STUDIES ON THE EFFECT OF ASCORBIC ACID WITH CYCLOPHOSPHAMIDE AGAINST MURINE ASCITES DALTON'S LYMPHOMA

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DECLARATION

I, Bonnie M Nicol, hereby declare that the subject matter of this thesis is the record of the 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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Dedicated with love
to my dear mum

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CONTENTS

ABBREVIATIONS

AA	Ascorbic acid
AP	Aldophosphamide
BCNU	*1, 3-bis (2-chloroethyl)-1-nitrosourea*
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CAT	Catalase
CISP	Cisplatin
CP	Cyclophosphamide
DAB	Dimethyl amino-azo-benzene
DEM	Diethylmalonate
DL	Dalton's Lymphoma
DMH	Dimethyl hydrazine
DOX	Doxorubicin
DTNB	5, 5'- dithiobis - (2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
GPx	Glutathione peroxidaes
GR	Glutathione reductase
GSH	*Reduced glutathione*
GSSG	Glutathione disulphide
GST	Glutathione-s-transferase
i.p.	Intraperitonially

LDH	Lactate dehydrogenase
LPO	Lipid peroxide
O.D.	Optical density
PBS	Phosphate buffer saline
PCV	Packed cell volume
PEC's	Peritoneal exudates cells
PKC	Protein kinase C
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
TGSH	Total reduced glutathione
TSH	Total sulfhydryl

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INTRODUCTION

CANCER

What is cancer?

The multiplication of cells in the body is carefully regulated. In a young animal, cell multiplication exceeds cell death, so the animal increases in size, while in adult; the processes of cell death and cell multiplication are balanced to produce a steady state. Very occasionally, the exquisite controls that regulate cell multiplication breaks down, so that the affected cell begins to grow and divide in an unregulated fashion. The descendants of such cells inherit the propensity to proliferate without responding to regulation, thereby the clone of cells are able to expand indefinitely, forming a mass called as **tumor**. A tumor is initiated by the transformation of a normal cell into one that escapes the host's usual controls on growth and differentiation (Nicolson, 1979). A 'tumor' may be '**benign**' or '**malignant**'. Benign tumors are characterized by the complete absence of invasion of surrounding tissues and are generally not lethal. The word 'benign' refers to form of behaviour and is characterized by inability to metastasize (Vincent, 1985). Benign tumor grows and remains similar in structure to the tissue from which it has been derived and grows gradually by simple expansion remaining encapsulated by a layer of connective tissue and is generally not lethal. However, malignant tumors have the potential to invade the surrounding tissues including blood vessels and lymphatic channels and metastasize to distant sites where they may replicate. Malignant tumors are commonly referred to as '**cancer**' suggesting its tendency to cling and reach out to adjacent tissues. A *malignant* tumor is usually atypical in tissue structure, grows rapidly and does not remain encapsulated, displays many abnormal nuclear divisions and chromosomes, invading the surrounding normal tissues and shedding cells that have the ability to colonize new sites. The word '**cancer**' is derived from the Latin

for 'crab', suggesting its capacity to cling and reach out to adjacent tissues. Cancer cells are usually less differentiated than the cells of the tissue where they arose. The development from a normal cell to a cancerous cell is usually a multistep process of clonal evolution driven by a series of somatic mutations that progressively convert the cell from normal growth to a precancerous state finally to a cancerous state. In carcinogenesis two types of genes i.e. tumor **suppressor genes and oncogenes** may be implicated. Suppressor genes 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 inactivation 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 cells proliferation which may be transcription factors (p53 and WTI), cell cycle regulators (Rb and p16), components regulating signaling pathways (NFI), regulating RNA polymerase II elongation (VHL). Thus, their elimination contributes and promotes uncontrolled cell growth. The p53 gene product normally senses DNA damage and either halts the cell cycle until it can be repaired or, if the damage is too massive; it triggers apoptosis.

More than hundred oncogenes have been identified from viruses and cells that are involved in different types of cancer, and they are also referred to as **cancer-critical genes** because mutation(s) in these oncogenes contributes to the causation of cancer. When tumor suppressor genes malfunction due to mutations, the rapidly dividing cells ignores messages from its neighboring cells to stop dividing. Malfunctioning tumor suppressor genes are not enough to cause cancer- the cell still must overcome a host of other safety mechanisms before it can cause truly significant damage. The cell nucleus contains a collection of interacting proteins that

control cell division, which interpret the incoming messages at several check points in the cell cycle. The unique receptors on the surface of a cell may also play a role in where tumors metastasize. Specialized molecules on the cell's surface, identify where in the body, the cell belongs. Similar cells adhere to one another when their surface receptors are compatible. Under normal circumstances, the production of cells is so regulated that the numbers of any particular types of cell remain constant. Occasionally, cells arise that are no longer responsive to normal growth control mechanisms. In a broad sense, cancer refers to the full spectrum of malignant neoplasm and can be defined, as processes by which the normal controlling mechanisms that regulate cell growth and differentiation are impaired, resulting in progressive growth under the influence of various oncogenic and carcinogenic agents. The tumor suppressor genes encode proteins that restraint cell growth and prevent cells from turning malignant. In contrast to tumor suppressor genes, oncogenes encode proteins that promote the loss of growth control leading to malignancy. It has been found that normal cells have oncogenes, which are the altered versions of normal genes and their expression can lead to cancer growth (Bishop, 1982). On a dynamic approach to tumor development, what is generally recognized as the cancer cell is in reality but a late product of a complex set of biological interactions which gives rise to an almost continuous spectrum of cellular changes.

Cairns (1986) classified cancers into three broad groups - **Carcinomas, Sarcomas and Leukemias/Lymphomas**. Carcinomas are tumors that arise from the endodermal or ectodermal tissues such as skin or the epithelial lining of internal organs and glands. Sarcomas are derived from mesodermal connective tissues such as fat and cartilage, whereas, leukemias and lymphomas are malignant tumors of the

haematopoietic cells of the bone marrow. Leukemias develop in blood cells, and lymphomas originate in the lymphatic system. Lymphomas interfere with the function of healthy lymphocytes and spread to organs in the body, where they compress and destroy healthy tissue. The risk of lymphoma increases with decreasing immune function. Lymphomas are divided into two general types- Hodgkin's disease and non-Hodgkin's lymphomas. Scientists suggest that some cancer is caused by genetic factors, while other forms of cancer may be caused by environmental factors.

Causes of Cancer:

Cancers are caused by:

- Radiation that can penetrate to the nucleus and interact with DNA (ultraviolet rays, γ-rays, X-rays, electromagnetic radiation). Most cancer deaths from radiation are from skin cancer, which is triggered by too much exposure to sunlight.
- Carcinogens that can damage the DNA genome (Benzopyrene, acetyl aminoflourene (AAT), dimethyl amino-azo-benzene (DAB), carbon tetrachloride, asbestos, aflatoxin B1, lead). One of the greatest risk factors for cancer is prolonged or repeated exposure to carcinogens-chemical, biological, or physical agents that cause the cellular damage that leads to cancer. The details of how carcinogens cause cancer remain unclear. One theory is that exposure to carcinogens, when combined with the effects of aging, causes an increase in chemicals in the body called free radicals. An excessive number of free radicals cause damage by taking negatively charged particles

(electrons) from key cellular components of the body, such as DNA. This may make genes more vulnerable to the mutating effects of carcinogens.

- Anything that is mutagenic and damages DNA and anything that stimulates the rate of mitosis
- Certain steroid hormones (e.g. hormones that stimulate mitosis in tissues like the breast and the prostate gland)
- Agents that cause inflammation (which generates DNA-damaging oxidizing agents in the cell)
- Hereditary factors-evidence suggests that hereditary plays a role in developing cancer as some gene mutation associate with cancer are inherited. For example inheritance of mutated tumor suppressor genes BRCA1 or BRCA2 greatly increases the risk of breast cancer.
- Pathogens-certain viruses (Simian virus 40, Rous sarcoma virus, polyoma virus), bacteria and parasites. Cancer causing viruses include the human papilloma virus (HPV). Hepatitis B and C viruses cause almost 80 % of liver cancer in the world. Epstein-Barr virus can also be carcinogenic, causing cancer of the lymphatic system.

Cell surface in Malignancy:

The intracellular microenvironment plays a vital role in the regulation of the cellular growth and development and interaction with the extra cellular matrix via the external surface of its plasma membrane (Gallagher, 1985) probably by receiving and transmitting regulatory signals from the microenvironment. Cancer, being associated with abnormalities both in the cell growth and cell development and defects in the cell-to-cell recognition underlie the uncontrolled growth and motility

which are the characteristic properties of neoplastic transformation and metastasis. The alterations in the cell surface are involved in various altered behaviour of the tumor cell. Many structural and functional properties of malignant cells are related to changes in the cell surface-membrane (Hynes, 1979; Gallagher, 1985). The presence of cell-cell contacts has been described in solid tumors (Hoshino, 1963) and a few ascitic tumors (Hayashi and Ishimaru, 1981; Gupta *et al.*, 1985). Cell association in malignant cells has been suggested to regulate the pattern of growth and malignancy in tumors (Curtis, 1973). Cell connections form the channels, which probably regulate the hydrophilic pathway between adjacent cells and thus help in transport of ions and small molecules from one cell to another (Loewenstein, 1975; Goodenough, 1976). Cancer cells are reported to have shown high agglutination property with lectins while normal cells do not agglutinate appreciably (Prasad and Sodhi, 1981). An increase in the membrane fluidity has been reported in cancer cells (Rule *et al.*, 1979). Prasad (1986) reported that the agglutination behaviour of normal and malignant cells depends upon the sialic acid moieties present on the cell surface. Warren *et al* (1978) suggested that changes in bound carbohydrates at the cell surface might result in persistent cell division, decreased intercellular adhesiveness, and altered transport, altered/diminished/masked immunogenicity and other specialized functions accompanying malignant transformation. Lack of adhesiveness between the tumor cells is due to a high electrical charge resulting in electrostatic repulsion between the surfaces membranes which have a reduced capacity to bind positive ions, notably calcium.

The surface properties of tumor cells differ from those of their normal counterparts that may partly be due to altered sialoglycoconjugates expressed on the plasma membrane. Sialic acid is an important biological tumor marker of high

sensitivity and specificity in diagnosis and response to treatment of cancer (Chen *et al.*, 1979; Shamberger, 1984; Pulcinsky *et al.*, 1986; Stringou *et al.*, 1992). It was reported by Yogeeswaran and Salk (1981) that the differences in the surface charge as a result of the differential expression of sialic acid may have a direct effect on cell-cell as well as cell- substrate adherence and in turn could have a direct effect on the cellular behaviour *in vivo*. With the progress of malignancy and metastasis, the cell surface glycoproteins and glycolipids (gangliosides) show marked elevations and are referred to as 'tumor markers' (Stringou *et al.*, 1992). It has been reported that with the progression of tumor development there is an increase in the sialic acid content of Yoshida ascites sarcoma cells (Rao and Sirsi, 1973). Although elevated levels of sialic acid have been associated with malignancy (Ingraham and Alhadeff, 1978)), a clear correlation of changes in sialic acid concentrations and malignancy has not emerged, because some reports (Onodera *et al.*, 1976) have shown a decrease and not an increase in sialic acid in association with malignancy. Thus evaluation of sialic acid changes could be very helpful by contributing both the diagnosis of patients and to monitoring their progression and response to treatment. Numerous studies have demonstrated qualitative and quantitative changes in cell surface carbohydrate composition accompanying malignant transformation and neoplastic progression (Yogeeswaran and Salk, 1981; Altevog, *et al.*, 1983; Tao and Burger, 1977), though, in most cases, the consequences and functions of the altered pattern of carbohydrate expression are unclear. Studies on a series of murine tumor cell lines of varying malignant potential have revealed a differential expression of alpha- D-galactopyranosyl groups (alpha-D-Galp) and malignant behaviour. Additional studies have shown that laminin, a particular glycoprotein accounts for a significant portion of the total alpha-D-Galp groups on the highly malignant cells. The internal structure

and composition of the membrane may be changed during tumor growth is suggested by observations of cell permeability. Cell surface components could play a vital role in the malignant transformation of a variety of cells. The study of some cell surface biochemical parameters in tumor cells treated with / without anticancer drugs may give some useful information to understand the chemotherapeutic mechanisms. Evaluation of the macromolecular concentration could be very helpful by contributing both to the diagnosis and to monitoring their progression and response to treatment.

Cancer and Enzymes:

The overall metabolic changes that occur during malignancy may be manifested through the enzymatic changes. The internal structure of the membrane as opposed to the cell surface may be changed during carcinogenesis is suggested by observations in the cell permeability. Particularly striking have been the observations indicating that a considerable leakage of enzymes takes place from the cytoplasm in growing tumors. The release of enzyme into the interstitial fluid plays an important role in tumor invasiveness, particularly the catheptic and peptidase activity which can break down the normal stroma. Necrosis results from loss of osmoregulation, with random DNA degradation by lysosomal enzymes at a later stage Enzymatic changes have also been implicated in the mechanism of action of cisplatin (Aggarwal, 1993). Aggarwal and Meara (1996) reported the cisplatin induced inhibition of variety of dehydrogenases (isocitrate dehydrogenese, β-hydroxybutyrate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase, succinate dehydrogenase and lactate dehydrogenase) in liver and kidney of rats, and suggested that the inhibition of dehydrogenase activity may be involved as a mechanism behind

cisplatin-induced toxicities. Stefanini (1985) has discussed the changes in various enzymes in patients with malignancies and suggested that enzymes, isozymes and enzyme variants could be very useful in the detection and evaluation of biomarkers in the malignancies. Enzymes involved in the synthesis of nucleic acid (thymidine kinase, uridine kinase), of essential amino acids (phosphoserine phosphatase), or in glycolysis (hexokinase, aldolase) have been reported to be elevated in malignant tissue (Yesher, 1978).

Various studies demonstrating significant changes both in the quantitative and molecular structure of enzymes in malignant diseases along with changes in the activity of ecto- as well as endoenzymes and appearance of isozymes and enzyme variants have been reviewed (Stefanani, 1985). The changes triggering biochemical processes aiding cancerous cells over normal surrounding tissue cells may be related to the aggressiveness of the tumor as reported in the activity of β- hexoaminidase activity in ovarian cancer and glucose-6-phosphate dehydrogenase in carcinoma of the prostate (Zampella *et al.*, 1982). Rogers *et al.*, (1981) demonstrated that the activity of LDH seems higher in the malignant tissues and in gastric juice fluid of patients with carcinoma of the stomach. Khynriam and Prasad (2002) have reported that the activity of glutathione related enzymes is variable in different tissues in Dalton's Lymphoma bearing mice. Leptomeningeal infiltration by carcinoma is accompanied by enhanced β- glucorinidase and LDH-5 activity in the cerebro-spinal fluid (Tiez, 1980). A specific isozyme of acid phosphatase in serum was marked in metastatic adenocarcinoma of the prostate (Holyoke *et al.*, 1981). Aldolase is present in three isozyme forms (A, B, C), out of which aldolase A predominates in cancer tissue and is elevated in carcinomas of the gut and pancreas and in hepatomas (Asaka *et al.*, 1980).

LDH catalyses a reversible reaction of pyruvate to lactate in cells existing in five isozyme forms. The use of LDH isozymes as a diagnostic aid has been amply documented (Wilkinson, 1970; Dito, 1973). Malignant cells have distinct type of metabolism in which enzymes of the TCA cycle are poorly integrated. Hence, the cells tend to utilize 5-10 times more glucose. Increased LDH activity has been reported in testicular cancer (Lippert and Javadpour, 1981) leukemias and lymphomas, Burkitt's lymphoma and metastatic tumors (Schneider *et al.*, 1980). There are also reports showing low LDH activity in some patients with cancer possibly due to mutational inactivation. Various workers have studied the metabolic activities of tumor in relation to glucose-6-phosphatase activity. Schull *et al.* (1956) reported an increase in the glucose-6-phosphatase activity in mice bearing corticotrophin-secreting tumor. An increased level of arginase activity was reported during blastogenesis (Klein and Morris, 1978). Chatterjee *et al.*, 1981 have found low level of 5'- Nucleotidase activity in ovarian cancer and suggested that the decreased leveling of enzyme activity may serve as a marker for the said tumor. Therefore no consistent change in the overall activity of these enzymes could be associated with malignant transformation.

CANCER THERAPY:

The fundamental goal of cancer research is to understand how normal cell undergo neoplastic transformation and develop into benign and malignant tumors. Neoplastic transformation results from the dysregulation of one or more genes responsible for growth and /or differentiation of cells. At the outset it is important to consider the very real possibility that neoplastic transformation may result from multiple distinct pathogenetic pathways all of which cause dysregulated growth.

There may be multiple mechanisms of carcinogenesis, each of which may operate on any cell sensitive to neoplastic transformation. Cancer metastasis comprises of a series of events involving interactions among malignant cells from the primary tumor and between these cells and the body's normal cells. A thorough understanding of the cancer cells and its interaction with its microenvironment still remains one of the foremost challenges to mankind today. Oncologists select from a number of options when treating cancer, depending on the type and stage of the tumor involved. The major treatments currently available are surgery, radiation therapy, chemotherapy, hormone therapy, and immunotherapy. Often, targeting cancerous tumors requires the artful combination of more than one type of cancer therapy.

Treatment for cancer ranges from rounds of powerful chemicals to focused burst of radiation to complete surgical removal of the tumor and surrounding tissue. Each of these treatment types bring a certain level of risk and pain to the patient, but cancerous cells if left untreated will almost inevitably choke off vital organs and circulation. Surgery is the most effective and fastest treatment for tumors and can lead to a permanent recovery, but undetected malignant cells may metastasize to other organs. Some cancers can be treated surgically with less-invasive techniques, such as laser surgery. Laser surgery uses a powerful beam of high-energy light to vaporize certain tumors of the cervix, larynx, and skin. Therapeutic radiology utilizes heat energy to literally burn off malignant cells, inflicting genetic damage that kills cancerous cells. Radiation therapy damages rapidly dividing cells, mostly cancer cells but also healthy cells that reproduce quickly. This leads to side effects such as fatigue, skin changes, and loss of appetite. Approaches to improving the therapeutic index of cure of neoplastic diseases during radiotherapy have included reducing toxicity of normal tissues leaving tumor

resistance unchanged. A considerable effort has been focused on the potential use of chemical radiation protectors in the clinic as adjuvant in cancer treatment. Thiols, molecules containing free or potential sulfhydryl groups (SH) in their structure have received a great deal of attention in radioprotection. Unlike surgery, radiation can destroy microscopic cancer cells that have moved into surrounding tissues. Oncologists may use radiation to shrink the tumor, making surgery feasible. Although surgery and radiation therapy destroy or damage cancer cells in a specific area, chemotherapy works throughout the body. Chemotherapy is an effective treatment against cancers either singly or in combination with surgery and/or radiotherapy. In chemotherapy, drugs like cisplatin, carboplatin, cyclophosphamide, doxo-rubicin, melphalan, mitomycin-C, gemcitabine, etc. have been used for the treatment of cancers (Black & Livingston, 1990). However, therapeutic efficacy of most of them is limited due to the development of various side effects in the host and/or the acquired drug resistance by the cancer cells (Black & Livingston, 1990; Kartalou & Essigmann, 2001). Conventional cytotoxic anticancer chemotherapeutic drugs were developed with the intent of treating cancer by direct killing, or inhibition of growth, of cycling tumor cells. Recently, however, there has been considerable interest in the notion of exploiting such drugs as angiogenesis inhibitors (Miller *et al.*, 2001; Gately and Kerbel, 2001). For the development of more selective cytostatic agents, it is necessary to utilize biochemical differences at the molecular level of malignant and normal cell populations or in the metabolism of the cells (Bodansky, 1975). A high content of glucuronidase and potassium cations, and the lack of asparagines synthetase in malignant cells have been observed (Gros *et al.*, 1981).In some cases of leukemia, an RNA-dependent DNA polymerase is found. One main

difference in the metabolism between normal and malignant cell population common for nearly all types of cancer is an increased rate of glycolysis in malignant cells.

Chemotherapy uses powerful anti-cancer drugs that travel through the blood stream, making it potentially useful for cancers that have spread and target fast growing cells. Chemotherapeutic compounds have different sites of attack, but in all cases, the differentiation between normal and malignant cells is based only on the difference in the amount of proliferating cells in the tumor and normal cell population. Oncologists used different chemotherapeutic drugs to combat cancer, generally administering more than one drug at the time because these drugs are more powerful when combine. Whether, taken orally or injected into the blood stream; chemotherapeutic drugs interfere with the cancer cells' ability to make new DNA or to undergo division. In some cases, the drugs cause programmed cell death. More than 100 chemotherapy drugs are used in various combinations. Much Leukemia, Lymphomas and cancer of the testicles are successfully treated with chemotherapy. A combination of drugs with different actions can work together to kill more cancer cells and reduce the chance to become resistant to a particular chemotherapy drug. However, chemotherapy often causes severe side effects, particularly reduced resistance to infection, internal bleeding, diarrhoea, nausea, vomiting, hair loss and anaemia. Bone marrow suppression and depletion circulating leucocytes are major effects of cancer chemotherapy (Hoagland, 1982). Chemotherapy-induced leucopenia and resultant immune suppression may become life-threatening for the possibility of opportunistic infection. High dose chemotherapy for tumoricidal effects often could not be administered to cancer patients because of these hematological toxicities (Pizzo, 1984). Some develop resistance to many drugs after exposure to one drug, leading to multi drug resistance. Chemotherapeutical studies

were done by injecting tumor cells pre-incubated *in vitro* with different drugs and glucose. The main goal in the development of new antitumor agents is therefore not to find substances of higher activity, but to increase their therapeutic index. There are some new approaches like immunotherapy also called as biologic therapy using the body's own immune system to fight cancer cells or protect the body from side effect. Interferons slow down the growth of tumor cells and stimulate the immune system to attack the cancerous cells. However a breakthrough in this area has not been obtained so far. Hormone therapy prevents cancerous cells from receiving or using the hormones required. Pharmacokinetic modeling has suggested that i.p. administration of cytostatic drug might result in a significantly greater drug concentration difference in the peritoneal cavity than in the plasma (Dedrick et al., 1978). This concentration difference offers a potential advantage in the treatment of malignancies confined to the peritoneal cavity.

Alkylating agents affect the mammal genome by forming DNA lesions and thus causing base substitution mutations, or preventing DNA replication. DNA is generally considered as the most critical cellular target by carcinogen and mutagenic effects of drugs, radiation and environmental chemicals. The development of cancer is a multistep process involving sequentially initiation, promotion, transformation and progression of the disease. During initiation i.e. the first stage of development, the effect is on a critical DNA segment. The latter may be a proto-oncogene, which regulates cell proliferation and differentiation, altering to an oncogene or a tumor suppressor gene. Cancer cells escape immune destruction and grow undisturbed by suppressing the immune response. It is well known that apoptotic cell death is induced by DNA- damaging agents.'Apoptosis' (programmed cell death) is accepted as an active and predominant process of cell death observed during chemotherapy.

After exposure of the cells to the toxic agents, in contrast to the interphase apoptotic cell death, the post-mitotic apoptosis occurs as a result of irrepairable damages. Although numerous investigations dedicated to the elucidation of apoptosis, initiation and regulation, many molecular and biochemical mechanism have been studied in detail. Apoptosis is characterized by DNA fragmentation caused by activation of endonuclease. Cyclophosphamide (CP), bleomycin (BL), doxorubicin (DOX) and cisplatin (CISP) are potent antitumor drugs used worldwide against many forms of cancer. As with most such agents, there can be physiological side effects and the possible induction of mutations and other genotoxic effects in non-tumor cells.

Although many cytotoxic agents are being used for the treatment of various forms of cancer, their use is limited due to inherent or acquired drug resistance by the cells and these agents are often associated with severe toxicities. In the 1980's, studies revealed that dietary factors play an important role in cancer risk. Chemoprevention is emerging as a promising strategy of cancer controls and many antioxidants are receiving increasing attention. The aim of chemoprevention is to circumvent the development and progression of precancerous cells through the use of non-cytotoxic nutrients and/ or pharmacological agents. Chemoprevention involves inhibition of neoplastic progression by chemical agents or biological agents and has different mechanisms of action, which can block or suppress the effect of mutation. Most antimutagens act by inducing enzymes, which mediate reactions that enhance the elimination of carcinogens. A growing field of cancer preventing research is chemoprevention, or the use of natural or synthetic compounds to decrease the number of mutations that may lead to cancer. Chemoprevention research seeks to identify those compounds that reduce risk and use them in pills or food additives as a

prevention measure for those who are at high risk for cancer. Anticarcinogenic and chemopreventive activities by a variety of agents that have shown promising chemopreventive activity include antioxidants, anti-inflammatory, anti-oestrogens and anti-androgens. In many instances the effect of an antioxidant compound with a certain therapeutic agent may be specific to a particular tumor type, or may vary with dosage of both antioxidants and chemotherapy. Chemotherapy of malignant lymphomas has entered the exciting era in which cure can be expected in a large portion of untreated patients, even those with advanced- stage disease. Chemotherapy is used for an extensive spectrum of solid tumors either as adjuvant therapy in the early stages of malignancy or as a palliative therapy for patients with advanced extensive- stage and/or rapidly progressive disease. Alkylating agents affects the mammal genome by forming DNA lesions and thus causing base substitution mutations, or preventing DNA replication. The major obstacle to complete remission is selection and overgrowth of a permanent, drug-resistant, neoplastic cell population. For this reason, a number of investigators have tested the efficacy of two or more non-cross resistant regimens delivered in alternating fashion. A key requirement in the design of anti-tumor drugs is that they should be able to move from the point of administration (usually the blood stream) to their molecular targets in the tumor cells. This process involves both diffusion and movement across cell membranes, and each step strongly depends on the structure of the drug.

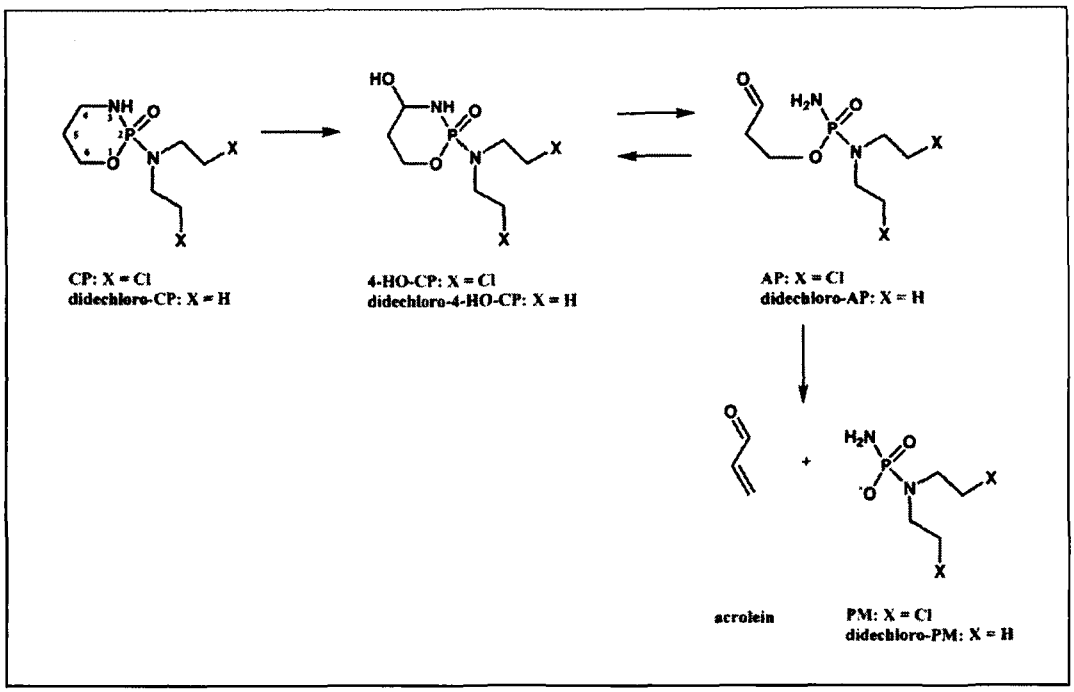
CYCLOPHOSPHAMIDE

Cyclophosphamide as a chemotherapeutic agent:

Cyclophosphamide (2-[bis-/2-chloroethyl/-amino]-tetra hydro-2H-1, 2, 3,-oxazaphosphoric -2 –oxide, CP) also known as *Cytoxan*, an antineoplastic agent is an alkylating agent used for the treatment of malignant and non malignant disorders. This compound is effective against a wide spectrum of malignancies such as leukemia, breast cancer, lymphoma, lung cancer, prostate cancer and ovarian cancer (Moore, 1991; Khan *et al.*, 2004).It is also known as a potent immunosuppressive drug in human and experimental animals (Maguire Jr *et al.*, 1996; Santos *et al.*, 1982) and the immunological augmentation is observed by administration of much lower and minimal dose of CP (Berd *et al.*, 1982). The cytostatic agent CP is a frequent component of cytotoxic combination regimens for tumor patients commonly used in clinical trials. CP exhibits relatively high emetic potency.

Pharmacodynamics and pharmacokinetics of CP:

CP is an alkylating agent which was derived from nitrogen mustard (Arnold and Bourseaux,1958).The prodrug CP, is a bisalkylating chemotherapeutic agent used to treat many types of cancer (Colvin and Chabner, 1991) and requires bioactivation to show its therapeutic index. The parent compound is inactive *in vitro* and *in vivo*.



Schematic diagram showing the metabolism of CP

As shown in the schematic diagram, the metabolism of CP is initiated through an oxidation at the C-4 position by a hepatic cytochrome P-450. Cytochrome P-450 enzymes, expressed at high levels in liver, catalyze the oxidation metabolism of numerous drugs and other foreign chemicals (Guengerich, 1997), including antitumor drugs such as cyclophosphamide (CP). (Le Blanc and Waxman, 1989). Liver tissue has a high content of P-450 enzyme activity towards CP and is the main organ for CP activation.The product of this activation, 4-hydroxycyclophosphamide (4-OH-CP), rapidly undergoes a reversible ring-opening reaction to give aldophosphamide (AP). Aldophosphamide (AP) undergoes an irreversible fragmentation of AP resulting in the formation of acrolein and phosphoramide mustard (PM), a polar molecule, which enters, cells poorly. Therefore, the antitumor activity of CP depends on the readily diffusible and non-polar 4-hydroxy CP (Brock, 1983; Yule *et al*, 1996). The mustard metabolite generates a highly electrophilic aziridinium species that forms DNA cross-links, proposed to be the key cytotoxic lesions induced in tumors treated with cyclophosphamide (Sladek, 1988; Fleming,

1997). The alkylating metabolite(s) can bind to a variety of molecules including amino acids, proteins and peptides, but the most important binding site is DNA where cross-linking occurs. This can lead to DNA strand breaks, cessation of DNA synthesis, inhibition of cell proliferation, and ultimately to cell death (Wang *et al.*, 1993). Multiple biochemical factors and enzymatic steps, some of which are amenable to pharmacological modulation, influence the antitumor activity of CP. (Brain *et al.*, 1998). It has been suggested that the rapid improvement in renal vasculitis, which is often noted with the introduction of CP, may be due to an affect on neutrophils (Cochrane and Aiken, 1966; Cochrane, 1968). CP may directly affect t-cells, and production of adhesion molecules and cytokines by all groups of immune cells may potentially be affected. However, it is also known that not all cells are equally sensitive to CP. *In vivo* experiments in humans and animals have shown that at lower doses CP has a greater effect on B cells, less on CD 8+ cells and its weakest effect on CD 4+ T-cells (Bast *et al.*, 1983; Hemendinger and Bloom, 1996). This balance of resistance and sensitivity of cells of the immune system may have implications for our understanding of a variety of auto immune conditions and infections (Miller and North, 1981) and potentially may determine the effectiveness of CP as an anti cancer drug. There is good evidence that CP is a potent inducer of apoptosis with both high (200 mg/kg) and low (25mg/kg) pulses of CP during apoptosis in urine tumors within the first 24 hours of treatment (Meyn *et al.*, 1994). One of the metabolites of CP, phospharamide mustard, induces apoptosis when applied directly *in vitro* to rat embryos (Chen *et al.*, 1995). 4- Hydroperoxy - CP, a preactivated analogue of CP, likewise induces apoptosis when applied to the rat limb bud *in vitro* (Moallem and Hales, 1995). However, selective resistance to

cyclophosphamide- induced apoptosis is seen in different lymphocyte population in the non-obese mouse with diabetes mellitus (Colucci *et al.*, 1996).

