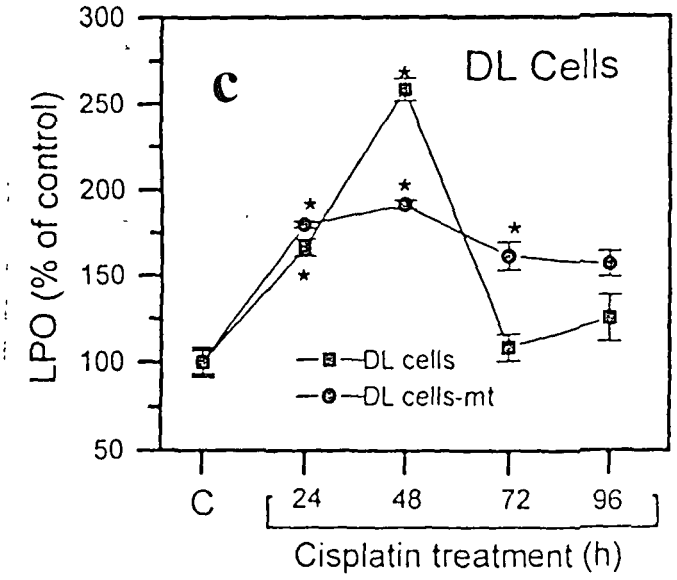
Tissues	Treatment	Tissue LPO	mt-LPO
Liver	Normal	0.29 ± 0.01	0.67 ± 0.02[a]
	Tumor-bearing(control)	0.34 ± 0.02	1.19 ± 0.07
	Cisplatin (24h)	0.36 ± 0.01	0.88 ± 0.05[a]
	Cisplatin (48h)	0.34 ± 0.02	1.68 ± 0.11[a]
	Cisplatin (72h)	0.36 ± 0.01	1.35 ± 0.06
	Cisplatin (96h)	0.37 ± 0.02	1.48 ± 0.12[a]
Kidney	Normal	0.35 ± 0.01	1.31 ± 0.08
	Tumor-bearing(control)	0.44 ± 0.02	1.21 ± 0.15
	Cisplatin (24h)	0.51 ± 0.01	1.16 ± 0.12
	Cisplatin (48h)	0.55 ± 0.02[a]	1.71 ± 0.09[a]
	Cisplatin (72h)	0.45 ± 0.01	1.76 ± 0.15[a]
	Cisplatin (96h)	0.43 ± 0.01	1.69 ± 0.16[a]
DL cells	Tumor-bearing(control)	0.12 ± 0.01	0.60 ± 0.04
	Cisplatin (24h)	0.20 ± 0.01[a]	1.08 ± 0.02[a]
	Cisplatin (48h)	0.31 ± 0.02[a]	1.15 ± 0.03[a]
	Cisplatin (72h)	0.13 ± 0.01	0.97 ± 0.08[a]
	Cisplatin (96h)	0.15 ± 0.02	0.94 ± 0.07
Testes	Normal	0.61 ± 0.04	1.64 ± 0.15
	Tumor-bearing(control)	0.35 ± 0.01	2.24 ± 0.05
	Cisplatin (24h)	0.60 ± 0.02[a]	1.46 ± 0.07[a]
	Cisplatin (48h)	0.43 ± 0.01	1.68 ± 0.10[a]
	Cisplatin (72h)	0.62 ± 0.02[a]	2.76 ± 0.13[a]
	Cisplatin (96h)	0.58 ± 0.01[a]	2.53 ± 0.09
Spleen	Normal	0.87 ± 0.05	1.61 ± 0.10
	Tumor-bearing (control)	0.43 ± 0.06	0.99 ± 0.11
	Cisplatin (24h)	0.66 ± 0.01[a]	1.30 ± 0.06[a]
	Cisplatin (48h)	0.65 ± 0.02[a]	1.45 ± 0.08[a]
	Cisplatin (72h)	0.52 ± 0.01	1.33 ± 0.08[a]
	Cisplatin (96h)	0.56 ± 0.04[a]	1.28 ± 0.07[a]

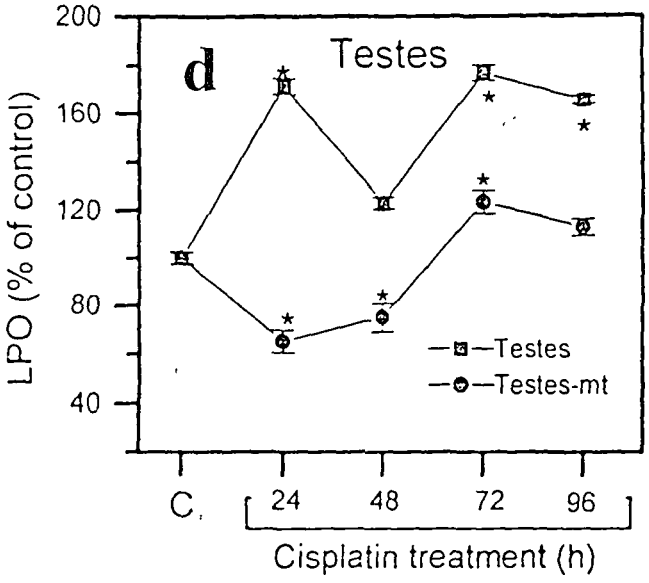
Cisplatin (8mg/kg body wt) was administered to tumor-bearing mice. Student's t-test, n = 6, as compared to the respective control, [a]$P \leq 0.05$. The significance of total changes between the control and cisplatin-treated groups was tested by ANOVA and it showed that mitochondrial lipid peroxidation increased significantly ($P \leq 0.05$) in all the tissues after cisplatin treatment.

Fig. 4. Graph showing the comparative percent changes in Lipid peroxidation (LPO) in the tissues (Liver, Kidney, Dalton's lymphoma cells, Testes and Spleen) and the respective mitochondrial fractions of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). *$P \leq 0.05$. It may be observed that mitochondrial lipid peroxidation (LPO) increased in all the five tissues studied.


Liver
LPO (% of control)
160
140
120
100
80
60
40
20
a
—Liver
—Liver-mt
C
24
48
72
96
Cisplatin treatment (h)


Kidney
LPO (% of control)
160
140
120
100
80
60
40
20
b
—Kidney
—Kidney-mt
C
24
48
72
96
Cisplatin treatment (h)


DL Cells
LPO (% of control)
300
250
200
150
100
50
c
—DL cells
—DL cells-mt
C
24
48
72
96
Cisplatin treatment (h)


Testes
LPO (% of control)
200
160
120
80
40
d
—Testes
—Testes-mt
C
24
48
72
96
Cisplatin treatment (h)

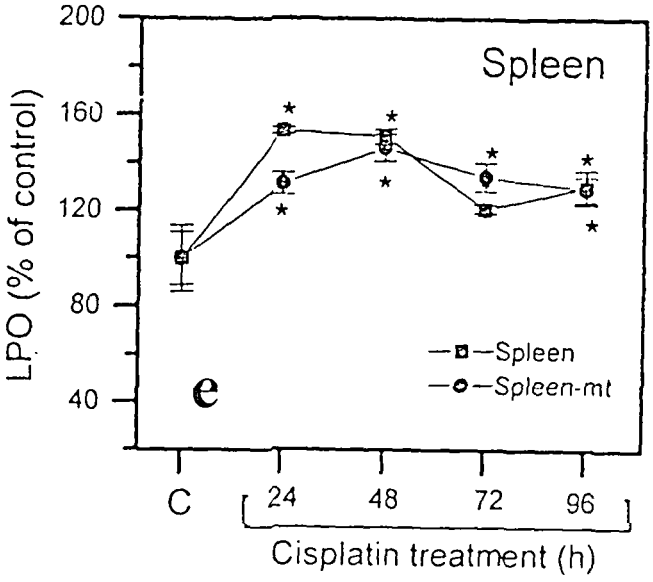

Spleen
LPO (% of control)
200
160
120
80
40
e
—Spleen
—Spleen-mt
C
24
48
72
96
Cisplatin treatment (h)

Table 4: Protein, GSH and LPO in the ascites supernatant of tumor-bearing mice (mean ± SD) .

Treatment	Protein	Lipid peroxidation	TSH	NPSH
Tumor-bearing (Control)	39.86±4.51	2.11 ± 0.08	0.30±0.10	0.01±0.001
Cisplatin (24h)	36.91±4.47	1.93 ± 0.10[a]	0.16±0.03[a]	0.004±0.001[a]
Cisplatin (48h)	33.70±4.53[a]	1.79 ± 0.12[a]	0.20±0.01[a]	0.003±0.001[a]
Cisplatin (72h)	37.15±4.52	2.63 ± 0.33	0.22±0.04[a]	0.004±0.001[a]
Cisplatin (96h)	36.43±3.25	1.87 ± 0.11[a]	0.29±0.01	0.006±0.001[a]

Cisplatin (8mg/kg body wt) was administered to tumor-bearing mice. Student's t-test, n = 6, as compared to the respective control, [a]$P \leq 0.05$. The significance of total changes between the control and cisplatin-treated groups was tested by ANOVA and it showed that protein, thiol contents and lipid peroxidation decreased significantly ($P \leq 0.05$) in the ascites supernatant after cisplatin treatment.

Fig. 5. Histogram showing the percent change in protein, LPO and total GSH in the ascites supernatant of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). $P \leq 0.05$. It may be observed that cisplatin treatment of tumor-bearing mice resulted in a significant decrease in protein concentration in the ascites supernatant. Lipid peroxidation decreased initially while thiol content decreased significantly after cisplatin treatment in the supernatant.

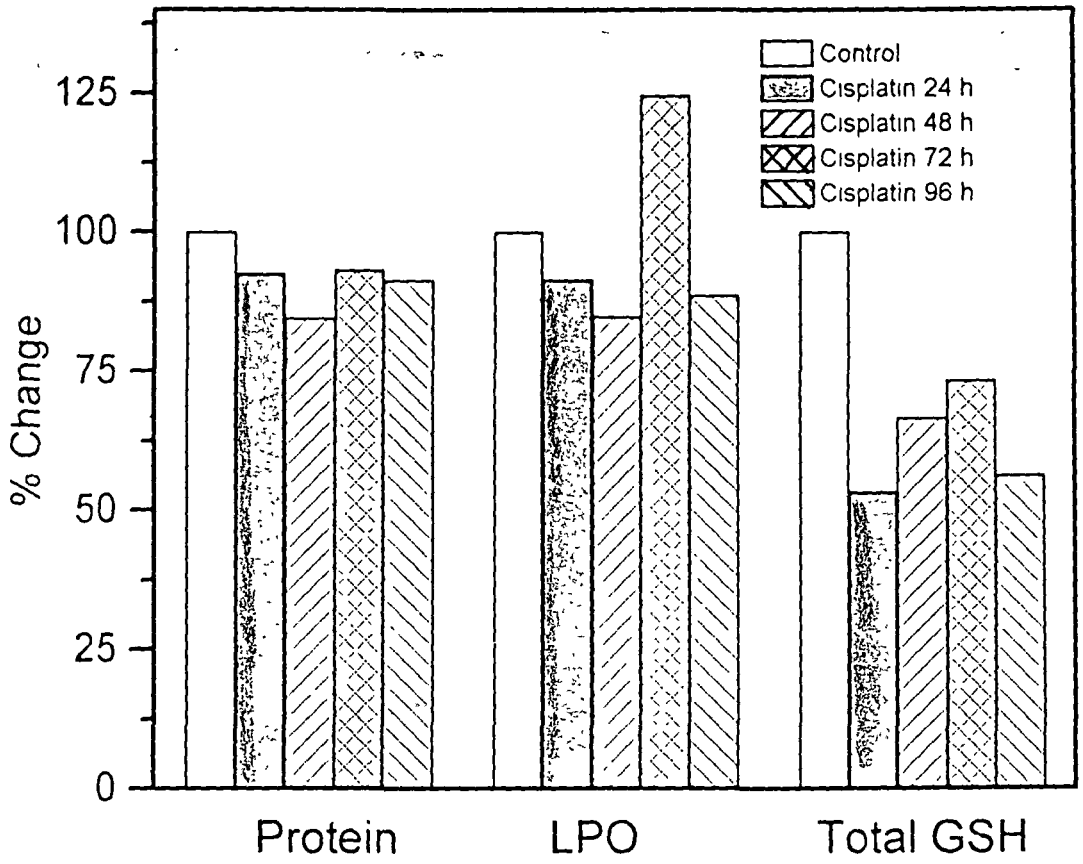

Control
Cisplatin 24 h
Cisplatin 48 h
Cisplatin 72 h
Cisplatin 96 h
% Change
125
100
75
50
25
0
Protein
LPO
Total GSH

Table 5 : Oxygen consumption (nmol/mg protein/min) in the DL cells and their mitochondrial fraction (mean ± SD).