CP has the advantage of being able to be administered orally, via the intraperitoneal as well as the intravenous route. Cyclophosphamide itself has low plasma protein binding, but that of its metabolites is significantly higher (Bagly *et al.*, 1973). Struck *et al.* (1987) reported no difference in the plasma concentration of the active metabolites of CP (4-hydroxy CP and phosphoramide mustard) when given either by the oral or intravenous route, thereby suggesting equal effectiveness. As a potent inducer of hepatic microsomal enzymes, CP therefore, shortens its own half-life overtime, but as this also results in greater production of active metabolites, there are no significant alterations to its biological effects (Bagley *et al.*, 1973). Cytochrome P-450 enzymes, expressed at high levels in liver, catalyze the oxidative metabolism of numerous drugs and other foreign chemicals (Guengerich, 1997), including antitumor drugs such as cyclophosphamide (Le Blanc and Waxman, 1989). Although, at high doses, CP is immunotoxic, at lower doses it has been found to cause a wide range of immuno - modulating effects. As reviewed by Ehrke *et al* (1989) and Mihich and Ehrke (1991), it was found that different subsets of lymphocytes express different sensitivities to CP and that appropriate CP administration may induce the augmentation of the immune response by selective deletion of down – regulating "suppressor", mainly T cell mechanisms. Thus CP has anti tumor efficacy when used either at high doses as a potent cytotoxic drug as suggested by Colvin (1997) or at low doses as an effective component of immuno modulating combination treatments. The cytotoxicity induced by CP is directly connected with its metabolism. Active metabolites of CP, 4- hydroxy CP, phosphoramide mustard and acrolein are involved in toxicological reactions -

induced DNA adducts, single strand breaks.Alkylating agents can bind to a variety of cellular molecules, but the most important site of binding is DNA where cross-linking occurs (Bishop *et al.*, 1997; Murata *et al.*, 2004). Damage of DNA caused by the covalent bonding of alkyl groups to phosphate, hydroxyl and amino groups of the bases of nucleic acid may result in DNA strand breaks, formation of micronuclei and ultimately to cell death (Brookes, 1990; Fraiser *et al.*, 1991; Moore, 1991). CP has been known to induce apoptosis in the tissues and in neoplastic tumors (Havrilesky *et al.*, 1995; Meyn *et al.*, 1995; Hiramine *et al.*, 1996). Cyclophosphamide and cisplatin have been reported to show synergistic antitumor activity in an *in vivo* model of mouse leukemia (Gale *et al.*, 1976). Peters and Stuart (1990) reported that the synergism between 4-hydroxycyclophosphamide (a metabolite of CP) and cisplatin is sequence dependent. Little attentions have been paid to potential drug–to-drug interaction between chemotherapeutic agents and drugs used for supportive care.

Cyclophosphamide and its side effects:

Cyclophosphamide, despite of its wide spectrum of clinical uses also possesses cytotoxicity to normal cells (Fraiser *et al.*, 1991; Moore, 1991). The cellular toxicity caused by CP, an alkylating drug is connected primarily with DNA damage (Brookes, 1990; Ferguson and Pearson, 1996). Common side effects of cyclophosphamide chemotherapy include nausea, vomiting, hair loss, skin rash or acne. CP may also increase the risk of developing some kinds of infections, especially herpes zoster, also known as "shingles." Cyclophosphamide can have significant effects on the blood cells, most often causing a reduction in the number of white blood cells, a key component of the body's immune system. Complications are not uncommon in patients receiving CP for the treatment of vasculitis and other

disorders. CP therapy includes complications such as sustained amenorrhoea (Boumpas *et al.*, 1993), haemorrhagic cystitis and bladder cancer, (Hoffman *et al.*, 1992; Stillwell *et al.*, 1988). One byproduct known as *acrolein* can cause an irritation of the bladder, or "cystitis," which may result in blood in the urine or scarring of the bladder causing bladder toxicity associated with CP use (Moore, 1991), which is normally manifested as sterile hemorrhagic cystitis. In addition to short-term side effects like leukopenia with an increased rate of infection and haemorrhagic cystitis, CP has also shown to increase the risk of malignancies of the haematopoietic system and the skin (Baker *et al.*, 1987; Pederson-Bjergaard *et al.*, 1988). The induction of reactive oxygen species production by CP leads to lung damage (Patel *et al.*, 1984; Patel, 1987). On the other hand, the reactive oxygen species can be apoptosis inducers (Ratan *et al.*, 1994; Wood and Youle, 1994). CP causes lung injury in rats through its ability to generate free radicals with subsequent endothelial and epithelial cell damage. Besides lung toxicity, CP is also known to cause embryotoxicity. Cyclophosphamide treatment at its clinical dose is associated with antigonadal activities as well as induction of oxidative stress in the testes. As with most such agents, there can be physiological side effects and the possible induction of mutations and other genotoxic effects in non-tumor cells.

Cyclophosphamide and glutathione:

The intracellular thiol levels are accepted to be important in determining the extent of cellular damage induced by chemotherapeutic agents. Glutathione is an endogenous intracellar thiol-containing tri-peptide (L-γ-glutamul-L-cysteinyl-glycine). In mammalian cells under normal physiological conditions, more than 98% of glutathione exists in the reduced form (Wang and Ballatori, 1998) and functions as

an important cellular antioxidant.GSH is involved directly or indirectly in a variety of biological phenomena including the bioreductive reactions, maintenance of enzyme activity, amino acid transport, protection against oxidative stress, detoxification of xenobiotics and drug metabolism (Wang and Ballatori, 1998). The intracellular thiol concentration may determine the sensitivity of cells to damage produced by these drugs (Stewart, 1989; Donnerstag *et al.*, 1996). It has been demonstrated that 4-hydroxy CP can react with the cellular thiols to form an alkylthio derivative that is resistant to enzymatic degradation by aldehyde dehydrogenase. The alkylthio derivative exists in equilibrium with 4-hydroxy CP and can act as a storage depot for Alkylating activity with the rate of release of 4-hydroxy CP directly proportional to cellular thiol concentration. The changes in the amount of cellular thiols and especially in the nuclear thiol concentration are important when considering DNA as the critical target for the alkylating agent. Exposure of K-562 cells to 4-hydroxycyclophosphamide resulted in a concentration and time dependent depletion of important cellular antioxidant, reduced gluthathione (GSH) and it was suggested that the intact GSH store is required for the synergism and the cytotoxic synergism between 4-hydroxy CP and cisplatin is modulated by GSH level (Peters *et al.*, 1991). Preliminary evidence suggests that glutathione might have a protective action on the bladders of patients undergoing CP therapy (Nobile *et al.*, 1989).

ASCORBIC ACID AND ITS CHEMOPROTECTIVE ROLE:

Vitamin C (L, 3-ketothreohexuronic acid lactone) commonly referred to as ascorbic acid is a vital, ubiquitous substance in the life process and performs anti-oxidative function *in vivo* by serving as a hydrogen ion donor at various metabolic sites and plays important roles in the metabolism and detoxification of many

endogenous and exogenous compounds (Holloway and Peterson, 1984). Chemically, ascorbic acid is a simple carbohydrate material, related to glucose, of rather unique properties. The presence of the -ene-diol group in the molecule confers electron ability, which makes it a member of an oxidation- reduction system. Evidences continue to accumulate that ascorbic acid has numerous biological effects including some that may relate to prevention and treatment of cancer (Benedict *et al.*, 1983; Kao *et al.*, 1993). The relationships between vitamins and cancer are being much explored in recent times. The role of ascorbic acid in the maintenance of tissue integrity (Wilson, 1977) and host defence (Firasen *et al.*, 1981) provides a rational basis for examining their relationship to cancer. The antioxidant β-carotene and vitamin C are regarded as having shown considerable anticarcinogenic property. Information on diet and cancer indicate that dietary factors may modify the risk of cancer to a significant level. Since cancer is a multi stage process, the protection afforded by dietary factors may be at more than one stage through more than one mechanism. The antioxidant and scavenging properties of vitamin C holds promising prospect in cancer prevention and treatment. Antioxidants represent a first line body defense against oxidative stress produced by generation of free radicals and reactive oxygen species (ROS). The chemotherapeutic / therapeutic role of ascorbic acid against cancers has been widely reported (Benedict *et al.*, 1983; Cameron *et al.*, 1979; Ghosh & Das, 1985; Leibovitz & Schlesser, 1983). However, the definite role of ascorbic acid in cancer treatment still remains controversial (Chen *et al.*, 1988). Ascorbic acid is a unique chemical entity that functions both as an essential nutrient and as an active reducing agent, commands an important role in the metabolism and detoxification of many endogenous and foreign compounds. Proposed mechanisms of ascorbic acid activity in the prevention and treatment of cancer include: (1)

enhancement of the immune system by increased lymphocyte production; (2) stimulation of collagen formation necessary for "walling off" tumors; (3) inhibition of hyaluronidase, keeping the ground substance around the tumor intact and preventing metastasis; (4) inhibition of oncogenic viruses; (5) expedition of wound healing after cancer surgery; (6) enhancement of the effect of certain chemotherapeutic drugs, such as tamoxifen, cisplatin and others; (7) reduction of the toxicity of other chemotherapeutic agents, such as cisplatin, Adriamycin and (8) prevention of cellular free radical damage. The ever-increasing volume of published research concerning ascorbic acid and drug biotransformation attests to the vitamins' central involvement in the organism's response to pharmacological agents. Ascorbic acid has been known to be utilized by the animals for maintenance of their defence mechanism that include immunocompetence and phagocytosis. It has been reported that ascorbic acid status of the host is often low in malignant conditions. Ascorbic acid at a non-toxic concentration, in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition of tumor cells in culture *in vitro* (Prasad & Rama, 1983; Prasad *et al.*, 1979) and *in vivo* (Ghosh and Das, 1985; Prasad *et al.*, 1992). The subtherapeutical dose of cisplatin with ascorbic acid against murine ascites Dalton's lymphoma showed an effective sequence-dependent synergistic antitumor activity (Prasad *et al.*, 1992) leading to tumor regression with significant increase in the host's survival. Ascorbic acid is the most important water- soluble biological antioxidant, which can scavenge both reactive oxygen species (ROS) and reactive nitrogen species. The possible anticarcinogenic effects of ascorbic acid may be accounted for by its ability to detoxify carcinogens as well as its ability to block carcinogen processes through its antioxidant activity. The anticarcinogenic effect of ascorbic acid may be accounted

for by its ability to detoxify mutagens and carcinogens as anthracene, benzo [a] pyrene and heavy metals. High concentrations of ascorbic acid in gastric juice may reduce the risk of gastric cancer by inhibiting the formation of carcinogenic N-nitroso compounds. In an *in vitro* study, ascorbic acid has been shown to reduce the mutagenic actions of carcinogenic nitroso compounds (Guttenplan, 1977). Ascorbic acid may have cancer preventive activity, at least for certain types of cancer as suggested by evidences; however, the role of ascorbic acid remains unclear. A recent cell culture study of human breast carcinoma lines showed ascorbic acid to improve the neoplastic activity of doxorubicin, cisplatin and paclitaxel. Another study suggests that the pro-oxidant form of ascorbic acid may up regulate some of the enzymes involved in DNA repair. This possible activity may play some anticarcinogenic role. Patients with malignant conditions have shown to have low levels of tissue ascorbic acid (Krasner and Dymock, 1974). Several reports have revealed the growth inhibitory effect of ascorbic acid on tumor cells in experimental animals. Logue and Frommer (1980) reported that ascorbic acid inhibited 1, 2-dimethyl-hydrazine (DMH) – initiated colon carcinogenesis. In contrast to these observations, results of some animal studies indicate that ascorbic acid may increase tumor growth. Liotti *et al.* (1983) reported that administration of ascorbic acid potentiated the growth of transplanted solid tumors in mice. However, the effect of ascorbic acid against different cancers is variable depending on the carcinogen-induced cancer, doses, and route of administration and different species of animals (Migliozzi, 1977). The potential role of dietary ascorbic acid to reduce the activity of free radical-induced reactions and testicular/gametogenic disorders has drawn increasing attention (Das *et al.*, 2002). Preliminary human research found that adding antioxidants to CP therapy increased the survival of patients with small-cell lung

cancer treated with CP. (Prasad *et al* (1979) reported that sodium ascorbate potentiated the growth inhibitory effect of certain agents like 5-flourouracil on cultured neuroblastoma cells. Ascorbic acid is also involved in collagen biosynthesis, cytochrome p-450 dependent hydroxylase activities, maintenance of polysomes, stimulation of chemotaxis, phagocytosis, protection against infection, detoxification processes, stimulation of the immune system, wound healing, prevention of oxidation etc. (Kallistratos and Fasske, 1983). Pauling *et al* (1985) reported that although ascorbic acid at high doses inhibited tumor growth in mice; but low doses accelerated tumor growth. As far as the mechanism of the observed effect of ascorbic acid is concerned, individual researchers propose various possible mechanisms. Bishnu *et al* (1978) found that ascorbic acid decreased the rate of DNA synthesis in the cultured cell lines, i.e., Hep-2 and KB. Studies by Tsao *et al.* (1992) on human mammary xenografts in mice have revealed that ascorbic acid, dehydro-ascorbic acid, and the oxidation product of ascorbic acid and from among twelve other ascorbic acid derivatives analyzed, six of them showed antitumor activity. The ability of ascorbic acid to confer marked protection to the animals against many toxic chemical agents and heavy metals has been described (Holloway and Peterson, 1984). Ascorbic acid mediated inhibition of bacterial (Salmonella typhimurium induced TA 100) mutagenecity induced by N-methyl-N'-nitrosoguanidine (Tyrsina *et al.*, 1994) and rat mutagenecity induced by the alkylating agent, N-ethyl-nitrosourea (ENU) (Aidoo *et al.*, 1994) has been reported. Moreover, the protective role of ascorbic acid in 2, 4-dichlorophenol induced teratogenic / carcinogenic toxicity along with significantly increased liver ascorbic acid and GSH levels has also been reported (Nagyova and Ginter, 1995) and its role in preserving glutathione concentrations. The antioxidant

properties of ascorbic acid also accounts for its role in protecting against the tissue damaging effect of toxic chemicals and heavy metals.

In view of the various findings briefly mentioned, the present investigations on various cellular and biochemical / ultrastructural parameters in tumor cells as well as other tissues were undertaken in an attempt to understand:

- the antitumor activity of cyclophosphamide alone or in combination with dietary AA against murine ascites Dalton's lymphoma *in vivo*.
- to find the effect of ascorbic acid on CP-mediated biochemical and morphological changes in Dalton's lymphoma bearing mice.
- to analyze the development of CP-mediated mutagenic effects and evaluate the chemoprotective role of ascorbic acid on the cyclophosphamide - mediated mutagenicity in the tumor-bearing host.
- the possible use of cyclophosphamide in combination chemotherapy with reduced host toxicities.

MATERIALS AND METHODS

MAINTENANCE OF MICE AND TUMOR TRANSPLANTATION:

Swiss albino mice were used as the experimental model in the age group of about 10-12 weeks old of both sexes and weighing about 25-30 grams .All mice were maintained in an inbred colony under conventional laboratory conditions at 24±2°C and fed with standard pellet diet (Amrut Laboratory, Delhi) and given drinking water *ad libitum*. Dalton's lymphoma originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, M D, in 1947. Subsequently, an ascites form was developed by repeated i.p. transplantation of tumor (Goldie and Felix, 1951). Ascites Dalton's lymphoma used in the present study was initially obtained from Gauhati University in the year 1989.

Ascites Dalton's lymphoma tumor was maintained *in vivo* by serial intraperitoneal (i.p.) transplantations of approximately $1x10^7$ viable tumor cells per animal (0.25 ml in PBS, pH 7.4) The tumor-transplanted animals developed the ascites tumor and served as the tumor hosts usually surviving for about 20 days.

CHEMICALS:

Cyclophosphamide (CP), reduced glutathione, 5, 5'- dithiobis-2-nitrobenzoic acid (DTNB), ascorbic acid, ethylene-diaminetetraacetic acid (EDTA) were obtained from 'Sigma' Chemical Company., St.Louis, MO, U.S.A. Glutaraldehyde and sodium cacodylate were obtained from Agar Scientific Ltd., U.K. NAD, NADH, nitroblue tetrazolium, sodium lactate and polyacrylamide were purchased from Sisco Research Laboratories, Mumbai, India. Anthrone and other chemicals of analytical grade were bought from Hi Media Laboratories, Mumbai, India. All the other chemicals used in the experiments were of analytical grade.

Preparations of buffers, stains and solutions were done in double glass-distilled water.

TREATMENT PROTOCOL:

Based on the report from Czyzewska and Mazur (1995), the dose of Cyclophosphamide (200mg/kg b.w.) was selected and administered as a single dose to the tumor-bearing hosts on the 10^{th} day post-tumor transplantation, which is approximately mid/log phase of tumor growth, taking the day of tumor transplantation as day "0". These tumor-transplanted mice were divided into 4 sets consisting of 10 mice in each group accordingly;

Group-I - Tumor bearing mice (Control group) given NaCl (0.89%).

Group-II - This group of tumor mice were given only 1% ascorbic acid in their drinking water from the 5^{th} day of tumor growth.

Group-III - The tumor mice were given a single therapeutic dose of CP (200mg/kg b.w) on the 10^{th} day of post-tumor transplantation.

Group-IV - The tumor bearing mice were pre treated with ascorbic acid in drinking water from the 5^{th} day and were then subjected to CP treatment (200mg/Kg b.w.) on the 10^{th} day.

METHODOLOGY

A. ANTI TUMOR ACTIVITY STUDIES:

Tumor growth pattern and Host's survivability:

Taking the day of tumor transplantation as day '0', the treatment was given at the log phase of tumor growth (10-12 days) and the survivability was studied in all the different treatment groups and compared with that of the tumor control. Animal deaths and body weights were also monitored daily. Ascites Dalton's lymphoma was collected and centrifuged at 2000xg for 10 mins at 4°C. The pellet was used as the DL cells and the supernatant recovered was used as the DL supernatant. The packed cell volume was determined giving the ratio (v: v) between the ascites fluid supernatant and the tumor cell pellet.

The **antitumor activity** was assessed from the mean survival time of both the control and treated groups from the changes depicted in the average body weight as an indicator of ascites tumor growth. The antitumor activity was thereby evaluated by determining the increase in life span (ILS) and growth inhibition rate (GIR) respectively as shown in the equations below:

ILS (%) = (T/C x100)-100, where T is the mean survival rate of the treated group and C is the mean survival rate of the control group.

GIR (%) = $1-V/V_0$, where V is the mean tumor weight of the treated group and V_0 is the mean tumor weight of the control group.

The **tumor pH** of the ascites Dalton's lymphoma was recorded using a pH meter. The treatment schedule was carried out for 4 days i.e. 24, 48, 72 and 96 hours of time interval. The animals from the different experimental groups were

sacrificed by cervical dislocation and the tissues (liver, kidney, spleen, testes, and DL cells) were excised instantly along with serum and ascites supernatant to be used for different experiments. All these samples from both the control and different treatment groups were analysed and studied using biochemical, microscopical and mutagenic parameters.

B. BIOCHEMICAL STUDIES:

Protein Estimation:

The protein content in the different tissue samples was determined following the protocol of **Lowry *et al.*, (1951)**. Homogenization of the tissues and DL cells was done in 0.25-mol/L sucrose solution, while 10 times dilution was done in case of SN and serum. To 1 ml of the homogenate sample taken in duplicate, 5 ml of 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 (15 g/l $CuSo_4.5H_2O$ in 10g/l Na, K tartarate freshly prepared) was added, thoroughly mixed and kept undisturbed at room temperature for 15 mins, after which 0.5 ml of diluted (1:1, v/v) Folin-Ciocalteau reagent was added and mixed instantly. The tubes were then kept at room temperature for 30 mins for the completion of the reaction and the O.D was measured at 750 mm against the reagent blank. The protein concentrations were determined from a standard curve obtained by using BSA as the standard.

Carbohydrate Estimation:

The analysis of carbohydrate content was done following the method of **Plummer (1987)**. A 10% homogenate was prepared in case of tissues and DL cells with distilled water and 10 times dilution for SN and Serum. To this 0.6 N HCl was added in the ratio 1:1 by volume. The homogenate was then boiled in hot water bath for 2 hours, followed by centrifugation at 4000rpm. The supernatant was taken for estimation in duplicate and the volume was made up to 1 ml. To this, 2ml of anthrone (2g/l in conc. H_2So_4) was added and mixed thoroughly. The samples were then incubated in a boiling water bath for 10 mins, cooled immediately and the optical density was taken at 620 nm against a reagent blank and the carbohydrate concentration was determined from the standard curve.

Sialic Acid Estimation:

The sialic acid concentration was determined using the method of **Warren L (1959)**. A 5% homogenate was prepared in case of tissues in 0.1 N H_2SO_4, followed by incubation of the homogenates for 1 hour at 80°C. The homogenates were then centrifuged at 8000 r.p.m. for 15 mins and the clear supernatant was used for sialic acid estimation.0.2 ml of the sample supernatant was taken in duplicates and to this 0.1ml of periodate solution (sodium periodate, 0.2M in 9 M phosphoric acid) was added, mixed and this sample solution was allowed to stand at room temperature for 20 mins. This was followed by the addition of 1 ml of arsenite solution and 3 ml of thiobarbituric acid, mixed properly and kept in a boiling water bath for 15 mins. After cooling the chromophore was extracted in an equal volume of cyclohexanone by vigorous shaking and then centrifuged at 2000rpm for 10 mins. The clear upper cyclohexanone phase was removed carefully and the O.D.

was recorded at 532 nm and 549 nm. The sialic acid concentrations were determined from the extinction coefficient using the formula;

Sialic acid (μmoles) $=0.090 \times O.D_{549} - 0.033 \times O.D_{532}$

Glutathione Estimation:

GSH was determined as total GSH (TSH) and non-protein thiol (NPSH) contents using the method of **Sedlak and Lindsay (1968)**. A 5% homogenate of tissues were prepared in 0.02M EDTA (pH 4.7) in motor-driven Teflon – pestle homogenizer. Total glutathione (TSH) was determined by adding the homogenate (100μl) to 0.2 M Tris –EDTA buffer (1 ml, pH 8.2) and 0.02 M EDTA, (0.9 ml, pH 4.7) followed by 20 μl of Ellman's reagent. After 30 minutes of incubation at room temperature, the reaction mixture was centrifuged and the absorbancy of the clear supernatants were read against a reagent a reagent blank at 412 nm in a Beckman DU-640 spectrophotometer. For the determination of NP-SH, the homogenate (500μl) was precipitated with 10% trichloroacetic acid (500μl) and centrifuged at 3000g for 15 minutes at 4°C. To the supernatant (800μl), 1.6 ml of Tris-EDTA buffer (0.4 M, pH 8.9) was added before the addition of Ellman's reagent(25μl).The reaction mixture was incubated at room temperature for 30 mins and the absorbancy was also taken at 412 nm against a reagent blank.

Ascorbic acid Estimation:

The ascorbic acid concentration in the different samples was estimated using the method of **Omaye *et al.*, (1979)**. Briefly, the tissues were homogenized in 9 ml of ice cold 5% metaphosphoric acid per gram of tissue while serum and supernatant samples were deproteinized by addition of 1 ml of sample to 1 ml of ice cold 10% metaphosphoric acid thoroughly mixed. The samples were then centrifuged for twenty minutes at 4000 r.p.m. at room temperature. The precipitate was discarded and 0.6 ml aliquot of the supernatant was used for analysis. 3/10 ml of citrate – acetate buffer (22 g of tri sodium citrate dihydrate in 40 ml distilled water; pH-4.15 total volume brought to 100 ml with distilled water) was added to each sample and any turbid sample was centrifuged. 0.3 ml of DCIP (0.1 mg / ml in distilled water). was added to the sample and exactly 30 sec, read against distilled water at 520 nm. A few crystals of ascorbic acid were added to bleach the dye by reducing it completely and the absorbance was measured again at 520nm. This value serves as the blank. The change in absorbance (ΔA) due to the reaction of the dye by ascorbic acid is calculated from the following equation:

$$\Delta A = (RB - RB_b) - (S - S_b)$$

Where RB= absorbance of reagent blank; RBb= absorbance after bleaching.

S= absorbance of the sample; S_b= absorbance after bleaching.

ΔA is linearly related to ascorbic acid concentration and the concentration in the sample is obtained by comparison of ΔA with the standard curve constructed with standard ranging between 0 and 20μg of ascorbic acid per ml of 5% metaphosphoric acid.

Lipid Peroxidation Assay:

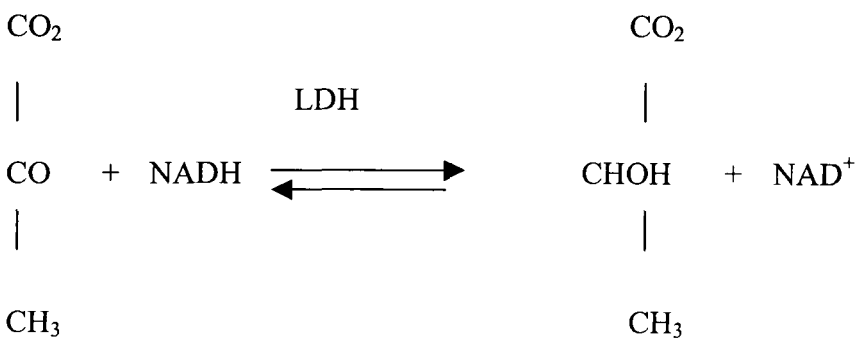
Following the protocol of **Buege and Aust (1978)**, the concentration of thiobarbituric acid-reactive substances (TBARS), mainly malondialdehyde, in the different samples was carried out. A 5% tissue homogenate was prepared in 0.15 mol/l NaCl, while serum and supernatant samples were diluted 10times .To 1 ml of the sample, 2 ml of the trichloro acetic acid (15%) –thiobarbituric acid (0.375%) – HCl reagent (0.25mol/l HCL) was added and thoroughly mixed. The samples were then heated in a boiling water bath for 15 minutes, and then cooled at room temperature. The precipitate was removed by centrifugation at 1000xg at 4^0C for 10 minutes. The absorbance of the clear supernatant was then read at 535nm.

The malondialdehyde concentration in the samples was calculated using an extinction coefficient of 1.56x 10^5 L/mol and expressed as n mol/mg protein.

Lactate dehydrogenase (LDH) activity: (EC: 1.1.1.27)

The activity of LDH (L-lactate: NAD+ oxido reductase) was ascertained following the method of Vorhaben and Campbell (1972). Lactate dehydrogenase catalyses the reversible reduction of pyruvate to lactate with the quantitative oxidation of NADH by the following reaction:

Lactate dehydrogenase catalyses the reversible reduction of pyruvate to lactate with the quantitative oxidation of NADH by the following reaction:



The assay mixture without the sample was pre incubated for 5 minutes at 38^0C in a 1ml quartz cuvette having 1cm light path directly in an UV visible spectrophotometer (Beckman, Model DU 640), having a temperature regulator. In order to start the reaction, the sample was added to the preincubated reaction mixture. The decrease in the absorbancy was recorded at 340nm at an interval of 10seconds, and the duration of linear decrease in the O.D. value was noted for calculating the activity of LDH. The molar extinction coefficient value of NADH at 340nm is $6.22x10^6$ .The enzyme activity of LDH (1 unit) is expressed as the amount of enzyme which catalyses the oxidation of 1 mole of NADH to NAD per minute at 30^0C.

LDH isozyme pattern:

LDH isozymes were separated by electrophoresis on polyacrylamide gels following the method of Davis (1964). The enzyme activity was visualized by histochemical staning. The gel tubes (0.5cmx7cm) were washed with detergent solutions and rinsed with distilled water prior to use. After drying the tubes were kept vertically in a gel-casting stand with their lower ends tightly closed with parafilm. Then 2 ml of small-pore gel containing 7% acrylamide, 0.18% N, N-methylene bis acrylamide, 0.06% N, N, N', N'-tetramethyl ethylene diamine (TEMED) and 0.08% ammonium persulphate was added to each tube. The surface of the gel was then covered with a few drops of distilled water and kept for polymerization. After 30-40 mins. of polymerization, the water layer was removed and the gel tubes were rinsed with the large-pore gel contaning 2.5% acrylamide, 0.62% N, N-methylene bis acrylamide, 0.12% TEMED, 0.001% riboflavin, and 20% sucrose. This was followed

by addition of 0.2 ml of the same solution and the surface was again covered with few drops of distilled water. The tubes were then placed under a mercury lamp for photo polymerization of the large-pore gel. After polymerization and removal of the water layer, the gel tubes were placed in the electrophoretic chamber with the large-pore containing ends in the upper chamber. The electrophoresis buffer containing Tris-glycine buffer, pH 8.2, I=0.02, was added to both the upper and lower chambers. The lower end of the gel tubes were kept in contact with the buffer.

10% homogenate prepared in PBS in case of tissues 6 fold dilute in case of SN and serum. Centrifuged at 8,000g for 20mins (40C). 50 μl of the supernatant was loaded on the polymerized gel. After loading the sample, the electrode buffer was filled gently in the upper chamber covering the gel tubes just on the surface. After the samples were loaded a few drops of marker dye (bromophanol blue 0.01% solution) was added to the upper chamber. Electrophoresis was run at a constant current of 50mA at 4° C (3-4 hrs.). The gel tubes were flushed out with the help of a syringe with distilled water and stained for LDH in LDH staining solution (2.5 ml of 1 M tris-HCl buffer (pH 8.3), 0.5 ml of 1 N lithium lactate solution, 80 mg of nicotinamide adenine dinucleotide (NAD), 1.2 mg of phenazine methosulfate (PMS), 0.8 mg of p-nitrobule tetrazolium chloride (NBT) and 47 ml of double distilled water) for 15 mins at 37° C. The gels were rinsed once in fixative (7% acetic acid). And then stored in the fixative and photographed.

C. MICROSCOPICAL STUDIES:

Light Microscopy

The tumor (control) and the different treated group of mice were sacrificed by cervical dislocation. The ascites tumor was collected and centrifuged at 1000 rpm for 5 mins. at 4°C, washed in PBS (0.15M NaCl,0.01 M sodium phosphate buffer, pH 7.4). The cell pellet was suspended in PBS (1:4) and a drop of the cell suspension was taken on a clean slide and a thin smear was made. The smear was air-dried, fixed in absolute methanol for 15 mins. and stained the following day with Leishman's stain. The slides were then studied under 40X magnification for morphological changes and photographed.

Transmission Electron Microscopy (TEM)

The DL cells pellet collected from the animals under varying experimental conditions were used for transmission electron microscopy. The tumor ascites collected from the peritoneal cavity was centrifuged at 1000 r.p.m. for 10 mins. at 4 C. The cell pellet was washed in PBS and collected by centrifugation at 500 r.p.m. for 5 mins at 4 C. The cell pellet was resuspended in in PBS (1:4, w/v). The DL cells were fixed in 3% glutaraldehyde for 2 hrs at 4°C and then washed in 0.1 M cacodylate buffer. The cells were then cut into small pieces and post fixed in 1 % osmium tetroxide for 15 mins at 4° C. This was followed by dehydration with an ascending grade of acetone (30-50-70-80-90-95%, twice at each concentration for 15 mins each) and finally kept in dry acetone (prepared by adding CuSO4 crystals in excess to absolute acetone and filtered). These were then kept twice in propylene oxide for 1hr at room temperature and at different ratio of propylene oxide and

embedding medium (1.0 ml araldite Cy212,10ml dodecenyl succinic anhydride, 0.4ml tridimethylamino methyl phenol and 1.0 ml dibutylphdyhalate). Ultra thin sections (60-80 nm) were cut in an ultramicrotome (ultratome-RMC, MTX, USA) and collected on copper grids. The sections were stained with lead citrate (5%) and uranyl acetate (5%) (1:1 v/v). Viewing was done in the electron microscope (Joel electron microscope) operated at a voltage of 80KV. The sections were scanned and photomicrographs were taken after thorough observation.

D. MUTAGENIC STUDIES:

Micronucleus assay (Following the method of Schmid, 1976):

Both the femora were removed and the bone marrow was flushed into a centrifuge tube with 1% sodium citrate solution (20^0C) from a syringe. The bone marrow was then gently flushed, agitated and immediately centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was decanted off and the precipitate was resuspended in fetal calf serum in PBS. A drop of the material was smeared on a clean slide. The peripheral blood samples were also collected after different treatment conditions and blood smears were prepared. The smears were then air dried, fixed in absolute methanol for 15 min and stained the following day in Leishman's stain. Two thousand each of polychromatic erythrocytes (PCE's), normochromatic erythrocytes (NCE's), reticulocytes (RET's), and immature white cells were scored/animal).

Chromosomes Aberration Studies:

Mice from different experimental groups were subjected to mitotic arrest by administering colchicine (i.p. 4mg/kg b.w.) 1.5h prior to sacrificing. The mouse was killed by cervical dislocation and the femur was dissected and cut out at both the ends. The bone marrow was flushed out from the femur with warm (37°C) sodium citrate solution (1%) with a syringe. Bone marrow cells were collected by centrifugation (1000 rpm, for 5 min at 4°C) and cells were washed once with Hank's balanced salt solution and to the cell pellet 9-10 ml of warm sodium citrate solution was added and incubated for 10-15 min. The sample was again centrifuged and the cell pellet was fixed in acetic acid: methanol (1:3; v/v), and repeated twice with an interval of 30 min. The cells were then finally resuspended in a small volume of the fixative and flushed gently until a cloudy suspension resulted. Two to three drops of this suspension was dropped on a clean slide pre-chilled in 50% ethanol, burnt over a flame for sometime and then air-dried. This was stained the following day with Giemsa stain (5.0 ml of stock stain + 4 ml methanol + 91.0 ml phosphate buffer, pH 6.8), for one hour, washed and finally mounted in DPX. One hundred good metaphase spreads were examined per animal and chromosome abbreviations in the categories of breaks/gaps, exchanges and sister chromatid unions were noted. Gaps have not been considered under statistical analysis due to their controversial genetic significance (Preston et al., 1981; WHO criteria 46, 1985).

E. DETECTION OF TRACE ELEMENTS IN THE TUMOR ASCITES BY ATOMIC ABSORPTION (VALKOVIC, 1983):

The experimental mice from the different groups mentioned earlier were used. The tumor ascites was collected from the peritoneal cavity using a 1.0 ml glass syringe with a 24G x1" disposable needle. The DL cells were then collected by centrifugation (5000g for 10 min) and washed in cold PBS. The pellet was weighed and kept in clean conical flasks. 0.5 g of the sample was digested in 5.0 ml nitric acid and a few drops of hydrogen peroxide with gentle heating to near dryness. To this 5 ml of perchloric acid was added to the digest and again heated to near dryness to remove excess nitric acid. This was repeated until a clear solution resulted. The digests were finally dissolved in distilled water maintaining the acidity of approximately 5%. The filtrate was stored in polypropylene bottles for analysis in an Atomic Absorption Spectrometer (AAS), Model: Perkin Elmer 3110. After calibarating the instrument with the appropriate standard solutions.

Statistical Analysis:

The significance of the various changes at a particular time point of treatment in comparison to the respective control was determined by Student's 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

ANTITUMOR ACTIVITY AND HOST SURVIVAL:

Following tumor transplantation, the increase in the belly size and body weight, with sluggish movement of the animal was noted from the 3rd - 4th day onwards depicting an early sign of tumor development. Control tumor-transplanted mice (group-I) survived for 19-21 days. In the group of mice treated with AA (group-II) or CP alone (group-III), survival time was significantly increased to about 47.7 days and 58.0 days respectively. The host's survivability was further increased to more than 68 days in the group-IV of mice treated with AA plus CP. The percent increase in the life span (ILS) in the CP-treated group and the combined treated group was 199 and 254.12 respectively (Table 1; Figure 2 & 3).

With the progress of tumor, there was a regular increase in the ascites tumor volume in the control (group-I), which reached to about 11 ml by the 20th day of tumor growth. CP treatment of tumor-bearing mice (group-III) or AA + CP treatment of tumor-bearing mice (group-IV) caused a significant decrease in the tumor volume (Figure 4). By the 5th day following CP treatment very little (0.5-1ml) ascites tumor could be recovered and in the combined treated (group IV), a very negligible amount of ascites tumor was recovered, indicating an effective tumor regression in both the treated groups (III and IV) as compared to the control.

Further, after the assessment of the changes in the ratio of ascites fluid to cell pellet in the tumor under treatment conditions, the value of the packed tumor cell volume was about 1 in the beginning of tumor growth. However, during the middle phase an increase in the volume of ascites fluid to tumor cells was observed. Following CP treatment, the ratio decreased sharply (~0.5) after which the tumor was almost in the viscous form. After combined treatment, little or no ascites (ratio ~0.2) was recovered and the tumor was in the viscous form (Figure 5).

Recording the pH of the tumor ascites showed that the pH of the control tumor was about 7.15. Following CP treatment alone and combined treatment of AA+CP, the pH of the tumor was noticed to decrease in the later periods i.e.72-96h of treatment (Table 2).

BIOCHEMICAL STUDIES

PROTEIN:

Protein concentration in the tissues and serum of normal and tumor-bearing mice:

Analysis of total protein in different tissues of normal mice revealed that the protein content was highest in kidney (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 that in normal mice, the tumor-bearing mice showed a decrease in the protein contents of liver and kidney to about 16% and 38% respectively, and an increase in the testes and spleen ~14% and ~ 45% respectively (Table 3; Figure 6 & 7).

Protein concentration after Cyclophosphamide (CP) treatment of tumor-bearing mice:

After the administration of CP to the tumor-bearing mice, the protein concentration in the liver decreased (172.4 mg/g) after 48 h of treatment. In kidney and spleen the concentration of protein decreased (163.5 mg/g & 150.7 mg/g respectively) after 48h of post CP treatment, which later showed a slight increase (178.3 mg/g & 178.1 mg/g respectively). An overall protein decrease was noticed in the ascites supernatant and serum .However, the maximum decrease was encountered

in the testes and in the Dalton's lymphoma cells (27%) after CP treatment (Table 3; Figure 6 & 7).

Protein concentration after Ascorbic acid (AA) and CP treatment of tumor-bearing mice:

An overall increase in the protein concentration was noted after the combined treatment of ascorbic acid (AA) and CP in the liver with an increase of ~ 24% after 96 h of AA and CP treatment of tumor-bearing mice. In kidney, the protein concentration increased significantly (~40%) after combined treatment whereas in spleen it showed an overall decrease (~12%). The comparative analysis of protein concentration revealed a significant decrease of ~23% in the testes after 24h of treatment. Dalton's lymphoma cells and ascites supernatant showed a maximum decrease of ~ 48% and ~ 56% respectively. A decrease of ~20% was seen in the serum after 72 h of treatment (Table 3; Figure 6 & 7).

Protein content in the liver and kidney did not show much significant change in the different treated groups (III, IV) as compared to control (group I). However, significant changes were noticed in the spleen, testes, DL, SN and serum ANOVA ($P \leq 0.01$) in both the groups of animals treated with CP alone as well as combined treatment of AA + CP.

CARBOHYDRATE:

Carbohydrate concentration in the tissues and serum of normal and tumor- bearing mice:

The determination of carbohydrate concentration in the tissues showed that in normal mice the carbohydrate contents were highest in liver (8.34μg/g),

followed by spleen (7.9μg/g), kidney (3.22μg/g) and testes (2.46μg/g). Total carbohydrate level in the respective tissues of tumor-bearing mice, liver (10.12 μg /g), kidney (3.97μg/g) and spleen (9.73μg/g) increased as compared to that of respective tissues of normal mice. On the other hand, a decrease of ~28% in the testes and ~ 29% in the serum was accounted in the tumorous condition as compared to the normal. However, as compared to the controls (group-I), a significant reduction in carbohydrate concentration in liver, kidney and spleen of mice receiving AA (group-II) was noted (ANOVA, $P \leq 0.01$), but not in spleen and DL cells. The tumor-bearing mice receiving 1% AA (group-II) also revealed a similar trend with a decrease of carbohydrate concentration, ~42% in the liver and 35% in kidney (Table 4; Figure 8 & 9).

Carbohydrate concentration after Cyclophosphamide (CP) treatment of tumor-bearing mice:

Following 72h-96h of CP-treatment, the carbohydrate contents decreased in the liver (~20%), kidney (~30%) and spleen (~33%). The maximum increase was noticed in the serum (~ 50%) and ascites supernatant (76%) at 72h of treatment (Table 4). As compared to the control (group-I), a significant reduction in the carbohydrate concentration in liver, kidney and spleen of mice after CP treatment (group-III) was noted (ANOVA, $P \leq 0.01$).

Carbohydrate concentration after Ascorbic acid (AA) and CP treatment of tumor-bearing mice:

As compared to control (group-I), combined treatment of the tumor-bearing hosts depicted a decrease of up to 42% in the liver and 47% in the kidney. The carbohydrate content in the testes, Dalton's lymphoma cells, ascites supernatant

and serum showed an increase during the course of post-treatment (24h-96h). It was also noted that as compared to the group of mice treated with CP alone (group III), the combination treatment of mice with AA plus CP (group IV) caused further significant decrease in the carbohydrate levels in liver and kidney but not in spleen and DL cells (Table 4; Figure 8 & 9). Carbohydrates content in testes, SN and serum in group-IV showed significant changes (ANOVA; $P \leq 0.01$) as compared to control (group-I).

SIALIC ACID:

Sialic acid concentration in the tissues and serum of normal and tumor-bearing mice:

The determination of sialic acid in the tissues revealed that sialic acid content was much higher in brain (~3μ mol/g) than in liver, kidney, testes and serum (Table 5). It was noted that sialic acid content increased in the tissues of tumor-bearing mice when compared to the respective tissues of normal mice and this increase was predominant in the brain (~58%) (Table 5; Figure 10 & 11).

Sialic acid concentration after Cyclophosphamide (CP) treatment of tumor-bearing mice:

The measurement of sialic acid in the tissues and DL cells showed that as compared to control (group-I), the sialic acid level decreased significantly in the group of mice treated with CP alone (group-III). The decrease was noted to be more in liver (35%) and brain (38%) at 48 h and 96 h respectively. while it was 27% in kidney, 24% in spleen and 22% in testes. The DL cells and ascites supernatant also showed a decrease of ~29% and ~27% respectively in the sialic acid concentration.

Interestingly, the sialic acid content in the serum was noticed to decrease by ~33% after 96h of CP treatment (Table 5; Figure 10 & 11).

Sialic acid concentration after Ascorbic acid (AA) and CP treatment of tumor-bearing mice:

The combined treatment with AA and CP resulted in an overall decrease in the sialic acid content in the tissues, DL cells and serum, although initially (24h-48h), there was an increase of about 9% in liver, 13% in kidney and 26% in case of testes. The most significant decrease (~47%) was encountered in case of Dalton's lymphoma cells. On the other hand following treatment, in the ascites supernatant sialic acid concentration was increased to about 28%. (Table 5; Figure 10 & 11).

Analysis of significant of variation through ANOVA showed that following treatment significant change ($P \leq 0.001$) was noticed in the liver, brain and serum as compared to the respective tissues in the control (group-I). Comparison of group-I and III, or group-I and IV revealed that there was not much change in the sialic acid level in kidney, testes and ascites SN. Further, it was noted that as compared to CP treatment (group-III), the combination treatment (group-IV) of mice resulted in more pronounced decrease in sialic acid level in DL cells (ANOVA, $P \leq 0.01$).

TOTAL REDUCED GLUTATHIONE (TGSH):

TGSH concentration in the tissues and serum of normal and tumor-bearing mice:

Total GSH level in Dalton's lymphoma cells (group-I) increased significantly during 5-15 days of tumor growth *in vivo*. Comparison of TGSH level in the respective tissue of normal and tumor-bearing mice showed that the glutathione concentration did not change significantly except for an increase of 27%

in the spleen of tumor- bearing mice (group-I). Liver has the highest glutathione concentration (11.72μ moles/g) followed by testes, kidney and spleen. Serum revealed a significant decrease of ~44% in the tumor condition as compared to the normal (Table 6; Figure 12 & 13).

TGSH concentration after Cyclophosphamide (CP) treatment of tumor-bearing mice:

As compared to controls (group-I), total GSH concentration in the liver did not change significantly following CP treatment of mice for 24-96h (group-III), whereas in kidney an increase, and in a significant decrease in DL cells (~49%) was noticed (ANOVA, P≤0. 01). After 96h of treatment serum showed a significant increase (~33%) in the TGSH level (Table 6; Figure 12 & 13).

TGSH concentration after AA and CP treatment of tumor-bearing mice:

The combined treatment of mice with AA plus CP (group-IV) caused more pronounced effects on the changes /decrease in TGSH concentration in liver and kidney as compared to CP alone (group-III). Following combined treatment of AA and CP, the TGSH concentration decreased significantly in Dalton's lymphoma cells (38%), ascites supernatant (~43%), liver (~20%), spleen (~21%) and testes (~22%) at 48h of treatment. However, there is maximum increase (~55%) in the TGSH concentration in serum at 96h of treatment. Analysis of significant of variation through ANOVA showed that following treatment significant change was noticed in testes (P≤ 0.01), SN (P≤0.001) but not in serum (Table 6; Figure 12 & 13) as compared to control (group-I).

ASCORBIC ACID:

Ascorbic acid concentration in the tissues and serum of normal and tumor-bearing mice:

The normal Swiss albino mice showed a high concentration of AA in the liver (174μmoles/g) followed by spleen (166.4 μmoles/g), testes (143.5 μmoles/g) and kidney (124μmoles/g). Normal serum showed a concentration of 10.38 μmoles /ml. A significant decrease was observed in all the tissues and serum of tumor-bearing animals as compared to its corresponding tissue in the normal animal. On the other hand, mice receiving 1% AA in drinking water from the 5^{th} day of tumor transplantation showed a significantly ($P \leq 0.05$) higher serum ascorbic acid level (9.50 μmoles/ml) than those of the tumor bearing hosts and was more or less close to the normal concentration (Table 7; Figure 14 & 15).

Ascorbic acid concentration after CP treatment of tumor-bearing mice:

As compared to control (group-I), the treatment with CP did not reveal significant change in AA level in tissue. DL cells showed a significant increase in AA level after 96h of CP treatment. It is interesting to note that the serum ascorbic acid level increases significantly after CP treatment (Table 7; Figure 14 & 15).

Ascorbic acid concentration after AA and CP treatment of tumor-bearing mice:

In the ascites supernatant there was a significant increase of ~61% in AA after the administration of AA alone. As compared to control (group-I), AA concentration in serum revealed a significant increase (11.06μmoles/g) after the combined treatment of AA and CP (Table 7; Figure 14 & 15). As compared to CP

treatment alone, the concentration of AA increased in liver (172.3 μmoles/g), kidney (126.85 μmoles/g), spleen (155.2 μmoles/g) and testes (143.7 μmoles/g) of the mice treated with AA plus CP (group-IV). As compared to respective control, combined treatment with AA plus CP did not show significant changes in AA level in the liver, kidney, spleen and testes but it showed significant increase in DL (ANOVA $P \leq 0.01$), SN (ANOVA $P \leq 0.02$) and serum (ANOVA $P \leq 0.001$).

LIPID PEROXIDATION (LPO):

LPO concentration in the tissues and serum of normal and tumor-bearing mice:

The LPO was measured in terms of malondialdehyde concentrations. It revealed a high concentration of LPO in the spleen (0.87nmol/mg protein) followed by testes (0.61nmol/mg protein), kidney (0.35nmol/mg protein) and liver (0.29nmol/mg protein) of normal mice. Comparing the LPO in the tissues of tumor-bearing animal to its normal counterpart, an increase of 17% in the liver and 25% in the kidney was noted, while a decrease was noted in the spleen (~50%) and testes (~42%). Normal serum showed an LPO concentration of 0.34nmol/mg protein with a decrease of 35% in the tumor condition (Table 8; Figure 16a, 16b & 17).

LPO concentration after CP treatment of tumor-bearing mice:

CP treatment of the tumor-bearing mice resulted in a significant increase in the LPO concentration in the liver and spleen (0.53 and 0.78 nmol/mg protein respectively) after 48h of treatment, in the kidney (~50%), testes (~143%) after 24h of treatment. In DL cells and serum there is a significant increase in LPO

concentration after 24-96h of treatment. However there is a significant decrease in the ascites SN at 24h-96h of CP treatment (Table 8; Figure 16a, 16b & 17).

LPO concentration after AA and CP treatment of tumor-bearing mice:

The administration of 1% AA caused to lower concentration of LPO in the liver, kidney, spleen and ascites SN while an increase in the LPO concentration was seen in the testes, DL cells and serum (Table 8). Combined treatment of the tumor-bearing mice with AA and CP (group-IV) revealed an increase in the LPO content in liver (~12%), kidney (~29%) and spleen (~34%), with the most significant increase in the testes (~158%) and serum (~222%) at 24 h after CP treatments. Following 48h-96h CP treatments, there was a gradual decrease in LPO concentration in the liver and kidney, while in spleen, testes, DL cells and serum there was an overall increase during the treatment. The DL cells interestingly showed a significant increase in the LPO level at 48h, while in the ascites SN there is a slight increase initially (24-48h) followed by a decrease of about 24% after 96h of CP treatment (Table 8; Figure 16a, 16b & 17). As compared to the control (group-I), LPO content showed significant changes (ANOVA; $P \leq 0.001$) in the spleen, testes, DL, ascites SN and serum in both the treatment groups (III and IV).

LACTATE DEHYDROGENASE (LDH) ACTIVITY:

LDH activity in the tissues and serum of normal and tumor-bearing mice:

The LDH activity was higher in the liver (~70%) and spleen (~38%) of tumor bearing animals as compared to the respective tissue of normal animals. In the kidney of tumor-bearing mice, the LDH activity decreased (~26%) as compared to

the kidney of normal mice. Interestingly, the LDH activity in the serum of tumor-bearing hosts was found to be significantly higher (~62%) than that observed in the serum of normal animals (Table 9; Figure 18 & 19).

LDH activity after CP treatment of tumor-bearing mice:

As compared to control, CP treatment resulted in an increase in the enzyme activity in the liver during 24-48 hr of treatment and it decreased during later period (72h-96h). An initial increase in LDH activity (~19%) was noticed in the kidney after 24h of treatment after which there was an overall decrease. In the spleen as compared to control, an increase in the LDH activity (~27%) was noted at 48h of treatment which later (96h) decreased by ~15%. At 24h-48h of treatment, the LDH activity in the serum was found to increase (~ 20%). As compared to the control, in tumor supernatants, the LDH activity increased to about 2 fold following CP treatment. Tumor cells on the other hand showed an overall decrease in the LDH activity after CP treatment (Table 9; Figure 18 & 19).

LDH activity after AA and CP treatment of tumor-bearing mice:

As compared to the respective control, the pretreatment of the tumor-bearing mice with 1% AA resulted in an increase in LDH activity (~32% in the liver, ~18% in the DL cells and ~9% in the supernatant). However, LDH activity decreased in the kidney, serum and spleen (Table 9). As compared to control, the combined treatment with AA and CP caused a significant increase in LDH activity of 47% and 32% in the liver and kidney respectively at 24h of treatment. A gradual decrease was noted in the spleen showing a maximum decrease of ~45% after 96h of the treatment. Following combined treatment, overall decrease was also noted in the DL cells and

serum. In the ascites supernatant, there is almost a 2-fold increase in the enzyme activity after 48h followed by an overall increase during 72h-96h (Table 9; Figure 18 & 19). As compared to the control (group-I), the LDH activity after combined treatment (group IV) significantly decreased in the spleen (ANOVA $P \leq 0.001$), DL (ANOVA $P \leq 0.01$) and serum (ANOVA $P \leq 0.02$), but in the liver and kidney there is not much significant changes.

LDH ISOZYME PATTERNS:

The analysis of LDH isozyme patterns prominently revealed the presence of all the five isozyme forms (i.e. LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5) in the kidney and serum of both normal and tumor-bearing mice. Among all the isozyme forms, LDH-2 and LDH-3 were found to be more expressed and LDH-5 the least expressed form as indicated from the band intensities. In the kidney of normal mice (lane-1), LDH -2 and LDH-3 are more prominently expressed as compared to the tumor-bearing mice (lane-2) and CP treated mice (lane-3 to lane-6) hence reflecting higher activity. In the normal and tumor serum, LDH-1was the least expressed, with maximum expression of LDH-5. Following CP treatment, the LDH-4 and LDH-5 band activity (intensity) in the serum was noticed to increase apparently during 24h-96h of treatment (lane-3 to lane-6). The isozyme pattern in the liver, however, shows only 4 isozyme bands i.e.LDH-2, LDH-3, LDH-4 and LDH-5 of which LDH-5 seems to be the most predominant form. A distinguishing feature in this case is that after the first day of treatment, there is marked variations in the band intensities revealing the changes in the activity and after 96h of treatment the bands appear fainter with the disappearance of LDH-2 and LDH-1. In the tumor supernatant, LDH-5 is the most active form, LDH-4 and LDH-3 being the other

distinct form present, whereas the other forms vary considerably. The tumor cells show only two bands i.e.LDH-4 and LDH-5 bands, as the conventional forms present. After AA application, no significant variations could be noted; however, the combined treatment of both AA and CP showed some slight changes in the intensities of the bands in the serum and tissues. In the serum, the LDH-5 band intensity was not as high as in case of only CP treated mice. The LDH bands in the liver and spleen showed varying intensities, whereas no change was noticed in the kidney. Interestingly, the presence of one extra band near the cathodic position is the most peculiar feature seen in the serum; tumor supernatant and tumor cells of tumor bearing as well as the CP treated groups (lane-2-6). This additional band is absent in the liver, kidney, spleen and serum of normal animals (Figure 20a, 20b and 20c).

MICROSCOPICAL STUDIES

LIGHT MICROSCOPY:

The light microscopical observations showed the presence of more or less rounded tumor cells with a few leukocytes surrounding the cells. After CP treatment (group-III), leukocytes are noticed to surround and come closer to the tumor cells. The appearance of vacuoles in the plasma membrane of the tumor cells is also seen after the treatment. The combined treatment of mice with AA plus CP further resulted in an increase in the membrane vacuoles, formation and shedding of membrane vesicles and disintegration of the plasma membrane leading to the lysis of the tumor cells (Figure 21a, 21b, 21c and 21d)

TRANSMISSION ELECTRON MICROSCOPY:

The control tumors showed a more or less rounded shaped cell, presence of surface processes and ruffles over the surface, with distinct mitochondrial cristae.. During the treatments condition (group-III and IV), the nuclear changes, mitochondrial changes and the changes in the mitochondria have been highlighted here. CP treatment of Dalton's lymphoma bearing mice for 24h-48h led to the movement of blebs-ruffles from the top surface of the cells towards the peripheral/marginal areas. However, during 24 h of CP treatment, the appearance of fine microvilli like processes extending from tumor cells were also noticed with the irregularity in the organization of mitochondrial cristae. Following 72h of CP treatment, there was disappearance of the membrane processes and thickening of the cristae. After 96h of treatment, rounded shaped mitochondria with thickened membranes along with the reduction in the number of cristae was seen. The application of only AA (group-II), however, shows similar characteristic features to that of the control i.e. the presence of ruffles and processes on the cell surface. It also shows well-defined nuclear membrane and presence of well-arranged cristae in the mitochondria. The combined treatment of mice with AA and CP showed enhanced irregularities in the shape of the mitochondria, thickening of cristae and formation of vacuoles. The mitochondria acquire a roundish form with thickening in the cristae after 24h. A distinct feature observed after 48h was in the reduction in the number of cristae and formation of prominent vacuoles. During 72-96 h of the combined treatment, the presence of roundish mitochondria with thickened membranes, reduction and disruption in the mitochondrial cristae, deformation in the elongated structure of the mitochondria, presence of vacuoles along with the removal of some

cellular material and the disappearance of the cellular outgrowths are the prominent features observed (Figure 22 A-K).

MUTAGENIC STUDIES

Micronuclei assay:

The effect of ascorbic acid on the cyclophosphamide-induced micronuclei frequency was assessed by the analysis of micronuclei in polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs) and the nucleated cells in the bone marrow cells of the hosts. After cyclophosphamide treatment, the frequency of micronucleated PCEs in the bone marrow increased significantly as compared to the control (Figure 24), whereas not much change was observed in frequency of MN in the NCE. In comparison to the untreated control, the micronuclei increased ($P \leq 0.01$) in PCEs and NCEs in the bone marrow cells as well as in RET's and NCE's in the peripheral blood after cyclophosphamide treatment. However, the frequency of micronuclei was observed to decrease significantly in the group of animals pre-treated with ascorbic acid as compared to CP treatment alone in the bone marrow cells ($P \leq 0.001$) and peripheral blood cells ($P \leq 0.01$) (Figure 24). The incidence of micronucleated PCE's and NCE's in the combined treated group were significantly different than those of the respective controls (Table 10&11; Figure 23& 24).

Chromosomal Aberrations:

The chromosomal aberrations in the bone marrow cells of mice increased after CP treatment as compared to the control. The present study showed both chromatid and isochromatid type gaps, chromatid deletions that included breaks,

sister chromatid unions and exchanges were observed in the treated series of which chromatid breaks and gaps occurred more frequently. The total number of aberrations as well as the percent abnormal metaphases decreased appreciably with the duration of treatment from 24h-96h in both the treated groups. The frequency of various aberrations was seen to be maximum at 24h after CP treatment as compared to the control as well as ascorbic acid alone ($P \leq 0.05$). The assessment of the distribution of breaks and gaps tentatively revealed that both the long and short chromosomes were equally vulnerable to CP treatment and the distal regions were found to be more sensitive. Moreover, a comparative analysis at corresponding periods of treatment revealed that total aberrant metaphases were always significantly less in ascorbic acid plus CP treated group of mice ($P \leq 0.05$) than those receiving CP alone (Table 12; Figure 25).

STUDIES ON TRACE ELEMENTS

Analysis of Trace elements:

The concentration of the trace elements (potassium, calcium, magnessium and iron) varied significantly in the tumor cells. Comparing the concentration of the four trace elements analyzed (potassium, calcium, magnessium and iron); the concentration of potassium is noted to be the highest in the tumor cells, followed by calcium, magnesium and iron. Following CP treatment, a significantly low potassium concentration was noted in the Dalton's lymphoma cells ($P \leq 0.05$) as compared to the control. However, pre-administration of AA of the tumor-bearing mice resulted in a significant increase in the potassium concentration ($P \leq 0.05$). The combined treatment of AA and CP also showed a significantly higher concentration in the

initial hours of treatment (24h), which is followed by an overall decrease in the later period (72h-96h). The calcium concentration in the tumor cells shows a different pattern with a gradual increased concentration after CP treatment alone as well as after combined therapy with AA and CP. Analysis of the magnessium concentration in the tumor cells also revealed a somewhat similar trend as noted in the potassium concentration with a gradual decrease after CP administration and an increasing magnessium concentration after the combined treatment. No significant change was however, encountered in the concentration of iron in response to CP treatment alone as well as AA and CP treatment (Table 13).

TABLES

Table 1:

Antitumor activity of cyclophosphamide and ascorbic acid against murine ascites Dalton's lymphoma.

TREATMENT	DAY OF TREAMENT	ROUTE OF TREATMENT	NO. OF MICE IN EACH GROUP	SURVIVAL DAYS (MEAN ± SD)	ILS (%)
Control Group-I	-	-	10	20 ± 2.0	-
AA Group-II	5th day	Oral	10	47.7* ± 1.0	145.8
CP Group-III	10th day	i.p	10	58.0* ± 1.5	199
AA + CP Group-IV	5th day 10th day	Oral i.p	10	68.7* ± 2.3	254.12

Values represent the mean ± SD, n=5.Control = Tumor-bearing mice without AA or CP treatment; AA = ascorbic acid; CP = cyclophosphamide. Increase in life span (ILS) %; (T/C x 100) – 100; where T = mean survival time of the treated group of mice, C = mean survival time of the control. Students t-test n = 5, as compared to control, *$p \leq 0.001$. The significance of variation between different groups and control was tested by ANOVA, *$p \leq 0.01$.

Table 2:

Average tumor pH in the DL cells of tumor-bearing mice before and after treatment.

DL cells	pH
Control	7.15 ± 0.38
Ascorbic acid treated	6.84 ± 0.27
CP treated (24 h)	7.05 ± 0.41
CP treated (48 h)	6.80 ± 0.22
CP treated (72 h)	6.41* ± 0.18
CP treated (96 h)	6.30* ± 0.30
AA+CP treated (24 h)	6.93 ± 0.26
AA+CP treated (48 h)	6.75 ± 0.34
AA+CP treated (72 h)	6.34* ± 0.29
AA+CP treated (96 h)	6.21** ± 0.20

The result are expressed as mean ± SD *p≤ 0.05, **p≤0.02, the significance of the change in pH between the control and different treated group was tested by Student's t-test. Control = Tumor-bearing mice; CP = cyclophosphamide; AA = ascorbic acid; DL= Dalton's Lymphoma.

Table 3:

Total protein content in the tissues (mg/g wet weight), in the supernatant and serum (mg/ml) of Dalton's lymphoma-bearing mice after CP treatment (200mg/kg b.w.) and combination treatment of CP and AA (1%).

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	TESTES	DL	SN	SERUM
NORMAL	228.56±12.2	232.19±14.2	116.42±6.30	133.7±10.7	-	-	48.0±1.0
TB (CONTROL)	197.7±13.57	168.33±6.08	168.97±8.34	152.4±12.53	153.84±9.05	39.86±2.51	60.37±2.54
CP 24h	184.6±5.01*	154.7±2.81*	159.4±5.56*	100.3±5.58*	132.4±2.45*	32.2±2.13	42.08±1.74*
CP48h	172.4±6.57*	163.5±3.8	150.7±4.70*	118.7±4.92*	120.5±4.41*	28.4±1.27*	44.8±1.88*
CP72h	202.6±4.38	170.85±6.74	172.0±4.87	125.3±5.16*	112.56±2.52*	22.6±2.3*	49.0±1.96*
CP96h	213.4±4.28*	178.3±5.58*	178.1±3.8*	134.6±5.97*	103.8±3.09*	18.2±1.78*	50.1±2.01*
AA	201.6±3.3	169.35±4.82*	157.5±3.56*	139.23±3.52*	128.5±2.52*	30.95±1.02	55.46±1.95
AA+ CP 24h	208.5±7.42*	193.2±7.08*	146.6 ±7.35*	116.8±6.0*	115.75±3.11*	28.2±0.68	53.5±1.84
AA+ CP48h	217.4±12.15*	210.4±6.50*	148.3±7.23*	122.7±4.41*	97.19±4.97*	24.6±1.25*	51.2±1.86*
AA+ CP72h	225.6±6.52*	228.7 ±10.6*	155.6±7.47*	136.3±4.48*	85.8±4.09*	20.3±1.71*	48.3±1.71*
AA+ CP96h	245.2±5.78*	235.2±8.18*	163.3±7.10	140.2±6.3*	80.3±3.80*	17.5±1.50*	46.5±1.60*

The results are expressed as Mean ± S.D. Student's t- test, n=6 as compared to the respective control, *p≤0.05. The significance of the total changes between the control and different treated groups was also tested by ANOVA. TB = Tumor-bearing mice; CP = cyclophosphamide; AA = ascorbic acid; DL=Dalton's Lymphoma; SN= supernatant.