Treatment	DL Cells	DL Cells Mitochondria
Tumor-bearing (Control)	3.31 ± 0.22	10.00 ± 0.56
Cisplatin (24h)	5.47 ± 0.28[a]	17.40 ± 0.71[a]
Cisplatin (48h)	3.95 ± 0.20	11.35 ± 0.61
Cisplatin (72h)	2.81 ± 0.26[a]	9.57 ± 1.02
Cisplatin (96h)	2.75 ± 0.16	8.62 ± 0.73[a]

Cisplatin (8mg/kg body wt) was administered to tumor-bearing mice. Student's t-test, n = 6, as compared to the respective control, [a]$P \leq 0.05$. The significance of total changes between the control and cisplatin-treated groups was tested by ANOVA and it showed that oxygen consumption decreased significantly ($P \leq 0.05$) in the DL cells and its mitochondrial fraction after cisplatin treatment.

Fig. 6. Graph showing the comparative percent changes in the rate of oxygen consumption in Dalton's lymphoma cells and its mitochondrial fraction of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). *$P \leq 0.05$. It may be observed that cisplatin treatment of tumor-bearing mice resulted in an initial increase in oxygen consumption in DL cells and its mitochondrial fraction but decreased at later time points in a time dependent manner.

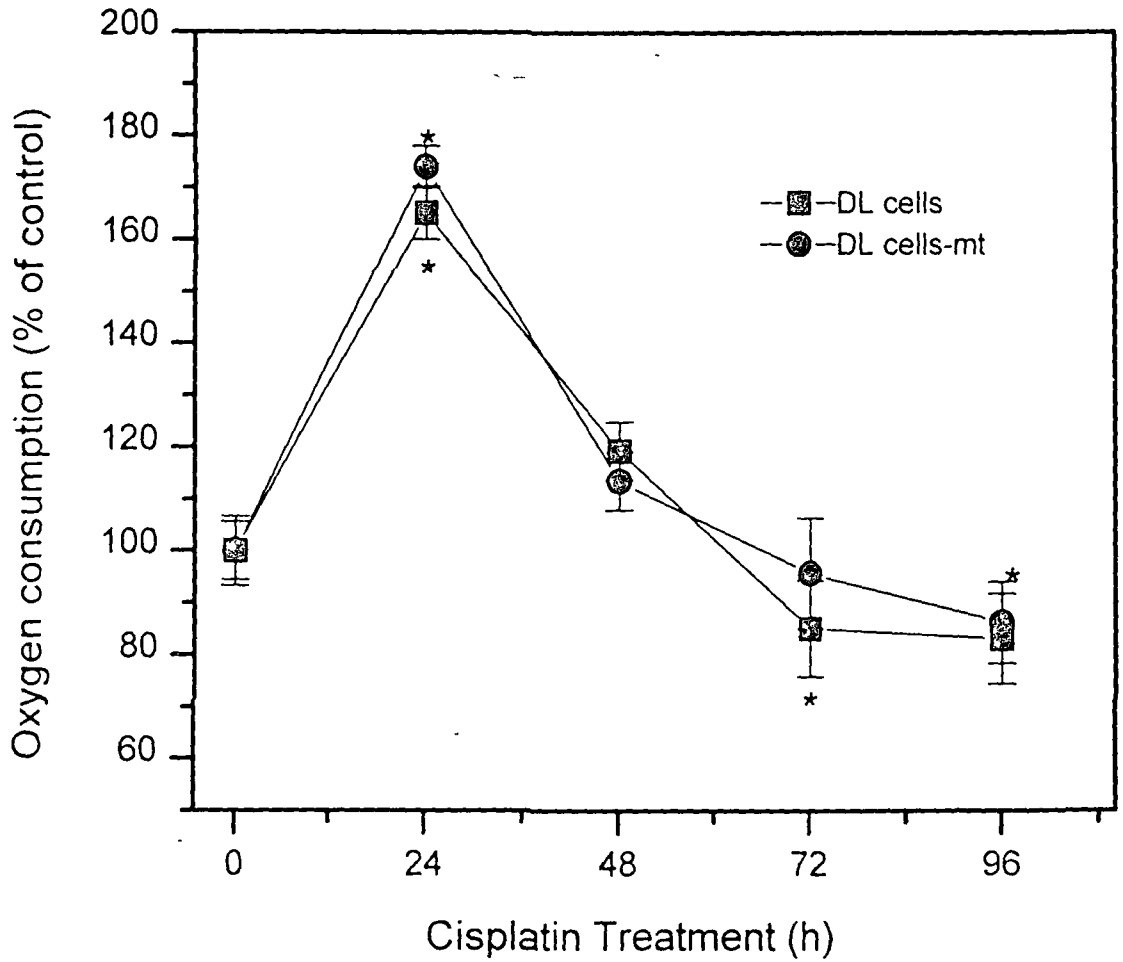
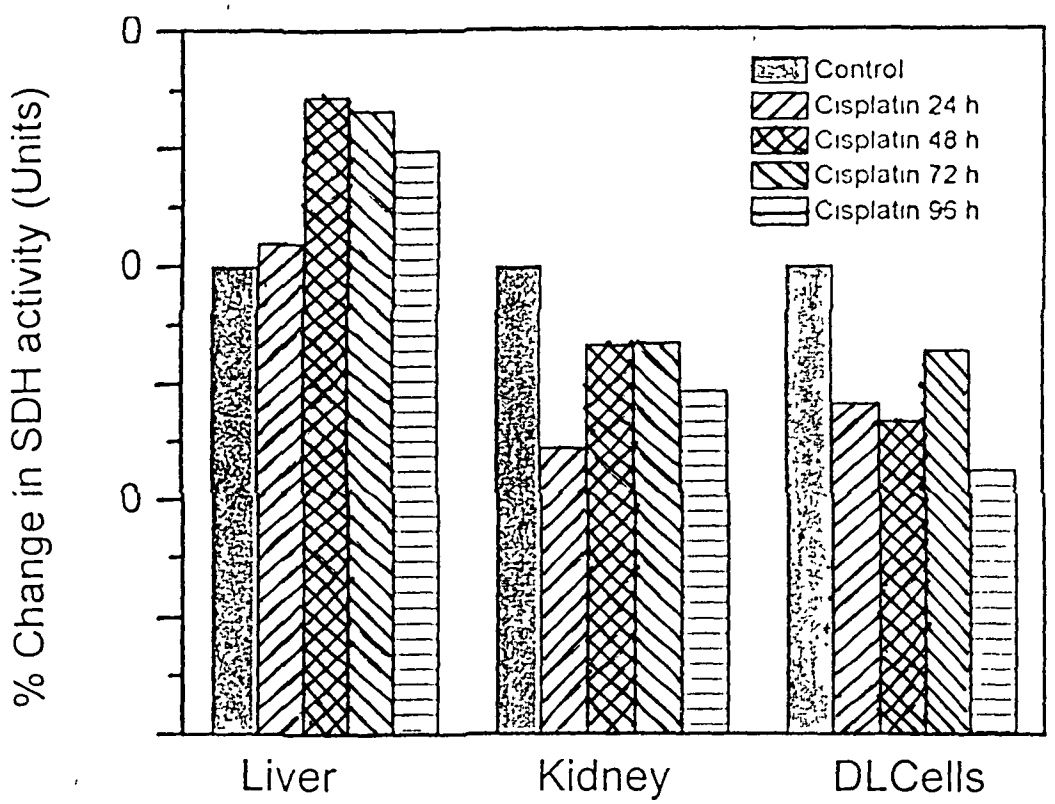

200
180
160
140
120
100
80
60
Oxygen consumption (% of control)
0
24
48
72
96
Cisplatin Treatment (h)
DL cells
DL cells-mt
*
*
*
*

Table 6: Units and Specific activity (mean ± SD) of succinate dehydrogenase (SDH) in the mitochondrial fraction of different tissues

Tissues	Treatment	Units	Specific activity
Liver	Normal	8.93 ± 0.64	25.89 ± 1.86
	Tumor-bearing(control)	7.16 ± 2.18	26.70 ± 8.14
	Cisplatin (24h)	7.52 ± 3.24	39.38 ± 16.96
	Cisplatin (48h)	9.75 ± 1.36[a]	51.33 ± 7.17[a]
	Cisplatin (72h)	9.54 ±1.41[a]	49.47 ± 7.31[a]
	Cisplatin (96h)	8.92 ± 2.32	47.83 ± 5.63[a]
Kidney	Normal	9.28 ± 0.72	47.66 ± 3.69
	Tumor-bearing(control)	11.26 ± 1.66	54.66 ± 8.05
	Cisplatin (24h)	6.89 ± 0.29[a]	41.43 ± 3.01[a]
	Cisplatin (48h)	9.38 ± 0.71[a]	59.73 ± 5.81
	Cisplatin (72h)	9.43 ± 0.45[a]	63.6 ± 3.76
	Cisplatin (96h)	8.28 ± 0.60[a]	64.65 ± 7.46
DL cells	Tumor-bearing(control)	10.49 ± 2.38	70.88 ± 16.10
	Cisplatin (24h)	7.42 ± 2.01[a]	76.36 ± 24.77
	Cisplatin (48h)	7.01 ± 0.21[a]	72.98 ± 2.87
	Cisplatin (72h)	8.65 ± 0.03	104.47 ± 7.56[a]
	Cisplatin (96h)	5.90 ± 2.12[a]	79.77 ± 8.86
Testes	Normal	10.15 ± 0.55	95.76 ± 5.21
	Tumor-bearing(control)	12.45 ± 0.62	122.07 ± 6.13
	Cisplatin (24h)	8.85 ± 0.43[a]	76.37 ± 3.70[a]
	Cisplatin (48h)	10.95 ± 1.09	127.33 ± 12.69
	Cisplatin (72h)	7.32 ± 0.64[a]	101.68 ± 9.01[a]
	Cisplatin (96h)	8.40 ± 0.95[a]	98.33 ± 7.52[a]
Spleen	Normal	3.70 ± 1.28	30.50 ± 2.12
	Tumor-bearing(control)	3.16 ± 0.96	20.38 ± 1.28
	Cisplatin (24h)	2.37 ± 0.45	12.00 ± 1.56[a]
	Cisplatin (48h)	2.12 ± 2.01[a]	18.13 ± 2.01
	Cisplatin (72h)	2.10 ± 1.36[a]	15.47 ± 1.72[a]
	Cisplatin (96h)	2.76 ± 1.84	17.52 ± 1.88

A unit of enzyme activity is defined as the nanomoles of succinate oxidized per minute. The specific activity is expressed as units/mg mitochondrial protein. Cisplatin (8mg/kg body wt) was administered to tumor-bearing mice. Student's t-test, n = 6, as compared to the respective control, [a]$P \leq 0.05$. The significance of total changes between the control and cisplatin-treated groups was tested by ANOVA and it showed that the units of SDH decreased significantly in kidney, DL cells, testes and spleen ($P \leq 0.05$) after cisplatin treatment.