Table 4:

Total carbohydrate concentration in the tissues (μg/gm), supernatant and serum (μg/ml) of Dalton's lymphoma-bearing mice after CP treatment (200mg/kg b.w) and combination treatment of CP and AA (1%).

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	TESTES	DL	SN	SERUM
NORMAL	8.34 ±0 .34	3.22 ± 0.20	7.9 ± 0.35	2.46±0.15	-	-	0.65±0.05
TB (CONTROL)	10.12±0.45	3.97±0.27	9.73±0.38	1.76±0.17	1.39±0.09	0.71±0.051	0.46±0.04
CP 24h	11.23±0.52	4.02±0.21	9.78±0.37	1.90±0.15	0.91±0.06*	0.80±0.06	0.80±0.06*
CP48h	9.27±0.32*	3.12±0.15*	7.30±0.42*	2.25±0.16*	1.09±0.08*	0.98±0.055*	1.05±0.07*
CP72h	8.12±0.27*	2.86±0.31*	5.80±0.20*	2.47±0.19*	1.32 ±0.12*	1.25±0.075*	0.72±0.05*
CP96h	8.08±0.25*	2.36±0.24*	4.30±0.21*	2.60±0.18 *	1.62±0.15*	1.20±0.061*	0.67±0.04*
AA	5.90 ± 0.25*	2.60±0.24*	3.20±0.15*	1.92±0.20	1.72±0.14*	1.00±0.09*	0.52±0.04
AA+ CP 24h	9.34±0.70	3.00±0.16*	4.42±0.26*	2.23±0.11*	2.38±0.17*	1.40±0.11*	0.85±0.07*
AA+ CP48h	7.81±0.35*	2.45±0.23*	5.65±0.38*	2.31±0.15*	1.47±0.15*	1.33±0.10*	1.13±0.09*
AA+ CP72h	6.88±0.24*	2.25±0.22*	6.35±0.32*	2.50±0.16*	1.10±0.14*	1.21±0.08*	0.90±0.08*
AA+ CP96h	5.92±0.23*	2.10±0.18*	7.50±0.55*	2.62±0.22*	0.98±0.12*	1.10±0.10*	0.75±0.06*

The results are expressed as Mean ± S.D. Student's t- test, n=6 as compared to the respective control, *P≤0.05.The significance of the total changes between the control and different treated groups was also tested by ANOVA . TB = Tumor-bearing mice; CP= cyclophosphamide; AA=Ascorbic acid: DL=Dalton's Lymphoma; SN= supernatant.

Table 5:

Quantitative changes in the sialic acid content of different tissues (μ moles/g), supernatant and serum (μmoles/ml)of Dalton's lymphoma-bearing mice after CP treatment(200mg/kg b.w.) and combination treatment of CP and AA(1%).

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	TESTES	DL	SN	SERUM	BRAIN
NORMAL	1.031±.0.047	1.078±0.092	2.12±0.143	0.786±0.045	-	-	1.141±0.11	3.213±0.243
TB (CONTROL)	1.482±0.051	1.183±0.11	2.64±0.21	1.053±0.08	1.441±0.11	1.72±0.17	1.85±0.098	5.083±0.344
CP 24h	0.985±0.081*	0.859±0.083*	2.01±0.18*	0.818±0.073*	1.091±0.090*	1.47±0.08	1.5±0.09	4.05±0.28*
CP48h	0.961±0.076*	0.872±0.091*	2.10±0.14*	0.85±0.078*	1.046±0.092*	1.39±0.074*	1.36±0.12*	3.7±0.16*
CP72h	1.213±0.103*	1.107±0.042	2.26±0.16	0.89±0.084	1.087±0.076*	1.3±0.08*	1.31±0.14*	3.32±0.15*
CP96h	1.067±0.088*	1.012±0.061*	2.40±0.19	0.92±0.07	1.031±0.083*	1.25±0.073*	1.24±0.08*	3.15±0.11*
AA	1.54±0.078	1.105±0.087	2.50±0.22	1.0±0.092	1.01±0.058*	1.75±0.11	1.8±0.16	4.48±0.27*
AA+ CP 24h	1.60±0.025*	1.34±0.033	2.34±0.2	1.33±0.10*	0.945±0.088*	1.80±0.15	1.44±0.09	4.35±0.13*
AA+ CP48h	1.39±0.04	1.192±0.028	2.2±0.21	1.25±0.093	0.885±0.041*	1.95±0.13*	1.36±0.12*	3.90±0.10*
AA+ CP72h	1.20±0.044*	1.13±0.036	2.15±0.19*	0.985±0.085	0.830±0.035*	2.10±0.15*	1.20±0.10*	3.51±0.14*
AA+ CP96h	1.08*±0.05*	1.10±0.03	2.08±0.14*	0.80±0.076*	0.772±0.032*	2.2±0.16*	1.15±0.08*	3.3±0.20*

Sialic acid content is reported as μmoles/gm wet wt in the tissues, μmoles/ml in the supernatant and serum. Data are reported as Mean ± S.D (n=4-5) Student's t- test as compared to the respective control *P≤0.05.The significance of the total changes between the control and different treated groups was tested by ANOVA. TB = Tumor-bearing mice; CP= cyclophosphamide; AA=Ascorbic acid: DL=Dalton's Lymphoma; SN= supernatant.

Table 6:

Total Reduced Glutathione content (TGSH) in the tissues (μ mole/g) supernatant and serum (μ moles/ml) of Dalton's lymphoma-bearing mice under different treatment conditions.

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	TESTES	DL	SN	SERUM
NORMAL	11.72±.0.94	8.03±0.68	7.86±0.63	10.16±0.38	-	-	0.48±0.022
TB (CONTROL)	11.91±1.06	8.50±0.61	9.95±0.74	9.28±0.25	4.63±0.26	0.30±0.016	0.27±0.02
CP 24h	13.82±0.49*	9.31±0.56	9.58±0.83	7.57±0.27*	2.25±0.13*	0.25±0.010	0.22±0.019
CP48h	12.19±0.39*	11.16±0.47*	8.97±0.44	7.22±0.44*	2.75±0.11*	0.21±0.014	0.29±0.023
CP72h	11.70±0.42	12.27±0.45*	8.27±0.61*	8.43±0.32	3.67±0.27	0.18±0.007*	0.31±0.018
CP96h	11.50±0.85	12.42±0.36*	8.10±0.35*	8.94±0.36	3.82±0.28	0.15±0.010*	0.36±0.025
AA	11.97±0.58	9.93±0.53	13.07±0.66*	7.96±0.24	2.07±0.25*	0.26±0.020	0.24±0.024
AA+ CP 24h	8.10±0.90*	7.85±0.54	7.25±0.69*	7.45±0.40*	2.33±0.13*	0.20±0.010*	0.28±0.014
AA+ CP48h	8.48±0.72*	8.61±0.38	8.18±0.23*	7.23±0.56*	2.77±0.12*	0.17±0.009*	0.35±0.023
AA+ CP72h	10.05±0.85*	10.58±0.55*	8.70±0.38*	8.86±0.62	3.18±0.18*	0.14±0.010*	0.39±0.028*
AA+ CP96h	10.62±0.73*	11.20±0.46*	8.80±0.58	9.15±0.58	3.30±0.26*	0.10±0.008*	0.42±0.034*

The results are expressed as Mean ± S.D. Student's t- test, n=6 as compared to the respective control, *P≤0.05. The significance of the total changes between the controls and different treated groups was tested by ANOVA. TB = Tumor-bearing mice; CP= cyclophosphamide; AA=Ascorbic acid: DL=Dalton's Lymphoma; SN= supernatant.

TABLE 7:

Quantitative changes in the concentration of Ascorbic acid in the tissues (µmoles/gm), serum and ascites supernatant (µmoles/ml) of tumor control and treated Dalton's lymphoma mice.

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	TESTES	DL	SN	SERUM
NORMAL	174.05±7.02	124.0±5.05	166.4±4.56	143.5±8.10	-	-	10.38±0.62
TB (CONTROL)	145.2±8.96	102.32±4.96	128.77±4.84	118.1±7.4	94.8±3.96	36.47±1.54	7.10±0.40
CP 24h	147.3±5.56	105.43±4.34	130.0±6.06	120.0±4.22	106.27±2.22*	33.71±2.2	7.60±0.45
CP48h	149.5±5.9	108.8±7	132.45±4.4	123.5±6.4	108.15±2.8*	35.2±1.66	8.40±0.50
CP72h	152.0±6.4	110.62±5.8	135.0±3.56	128.3±6.2	110.8±2.72*	37.6±2.4	8.63±0.53
CP96h	154.6±9.4	116.37±4.42*	136.8±4.6	130.6±7.6	111.5±6.6*	38.0±1.28	10.40±0.90*
AA	158.8±7.36	126.72±2.44*	145.2±10.2	123.80±3.46	144.5±7.06*	58.8±3.74*	9.50±0.80*
AA+ CP 24h	160.25±9.8	117.27±5.0*	138.3±8.0	125.9±10.0	117.0±3.5*	43.18±2.22*	9.80±0.95*
AA+ CP48h	162.45±10.2	120.7±2.36*	143.8±6.4*	127.0±8.2	127.45±3.16*	45.6±2.4*	10.30±1.0*
AA+ CP72h	166.0±4.28*	123.3±4.0*	146.0±7.8*	135.8±8.0*	134.07±3.44*	48.3±4.0*	10.45±0.88*
AA+ CP96h	172.3±6.4*	126.85±6.3*	155.2±8.7*	143.7±7.6*	138.3±4.2*	56.02±4.6*	11.06±1.0*

The results are expressed as Mean ± S.D. Student's t- test, n=6 as compared to the respective control, *P≤0.05. The significance of the total change between the control and different treated groups was also tested by ANOVA . TB = Tumor-bearing mice; CP= cyclophosphamide; AA=Ascorbic acid: DL=Dalton's Lymphoma; SN= supernatant.

Table 8:

Total lipid peroxide (LPO) content) in the tissues (n mol/mg protein), supernatant and serum (nmol/ml protein) of Dalton's lymphoma-bearing mice.

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	TESTES	DL	SN	SERUM
NORMAL	0.29±0.012	0.35±0.013	0.87±0.051	0.61±0.04	-	-	0.34±0.06
TB (CONTROL)	0.34±0.024	0.44±0.025	0.43±0.032	0.35±0.022	0.12±0.008	0.93±0.05	0.22±0.015
CP 24h	0.436±0.023*	0.72±0.044*	0.67±0.038*	0.96±0.054*	0.23±0.014*	0.58±0.035*	0.73±0.042*
CP48h	0.530±0.022*	0.64±0.032*	0.786±0.043*	0.76±0.051*	0.28±0.016*	0.68±0.031*	0.62±0.036*
CP72h	0.480±0.014*	0.53±0.025*	0.64±0.037*	0.69±0.046*	0.32±0.024*	0.73±0.021*	0.55±0.031*
CP96h	0.421±0.012	0.48±0.032	0.57±0.031*	0.52±0.048*	0.40±0.021*	0.78±0.015*	0.48±0.024*
AA	0.324±0.022	0.36±0.028	0.40±0.054	0.43±0.051	0.18±0.012	0.84±0.043	0.28±0.022
AA+ CP 24h	0.38±0.016	0.57±0.013*	0.58±0.047*	0.906±0.038*	0.22±0.015*	0.94±0.041	0.71±0.046*
AA+ CP48h	0.315±0.012	0.43±0.016	0.63±0.042*	0.84±0.043*	0.31±0.023*	0.98±0.027	0.64±0.037*
AA+ CP72h	0.26 ±0.011*	0.38±0.021	0.50±0.044	0.67±0.033*	0.25±0.024*	0.82±0.016*	0.52±0.028*
AA+ CP96h	0.22±0.016*	0.28±0.026*	0.47±0.04	0.48±0.044*	0.21±0.031*	0.71±0.014*	0.43±0.025*

The results are expressed as Mean ± S.D. Student's t- test, n=4-5 as compared to the respective control, *P≤0.05. The significance of the total changes between the control and different treated groups was also tested by ANOVA. TB = Tumor-bearing mice; CP= cyclophosphamide; AA=Ascorbic acid: DL=Dalton's Lymphoma; SN= supernatant.

Table 9:

Specific activity (mean ±SD) of lactate dehydrogenase (LDH) in the tissues, serum and supernatant of Dalton's Lymphoma-bearing mice.

TREATMENT CONDITIONS	LIVER	KIDNEY	SPLEEN	DL	SN	SERUM
NORMAL	0.2±0.03	0.46±0.04	0.29±0.03	-	-	0.42±0.04
TB (CONTROL)	0.34±0.04	0.37±0.03	0.40±0.034	0.38±0.017	0.23±0.02	0.68±0.043
CP 24h	0.44±0.032*	0.43±0.04	0.44±0.03	0.32±0.02	0.39±0.015*	0.74±0.045
CP48h	0.53±0.040*	0.35±0.024	0.51±0.035*	0.26±0.023*	0.46±0.03*	0.82±0.05*
CP72h	0.40±0.036	0.30±0.04	0.42±0.032	0.21±0.03*	0.53±0.035*	0.76±0.08
CP96h	0.32±0.021	0.27±0.03*	0.34±0.028	0.24±0.025*	0.50±0.027*	0.59±0.04*
AA	0.45±0.04*	0.33±0.03	0.28±0.03*	0.45±0.03	0.25±0.023	0.63±0.05
AA+ CP 24h	0.50±0.03*	0.49±0.038*	0.33±0.02	0.39±0.026	0.33±0.025*	0.70±0.046
AA+ CP48h	0.36±0.028	0.42±0.032	0.30±0.01*	0.33±0.03	0.43±0.028*	0.56±0.045*
AA+ CP72h	0.28±0.03	0.34±0.025	0.26±0.02*	0.27±0.02*	0.35±0.02*	0.50±0.04*
AA+ CP96h	0.23±0.02*	0.28±0.03*	0.22±0.01*	0.30±0.03*	0.26±0.025	0.44±0.03*

The results are expressed as Mean ± S.D. Student's t- test, n=4-5 as compared to the respective control, *P≤0.05.The significance of the total changes between the control and different treated groups was tested by ANOVA. TB = Tumor-bearing mice; CP= cyclophosphamide; AA=Ascorbic acid: DL=Dalton's Lymphoma; SN= supernatant.

Table 10:

Incidence of micronuclei in bone marrow cells after CP treatment in vivo.(values are mean % ± S. D)

TREATMENT CONDITIONS	TOTAL NO OF CELLS EXAMINED	% PCEs	% NCEs	% NUCLEATED CELLS
TB Control	2000	0.13 ± 0.06	0.06 ± 0.03	0.16 ± 0.05
CP	2000	1.82 ± 0.21*	0.78 ± 0.15*	0.62± 0.08*
AA	2000	0.28 ± 0.05*	0.18 ± 0.04*	0.15 ± 0.06
AA +CP	2000	0.45± 0.10*$^{\#}$	0.24± 0.08*$^{\#}$	0.18 ± 0.08$^{\#}$

Cyclophosphamide (CP) was administered as a single dose (200mg/Kg body weight; ip.1 % Ascorbic acid (AA) was given in drinking water from the 5th day of tumor transplantation ; TB = Tumor-bearing mice ; PCE = Polychromatic erythrocyte; NCE = Normochromatic erythrocyte; MN = Micronucleus. Results are expressed as Mean ± SD and statistical analysis was done using t-test, (n = 5) as compared to control (*$p \leq 0.01$) and as compared to CP treatment alone ($^{\#}p \leq 0.001$).

Table 11:

Incidence of micronuclei in the peripheral blood cells after CP treatment in vivo. (values are mean % ± S. D)

TREATMENT	TOTAL No. OF CELLS EXAMINED	% MN RET	% MN NCE	% NUCLEATED CELLS
TB Control	2000	0.05 ± 0.02	0.02 ± 0.01	0.08 ± 0.02
CP	2000	0.70 ± 0.19	0.21 ± 0.09	0.30 ± 0.06
AA	2000	0.11 ± 0.04	0.04 ± 0.01	0.05 ± 0.01
AA + CP	2000	0.32 ± 0.12	0.18 ± 0.07	0.14 ± 0.03

TB = Tumor-bearing mice; Cyclophosphamide (CP) was administered as a single dose (200mg/Kg body weight; ip). 1 % Ascorbic acid (1%) was given in drinking water from the 5th day of tumor transplantation; RET= Reticulocytes, MN RET= micronucleated RET, MNNCE= micronucleated normochromatic erythrocytes. Results are expressed as Mean ± SD and statistical analysis was done using t-test, (n = 5) as compared to control ($^{*}p \leq 0.01$) and as compared to CP treatment alone ($^{\#}p \leq 0.001$).

Table 12:

Frequency of chromosomal aberrations in bone marrow cells of mice under different CP and AA treatment conditions in vivo.

Treatment	No of metaphases scored	Mean % of aberrant metaphases	Chromatid %		Exchange %	Isochromatid %		SCU%	Total aberrations per cell Mean ± SD
			Breaks	Gaps		Breaks	Gaps		
TB Control	500	0.76	0.25	0.31	0.28	-	-	-	0.53±0.08
AA	500	0.83	0.28	0.46	0.30	-	-	-	0.58±0.11
CP24h	400	44.25	32.15	14.05	6.4	4.0	4.32	2.10	44.65±5.3$^{*\#}$
CP48h	400	35.0	20.6	8.3	4.1	2.3	2.52	1.60	28.6±3.88$^{*\#}$
CP72h	400	23.5	12.3	4.2	2.7	1.2	2.32	1.52	17.72±2.70$^{*\#}$
CP96h	300	14.0	4.8	1.8	2.1	0.8	1.09	0.34	8.04±0.97$^{*\#}$
AA+CP24h	300	25.5	27.9	8.6	6.12	2.56	3.85	1.25	37.83±4.42$^{*\#}$
AA+CP48h	300	11.5	14.3	4.2	3.08	1.93	2.56	1.83	21.14±2.14$^{*\#}$
AA+CP72h	300	7.03	3.74	2.0	1.83	0.94	1.86	1.24	7.75±1.03$^{*\#}$
AA+CP96h	300	2.74	1.38	0.8	0.93	0.41	0.43	0.56	3.28±0.068$^{*\#}$

TB = Tumor-bearing mice; CP=Cyclophosphamide was given as a single dose (200mg/kg body wt.i.p.).AA= Ascorbic acid (1%) was given in the drinking water on The 5th day after tumor transplantation, SCU, Sister chromatid unions. Gaps have not been included .Results are expressed as Mean± SD. Student's t-test, n=4-5; as compared to the control (*) and to respective treatment with ascorbic acid alone ($^{\#}$), $^{*\#}$ $p \leq 0.05$.

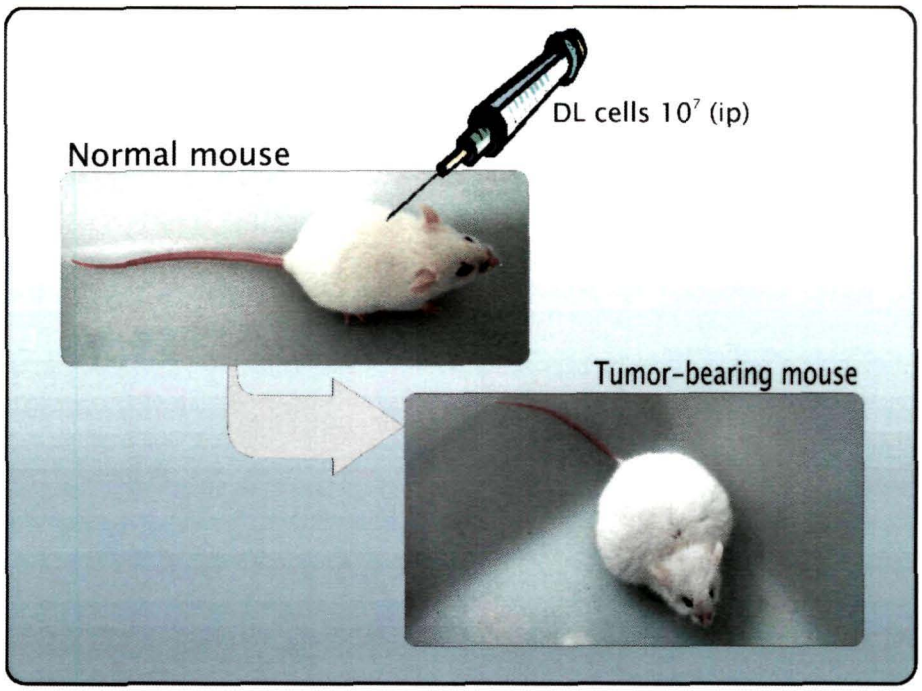
Table 13:

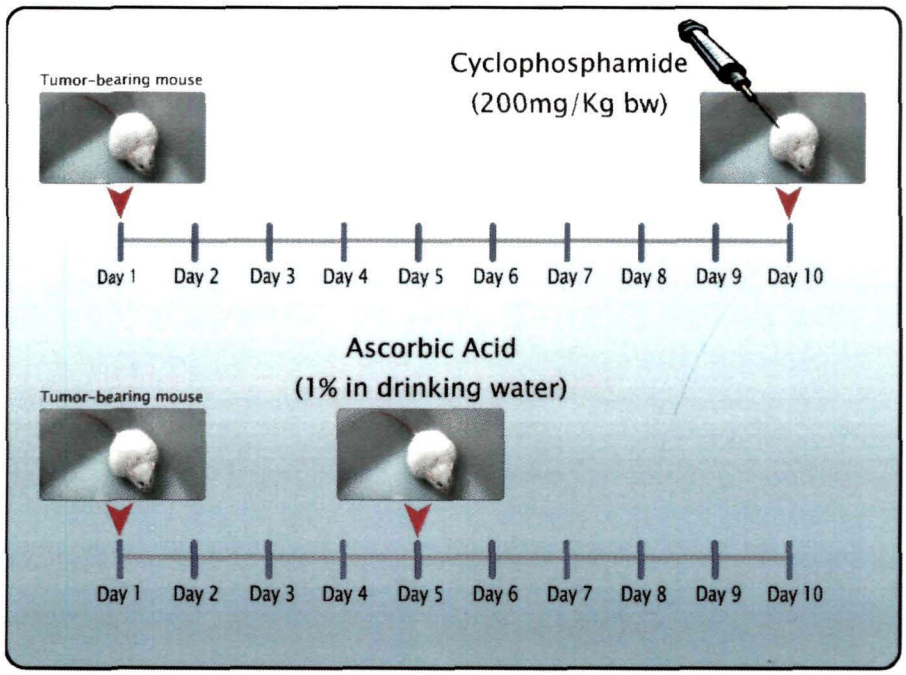
Concentration (μg /gm dry weight of tissue) of trace elements in Dalton's lymphoma with or with out treatment with Cyclophosphamide and Ascorbic acid.

DL Cell	Potassium	Calcium	Magnesium	Iron
TB CONTROL	1485± 26.3	158±8.3	139±8.7	15.60±0.95
CP 24h	854± 20.1*	180±9.7	145±10.8	20.53±1.13
CP48h	689±18.0*	200±11.4	94±6.8*	19.40±1.0
CP 72h	627± 13.5*	225±10.8*	88±9.5*	18.38±0.68
CP 96h	594± 14.7*	260±9.7*	84±7.6*	19.05±1.0
AA	1651±21.3*	164±8.6	148±10.4	17.00±0.74
AA+ CP 24h	1688±25.4*	193±10.1	192±15.3*	16.40±0.92
AA+ CP 48h	930±22.0*	210±11.3*	168±12.6	15.00±0.44
AA+ CP 72h	640±18.8*	242±21.0*	150±14.3	14.80±0.62
AA+ CP 96h	408±10.6*	290±18.2*	143±13.7	13.00±.51

TB = Tumor-bearing mice; CP=Cyclophosphamide was given as a single dose (200mg/kg body wt.i.p.).AA= Ascorbic acid (1%) was given in the drinking water on the5th day after tumor transplantation. Results are expressed as Mean± SD. Student's t-test, n=4-; as compared to the control*# $p \leq 0.05$.

FIGURES
&
GRAPHS


DL cells 10^7 (ip)
Normal mouse
Tumor-bearing mouse


Tumor-bearing mouse
Cyclophosphamide
(200mg/Kg bw)
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10
Ascorbic Acid
(1% in drinking water)
Tumor-bearing mouse
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
Day 10

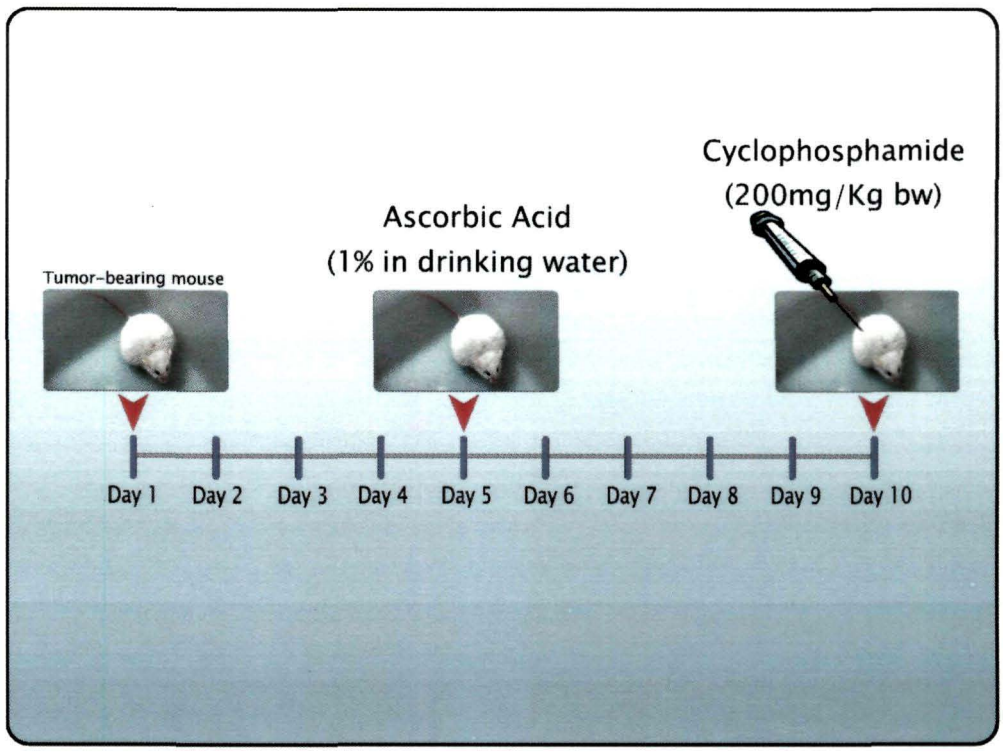


Figure 1. Photograph showing normal and tumor-bearing mouse (A) and different treatment schedule of the tumor-bearing mice (B, C).

The treatment schedule was carried out for 4 days i.e. 24, 48, 72, 96 hours of time interval. The tumor bearing mice (control) as well as the treated group of mice were sacrificed by cervical dislocation and the ascites SN, DL cells, tissues, bone marrow and blood samples were collected after treatment and processed for the various experiments respectively.

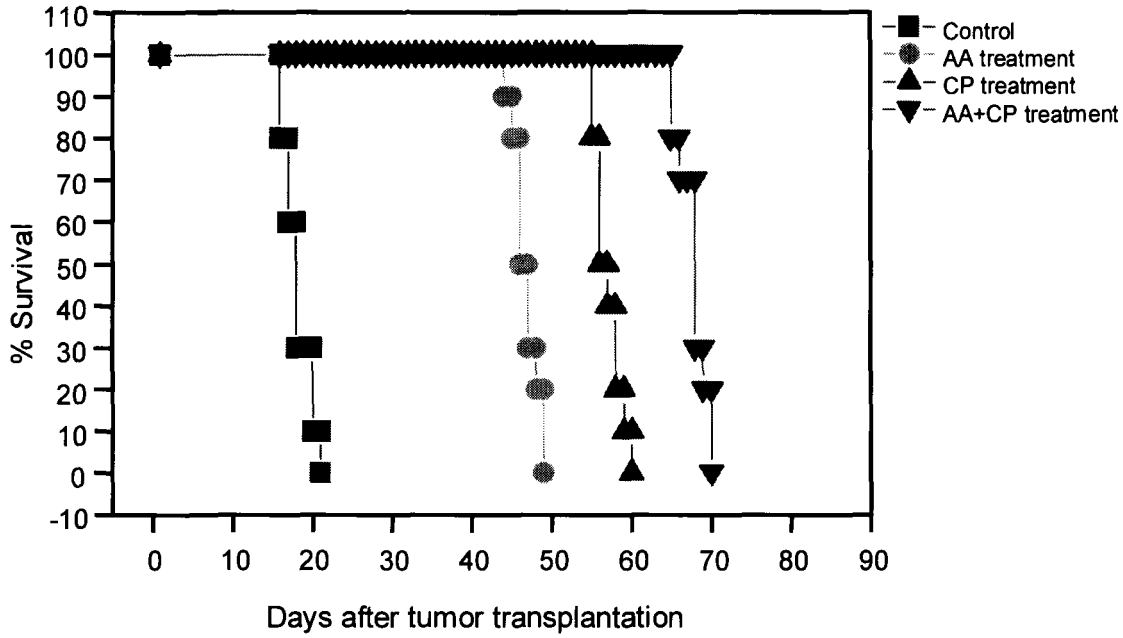


Figure 2.
Graph showing the survival pattern of the tumor bearing after different treatments. Control (tumor bearing mice without treatment). Treatment with CP (200mg/kg b.w.) on the 10th day of tumor growth, AA (1% in drinking water) from the 5th day onwards and the combined treatment with AA(1%) and CP(200mg/kg b.w.). Results are expressed as a mean of 5 independent experimental sets.

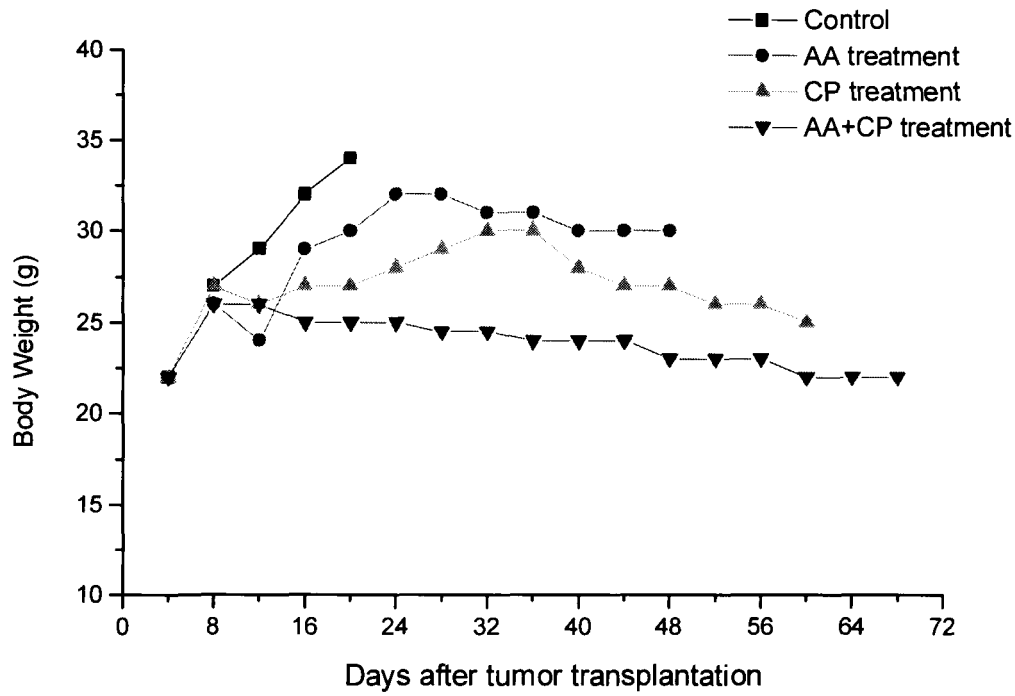


Figure 3.
Graph showing the change in the body weight (gram) in the control tumor mice and under different treatment conditions.