Fig. 7. Histogram showing the percent changes in units of SDH activity in the mitochondrial fraction of tissues of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control, tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). $P \leq 0.05$. It may be observed that cisplatin treatment decreased the units of SDH in all the tissues except liver.


% Change in SDH activity (Units)
0
0
0
Control
Cisplatin 24 h
Cisplatin 48 h
Cisplatin 72 h
Cisplatin 96 h
Liver
Kidney
DLCells

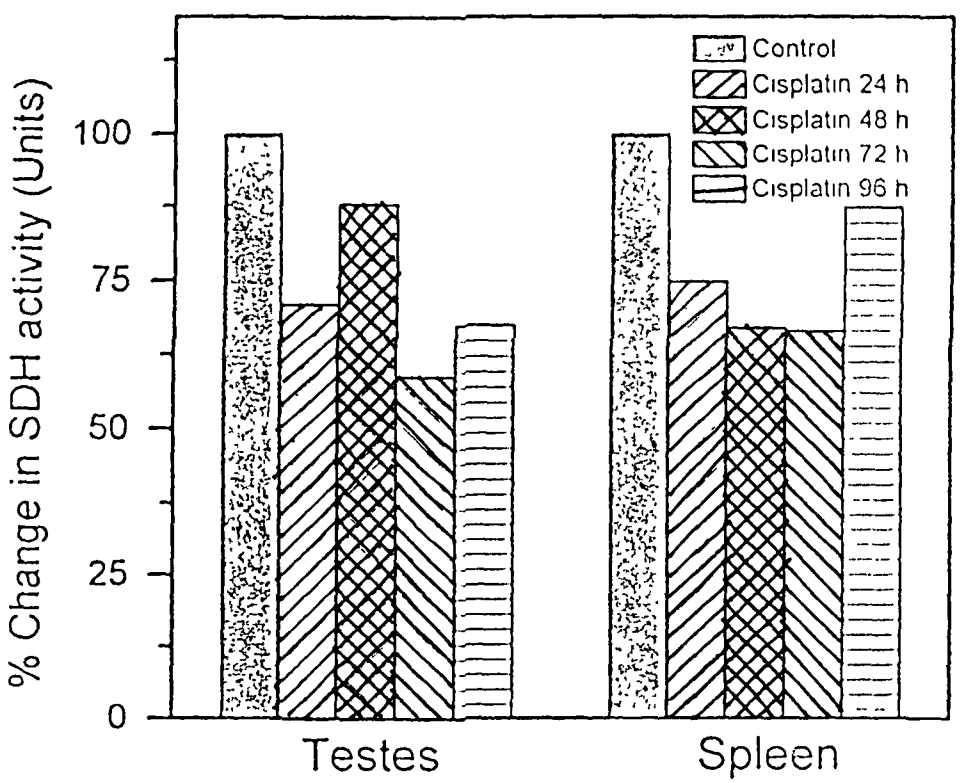
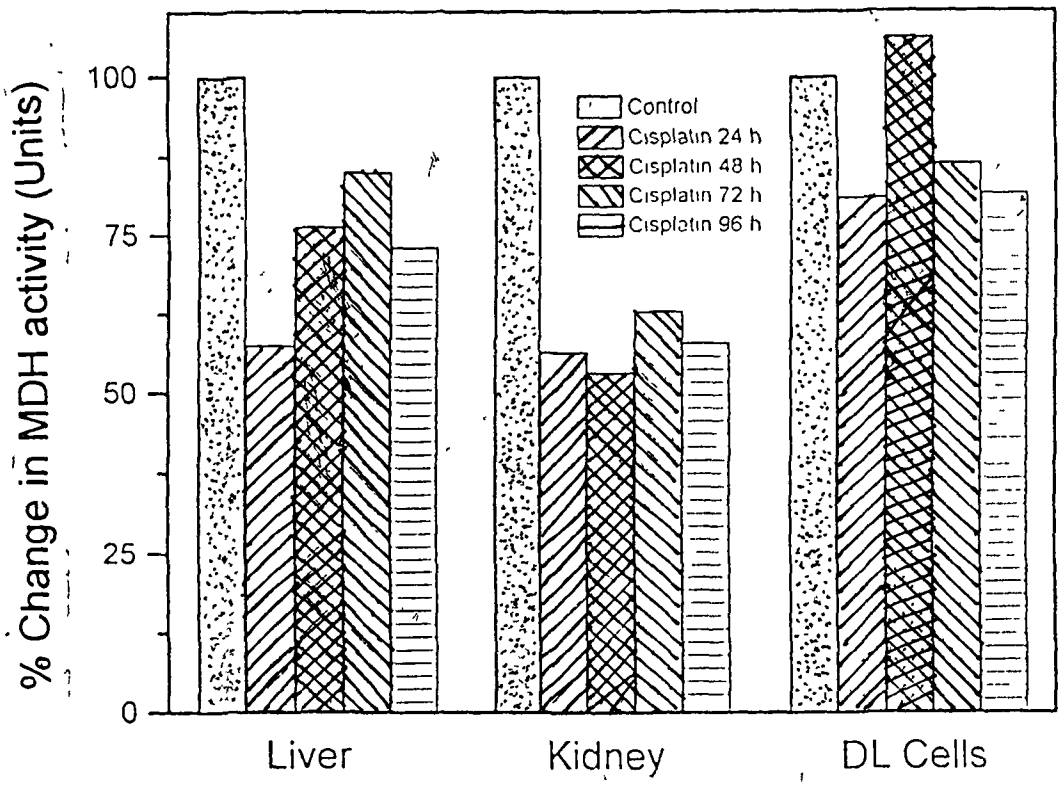

% Change in SDH activity (Units)
100
75
50
25
0
Control
Cisplatin 24 h
Cisplatin 48 h
Cisplatin 72 h
Cisplatin 96 h
Testes
Spleen

Table 7: Units and Specific activity (mean ± SD) of malate dehydrogenase (MDH) in the mitochondrial fraction of different tissues.

Tissues	Treatment	Units	Specific activity
Liver	Normal	2.52 ± 0.17[a]	0.73 ± 0.05
	Tumor-bearing(control)	2.19 ± 0.13	0.82 ± 0.06
	Cisplatin (24h)	1.26 ± 0.07[a]	0.71 ± 0.02
	Cisplatin (48h)	1.67 ± 0.10[a]	0.88 ± 0.05
	Cisplatin (72h)	1.86 ±0.08	0.94 ± 0.04
	Cisplatin (96h)	1.59 ± 0.11[a]	0.91 ± 0.06
Kidney	Normal	1.84 ± 0.14	0.95 ± 0.07
	Tumor-bearing(control)	2.46 ± 0.09	1.19 ± 0.04
	Cisplatin (24h)	1.39 ± 0.05[a]	0.81 ± 0.03[a]
	Cisplatin (48h)	1.31 ± 0.08[a]	0.83 ± 0.05[a]
	Cisplatin (72h)	1.55 ± 0.09[a]	1.09 ± 0.07
	Cisplatin (96h)	1.42 ± 0.13[a]	0.97 ± 0.10
DL cells	Tumor-bearing(control)	1.63 ± 0.18	1.10 ± 0.12
	Cisplatin (24h)	1.32 ± 0.21[a]	1.36 ± 0.22
	Cisplatin (48h)	1.73 ± 0.10	1.79 ± 0.10[a]
	Cisplatin (72h)	1.41 ± 0.11[a]	1.84 ± 0.12[a]
	Cisplatin (96h)	1.33 ± 0.15[a]	1.61 ± 0.23[a]
Testes	Normal	1.32 ± 0.08	0.88 ± 0.02
	Tumor-bearing(control)	1.24 ± 0.06	0.95 ± 0.07
	Cisplatin (24h)	1.49 ± 0.13[a]	0.76 ± 0.03
	Cisplatin (48h)	1.25 ± 0.01	0.94 ± 0.04
	Cisplatin (72h)	1.43 ± 0.25[a]	0.97 ± 0.08
	Cisplatin (96h)	1.40 ±0.16[a]	1.91 ± 0.90[a]
Spleen	Normal	1.10 ± 0.63	1.05 ± 0.12
	Tumor-bearing(control)	0.80 ± 1.01	1.36 ± 0.15
	Cisplatin (24h)	1.00 ± 2.01[a]	1.73 ± 0.12[a]
	Cisplatin (48h)	1.30 ± 0.72[a]	1.85 ± 0.10[a]
	Cisplatin (72h)	1.20 ± 1.18[a]	1.42 ± 0.12
	Cisplatin (96h)	1.12 ± 1.08[a]	1.65 ± 0.21

A unit of enzyme activity is defined as the amount of enzyme catalyzing the reduction of 1 nmole of oxaloacetate per minute and the specific activity units/mg mitochondrial protein. Cisplatin (8mg/kg body wt) was administered to tumor-bearing mice. Student's t-test, n = 6, as compared to the respective control, [a]$P \leq 0.05$. The significance of total changes between the control and cisplatin-treated groups was tested by ANOVA and it showed that the units of MDH decreased significantly in liver, kidney and DL cells ($P \leq 0.05$) after cisplatin treatment.

Fig. 8. Histogram showing the percent changes in units of MDH activity in the mitochondrial fraction of tissues of tumor-bearing mice after cisplatin treatment for 24-96h. Results are expressed as mean ± S.D., Student's t-test, n=6, as compared to respective control (C; control tumor-bearing mice without cisplatin treatment but treated with vehicle, normal saline). $P \leq 0.05$. It may be observed that cisplatin treatment of tumor-bearing mice decreased the units of MDH selectively in liver, kidney and DL cells.


% Change in MDH activity (Units)
100
75
50
25
0
Control
Cisplatin 24 h
Cisplatin 48 h
Cisplatin 72 h
Cisplatin 96 h
Liver
Kidney
DL Cells