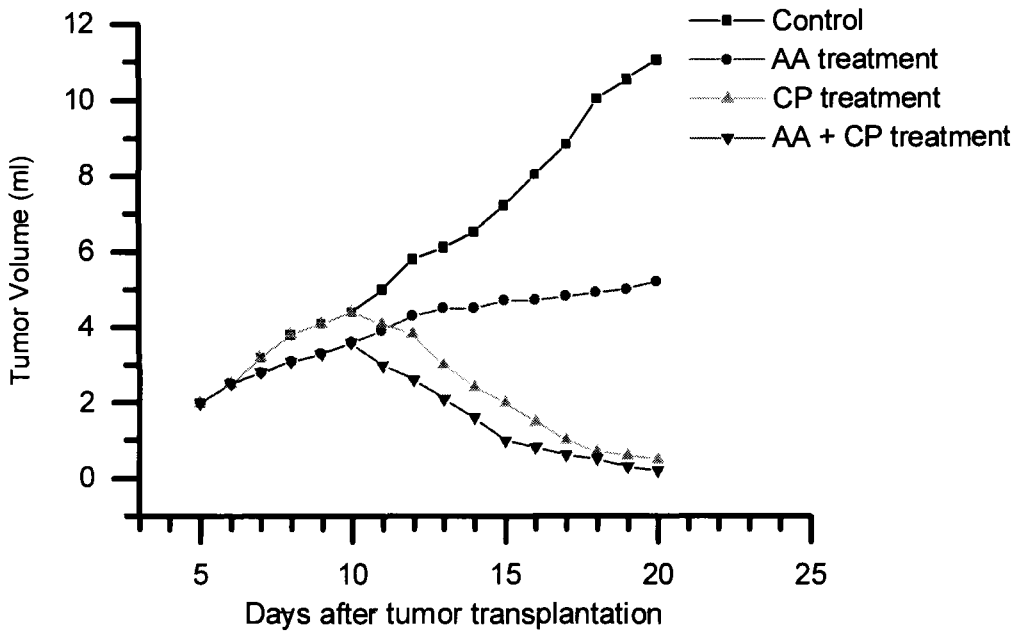


Figure 4.
Graph showing the changes in the total tumor volume (ml) in the control tumor mice and under different treated conditions.

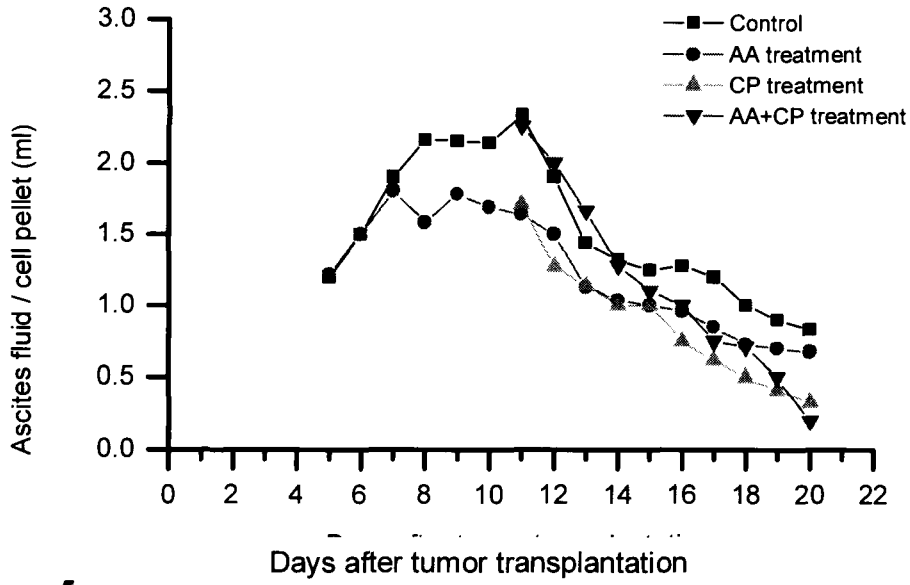


Figure 5.
Graph showing the ratio of the ascites fluid and cell pellet (ml) in the tumor control and under different treatment conditions.

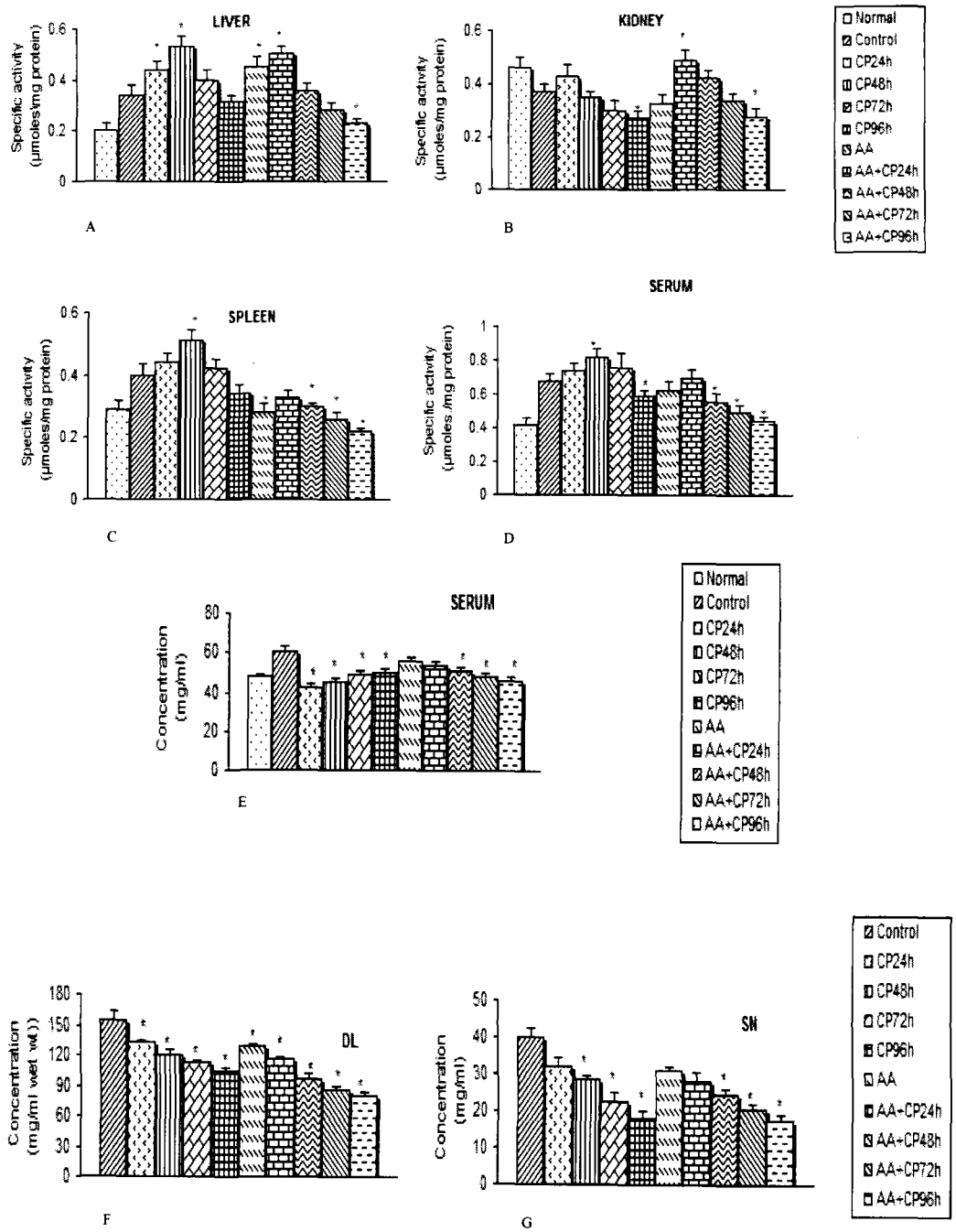


Figure 6.

Histogram showing the changes in protein concentration in the (A) liver, (B) kidney (C) spleen (D) testes (E) serum (F) DL and (G) ascites SN of tumor-bearing mice after treatment with CP and ascorbic acid. Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, $p \leq 0.05$.*

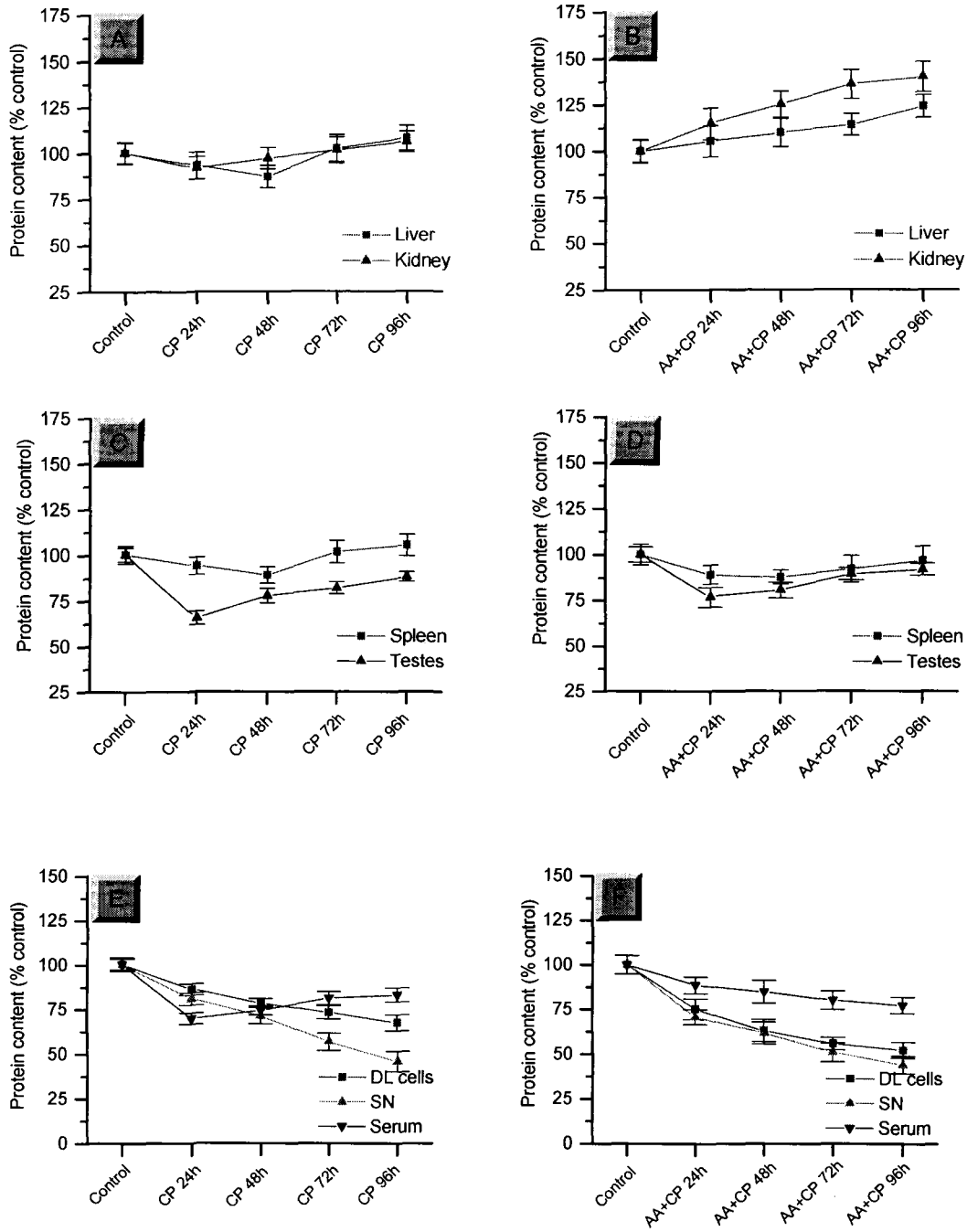


Figure 7.

*Graph showing the percent change in the protein content in the tissues(mg/g), DL cells, ascites supernatant and serum (mg/ml) of tumor-bearing mice after CP treatment (200mg/g) and combined treatment of CP and AA(1%). Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * p ≤ 0.05.*

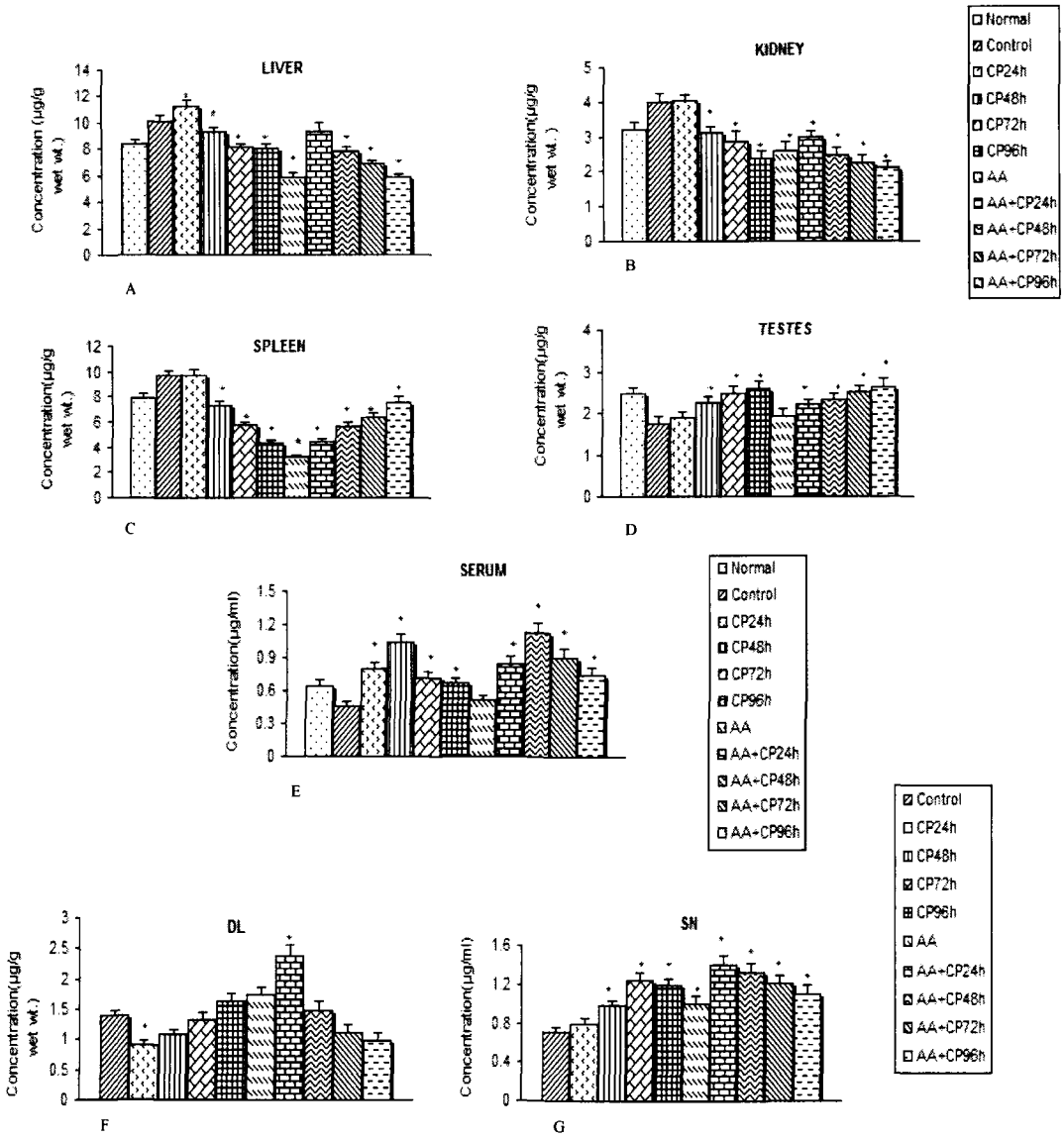


Figure 8.

Histogram showing the changes in carbohydrate concentration in the (A) liver, (B) kidney, (C) spleen, (D) testes, (E) serum, (F) DL and (G) ascites SN of tumor-bearing mice after treatment with CP and ascorbic acid. Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, $p \leq 0.05$*

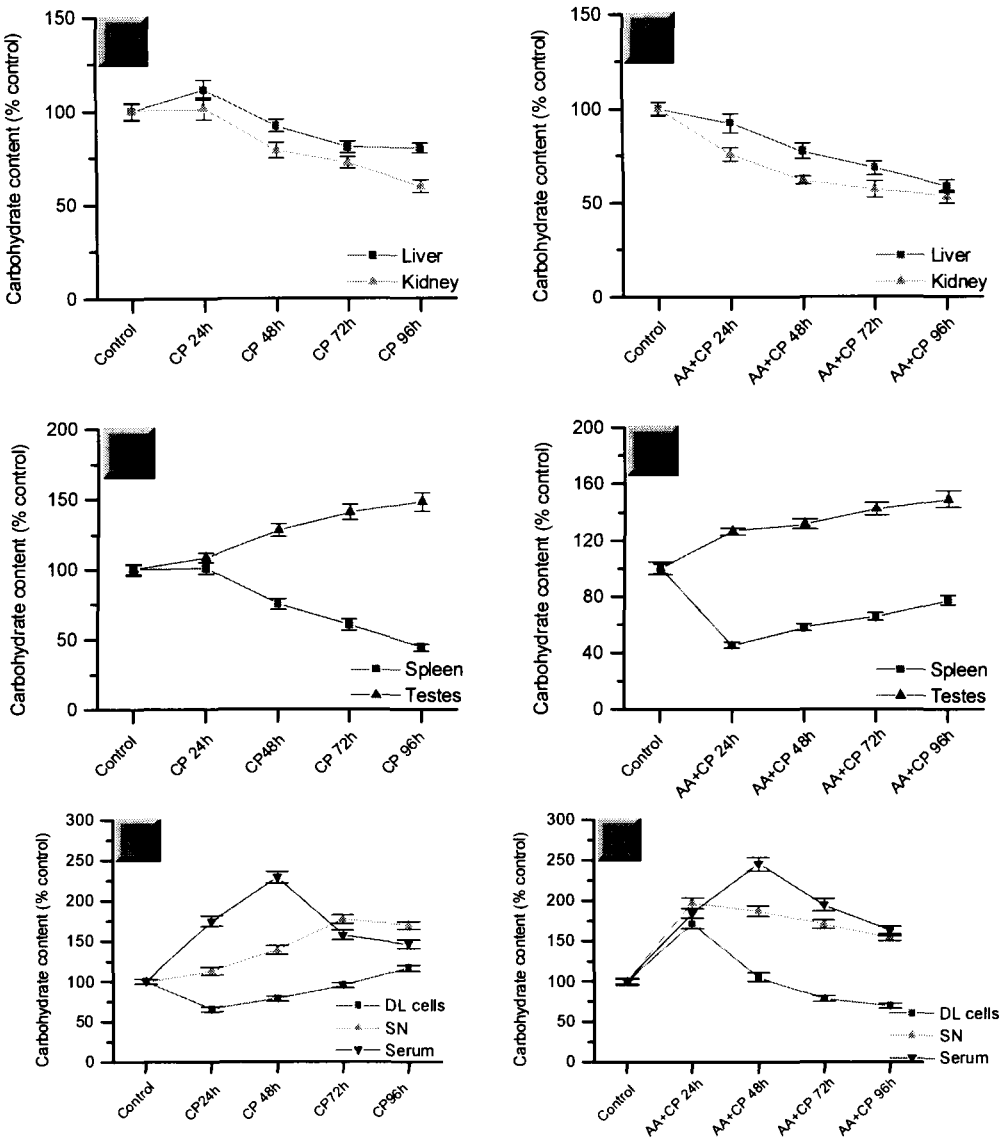
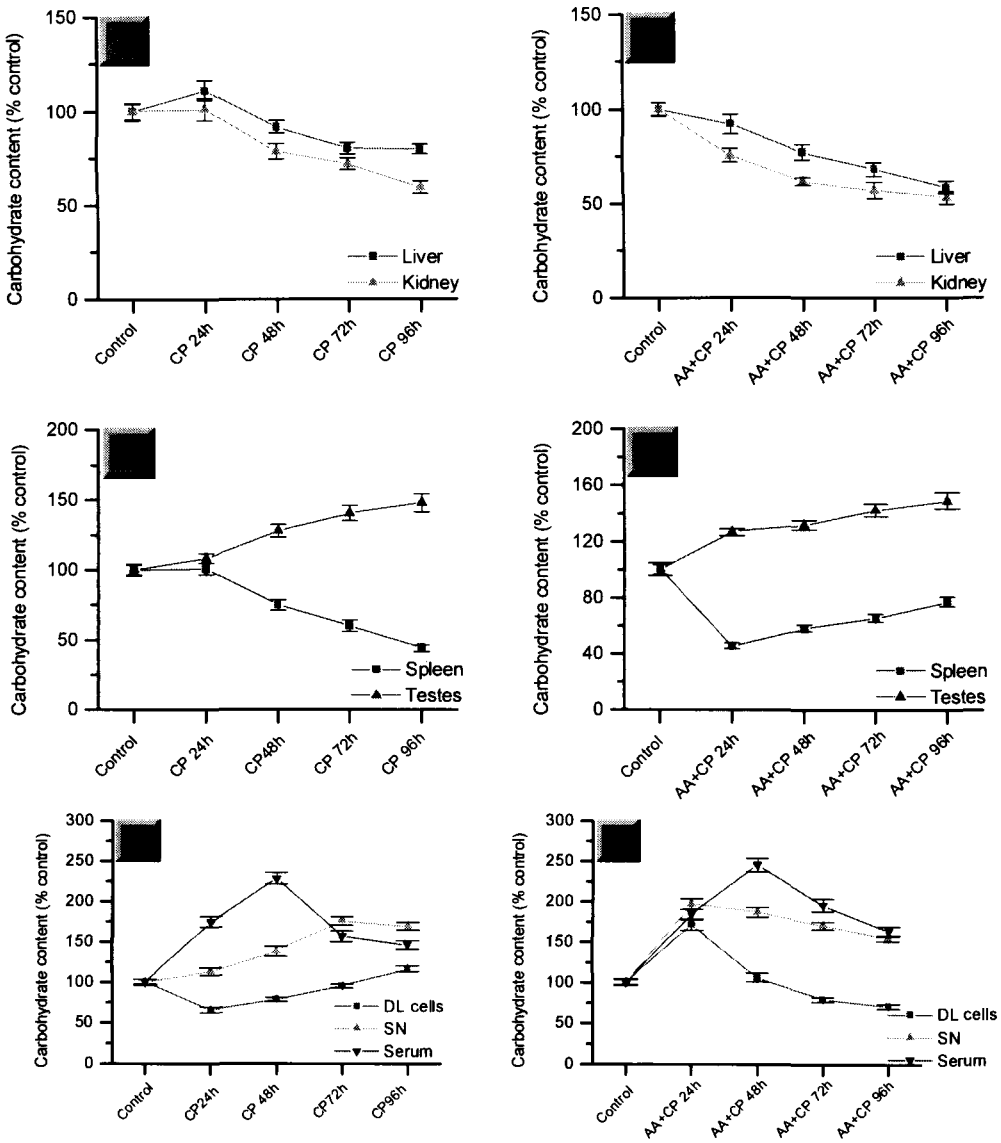


Figure 9.

Graph showing the percent change in the Carbohydrate content in DL cells(μg/g), ascites supernatant and serum(μg/ml) of tumor-bearing mice after CP treatment(200mg/g) and combined treatment of CP and AA(1%). Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, p ≤ 0.05*



Graph showing the percent change in the Carbohydrate content in DL cells(µg/g), ascites supernatant and serum(µg/ml) of tumor-bearing mice after CP treatment(200mg/g) and combined treatment of CP and AA(1%).

Figure 9.

Graph showing the percent change in the Carbohydrate content in DL cells(µg/g), ascites supernatant and serum(µg/ml) of tumor-bearing mice after CP treatment(200mg/g) and combined treatment of CP and AA(1%). Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, p ≤ 0.05*

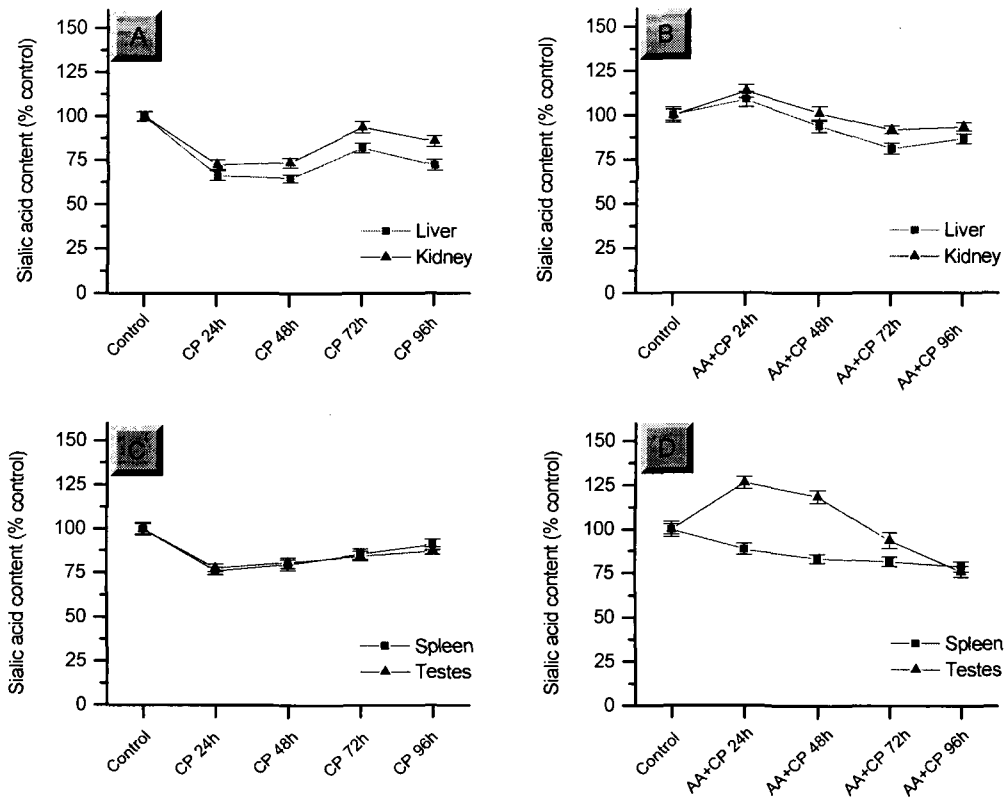


Figure 11a.

*Graph showing the percent change in the sialic acid concentration (µmole/g) in the liver, kidney, spleen and testes of tumor-bearing mice after cyclophosphamide (200mg/kg b.w.) treatment and combined treatment of ascorbic acid and cyclophosphamide. Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * $p \leq 0.05$.*

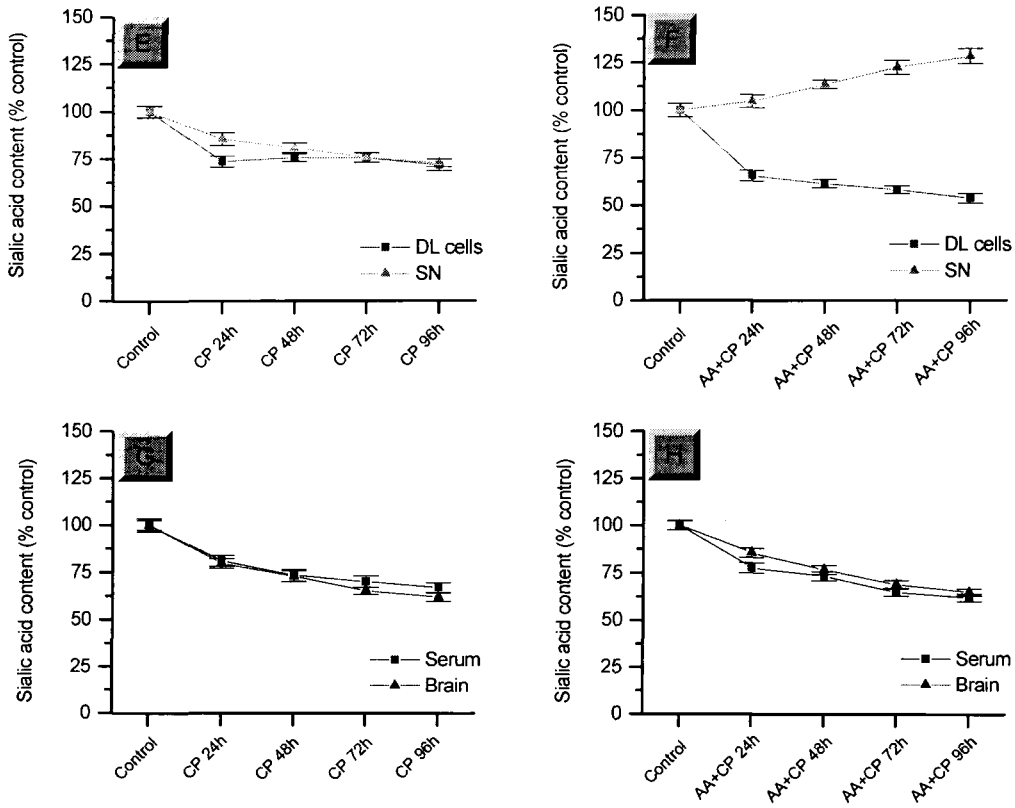


Figure 11b.

*Graph showing the percent change in the sialic acid concentration in the brain DL cells (µmole/g) and ascites supernatant, serum(µmole/ml)) of tumor-bearing mice after Cyclophosphamide (200mg/kg b.w.) treatment and combined treatment of ascorbic acid and cyclophosphamide. Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * $p \leq 0.05$.*

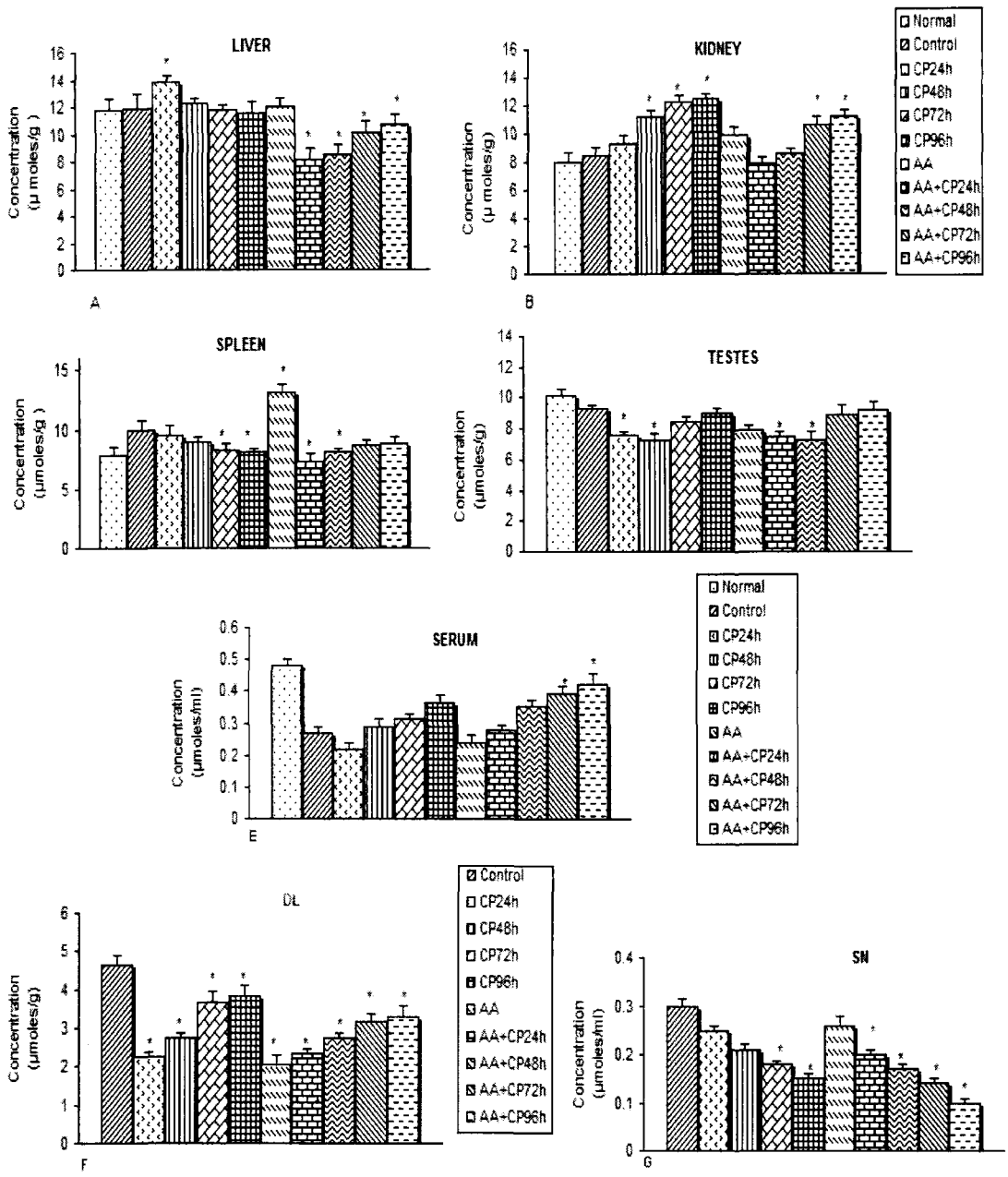


Figure 12.

Histogram showing the changes in the total glutathione concentration in the (A) liver, (B) kidney (C) spleen (D) testes (E) serum (F) DL cells and (G) ascites supernatant of tumor-bearing mice after treatment with CP and ascorbic acid Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, $p \leq 0.05$.*

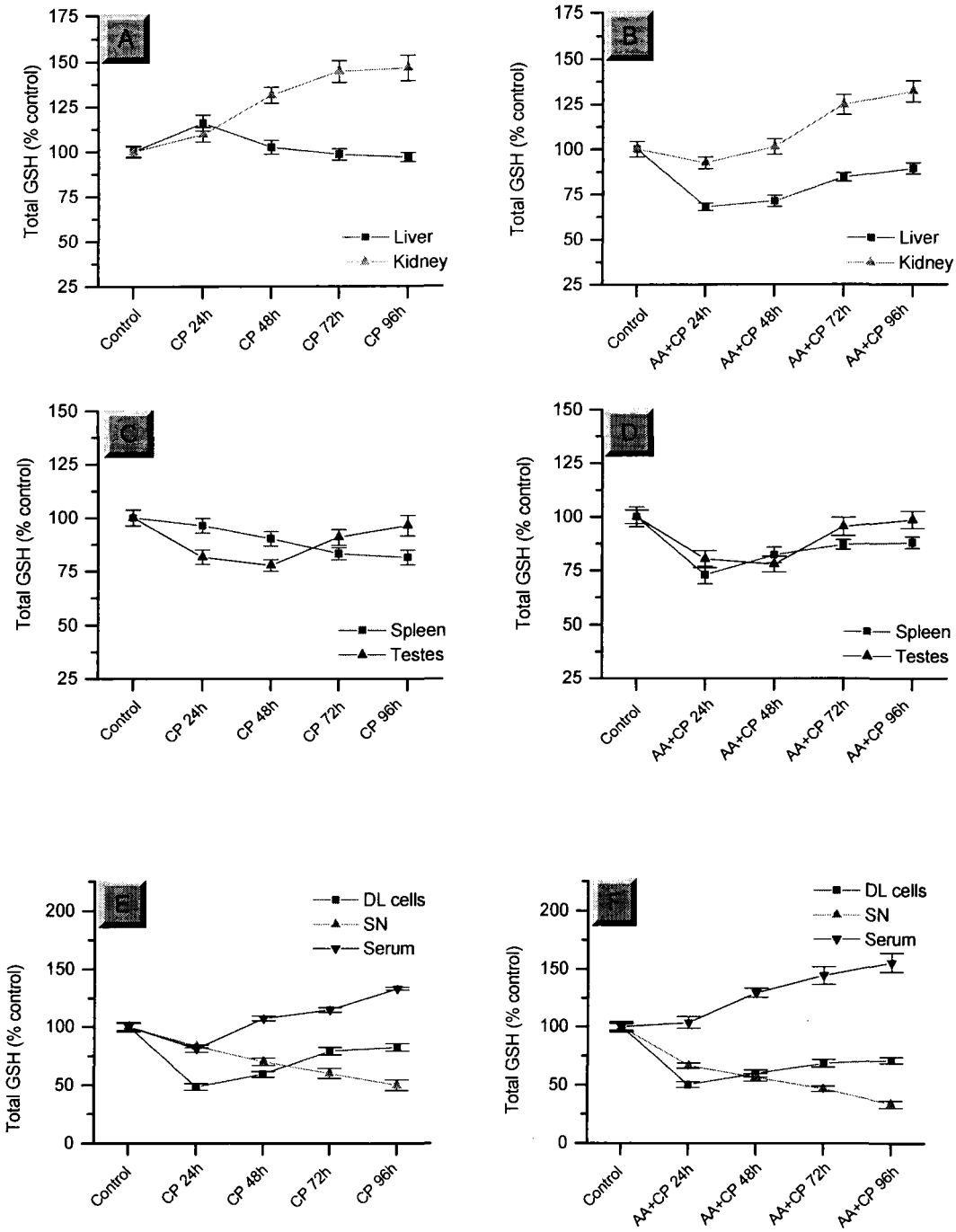


Figure 13.

Graph showing the percent change in the total GSH(TSH) content in DL cells(μ moles/g), ascites supernatant and serum(μ moles/ml) of tumor-bearing mice after CP treatment(200mg/g) and combined treatment of CP and AA(1%). Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, $p \leq 0.05$*

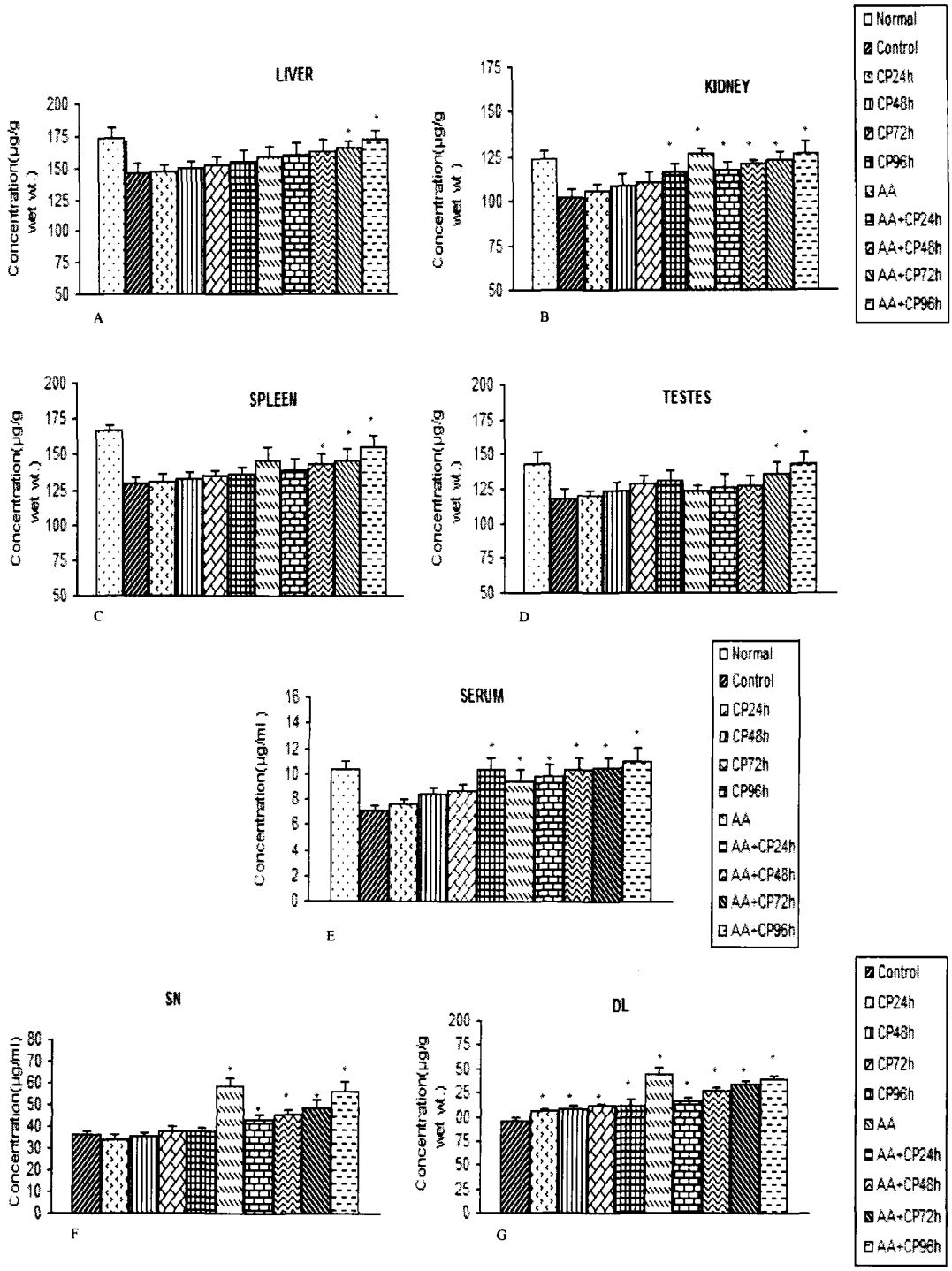


Figure 14.

*Histogram showing the changes in ascorbic acid concentration in the (A) liver, (B) kidney (C) spleen (D) testes (E) serum (F) ascites supernatant and (G) DL cells of tumor-bearing mice after treatment with CP and ascorbic acid Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * $p \leq 0.05$.*

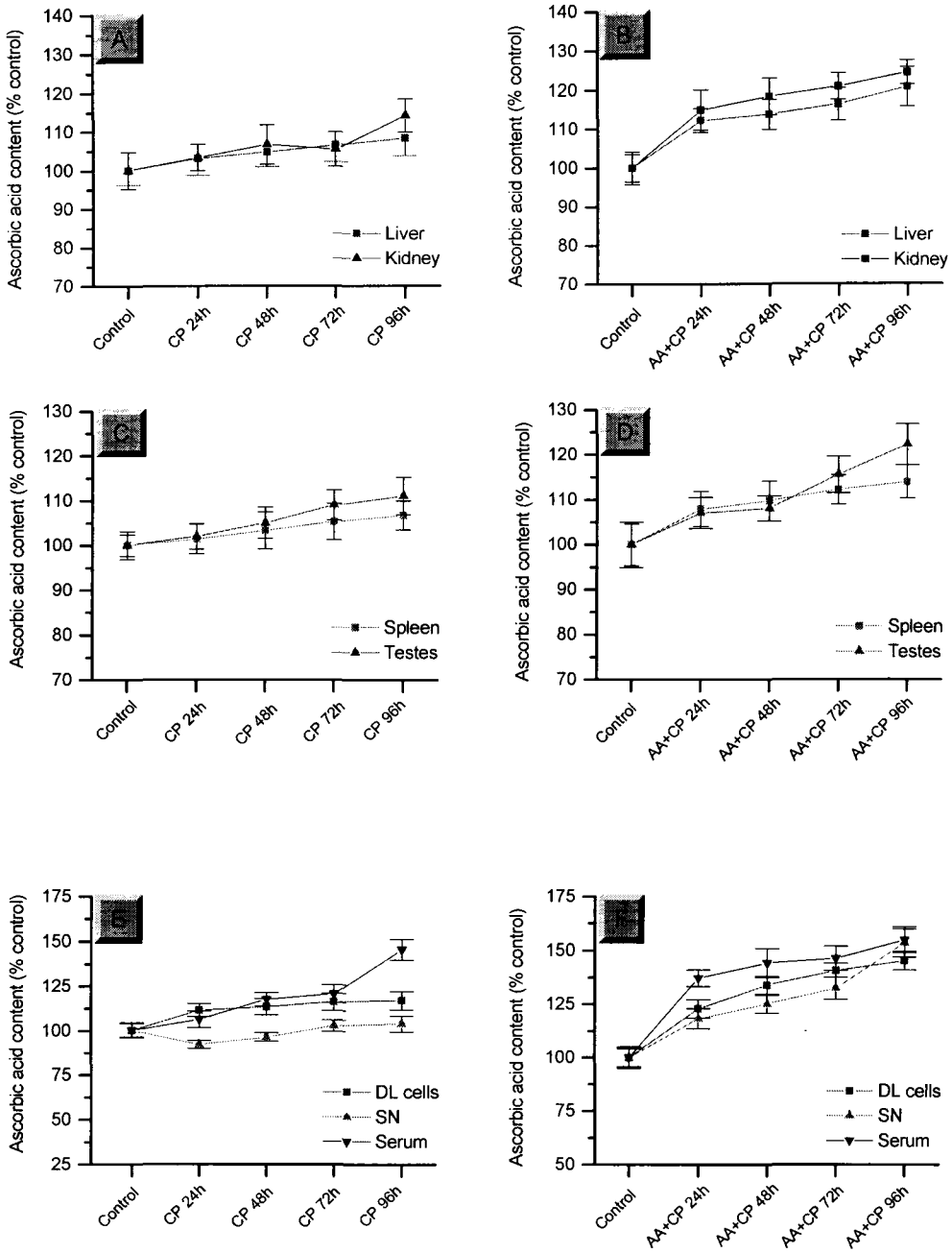


Figure 15.

*Graph showing the percent change in the ascorbic acid content in DL cells(μg/g), ascites supernatant and serum(μg/ml) of tumor-bearing mice after CP treatment (200mg/g) and combined treatment of CP and AA(1%). Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * $p \leq 0.05$.*

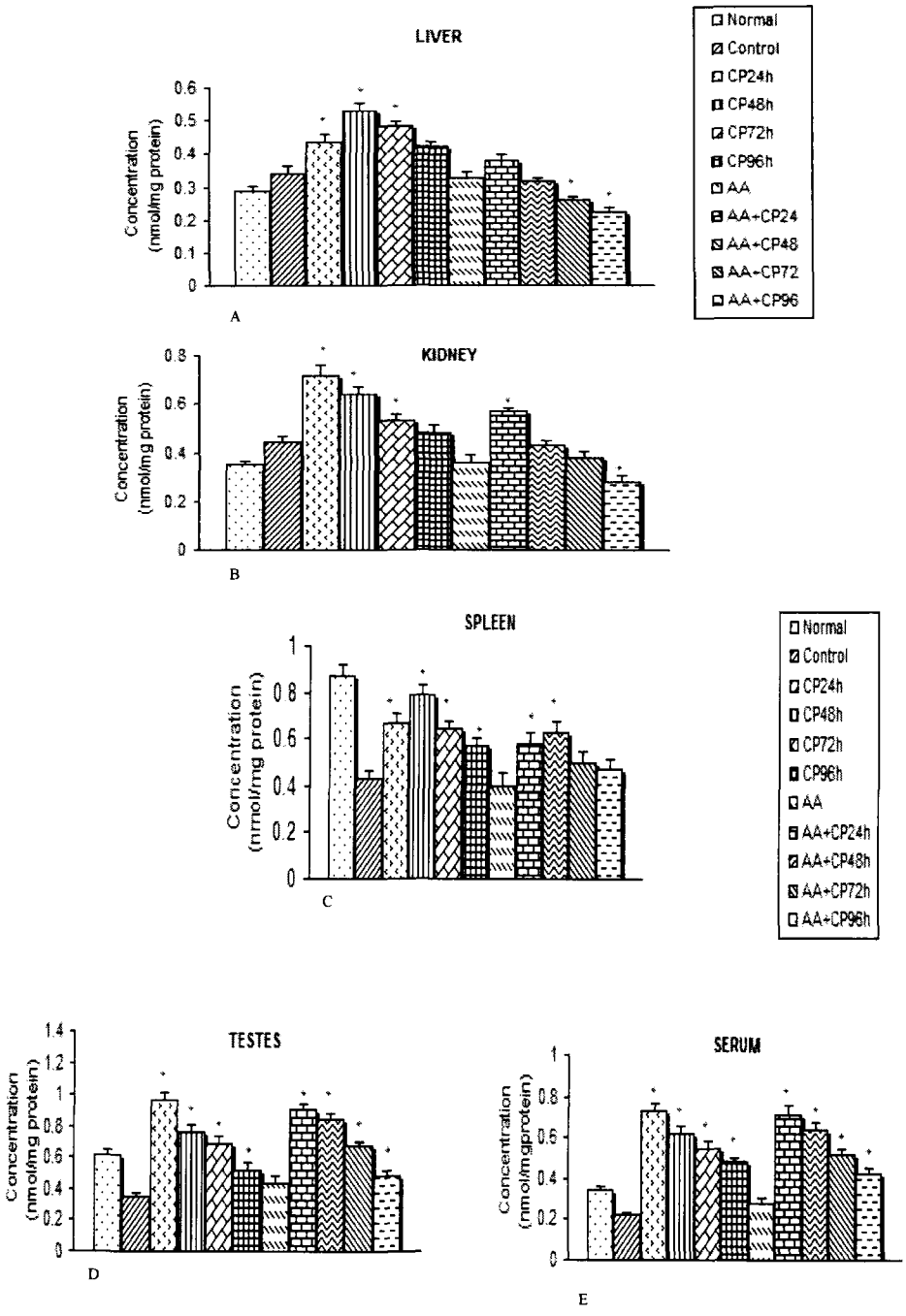


Figure 16a.

Histogram showing the changes in the LPO concentration in the (A) liver, (B) kidney (C) spleen, (D) testes and (E) serum of tumor-bearing mice after treatment with CP and ascorbic acid Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, $p \leq 0.05$.

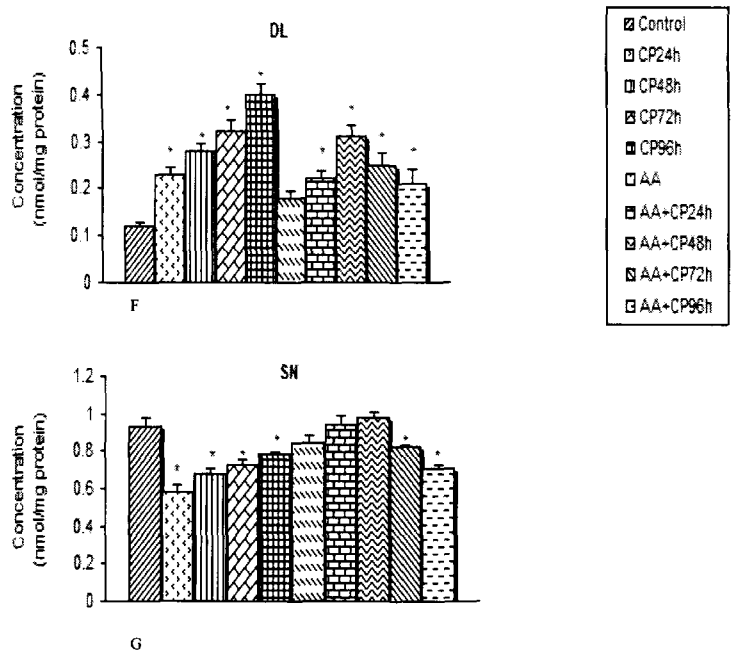


Figure 16b.

Histogram showing the changes in the LPO concentration in the (F) DL, (G) ascites SN of tumor-bearing mice after treatment with CP and ascorbic acid. Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, p ≤ 0.05.*

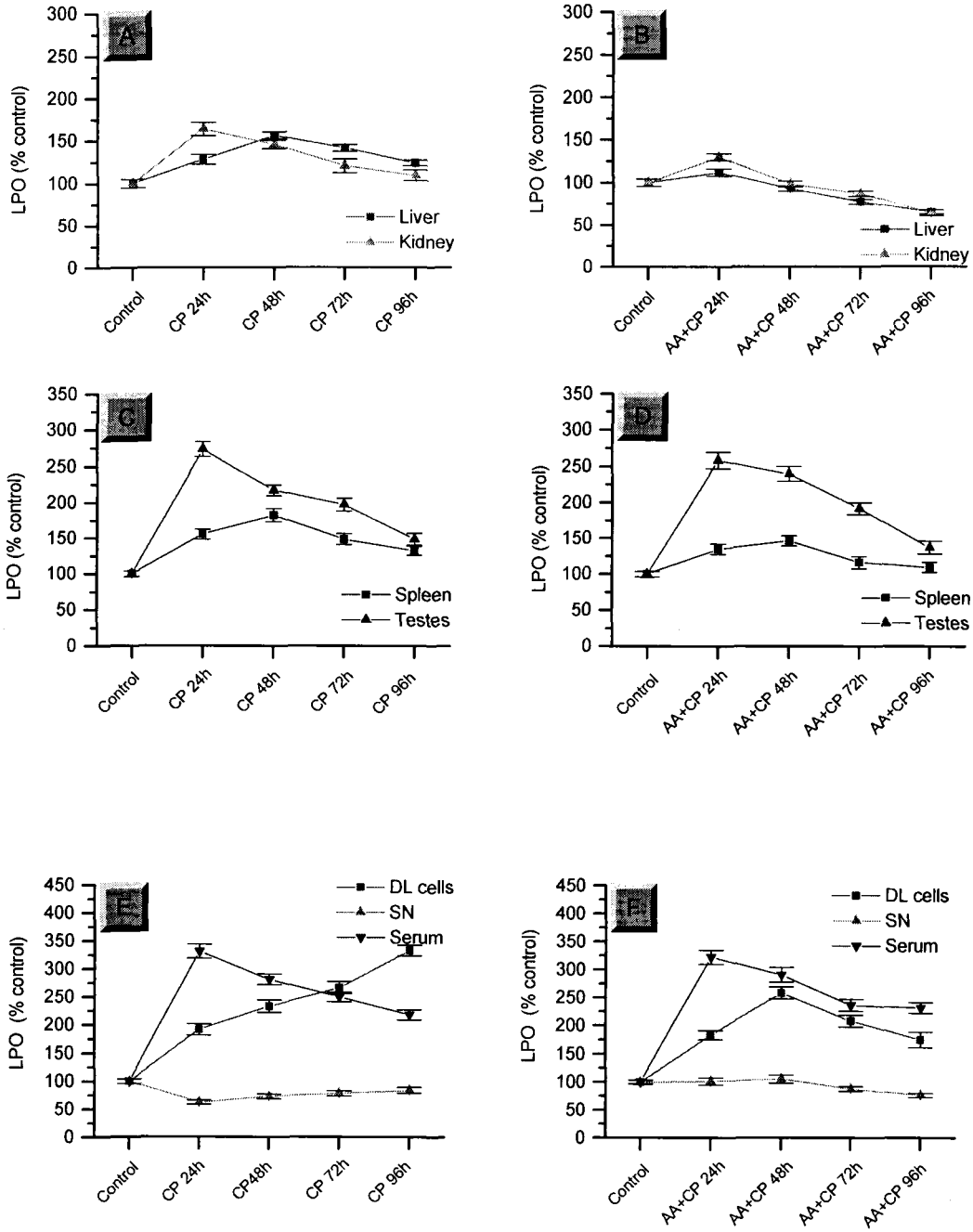


Figure 17

*Graph showing the percent change in the LPO (nmol/mg protein) in liver, kidney, spleen, testes, DL cells, ascites supernatant and serum of tumor-bearing mice after CP treatment(200mg/g) and combined treatment of CP and AA(1%). Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * $p \leq 0.05$.*

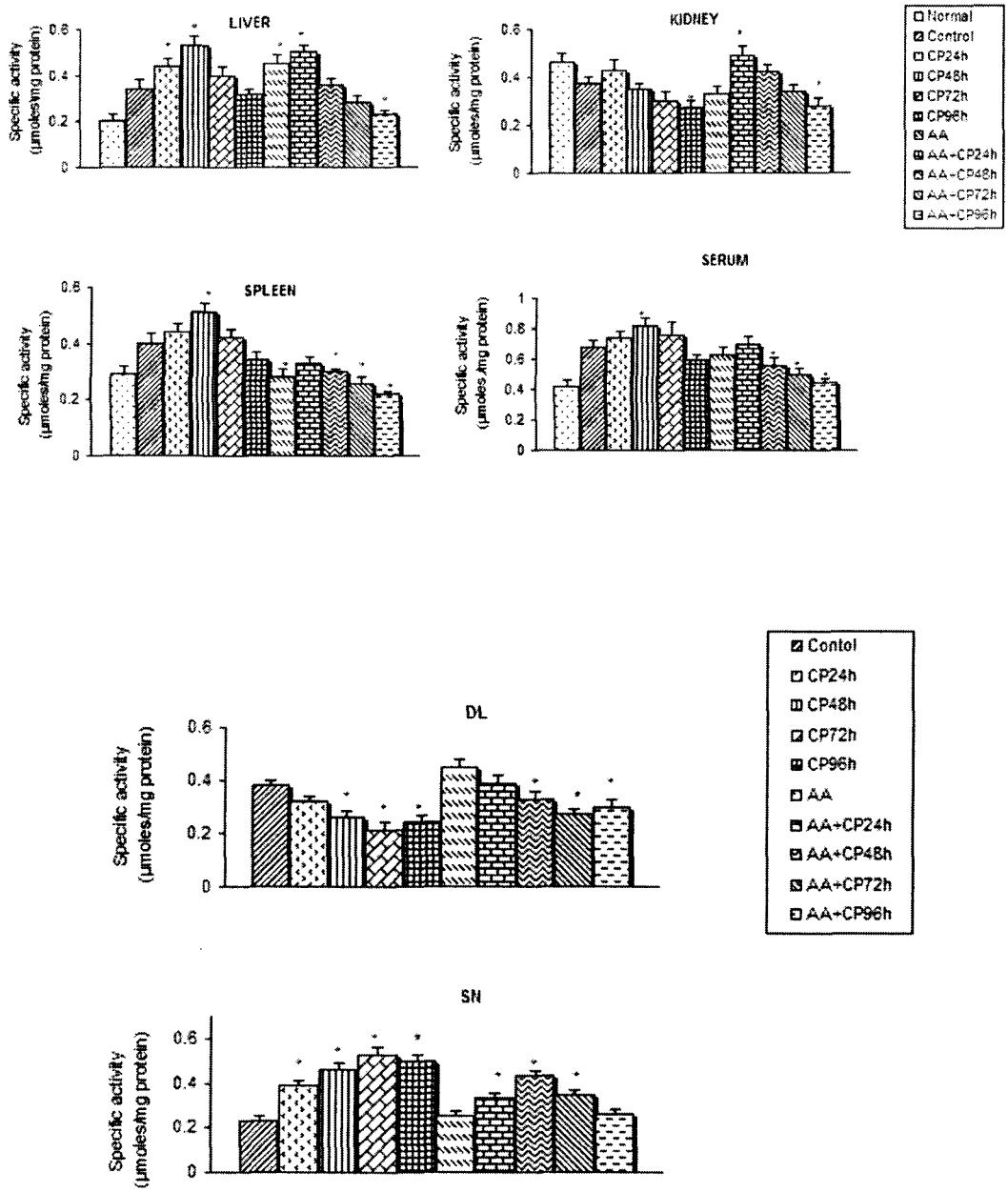


Figure 18.

Histogram showing the changes in the LDH activity in the (A) liver, (B) kidney (C) spleen (D) serum (E) DL cells and (F) ascites SN of tumor-bearing mice after treatment with CP and ascorbic acid Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, $p \leq 0.05$..