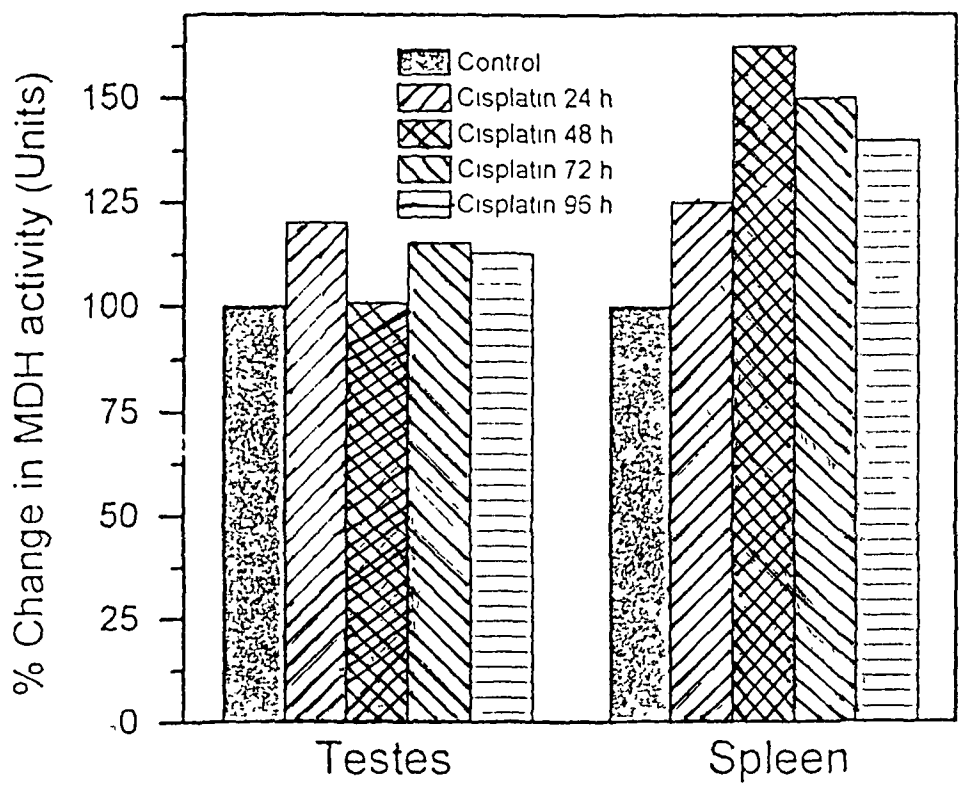
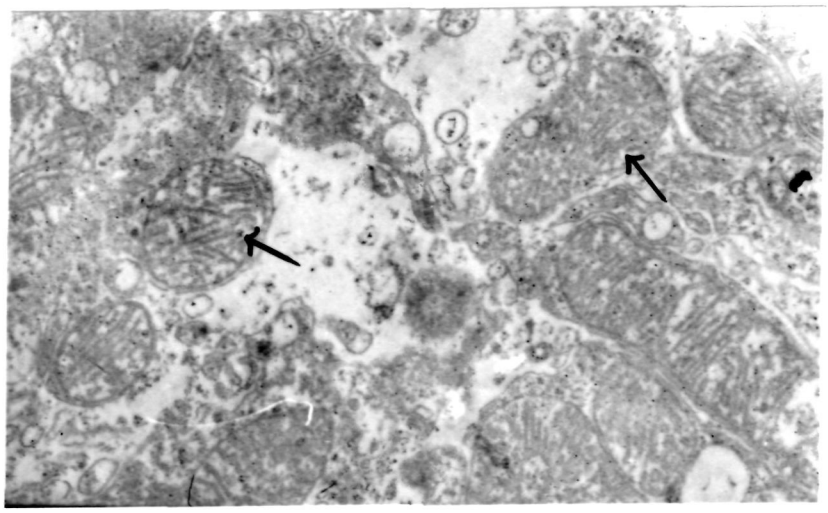
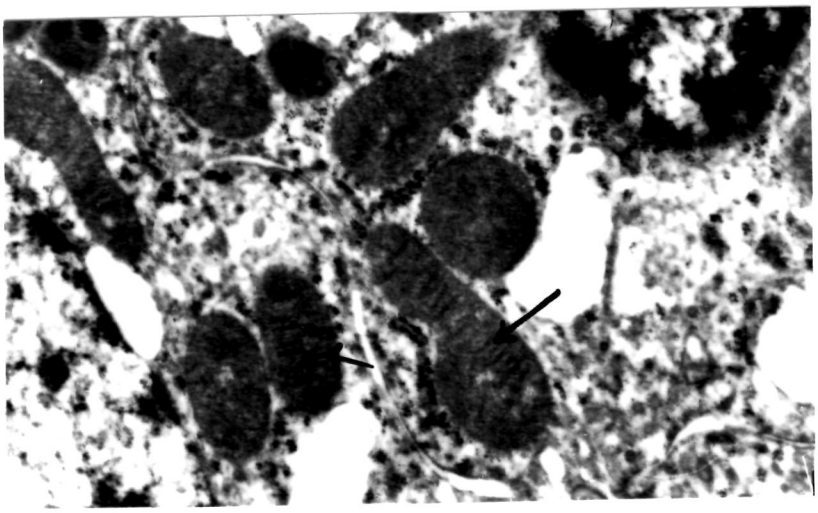

% Change in MDH activity (Units)
150
125
100
75
50
25
0
Control
Cisplatin 24 h
Cisplatin 48 h
Cisplatin 72 h
Cisplatin 96 h
Testes
Spleen

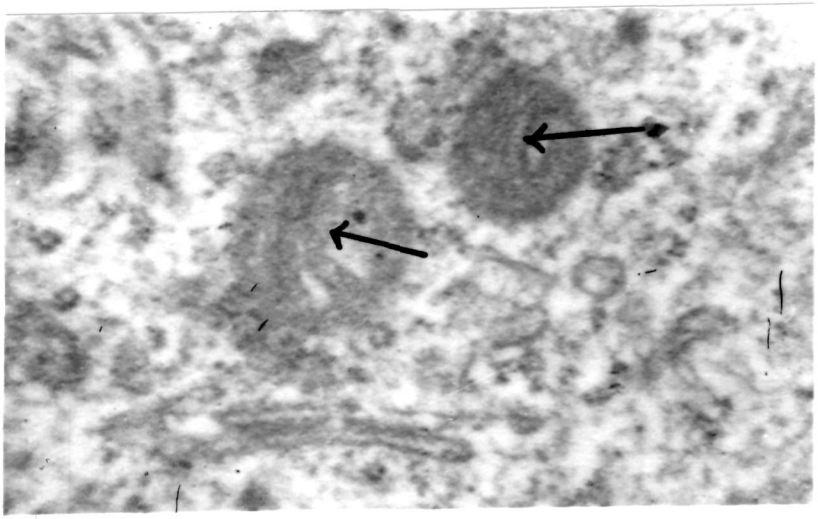
Fig. 9. Ultrastructural features of mitochondria of liver of mice under different experimental conditions. a: control ; b: cisplatin-treated mice, 24h ; c: cisplatin-treated mice, 48h. a & c, x14000; b, x20000. It may be observed that after cisplatin treatment of tumor-bearing mice irregularities in the arrangement of mitochondrial cristae developed along with its thickening (arrows).



a.



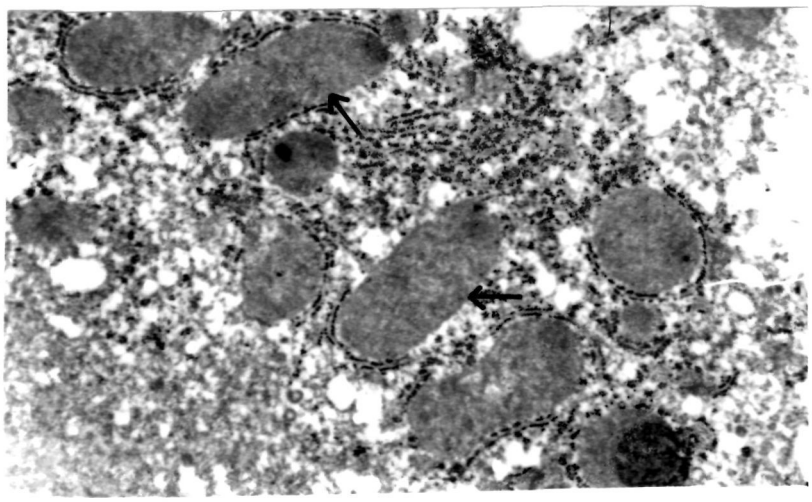
b.



c.

Fig. 9 Contd.

d: cisplatin-treated mice, 72h ; e: cisplatin-treated mice, 96h. d & e, x14000. It may be noted that cisplatin treatment of tumor-bearing mice resulted in the thickening of cristae and disruptions in some parts of the mitochondrial membrane (arrows).



d.

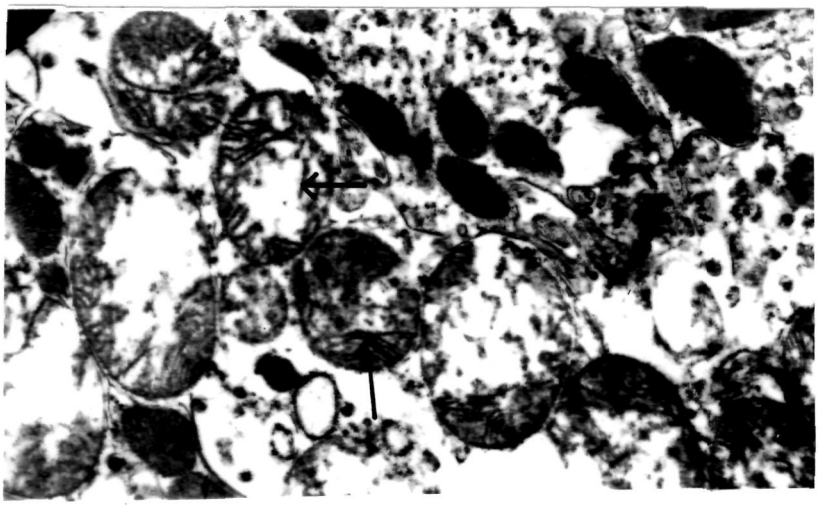
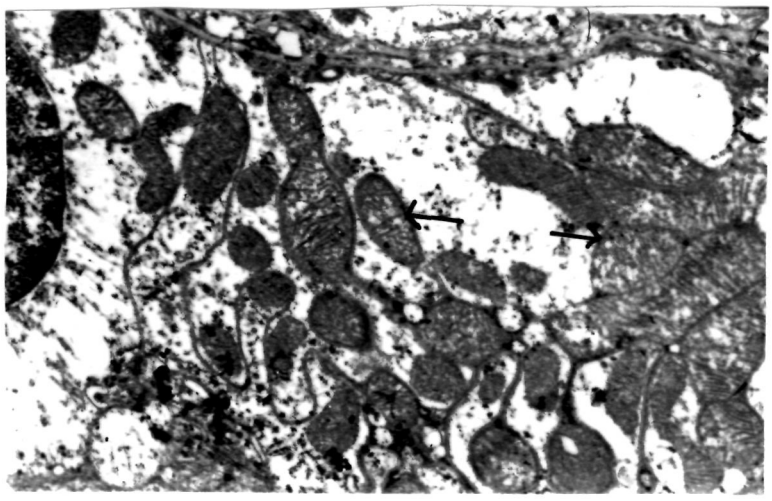
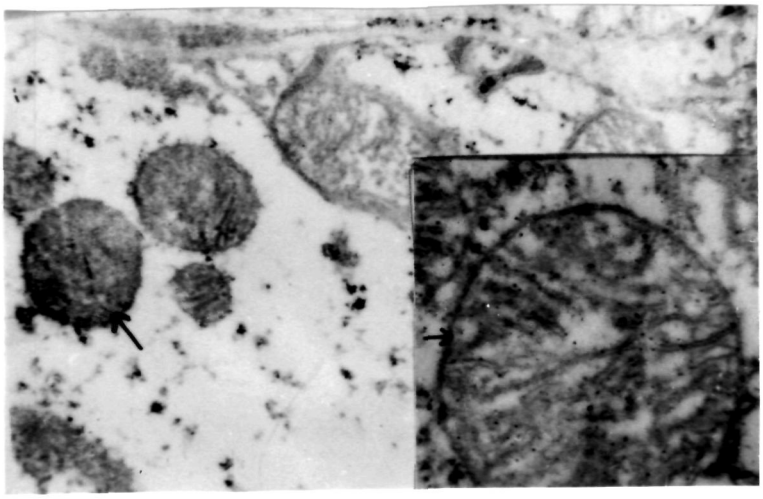


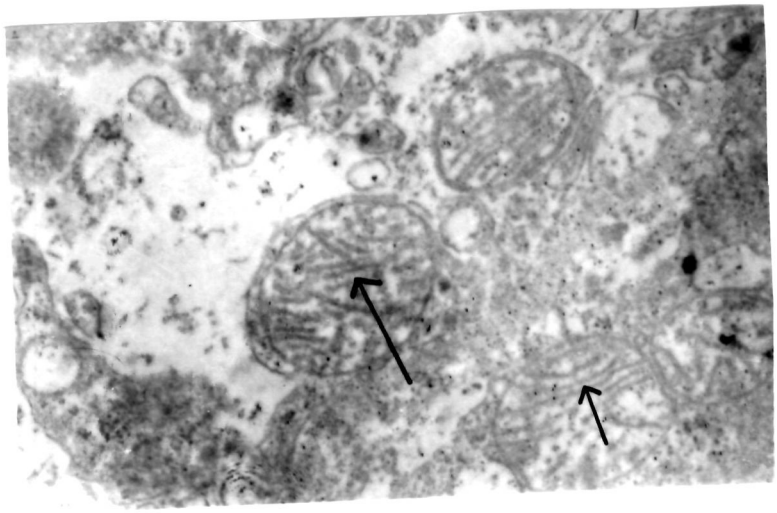
Fig. 10. Ultrastructural features of mitochondria of kidney of mice under different experimental conditions. a: control ; b: cisplatin-treated mice, 24h ; c: cisplatin-treated mice, 48h. a & b, x14000 (inset b, x 27000); c, x20000. It may be observed that after cisplatin treatment of tumor-bearing mice more roundish mitochondria with thickened membranes appeared along with the reduction in the number of cristae (arrows).



a.