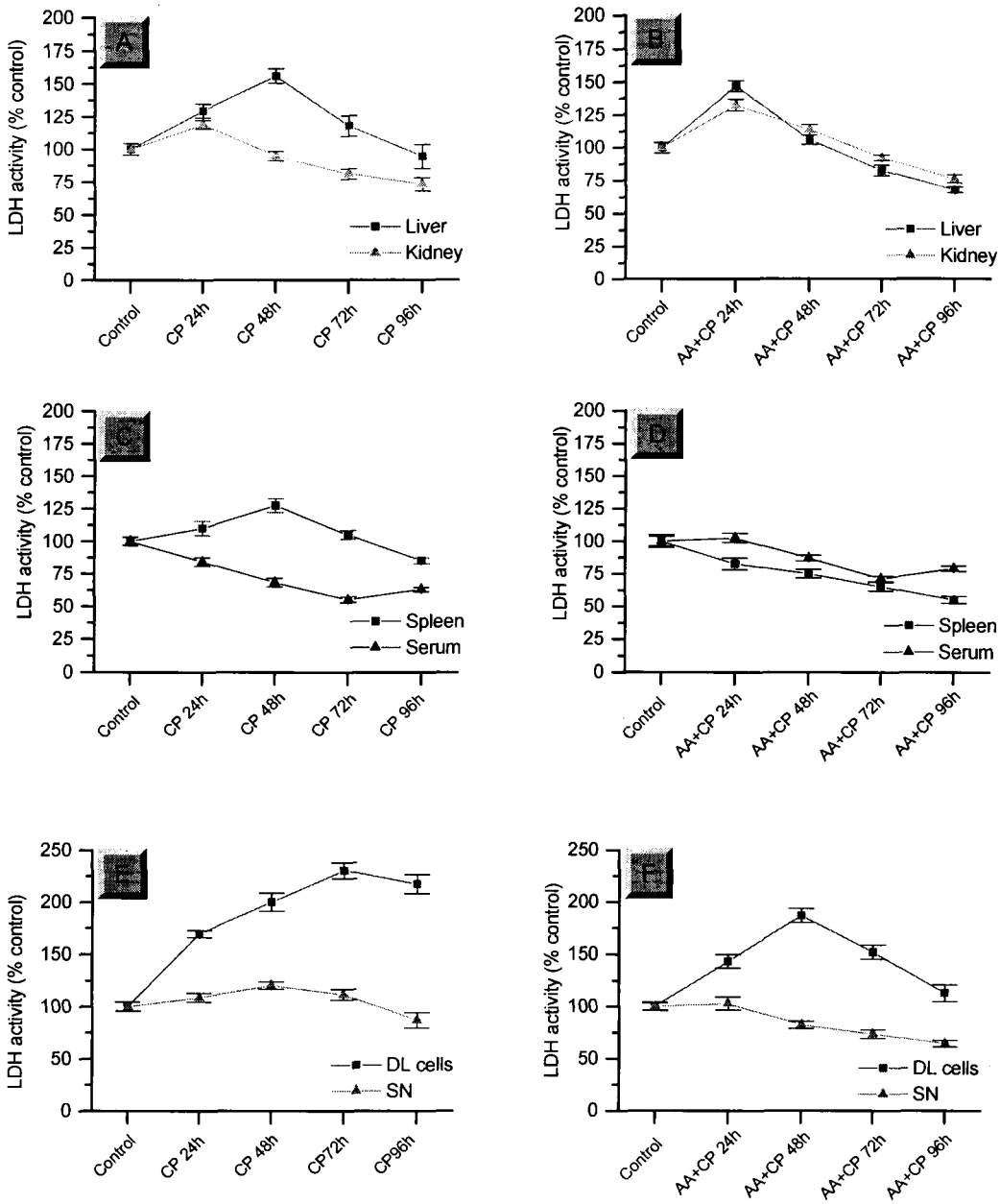
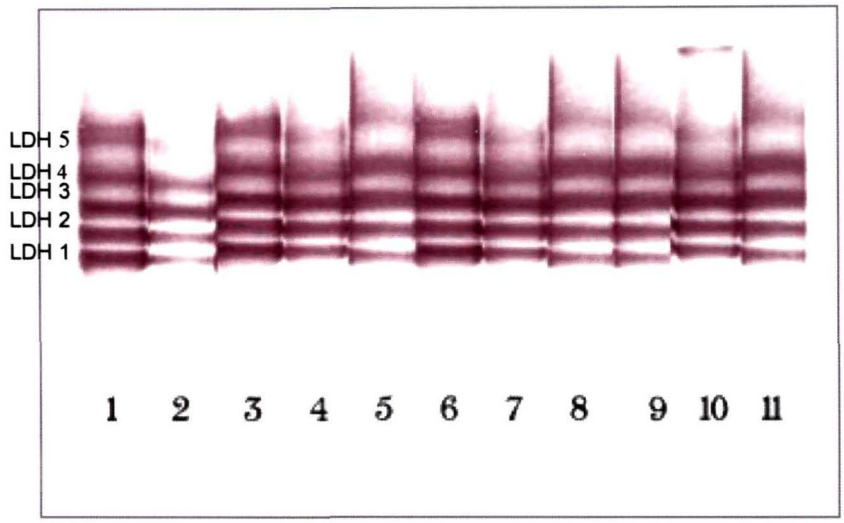
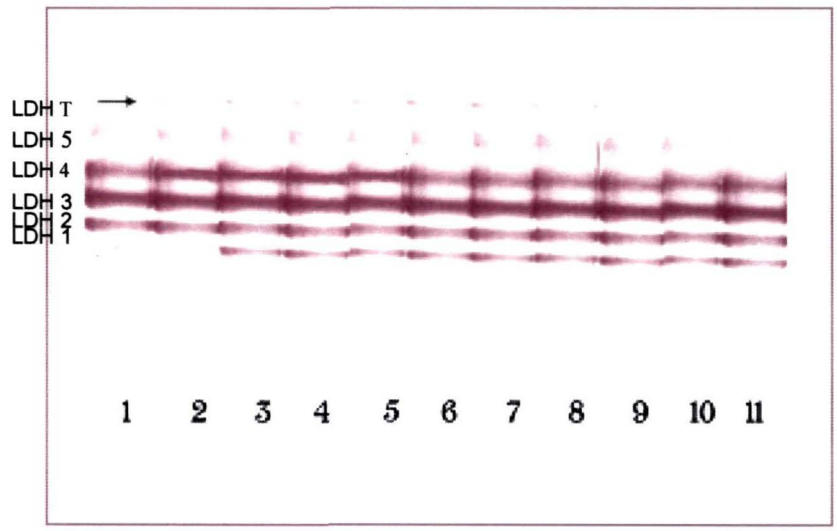


Figure 19.

*Graph showing the percent change in the specific activity of LDH (n moles/mg protein) in liver, kidney, spleen, testes, DL cells and ascites supernatant of tumor-bearing mice after CP treatment (200mg/g) and combined treatment of CP and AA. Results are expressed as ± S.D., Student's t-test, n=6, as compared to the respective control, * $p \leq 0.05$.*



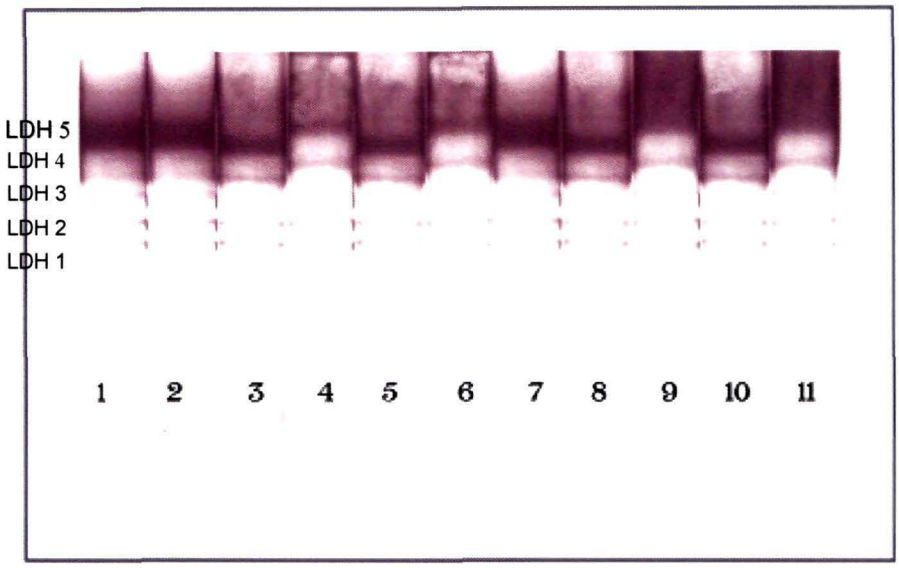
A. KIDNEY



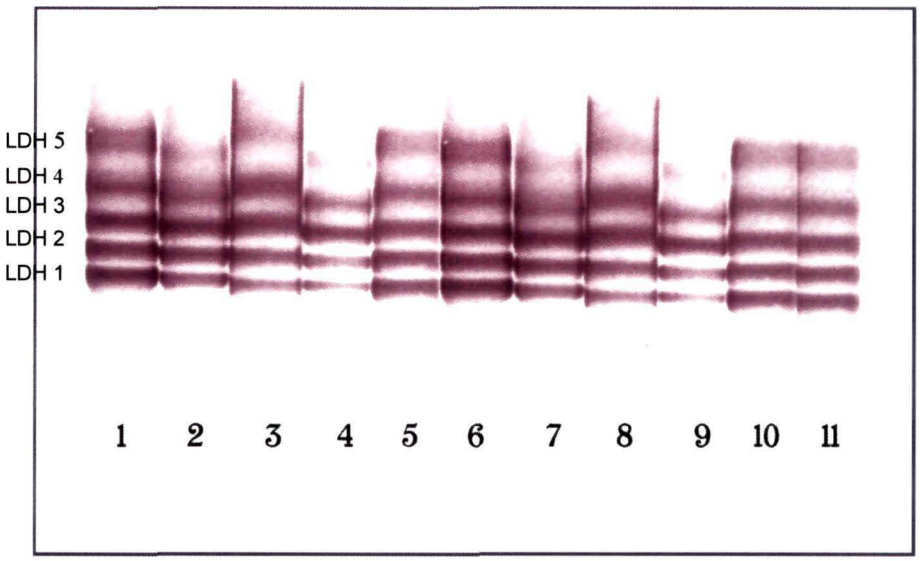
B. SERUM

Figure 20 a.

Micrographs showing the LDH isozyme patterns in (A) kidney and (B) serum with or without cyclophosphamide treatment alone or combined treatment with ascorbic acid and CP. Lane1 represents Normal, Lane2 Tumor, Lanes 3-6 CP 24h-96h, Lane7 AA treatment alone and Lanes 8- 11 represent AA+CP Treatment.



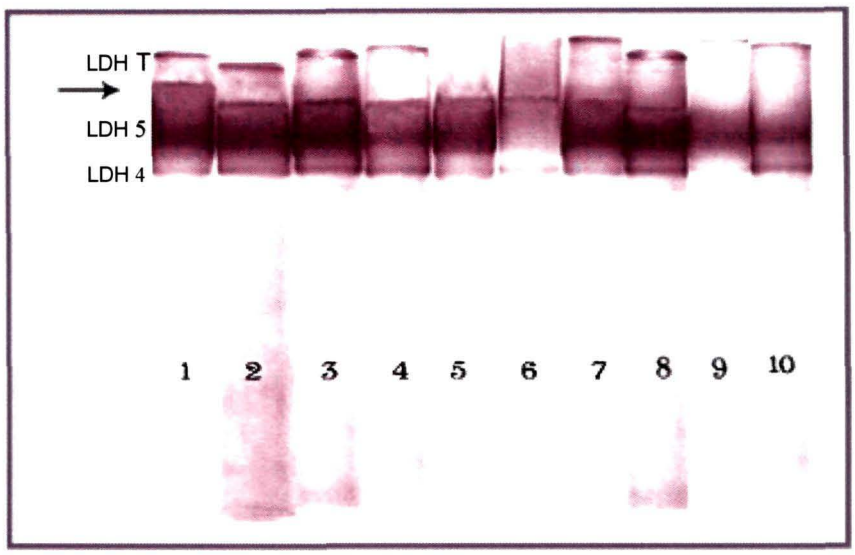
C. LIVER



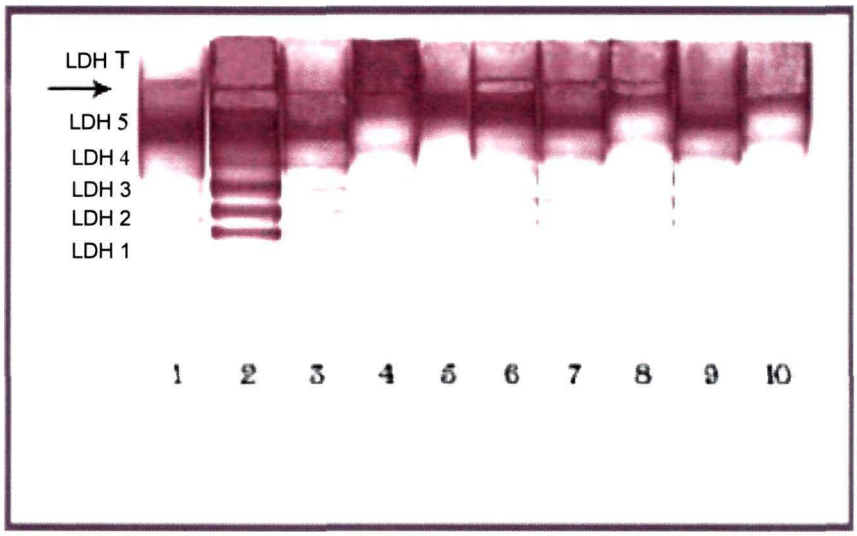
D. SPLEEN

Figure 20 b.

Micrographs showing the LDH isozyme patterns in liver and spleen with or without cyclophosphamide treatment alone or combined treatment with ascorbic acid and CP. Lane1 Represents Normal, Lane2 Tumor, Lanes 3-6 CP 24h-96h, Lane7 AA treatment alone and Lanes 8- 11 represent AA+CP treatment.



E. DL (TUMOR) CELLS



F. ASCITES SN

Figure 20 c.

Micrographs showing the LDH Isozyme patterns in DL cells and ascites SN with or without cyclophosphamide treatment alone or combined treatment with ascorbic acid and CP. Lane1 Represents Tumor, Lanes 2-5 CP 24h-96h, Lane 6 AA treatment Alone and Lanes 7-10 Represent AA+CP treatment.

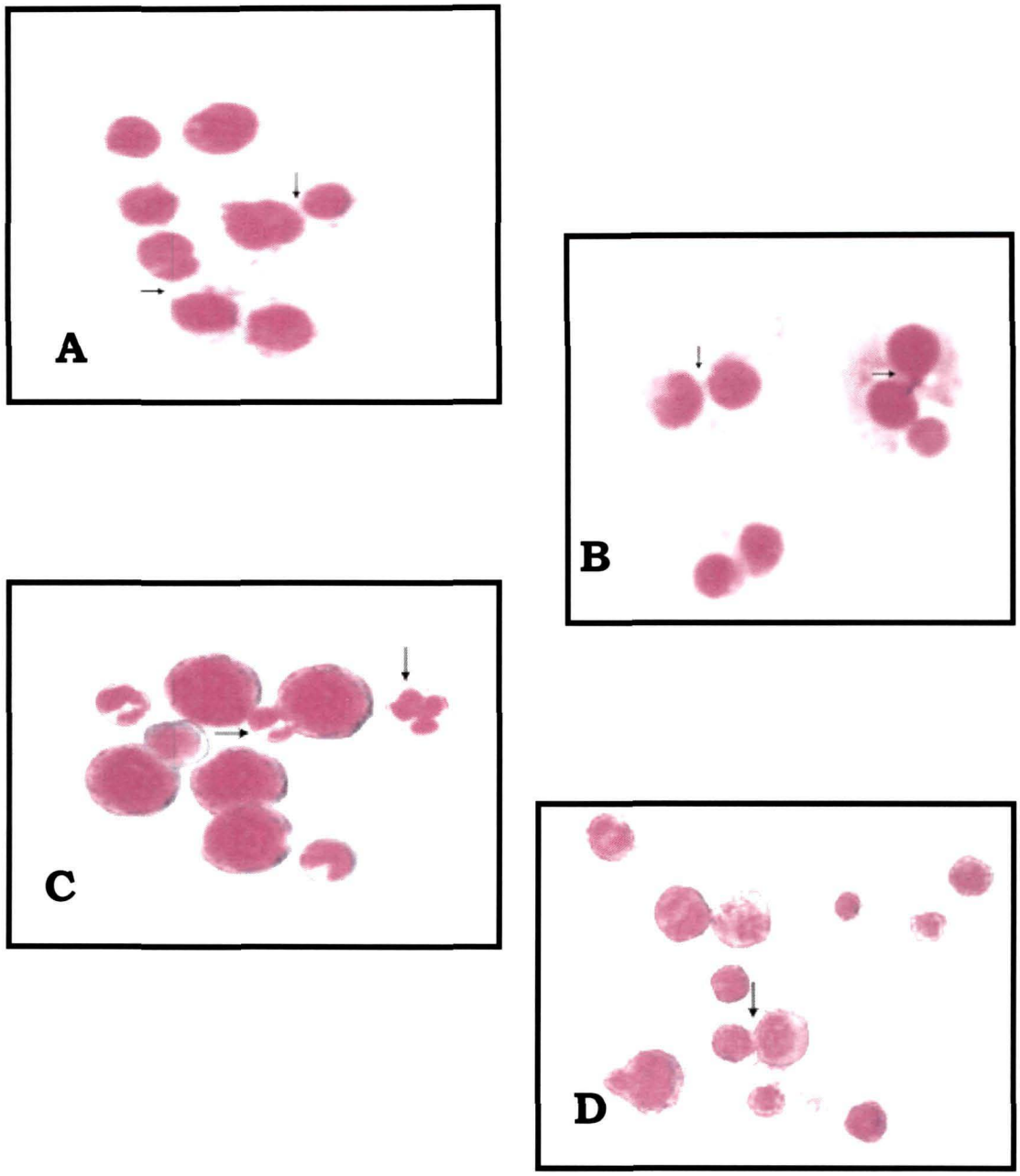


Figure 21 a.

Light micrographs of tumor cells treated with or without cyclophosphamide treatment alone or combined treatment with ascorbic acid and CP. Tumor cells (control) round in shape (A,B) with very few surrounding leukocytes, (C-D) CP treatment(24-48h) showing the infiltration of leukocytes towards the tumor cells.

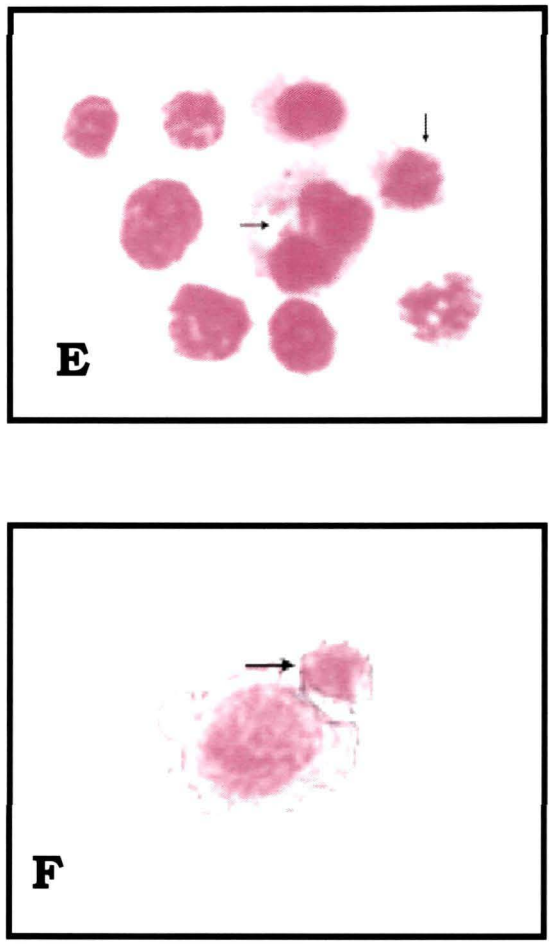


Figure 21 b.

(E,F) 72h-96h of CP. Appearance of membrane vacuoles on the plasma membrane of tumor cells, gradual disintegration of the plasma membrane and tumor cell lysis.

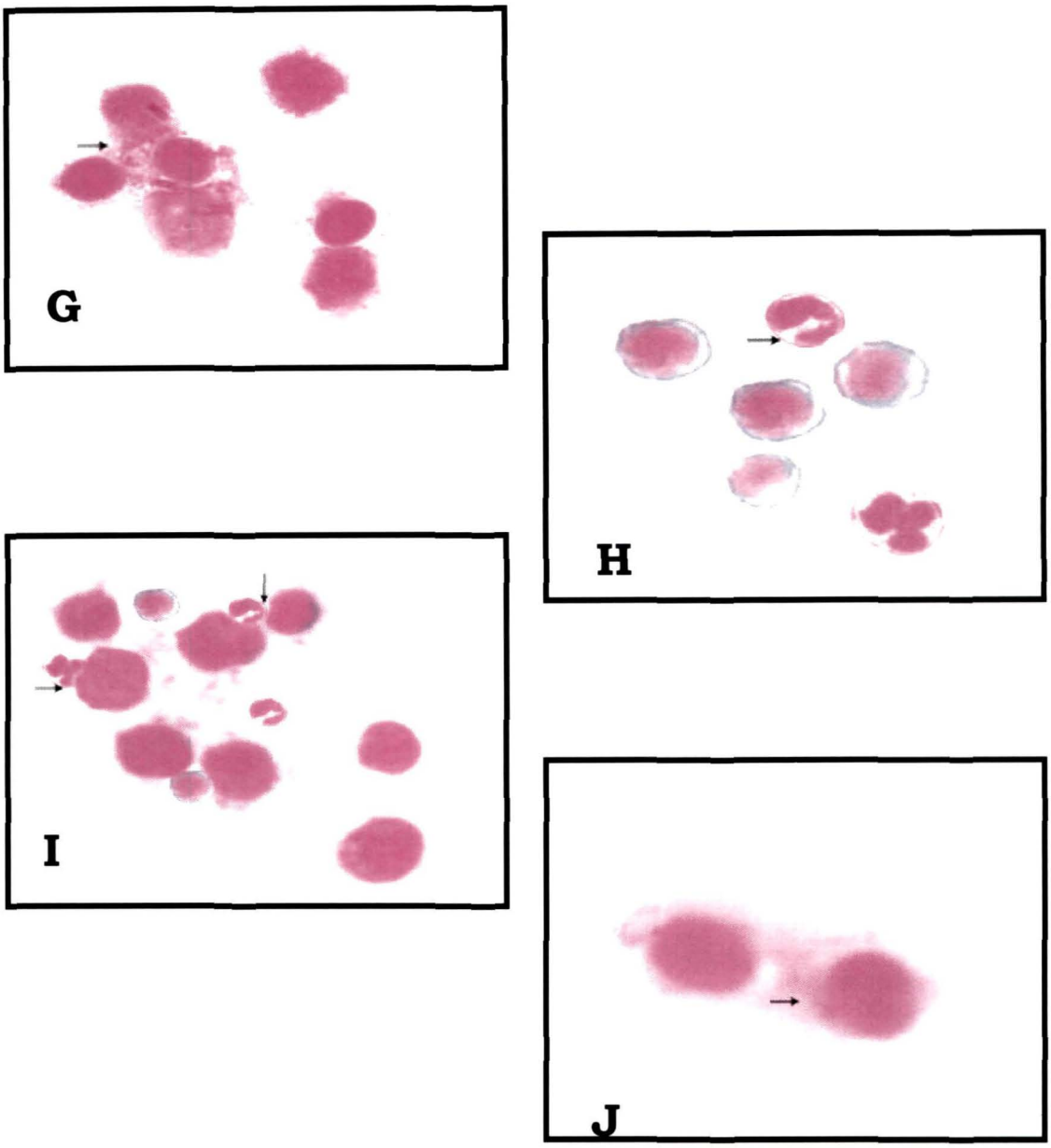


Figure 21 c.

(G,H) Tumor cells with very few leukocytes after AA treatment alone (I,J) 24-48 h of combined treatment with AA and CP showing infiltration of leukocytes and gradual disintegration of the plasma membrane.

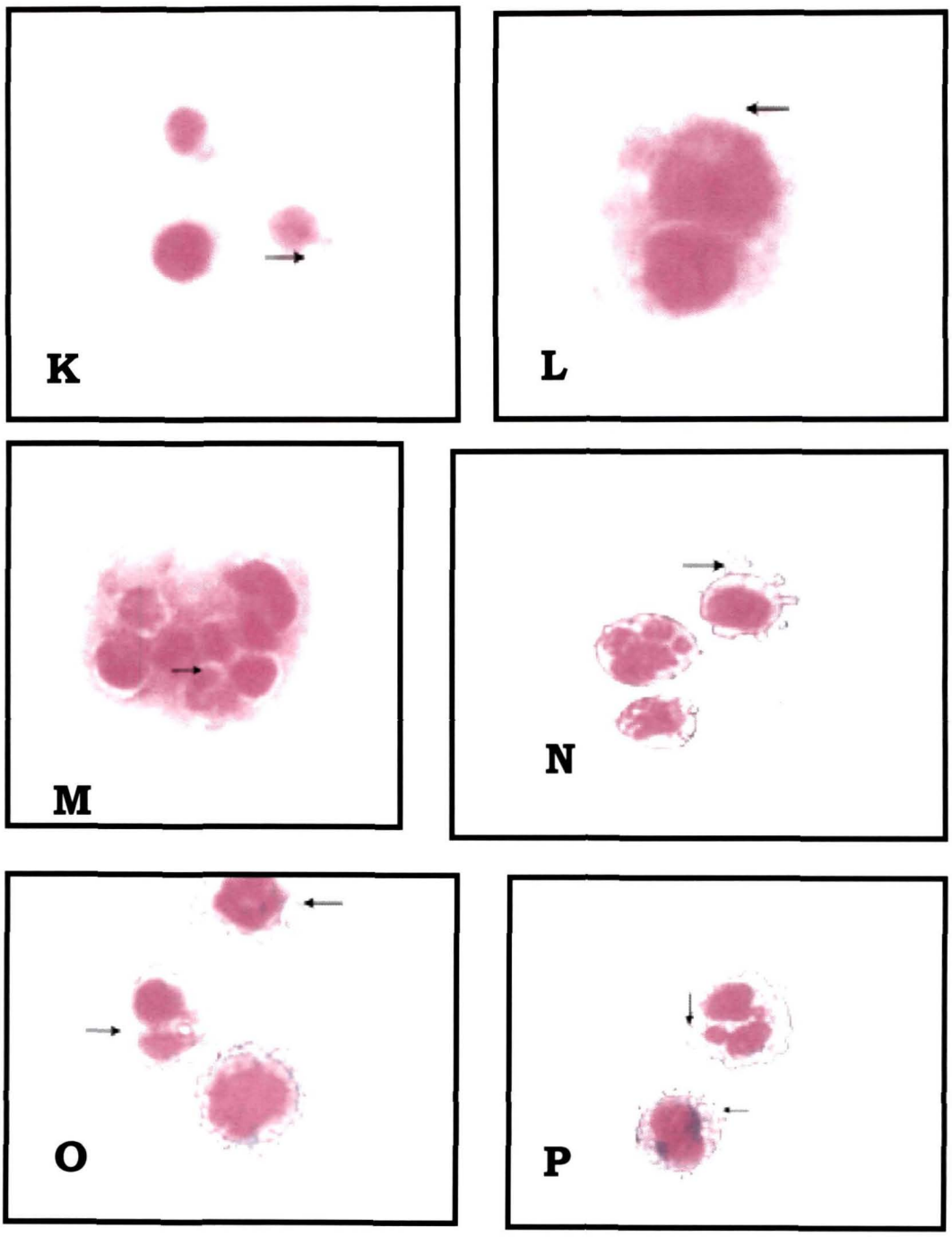
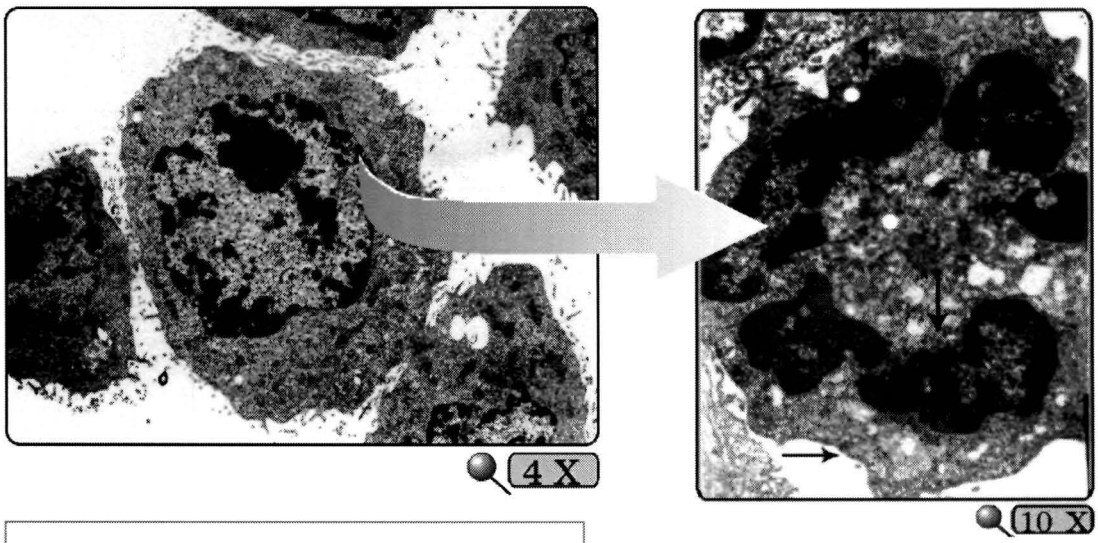


Figure 21 d.

(K-P)after72-96h of AA and CP treatment showing appearance of vacuoles, prominent surface blebs and tumor cell lysis.

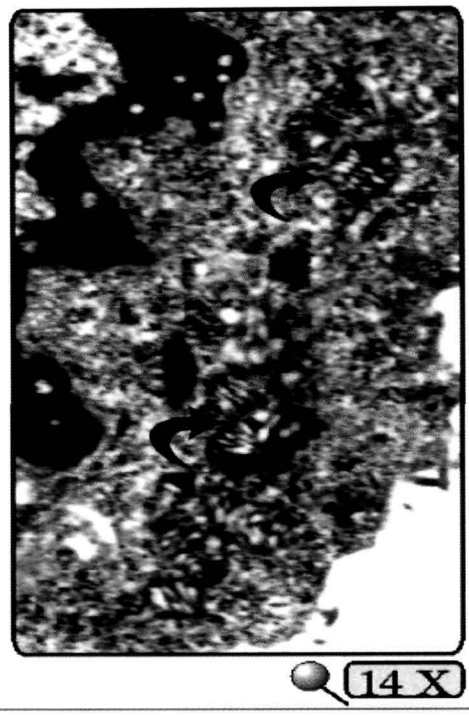
Figure 22.

*Micrographs showing **ultra structural features** of the tumor cells with or without cyclophosphamide treatment alone or combined treatment with ascorbic acid and CP.*



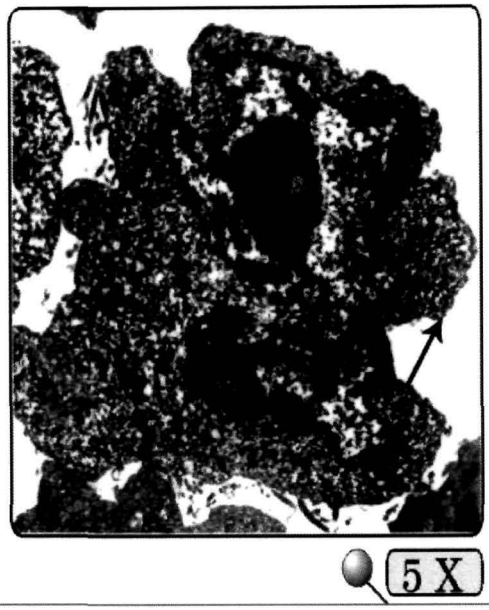
A. Control showing more or less rounded shaped cell, presence of few processes and ruffles over the cell surface.

B. Magnified view showing fewer membrane processes over the cell surface. The chromatin masses appear towards the periphery of the cell.



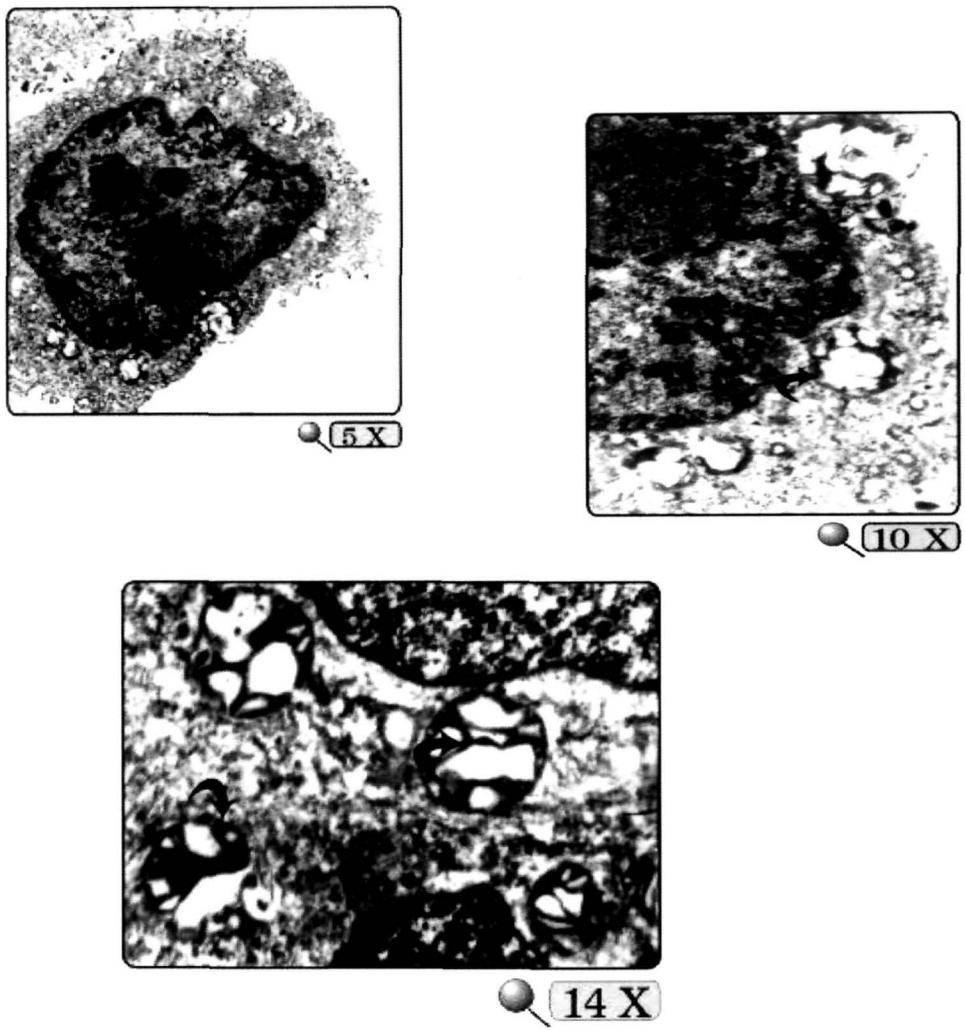
C. Magnified view of the mitochondria which begin to show abnormalities in the shape and arrangement of the cristae after 24hCP.