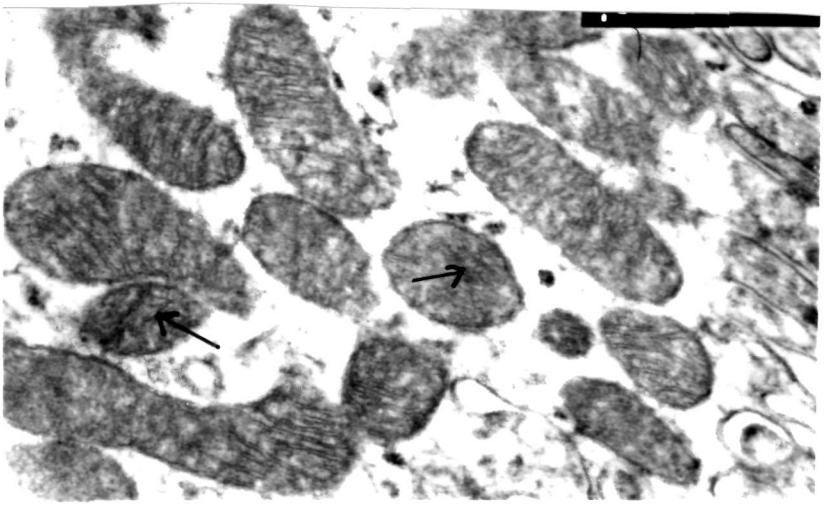
b.



c.

Fig. 10 Contd.

d: cisplatin-treated mice, 72h ; e: cisplatin-treated mice, 96h. d & e, x27000 (inset e, x 14000). It may be noted that cisplatin treatment of tumor-bearing mice resulted in the thickening of mitochondrial membranes and reduction in the number of cristae along with the formation of prominent vacuoles (arrows).



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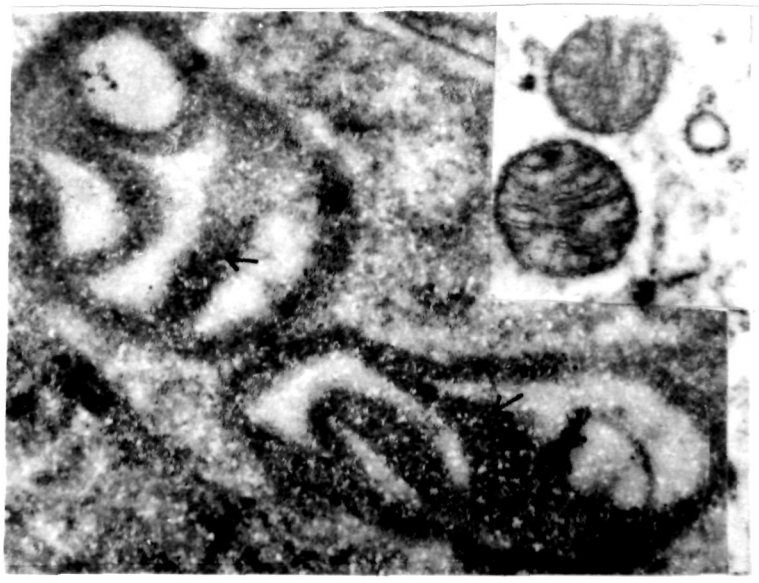
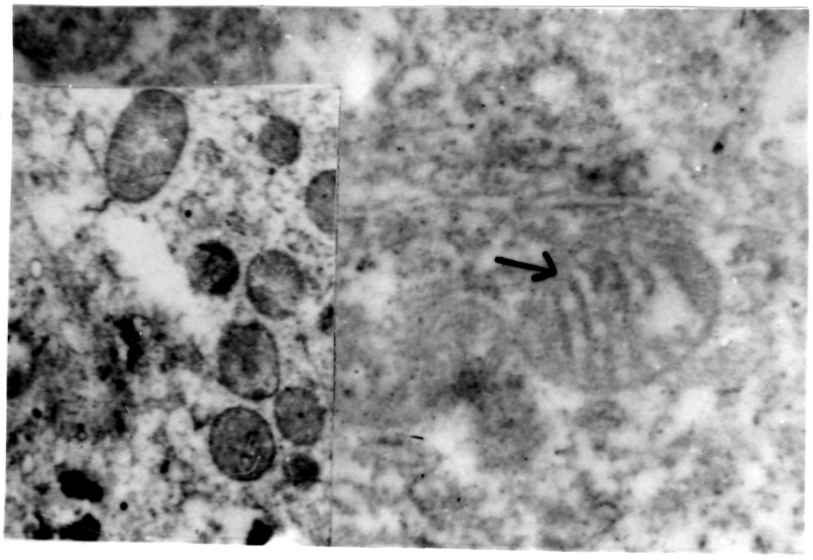
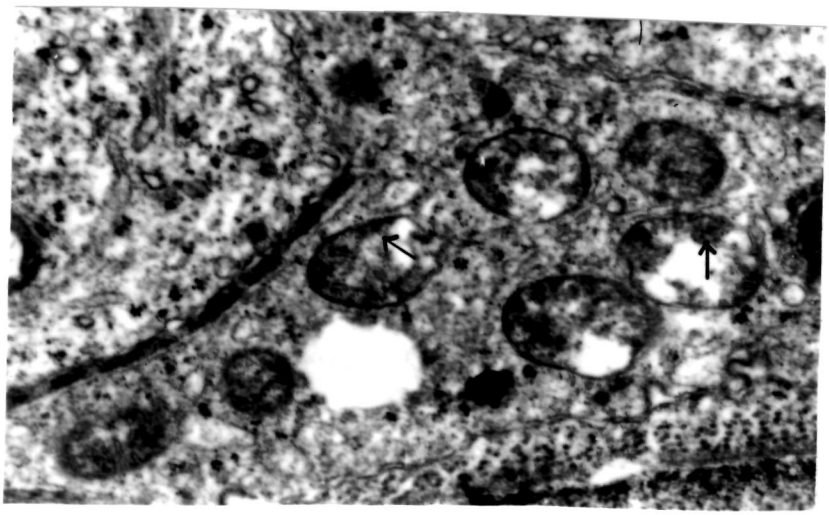


e.

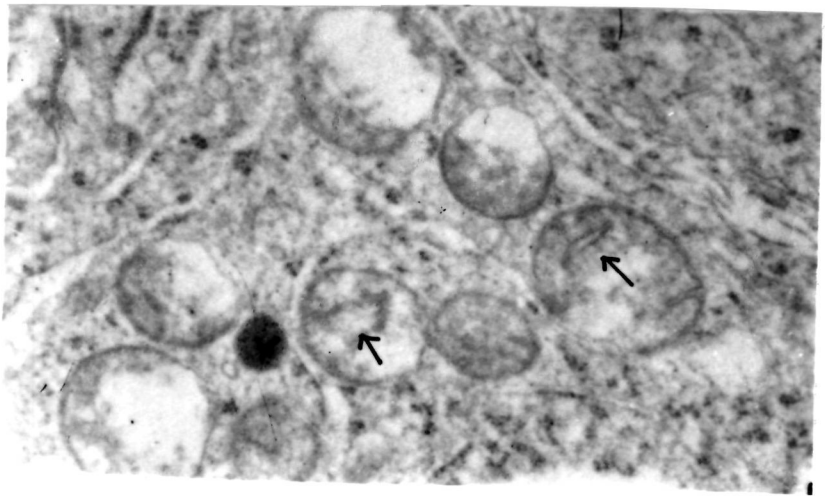
Fig. 11. Ultrastructural features of mitochondria of testes of mice under different experimental conditions. a: control ; b: cisplatin-treated mice, 24h ; c: cisplatin-treated mice, 48h. a-c, x 20000 (inset a, x 14000). It may be observed that cisplatin treatment of tumor-bearing mice resulted in early disruption or disappearance of cristae along with the disruptions in some parts of the mitochondrial membranes (arrows).



a.



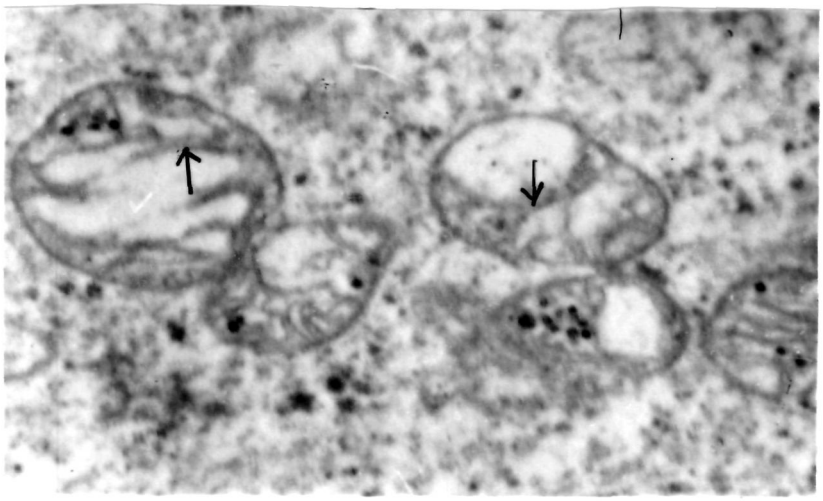
b.



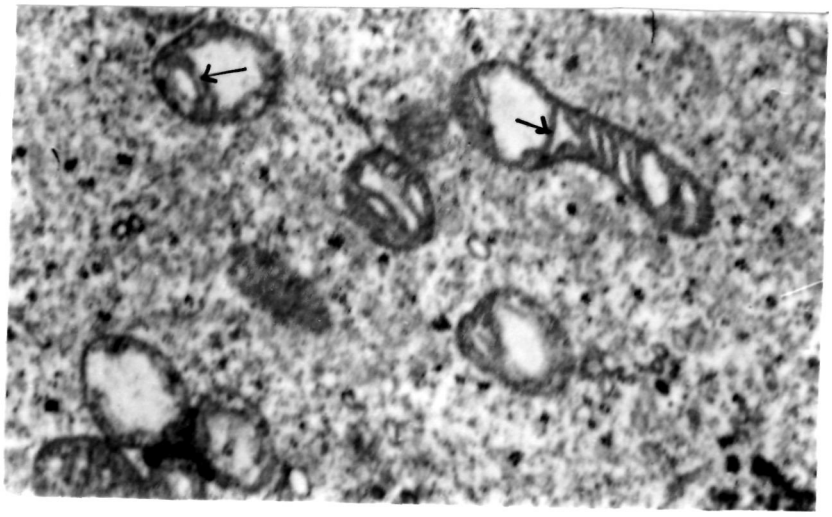
c.

Fig. 11 Contd.

d: cisplatin-treated mice, 72h ; e: cisplatin-treated mice, 96h. d, x27000;e, x 14000. It may be noted that cisplatin treatment of tumor-bearing mice resulted in the formation of vacuoles along with the thickening of cristae and deformations in the elongated structure of the mitochondria (arrows).



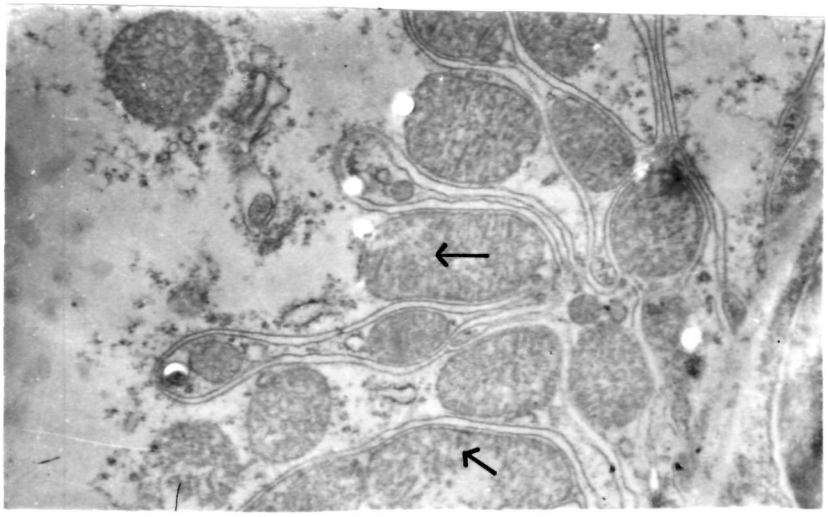
d.



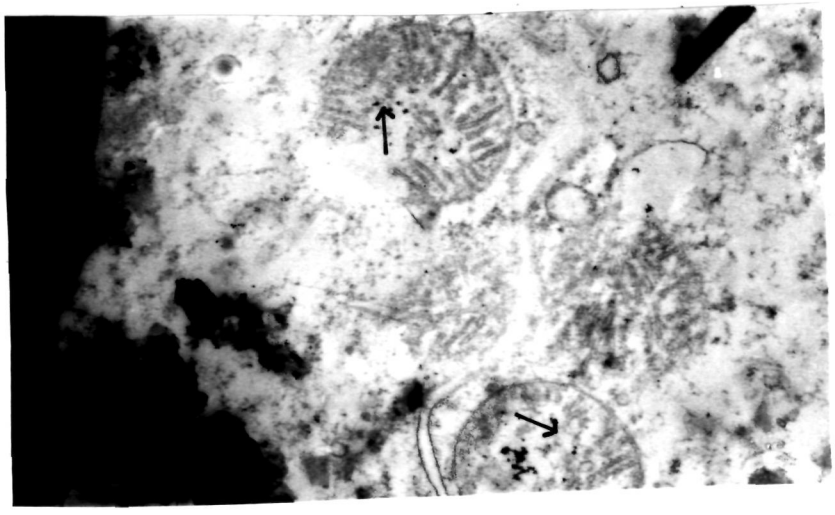
e.

Fig. 12. Ultrastructural features of mitochondria of DL cells of mice under different experimental conditions. a: control ; b: cisplatin-treated mice, 24h ; c: cisplatin-treated mice, 48h. a-c, x 27000. It may be observed that cisplatin treatment of tumor-bearing mice resulted in the irregular arrangement of mitochondrial cristae along with disruptions in some parts of the mitochondrial membrane (arrows).

a.



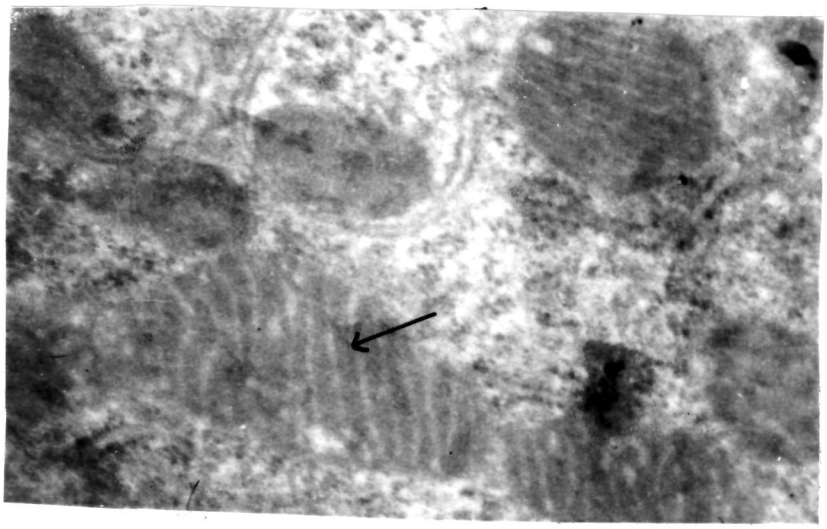
b.



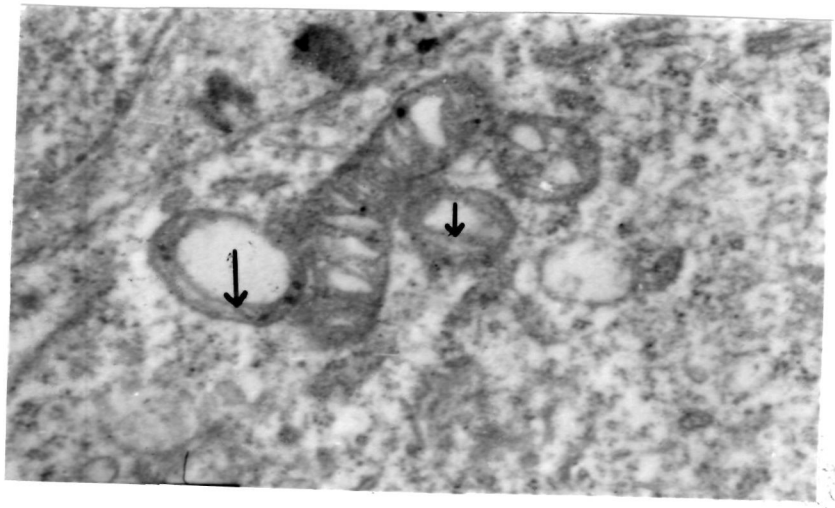
c.

Fig. 12 Contd.

d: cisplatin-treated mice, 72h ; e: cisplatin-treated mice, 96h. d, x 27000;e, x 20000. It may be noted that cisplatin treatment of tumor-bearing mice resulted in the thickening of cristae and formation of prominent vacuoles (arrows).



d.



e.

DISCUSSION

Mammalian cells typically contain a few hundred to several thousand mitochondria, each with 2 to 10 copies of the genome (Bogenhagen and Clayton, 1974). Mitochondrial genome encodes 13 proteins which are essential subunits of the electron transport chain and ATP synthase (Sawyer and Van Houten, 1999). Mitochondria play a pivotal role in cellular metabolism and are the sites of fatty acid β-oxidation, Krebs' cycle, electron transport and oxidative phosphorylation. Various recent reports have indicated that mitochondria may be involved in tumorigenesis, Ca^{++} signalling, aging, lipid peroxidation, maintenance of the malignant phenotype and control of apoptosis (Cavalli and Liang, 1998; Mignotte and Vayssiere, 1998; Smaili et al., 2000; Murphy and Smith, 2000, Penta et al., 2001). Alterations in mitochondrial structure and function including reduction in the mitochondrial transmembrane potential occur early during apoptosis before nuclear or chromatin structures are affected (Petit et al., 1995; Zamzami et al.,1995; Park et al., 2002) suggesting that mitochondria play a pivotal role in the process.

Cellular DNA has been indicated to be the primary target in the anticancer activity of cisplatin (Zamble and Lippard, 1995), but it has also been suggested that, because cisplatin is chemically very reactive, other biochemical targets, besides DNA, may exist in the cells and contribute significantly to cell growth inhibition and cytotoxicity (Just and Holler, 1991). Mitochondrial DNA (mtDNA) has been shown to be a preferential target for cisplatin causing 4- 6-fold higher DNA adducts with mtDNA as compared to nuclear DNA (nDNA) (Olivero et al., 1997). The early subcellular events in cisplatin's cytotoxicity are not well established and present findings on the cisplatin-induced biochemical and ultrastructural changes in the mitochondria of the tumor cells and other tissues provide a definite hint that mitochondria should be one of the targets of potency in cisplatin-mediated cancer chemotherapy and toxicity in the host.

The findings from the present studies show that cisplatin treatment causes definite mitochondrial biochemical injury which could be involved in developing cellular toxicity/cytotoxicity. Significant decrease of protein in the tissues as well as in their mitochondrial fractions was noted after cisplatin treatment (Table 1). Cisplatin treatment of tumor-bearing mice resulted in a significant decrease of protein concentration in the mitochondria of all the tissues. The decrease is more significant in liver mitochondria (~31%) at 96h, kidney mitochondria (~38%) at 96h and testes mitochondria (~49%) at 72h of cisplatin treatment. In DL cells mitochondria a very significant decrease (~45%) was observed at 96h of the treatment while in spleen, a decrease (~25%) was seen at 48h of treatment (Table 1). However, as compared to that in the corresponding tissues, the protein decrease was considerably larger in the respective mitochondrial fraction and the maximum decrease was noted in DL cells (Fig.2). This may involve inhibited protein transport from the cytosol to mitochondria, decreased mitochondrial protein synthesis and/or proteolysis by peptidases within mitochondria themselves. In the mitochondria three groups of peptidases i.e. processing peptidases, oligopeptidases and ATP-dependent proteases, have been known (Kaser and Langer, 2000). The observation of cisplatin-mediated decrease in mitochondrial protein and the idea of its possible involvement in the toxicity/cytotoxicity is in agreement with the other observations also. In carbon-tetrachloride exposed rats, decreased protein synthesis along with depressed oxidative phosphorylation and disruption in mitochondrial structure have been reported (Dewit and Brabec, 1985). Heminger et al. (1997) reported that cisplatin inhibits protein synthesis by causing an inhibition of elongation and also suggested that this may contribute to the cytotoxic/toxic effects of cisplatin during therapy. The products of mitochondrial protein synthesis are essential to the structure and function of the mitochondria. Three subunits of cytochrome-C oxidase (Rascatti and Parsons, 1979; Wilson et al., 1981; Kolarov et al., 1981), apocytochrome b (Krieke et al., 1979; Gellerfors and Nelson, 1981), and at least two of the ATPase subunits (Tzagoloff,1971; Orian et al.,1981; Kuzela et al., 1980;) have been identified

as products of mt-protein synthesis. Anderson et al. (1981) have identified the genes for these proteins on the human mitochondrial genome. Interruption of protein synthesis in yeast (Clark Walker and Linnane, 1967) and mammalian mitochondria (King et al., 1972) results in a depression of oxidative phosphorylation, swelling and disappearance of cristae. It was reported that acute carbon tetrachloride intoxication in rats results in an inhibition of oxidative phosphorylation of hepatic mitochondria (Theirs et al., 1960; Brabec et al., 1974), accompanied by swelling, loss of cristae (Bassi, 1960) and an increase in mitochondrial membrane permeability (Brabec et al., 1980). If mitochondrial damage is accumulated, electron transport chain function will diminish with a concomitant fall in cell bioenergetics and subsequent cellular dysfunction (Zamzami et al., 1995). It has been reported that mitochondrial injury is an important event during the early stages of cisplatin toxicity to renal proximal tubules (Brady et al., 1990). In carcinogen and virally induced tumors, mtDNA alterations in size and structure as well as deletions and insertions have been noted which could alter the ability of the mitochondria to transcribe or translate oxidative phosphorylation proteins from mtDNA (Yamamoto et al., 1992).

Glutathione is the most abundant intracellular thiol and plays an important role in the detoxification of reactive oxygen species (ROS) and xenobiotics (Meister and Anderson, 1983). In mammalian cells under normal physiological conditions more than 98% of glutathione exists in the reduced form (GSH) (Wang and Ballatori, 1998). Mitochondria are the major site of reactive oxygen species (ROS) production in the cells (Mignotte and Vayssiere, 1998) and the changes in mtGSH levels may be very helpful to understand the overall cellular GSH-mediated protective status because GSH is the main component of antioxidant defense system against the oxidative damage. Nutritional and biochemical studies on GSH turnover have shown that the GSH pool within many mammalian cell types such as liver (Meredith and Reed, 1982; Romero and Sies, 1984) and renal proximal tubule (Schnellmann et al., 1988) is divided into two distinct sub pools, one comprising the majority of total intracellular GSH (70-85%

of total) that turns over relatively rapidly ($t_{1/2}$ of 2 hr) and other which comprises a smaller portion of total intracellular GSH (15-30% of total) that turns over relatively slowly ($t_{1/2}$ of 30 hr). Investigations employing digitonin fractionation and marker enzymes showed that the larger pool of intracellular GSH is cytosolic in origin, whereas the smaller slowly turning over pool is predominantly mitochondrial in origin (Lash, 1995). Much less is known about the properties and regulation of the mitochondrial pool of intracellular GSH as compared with the cytosolic pool because the mitochondrial pool comprises a relatively minor fraction of total cellular GSH and because the cytosolic pool is more easily accessible to analysis and manipulation. GSH is taken up from the cytosol through a high-affinity multicomponent transport system(Martensson et al., 1990).

The highest mitochondrial-TSH concentration was noted in spleen, liver (~2.65 mmoles/g), followed by kidney (2.28 mmoles/g) and testes (0.82 mmoles/g). Mitochondrial-NPSH content was found to be highest in spleen (0.304 mmoles/g) followed by kidney (0.210 mmoles/g), testes (0.090 mmoles/g), and liver (0.071 mmoles/ g) (Table 2). As compared to the corresponding normal tissue, mitochondrial-TSH of tumor-bearing mice increased significantly in liver (43%), testes (36%) and spleen (12%) while in kidney a slight decrease was noted (4%). In comparison to the normal tissues, mitochondrial-NPSH in the respective tissue of tumor-bearing mice increased significantly in liver (125%) and testes (44%) while it decreased in kidney (39%) and spleen (21%) (Table 2, Fig.3).

The total GSH concentration of liver and testes showed no significant change after cisplatin treatment of tumor-bearing mice for 24-96h, but it resulted in a significant decrease in total GSH concentration in kidney (~29%) at 24h, DL cells (~21%) at 24h and in spleen (~19%) at 48h. However, after taking into account of all the treatment time points, over all decrease in GSH was noted (ANOVA; $P \leq 0.05$) indicating over all decrease in GSH concentration. The concentration of NPSH decreased in liver (~15%) and testes (~ 20%) at 24h, kidney (~27%) at 24h and spleen (~34%) after 48h of

cisplatin treatment (Table 2).

Cisplatin treatment of tumor-bearing mice resulted in a significant decrease of mitochondrial-TSH in liver (~60%), kidney (~35 %) after 48h and spleen (~42%) at 24 h of treatment. Contrary to the changes in other tissues, a significant increase in mitochondrial-TSH was seen in DL cells (~32%) and testes (~58%) at 24h of cisplatin treatment (Table 2). In spleen, there is no significant change in mitochondrial-NPSH after treatment while a significant increase was observed in DL cells (~66%) and testes (~21%) at 24h of treatment. Comparison of the tissue and its mitochondrial fractions revealed that cisplatin treatment resulted in a very significant decrease of GSH contents in liver mitochondria but not in liver tissue (Fig.3a). TSH contents decreased in kidney as well as kidney mitochondria after the treatment (Fig.3b). However, a sharp increase in kidney mitochondrial-NPSH was observed at 72h of cisplatin treatment (Fig.3b). In DL cells (Fig.3c) and testes (Fig.3d) the treatment resulted in an increase in mitochondrial-GSH. In spleen, mt-TSH level decreased initially at 24-48h and it recovered later at 72-96h of cisplatin treatment (Fig.3e).

The observed increase in GSH in the tumor cells may also suggest its involvement in facilitating proliferation and metabolism of tumor cells in the host. It has been reported that elevation of intracellular GSH in tumor cells is associated with mitogenic stimulation (Shaw and Chou, 1986) and GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH (Terradez et al., 1993). A decrease in the rate of cancer cell proliferation and a decrease of GSH level in the tumor has also been noted (Estrela et al., 1992) The resistance of many cells to oxidative stress is also associated with high intracellular levels of GSH. Thus, it may be suggested that the changes in GSH level in DL cells with tumor growth in the mice may reflect the changes in the rate of tumor cell proliferation accompanied by changes in the antioxidant machinery.

The glutathione determinations showed large variations in the response of DL cells and other tissues to cisplatin treatment. A decrease in GSH and NPSH concentration

was seen after cisplatin treatment in tissues and DL cells. After cisplatin treatment total GSH concentration decreased significantly in kidney (24 and 48h) and in spleen (48h). NPSH concentration decreases at 24h in liver, testes and DL cells and at 24 and 48h in kidney after cisplatin treatment. In spleen NPSH decreased from 24-96h of cisplatin treatment. Except in spleen, glutathione concentration was found to recover during the later periods of treatment. (72 and 96h). Cisplatin has been shown to be sufficiently electrophilic to react with glutathione directly (Eastman, 1987; Jones and Basinger, 1989) and the resulting glutathione-platinum complexes of both endogenous and exogenous sources is actively eliminated from cells by the glutathione S-conjugate export pump (Ishikawa, 1992; Ishikawa and Ali Osman, 1993). The observed decrease in glutathione concentration after cisplatin treatment suggests the possible involvement of cisplatin-glutathione complex formation which would result lesser cellular protective mechanisms. Although glutathione recovery at 72 and 96h of treatment was noticed (Table 2,Fig.3) any damage initiated on cells during early stage of treatment (24 and 48h) may be partially repaired or retained which may lead to develop drug's toxic/ cytotoxic effects.

Cell compartmentation of GSH is important because many of the free radical species generated in the cell are highly reactive and will attack preferentially those cell components that are close to the organelle in which the radicals are generated. This is especially important for mitochondria. GSH is critical to numerous mitochondrial functions, including membrane structure and integrity, ion homeostasis and intramitochondrial redox status (Le-Quoc and Le-Quoc, 1989; Beatrice et al., 1984; Yagi and Hatefi, 1984).

As compared to respective tissue, GSH decrease was more conspicuous in the mitochondrial fraction after cisplatin treatment (Table 2, Fig.3). It has been suggested that most chemical exposures that are associated with GSH depletion require depletion of mitochondrial rather than cytosolic, GSH to elicit cellular inquiry (Shan et al., 1993). Many studies have provided evidence that the mitochondrial pool of GSH is critical for

the maintenance of both mitochondrial and cellular function and that several types of chemically induced injury are associated with depletion or oxidation of mitochondrial, rather than the cytosolic pool of GSH (Beatrice et al., 1984; Le-Quoc and Le-Quoc, 1982; 1985; 1989; Sies and Moss, 1978; and Yagi and Hatefi, 1984). The decrease in mt-GSH should be related to oxidative damage to mitochondria. However, cisplatin treatment caused a more distinct decrease of GSH in DL cells than in their mitochondrial fractions where, in fact, a slight increase in GSH was noticeable (Table 2; Fig.3). This could be due to there being fewer and/or structurally altered mitochondria and the protective mechanism in DL cells against chemotherapeutic drugs, and may indicate that the change/decrease in cytosolic GSH is more important for cisplatin-mediated cytotoxicity in DL cells than in their mitochondrial fraction. The depletion of GSH is also believed to be related to perturbation of intracellular calcium homeostasis by the increased cytosolic calcium (Zhang and Lindup, 1996) which can lead to oxidative stress and cell injury (Olafsdottir et al., 1988). Prasad and Giri (1999) have shown that cisplatin treatment of tumor-bearing mice caused an increase in the concentration of calcium in kidney and DL cells and the change/rise in calcium could also be related to mitochondrial injury because mitochondria play an important role in maintaining calcium homeostasis (Nicotera et al., 1992, Smaili et al., 2000; Murphy and Smith, 2000). The inability to detect *de novo* synthesis of GSH within hepatic mitochondria (Griffith and Meister, 1985) suggested that transport of cytosolic GSH as the intact tripeptide across the mitochondrial inner membrane into mitochondrial matrix must occur to provide the organelle with GSH. Kurosawa et al.(1990) and Martensson and Meister (1989) described transport systems for GSH uptake into rat liver mitochondria. The hepatic mitochondrial system is dependent on membrane potential and the presence of a proton gradient. Two transport systems were distinguished based on kinetics, a high affinity, low-capacity system and a low-affinity, high capacity system (Martensson and Meister, 1989). Kaplowitz et al. (1991) have found that activity of the hepatic system is altered by chronic ethanol feeding of rats. Schnellmann (1991) found evidence for a GSH transport

system in renal cortical mitochondria. Lash et al. (1991) determined that this transport process is electroneutral and appears to involve exchange with dicarboxylic acids, such as malate and succinate.

Many tumors and rapidly dividing cells exhibit a remarkable preference for L-glutamine (Gln), the most abundant amino acid in blood, as repiratory fuel (Kovacevic, 1971; Newsholme et al., 1985; Souba, 1993). Goossens et al. (1995) hypothesized that mitochondrial glutathione (mtGSH) plays a key role in scavenging reactive oxygen intermediates (ROIs). The primary study by Martensson et al. (1990), indicated that glutamate, a direct product of Glutamine metabolism through glutaminase, is a potential inhibitor of GSH transport into the mitochondria.

The decrease in mt-GSH should be related to oxidative damage and to examine this hypothesis, mt-lipid peroxidation was also determined.Cancer cells can generate large amounts of hydrogen peroxide, which may contribute to their ability to mutate and damage normal tissues and moreover, facilitate tumor growth and invasion (Szatrowski and Nathan, 1991).Peroxide are formed naturally during mitochondrial respiration and it may be responsible for mitochondrial damage. The results of the lipid peroxidation (LPO) determinations showed that LPO increased significantly in the tissues and their mitochondrial fractions after cisplatin treatment and supports the view of cisplatin-mediated increase in oxidative damage to mitochondria (Table 3, Fig.4). As compared to those in normal mice, LPO concentration in liver and kidney of tumor-bearing mice increased to about 17% and 25% respectively while in testes and spleen it decreased to about 42% and 50% respectively (Table 3). The highest concentration of mitochondrial LPO (1.64 nmol/mg protein) was noted in testes and it is followed by the spleen (1.61 nmol/mg protein), kidney (1.31 nmol/mg protein) and liver (0.67 nmol/mg protein). As compared to the corresponding normal tissues, the mitochondrial LPO concentration of tumor-bearing mice increased significantly in liver and testes (78% and 37% respectively) while a decrease was noted in kidney and spleen (8% and 38% respectively) (Table 3).

Cisplatin treatment of tumor-bearing mice resulted in a significant increase in LPO concentration in kidney (~25%) at 48h, DL cells (~66%) at 24h, testes (~77%) at 72h and spleen (~50%) at 24-48h of cisplatin treatment. However in the liver, no significant change could be observed (Table 3). Cisplatin treatment of tumor-bearing mice resulted in a significant ($P \leq 0.05$) increase in mitochondrial-LPO concentration in liver (~41%) at 48h of treatment, kidney (~40%) at 48h of treatment, DL cells (~91%) at 48h, testes (~23%) at 72h and spleen (~30%) at 24-96h of cisplatin treatment (Table 3).

Thus, as compared to respective tissue, the more prominent increase in mitochondrial-LPO could be seen after cisplatin treatment in liver and kidney (Fig.4a and b). In DL cells there is an overall increase in mitochondrial-LPO although there is a sharp increase in tissue-LPO at 48h of treatment (Fig.4c). In testes and spleen, there is a decrease in mitochondrial-LPO as compared to that in the respective tissue (Fig. 4d and e). It may therefore be suggested that a decrease in mt-GSH and concomitant increase in mt-LPO could be an early and critical factor in cisplatin-induced toxicity in the liver and kidney and cytotoxicity in DL cells. It has been reported that mitochondrial dysfunction could be a major mechanism during drug-induced hepatotoxicity in liver and nephrotoxicity in kidney (Kruidering et al., 1997; Pessayre et al., 1999).

As in the ascites Dalton's lymphoma tumor, the tumor cells are in direct contact with the supernatant and as any changes in the supernatant and/or tumor cells should be affecting each other, some biochemical determinations were done in the supernatant also. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease in protein concentration in the ascites supernatant (~16%) at 48h with an overall decrease at different time of treatments (ANOVA; $P \leq 0.05$) . Lipid peroxidation was noted to decrease ($P \leq 0.05$) at different times of treatments (Table 4). Cisplatin treatment caused a significant decrease in the total GSH also showing about 26% to 46% decrease at different time points of treatment. The NPSH contents decreased significantly ($P \leq 0.05$) after cisplatin treatment showing over all decrease of about 40% to 70% (Table

4). Thus, cisplatin treatment of tumor-bearing mice resulted in a decrease ($P \leq 0.05$) in protein concentration, LPO and thiol content in the ascites supernatant (Table 4, Fig.5) and it may fairly indicate a disruption in these biochemical relationships of tumor cells with the ascites supernatant causing unfavourable conditions for the tumor growth. It may be mentioned that blood glutathione levels have also been noted to be decreased after cisplatin treatment and it has been suggested that the development of cisplatin-induced hematological toxicities and changes in blood glutathione levels are inversely related (Khynriam and Prasad, 2001).

Measurement of oxygen consumption has been used as a sensitive index of ATP utilization in the proximal tubule since this segment relies almost exclusively on mitochondrial oxidative phosphorylation for ATP synthesis. (Mandel, 1986; Soltoff, 1986; Silva, 1987). Further, to understand a relationship with changes in LPO etc and oxygen consumption, measurement of oxygen consumption (Qo_2) was done and it showed that Qo_2 increased initially to 15-35% of the control value after 24h of cisplatin treatment in the DL cells and its mitochondrial fraction. However at later periods after 48h of treatment it decreased consistently in a time dependent manner (Table 5, Fig.6). This may indicate the development of anoxic condition /oxidative injury which along with elevated calcium (Prasad and Giri, 1994) may contribute to result in extensive membrane blebbing and eventually cause lysis/death of tumor cells. Decrease in oxygen consumption have also been correlated with progressive loss of K^+ in the cells (Brady et al., 1990). Prasad and Giri (1999) also reported that potassium concentrations declined significantly and specifically in DL cells following cisplatin treatment which may involve the obvious selective cytotoxic effects of the drug in DL cells. It has been suggested that decreases in the mitochondrial Qo_2 may lead to decreased rates of the generation of ROS (Teranishi et al., 1999).

Succinate dehydrogenase (EC 1.3.99.1), a component of complex-II of the respiratory chain, is an important mitochondrial enzyme of the electron transport chain and oxidative phosphorylation. Thus, assay of SDH should give an indirect idea of the

oxygen consumption by mitochondria. Among the tissues of normal mice, testes showed the more SDH activity (10.15 units) followed by kidney (9.28 units), liver (8.93 units) and spleen (3.70 units) (Table 6). As compared to the respective tissue of normal mice, the unit of SDH activity in the tissue of tumor-bearing mice, increased in kidney and testes to about 21% and 23% respectively. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease of SDH activity in kidney (~38%) at 24h, DL cells (~30%) at 24-48h, testes (~41%) at 72h and spleen (~32%) at 48-72h of cisplatin treatment. In liver, an increase in the units of SDH activity (~30%) was observed after 48-72h of cisplatin treatment (Fig.7). The same trend of decrease or increase was noted for a particular tissue considering all the treatment time points also (ANOVA; $p \leq 0.05$). Thus, it may be said that the treatment caused a decrease in SDH activity in kidney, DL cells, testes and spleen but not in liver (Table 6, Fig.7).

The decrease of SDH activity, especially in kidney, testes and DL cells may therefore aid the development of some anoxic condition or mitochondrial dysfunction that along with the decrease in mt-GSH may contribute to lysis of cells. This could be supported by the selective increase of calcium observed particularly in kidney and DL cells after cisplatin treatment (Prasad and Giri, 1999). The slight increase in the specific activity after cisplatin treatment could be due to a larger decrease of other proteins rather than the SDH.

The cytosolic and mitochondrial forms of malate dehydrogenase (EC 1.1..37) are key enzymes in the malate aspartate shuttle. This shuttle has been shown to be the most important in the oxidation of cytosolic NADH produced during ethanol metabolism (Dawson, 1979). For the malate - aspartate shuttle to operate at a steady state, the rates of the mitochondrial and cytosolic dehydrogenases must be equal.

In the normal mice, liver was found to have higher MDH activity (2.52 units) than other tissues, kidney (1.84 units), testes (1.32 units) and spleen (1.10 units) (Table 7). As compared to the corresponding normal tissue, the units of MDH activity in the tumor-bearing mice increased in kidney to about 34% while it decreased in liver, testes

and spleen to about 13%, 7% and 27% respectively. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease in the units of MDH activity in liver (~42%) at 24h, kidney (~43%) at 24-48h and DL cells (~20%) at 24h. However, the units of MDH activity increased significantly in testes (~20%) at 24h and in spleen (~ 62%) at 48h of cisplatin treatment (Fig.8).

Cisplatin treatment of mice showed a decrease in enzyme activity in liver, kidney and DL cells while it increased in testes and spleen (Table 7, Fig.8). The decrease in MDH activity may assist the development of biochemical injury and mitochondrial dysfunction. The decrease in MDH activity particularly in kidney and DL cells along with the decrease in mt-GSH may contribute towards toxicity and lysis of cells. Prasad and Giri (1999) reported that the decline in GSH may be related to the selective increase in calcium in kidney and DL cells thus suggesting the possibility of developing selective toxicity in these tissues. The decrease in the enzyme activity may also indicate decreased synthesis and/or increased leakage of the enzyme from the cells due to injury

Mitochondria undergo various structural changes concomitant with changes in their functions under a variety of pathological conditions. When mitochondria become slightly to moderately enlarged they are termed 'swollen mitochondria' (Petit, 1996). When they become extremely enlarged sometimes reaching or even exceeding the size of the nucleus they are designated as 'giant mitochondria' or 'megamitochondria (MG)' (Wakabayashi et al., 1984). Polla et al. (1996) reported that preexposure to heat shock prevented, at least in part, the alterations in mitochondrial ultrastructure. The morphological mitochondrial alterations observed are swelling and disruption of cristae and protective effects of heat shock whether heat shock was administered *in vivo* or *in vitro*. It may suggest that mitochondria might represent the primary intracellular target for heat shock-induced protection against oxidative injury. Protection from oxidative injury has wide medical implications, including inflammation, cancer and AIDS (Pace and Leaf, 1995).

Cisplatin treatment of tumor-bearing mice resulted in the irregular arrangement of

mitochondrial cristae in liver along with the disruptions in some parts of the mitochondrial membrane (Fig.9d). In the kidney roundish appearance of the mitochondria and formation of vacuoles was observed (Fig.10c and d) after cisplatin treatment. In the testes, different morphological variations of the mitochondria (Fig.11a) and a deformation in the structure (Fig.11d) was noted after treatment. In the DL cells, cisplatin treatment resulted in the thickening of cristae and formation of prominent vacuoles (Fig.12c and d) Some other findings have reported that after cisplatin treatment, mitochondria vacuolalized or produced flocculi in hepatocellular carcinoma cells also (Wei et al., 2000) and it is very similar to present observations. Melendez-Zajgla et al. (1999) using fluorescent cation rhodamine 123 showed that cisplatin induced decrease in mitochondrial staining and resulted the damage to mitochondria in HeLa cells. Electron microscopic studies revealing fewer and structurally altered mitochondria in cancer cells have been suggested to indicate the respiratory impairment in cancer cells (White et al., 1974).

Prasad and Giri (1994) reported that cisplatin treatment brings about definite changes in the arrangement of surface membrane ruffles/blebs of DL cells with the appearance of membrane vesicles by 3-4 days of the treatment. Cabaud and Wroblewski (1958) have suggested that an increase in calcium concentration is involved in bleb formations. The swelling of mitochondria could result in decrease in the membrane potential of mitochondria leading to decreased rates of oxygen consumption and decreased phosphorylating ability of mitochondria.

Conclusions : Thus, based on these biochemical and ultrastructural studies involving mitochondria as the possible subcellular site for the cisplatin's activity some important conclusions may be derived as follows:

1. Cisplatin treatment of mice caused a decrease in mitochondrial protein in the Dalton's lymphoma cells as well as other tissues.
2. Glutathione content decreased in the mitochondria of liver and kidney while it increased in DL cells and testes, but showing no significant change in spleen after cisplatin treatment. The specific decrease of mt-GSH particularly in liver and kidney may be a significant step leading towards hepatotoxicity and nephrotoxicity in the host. The slight increase in mt-GSH in DL cells and testes may indicate that the change/decrease in cytosolic GSH may be more important in cisplatin-mediated cytoxicity in DL cells or toxicity in testes than in their mitochondrial fractions.
3. Contrary to the pattern of changes in mt-GSH in different tissues, mitochondrial lipid peroxidation (LPO) increased in all the five tissues studied after cisplatin treatment. It is suggested that depletion of mitochondrial glutathione and the increase in mitochondrial LPO, particularly in kidney and liver should be an early and critical event during cisplatin-induced toxicity. In other tissues the definite correlation between the changes in mt-GSH and mt-LPO could not be established.
4. Although an initial (at 24 h) increase in oxygen consumption in mitochondria and DL cells was observed after cisplatin treatment, it decreased very sharply at later time points in a time dependent manner. This may indicate the development of cisplatin-induced anoxic condition/oxidative injury in the DL cells.
5. Cisplatin treatment decreased the units of SDH in all the tissues except liver, while the units of MDH decreased selectively in liver, kidney and DL cells. This selective decrease of these enzymes particularly in kidney and DL cells

may also lead towards developing anoxic condition/mitochondrial dysfuntion and play a role in cisplatin-mediated nephrotoxicity and cytotoxicity.

6. Cisplatin treatment also caused a deformations in the mitochondria of different tissues of the host leading to their enlargement and/or irregularities in the mt-cristae with the appearance of thickened and decreased number of mt-cristae. The appearance of vacuoles and the damage to the mitochondrial membrane was also noted.
7. Further, these findings while supporting the view of the involvement of multilevel/multistep effects of cisplatin in the host, suggest that biochemical and ultrastructural changes in mitochondria could be an important factor in the development of toxic/cytototoxic effects in the host during cisplatin-mediated cancer chemotherapy.

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Publications

Full Papers:

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