D. Magnified view of the cell processes and mitochondria.

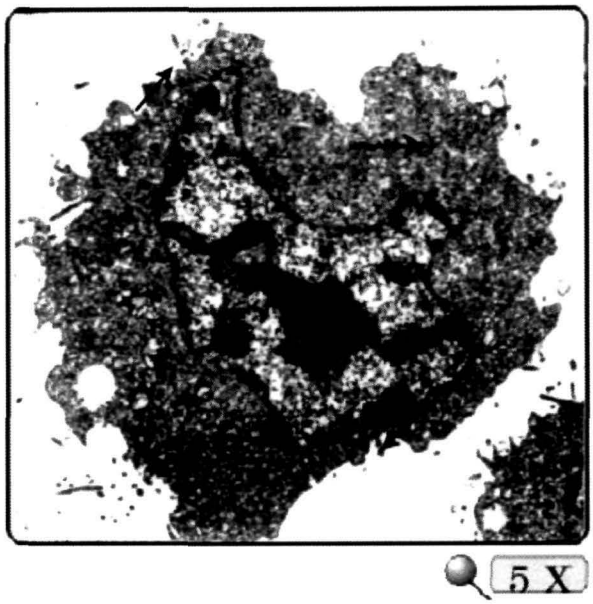


E. 48h of CP (200 mg/Kg b.w) treatment showing membrane and chromatin disintegration along with membrane processes.

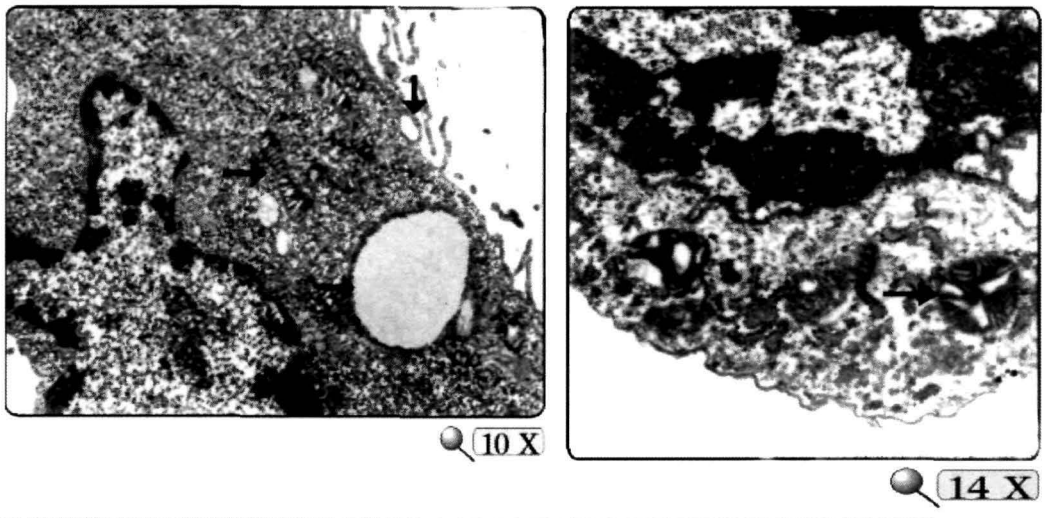
F. 72h of CP (200 mg/ Kg b.w) treatment showing the changes in the arrangement of the mitochondrial cristae along with thickening of the cristae.



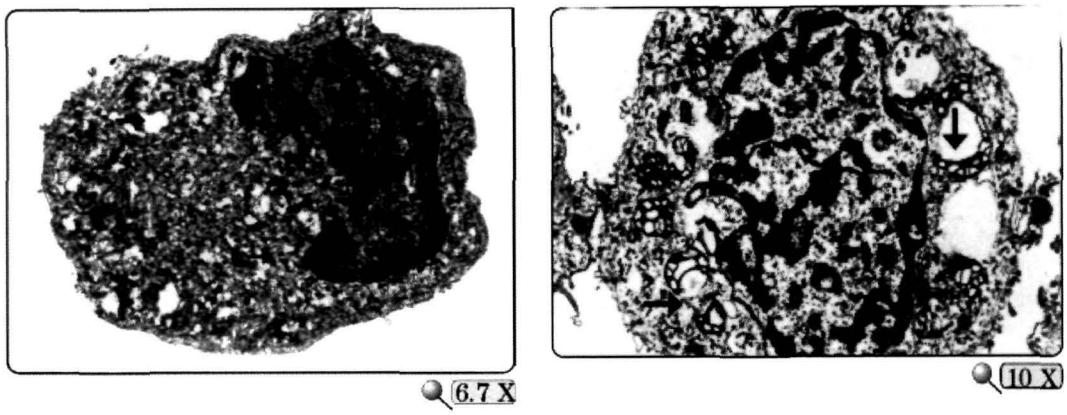
G. 96h of CP (200mg/ Kg b.w) treatment clearly depicting the round shaped mitochondria with thickened membranes along with reduction in the number of cristae and disintegration of the plasma membrane.



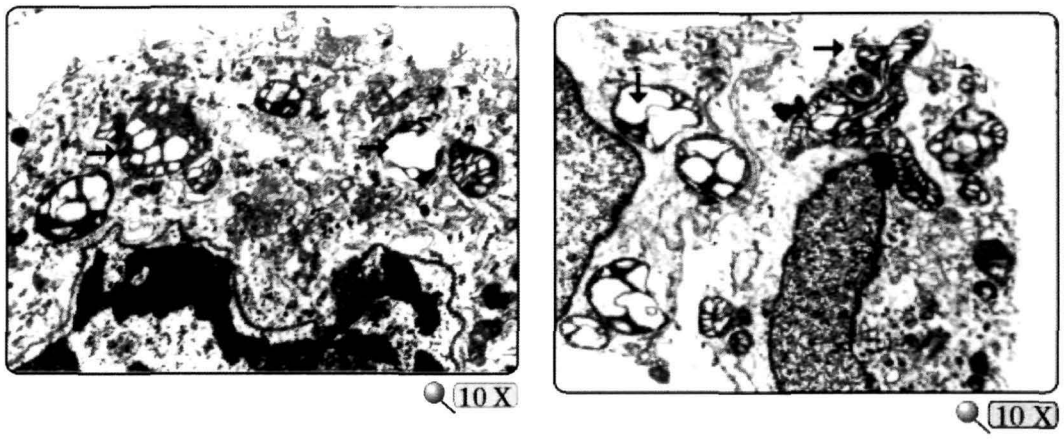
H. Tumor cell treated with ascorbic acid (1% in drinking water) from the 5th day of tumor transplantation. The micrograph shows more or less similar characteristics to that of the control; presence of ruffles and a few processes on the cell surface.



I. 24h after the combination treatment of ascorbic acid and CP (200 mg/Kg b.w). There are a few cell processes, the mitochondria acquire a roundish form and there is thickening in the cristae.



J. 48h after the combination treatment of ascorbic and CP, there is reduction in the number of cristae and formation of prominent vacuoles.



K. 72h - 96h after the combination treatment of ascorbic acid and CP showing disruption in the nuclear membrane and its presence towards the periphery. Presence of roundish mitochondria with thickened membranes, reduction and disruption in the mitochondrial cristae, deformation in the elongated structure of the mitochondria, irregular arrangement of cristae and presence of prominent vacuoles.

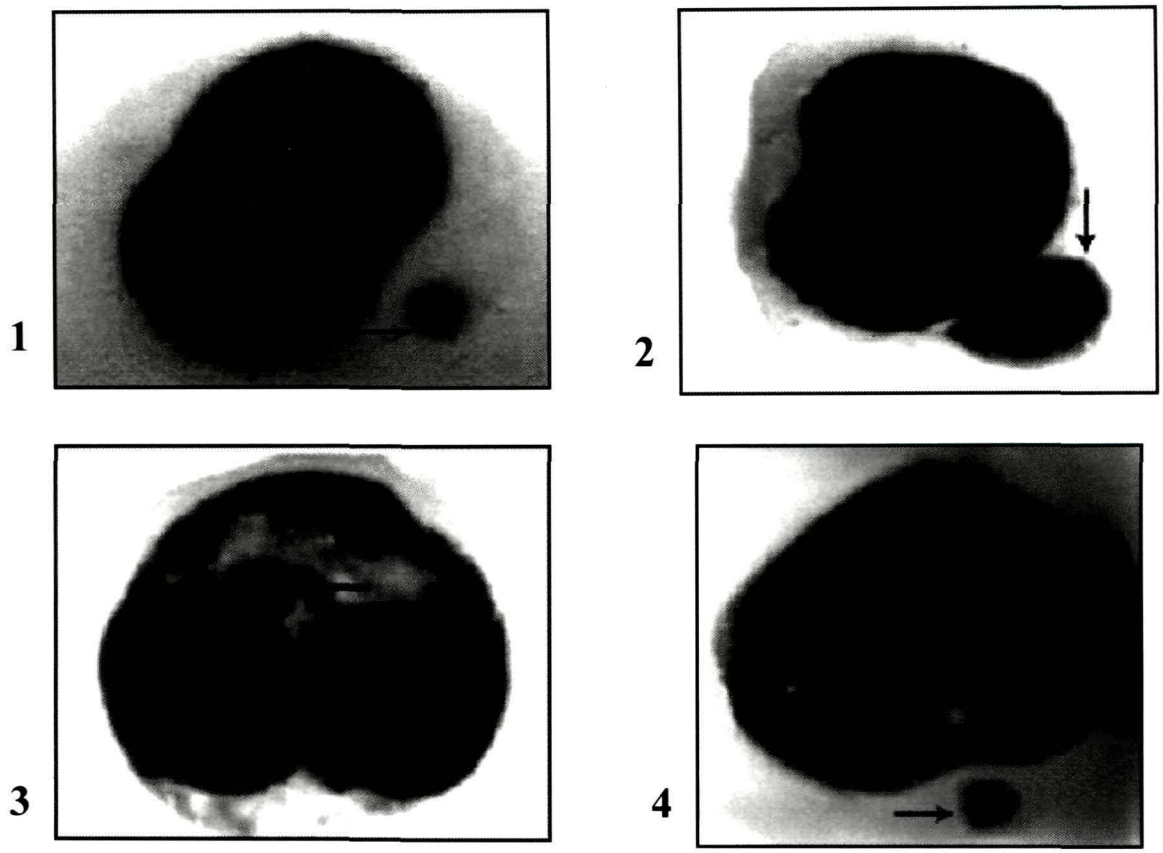


Figure 23.

Photomicrographs showing micronuclei in the bone marrow cells induced by Cyclophosphamide.

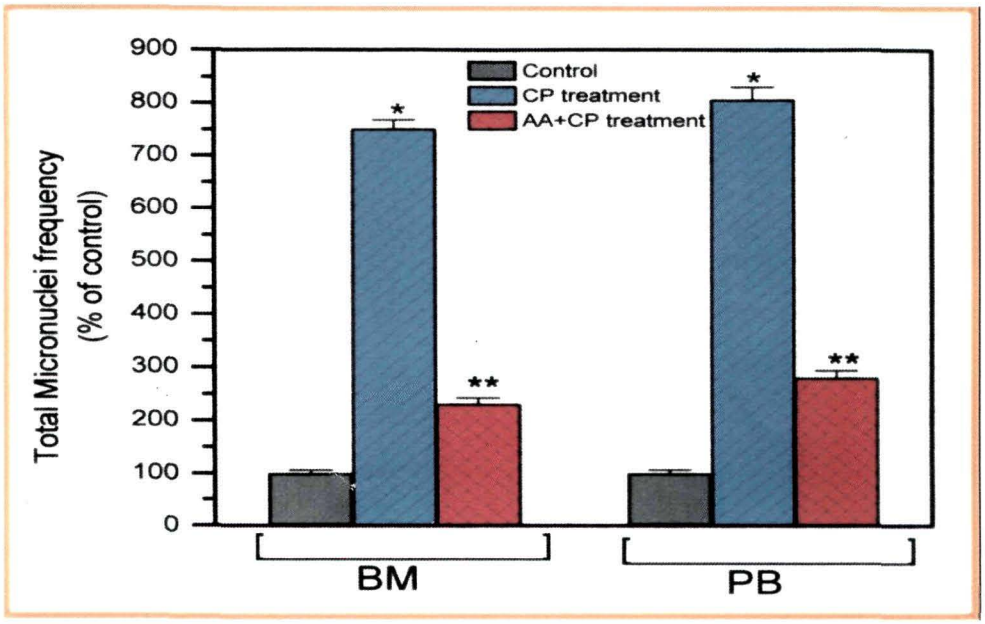


Figure 24.

*Graph showing the changes in micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) cells in tumor-bearing treated with cyclophosphamide (CP) or ascorbic acid (AA) plus CP. Student's test, n=5, *p ≤ 0.05, as compared to respective control, **p ≤ 0.05, as compared to respective CP treatment.*

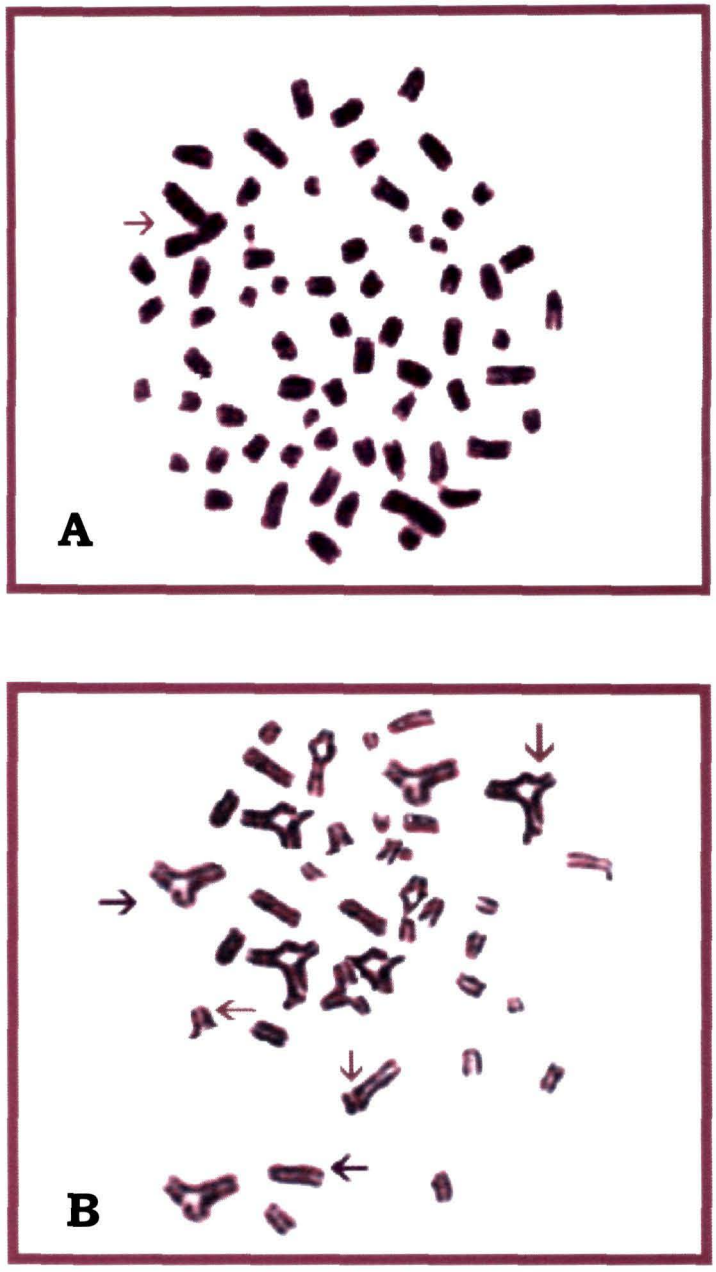


Fig 25 : Photomicrographs of bone marrow metaphase chromosome spreads showing different types of chromosomal aberrations induced by cyclophosphamide. A. Control bone marrow cell showing one metacentric chromosome. B. Cyclophosphamide treated bone marrow cells showing chromatid gaps, chromatid breaks, acentric fragments and chromatid exchanges (arrows).

DISCUSSION

The host survival data in the present study (Table 1; Figure 2) indicate very significant increase (ANOVA $P \leq 0.01$) in survivability of the group of the tumor-bearing mice treated with AA and CP combination (group-IV), as compared to the group of mice treated with either agent alone (group-II or group-III) suggesting additive/ synergistic antitumor activity of AA and CP on murine Dalton's lymphoma. The synergistic antitumor effect brought about by ascorbic acid and cyclophosphamide, which were administered via different routes into the animal, is noted to be sequence dependent. Thus, the treatment of tumor-bearing mice with AA first could be helpful in developing suitable conditions in the host potentiating CP's effect and more effective tumor regression. It has been reported that vitamin C may increase the effectiveness of CP without producing new side effects (Tapers *et al.*, 1987) and the AA treatment has been reported to cause a protective effect on the CP-induced testicular gametogenic and androgenic disorders (Das *et al.*, 2002). The changes in total body of tumor bearing mice was noted under different experimental conditions reveal definite changes in tumor growth and relate with the changes in host survivality pattern. A steady increase in body weight in the tumor bearing mice (group-I) was seen due to rapid growth of tumor until death of the animal. However, in CP alone and AA plus CP treated tumor bearing mice, less increase in body weight indicates retardation in the tumor growth rate leading to an increase in the survival time of the groups of mice. The group of tumor bearing mice treated with 1% AA plus CP, which showed the maximum survivality, showed the least increase in body weight /tumor weight due to effective regression of tumor growth.

Tumor cells are known to have a higher rate of glycolysis and it has long been accepted that this vigorous glycolytic activity of tumor cells decreases the extra and intracellular pH due to the production of excess amount of lactic acid after

incubation with glucose. It seems to be well established that a decrease in the pH in malignant tumors because of their higher rate of glycolysis can be obtained by an increase in the blood sugar level. In our present study, the low pH (Table 2) might be effective in enhancing the antitumor activity of CP thereby resulting in an increase in survival time.

The cell surface may alter in chemical constitution as a result of reaction with the surface molecules of the cell it contacts, diffusion of the substances out of or into the cell will be changed where it is in contact with another cell, thus altering the concentration on the surface or further inside the cell. It has been suggested that the internal structure and composition of the membrane may change during tumor-growth as a result of cell permeability. Particularly, striking have been the observations indicating that a considerable leakage of enzymes takes place from the cytoplasm in growing tumors. The release of enzymes may play an important role in tumor invasiveness, particularly the peptidase activity that may break down the normal stroma. According to the studies of De Grier and Van Deenan (1961), there is a direct correlation between permeability and phospholipids composition when erythrocytes from various species are compared, suggesting that the altered permeability of tumor cells may be related to changes in the phospholipids composition. Alkylating agents have been reported to alter the function and integrity of the plasma membrane (Grunicke *et al.*, 1985), damage DNA and inhibit DNA template functions.

As is evident from the present findings, there is a significant decrease in the protein concentration in the tissues of tumor-bearing mice as compared to the normal mice (Table 3; Figure 6 & 7). This decrease may involve inhibited protein synthesis and /or proteolysis by peptidases within the cell. The data are in agreement

with published reports indicating that cyclophosphamide has an inhibitory effect on protein synthesis (Fleming, 1997). The Lowry assay provides information about the protein content of the entire tumor sample; therefore, the total protein content could be directly correlated with the resolved changes in the protein level in the different tissues after CP treatment. Nonetheless, our data suggest that cyclophosphamide treatment may alter the protein content of the tumor. Cyclophosphamide is known to reduce protein synthesis by alkylating DNA, leading to cross-linking and strand breakage, which should lead to changes in DNA expression (*i.e.*, protein content). There may also be changes in protein content caused by alkylation after cyclophosphamide treatment. Our measurements of total protein content indicate that cyclophosphamide therapy may be altering the protein content of the tumor within 24-96h. Compared to the control (group-I), the significant decrease in the protein contents in the tissues (liver, kidney, spleen and testes) and serum during 24h-96h post-CP treatment (group-III) and combined treatment of AA+CP (group-IV), as revealed in the present study may involve changes in the rate of protein synthesis or decreased uptake of protein in these tissues. However, on the other hand, after analyzing the protein concentration in the DL and ascites supernatant, the significant decrease could involve decreased protein synthesis and/or proteolysis by peptidases. In CCl_4 exposed rats, decreased protein synthesis along with depressed oxidative phosphorylation and disruption in mitochondrial structure have been reported (Dewit and Brabec, 1985). 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 is reasonable to speculate that the decrease in protein content of the cyclophosphamide treated testis may be due to cell damage and lethality of spermatogonial cells (Devraj

et al., 1985). As mentioned earlier, CP is a prodrug that requires P-450 catalyzed metabolism to exhibit cytotoxic activity. The commonly used liver P-450 inducing agents and P-450 inhibitors can have a major impact on CP metabolism and pharmacokinetics. Tumor cells have a disproportionately lower or more irregular blood supply as compared to normal cells, which may in turn relate to the blood flow and /or membrane limited penetration of nutrients. The inhibitory effect of cisplatin on DNA, RNA and protein synthesis has been demonstrated earlier *in vitro* in mammal cells (Harder and Rosenberg, 1970).

Changes in carbohydrate level in tumor could also be an important parameter during antitumor effects. Warren *et al.,* (1978) believe that the bound carbohydrates at the cell surface might result in persistent cell division, decreased intercellular adhesiveness, altered transport, immunogenecity and other specialized functions accompanying malignant transformation. The changes in the carbohydrate level in the tumorous conditions could be used as an important parameter during antitumor effects. The carbohydrates are the important nutritional sources for the cells/tumor cells and any changes in the carbohydrates in the tumor cells as well as other tissues i.e. liver, kidney etc. under the different treatment conditions of mice (different groups) may help to understand the significance of nutritional involvement/changes as a parameter in tumor regression. As compared to the respective tissue of normal mice, total carbohydrate level of liver and spleen in the tumor-bearing mice (group-I) was observed to increase (Table 4; Figure 8 & 9). The comparatively higher carbohydrate level in the tissues of tumor-bearing hosts compared to that of normal mice might indicate a higher rate of carbohydrate (glucose) uptake in the tissues of tumor-bearing mice. The tumor-bearing mice receiving AA (group-II), showed a significant reduction in carbohydrate

concentration in the tissues of liver, kidney and spleen (ANOVA; $P \leq 0.01$). As compared to control (group-I), in the group of mice treated with CP alone for 24-96 hrs (group-III), a significant decrease of carbohydrates in the liver, kidney and spleen was noticed. Considering variations in time effects it was noticed that in DL cells as compared to control, CP treatment caused a significant decrease of carbohydrates at 24-48 h, which was recovered later at 72-96h of treatment. However, when compared to the control tumor-bearing mice (group-I), with the group of mice treated with CP for 24-96h (group-III) or combined treatment (group-IV), it did not show significant change in the carbohydrate level in DL cells. And as compared to CP treatment alone (group-III), the carbohydrates level in DL cells did not change significantly in combined treated group (group-IV) also (Table 4; Figure 8 & 9), which may suggest that these treatments may not involve much the changes in carbohydrates in DL cells during tumor regressions. The increase in the carbohydrate content in the DL cells and in the ascites SN after 96h post CP treatment and combined treatment of ascorbic acid and CP (24-48h), could be due to cell mortality as most dying cells are not able to utilize the carbohydrates present in the ascites fluid and due to release of surface mucopolysaccharides (Prasad, 1986; Prasad and Giri, 1994). Differences in the surface changes involving mucopolysaccharides may have a direct affect on the cellular properties such as cell to cell and cell to substrate adherence (Yogeeswaran and Salk, 1981: Prasad and Sodhi, 1982). These properties in turn have a direct effect on cellular behaviour in vivo. Numerous studies have demonstrated changes in the cell surface carbohydrate composition accompanying malignant transformation and neoplastic progression. Studies from a number of laboratories have reported qualitative and quantitative differences in the concentration of cell surface carbohydrates, which correlate with differences in *in vivo* behaviour (Yogeeswaran *et*

al., 1981; Altevog *et al.*, 1983; Tao *et al.*, 1977; Varani *et al.*, 1983). The study of altered tumor cell carbohydrates is not a new endeavour, although it is only recently that these studies have attracted broad attention. It is clear that the concept of altered carbohydrates or altered amounts of carbohydrates accompanying neoplastic transformation is complex.

Sialic acid, a derivative of N acetyl-neuraminic acid constitutes the common terminal side chains (Abercrombie and Ambrose, 1962). It has been reported that sialic acid influences many properties of the cell surface such as the determination of the cell surface negative charge and the loss of contact inhibition during malignancy and antigen masking agent. It is hypothesized that though the maximum release of sialic acid from tumor cells gives better results and suggests an increase in the tumor cells antigenicity. In many diseases such as Salla disease, free sialic acid storage disease and sialuria an increase concentration of free sialic acid in various tissues and fluids have been observed which may be due at least in part to defective *de novo* synthesis, transport, storage, catabolism, excretion and/or metabolism regulation of sialic acid in the cells. The widely distributed sialic acid moieties of glycoproteins are reported to have damping, protective and regulatory functions at the cell surface Warren *et al.*, (1978), Prasad (1986) reported that the agglutination behaviour of normal and malignant cells depends upon the sialic acid moieties present at the cell surface. Cell surface glycoproteins and glycolipids are susceptible to such elevations as soon as a malignant growth starts to develop, metastasize or recurs, that they are referred to as 'tumor markers' (Stringou *et al.*, 1992). Sialic acid is the main structural component of gangliosides and the terminal carbohydrate chain of glycoproteins and glycolipids. A number of co-workers have reported that sialic acid is an important biological tumor marker of high sensitivity

and specificity in diagnosis and response to treatment to cancer (Chen *et al.*, 1979; Shamberger, 1984; Pulcinsky *et al.*, 1986; Stringou *et al.*, 1992). Cisplatin is one of the leading chemotherapeutic drugs being used effectively against various malignancies (Rosenberg, 1985; Prasad and Giri, 1994). It has been reported that cisplatin has an effect on the surface of the cells, and brings about definite changes in cell lectin agglutinability and in the topographical pattern of lectin-binding sites on the cell surface (Prasad and Sodhi, 1982). The present findings showed an increase in sialic acid concentrations in the DL cells (Table 5; Figure 10 & 11) with tumor growth in mice, which may be an important feature of this tumor. The increase of sialic acid in DL cells may be due to enhanced activity of enzymes involved in sialic acid synthesis and/or transfer. Some reports have indicated a 3-5 times increased sialyl transferase activity in various virally transformed cells as compared to the corresponding normal cells, an event that may be associated with the increase in the amount of sialic acid in the transformed cells (Onodera *et al.*, 1976). The elevated sialic acid levels in malignant cells have also been observed for murine Yoshida ascites sarcoma (Rao and Sirsi., 1973). The influence of sialic acid on the oncogenecity of tumor cells may be based on : i) a negative charge determining constituent on the cell surface, resulting in the loss of contact inhibition, ii) an antigen-masking agent, and iii) a component of the cell surface involved in the adherence of tumor cells to the mesothelial membrane prior to their dissemination to form metastasis (Jeanloz and Codington.,1976).Furthermore the observation of increased sialic acid content in the tissues of tumor bearing mice could be helpful for DL cells in the host since sialic acid has also been known to be important in the transport of proteins, amino acids and ions to cancer cells. As far as the effect of CP on the quantitative changes in the sialic acid of DL cells and tissues is concerned, it

was noted that CP treatment of tumor-bearing mice for 24-96h caused a decrease of sialic acid in the DL cells and tissues (Table 5; Figure 10 & 11). The decrease was predominant in the brain. CP treatment may increase the antigenicity of the fibrocarcoma cells without much release of sialic acid by bringing about certain changes or reorganization of molecules on the surface of tumor cells. Cisplatin and CP treatment causes tumor regression, suggesting an effective anticancer activity of these drugs in this murine model (Nicol and Prasad, 2002). The decrease in sialic acid concentration in the DL cells after CP treatment may be associated with an enhancement of the immune response of the host. The increase in the sialic acid content in the ascites supernatant (SN) indicates increased release of sialic acid by the DL cells. Along with the sialic acid decrease in DL cells this drug-mediated decrease of tissue and serum sialic acid in tumor-bearing mice should also bring about restoration of the functional activity of the tissues and serum to normal in the host, thereby facilitating tumor regression.

Glutathione is a major cellular antioxidant that protects protein thiols and exhibits cellular damage due to oxygen free radicals. GSH levels are accepted to be important in determining the extent of cellular damage induced by chemotherapeutic agents and may determine the sensitivity of cells to damage produced by these drugs. As an important water-phase antioxidant and essential cofactor for antioxidant enzymes, it provides protection also for the mitochondria against endogenous oxygen radicals. The intracellular tripeptide thiol, glutathione (GSH) has facile electron-donating capacity, linked to its sulfhydryl (-SH) group. Its high electron-donating capacity combined with its high intracellular concentration endows GSH with great reducing power, which is used to regulate a complex thiol-exchange system. This functions at all levels of cell activity, from the relatively simple (circulating

cysteine/-SH thiols, ascorbate, other small molecules) to the most complex (cellular -SH proteins). It is the most abundant intracellular thiol and plays a vital 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 % glutathione exists in the reduced form (Wang and Ballatori, 1998).Glutathione plays a very important role in protection against tissue damage produced by oxidative stress, radiation and chemotherapy (Estrela *et al.*, 1992). The protective effect of the thiols against CP-induced cytotoxicity is most probably due to 4-OHCP formation and its ability to deliver PM to the appropriate targets. Thus, the changes in the amount of cellular thiols and especially in the nuclear thiol concentration are important when considering DNA as the critical target for the alkylating agents. The central role of GSH is in providing protection against endogenous oxiradicals and foreign pollutants. As an antioxidant, GSH is essential for allowing the lymphocyte to express its full potential, without being hampered by oxiradical accumulation during the oxygen-requiring development of the immune response. The reduction of dehydroascorbic acid to ascorbic acid by tissue GSH in the range of 1-10 mM has been known (Guaiquil *et al.*, 1997).Thus, evaluating the antitumor effect of the combination treatment of AA and CP against murine Dalton's Lymphoma and changes cellular glutathione level could be quite significant. Determination of GSH on the 5^{th}, 10^{th} and 15^{th} day after transplantation representing initial, middle and later stages of tumor growth in mice (group-I) showed that total GSH (TGSH) level in DL cells increase significantly with tumor growth being maximum on the day 10 and slight decrease thereafter over the next 4-5 days when tumor growth was declining. In Ehrlich ascites tumor cells, maximal GSH concentration was observed by the 7^{th} day, followed by a significant decrease on the

14^{th} day of tumor growth which was correlated with a decrease in cell proliferation and in the rate of GSH synthesis (Estrela *et al.*, 1992). Cancer cells can generate large amounts of hydrogen peroxide, which may contribute in their ability to damage normal tissues (Szatrowski and Nathan, 1991). The observed increase of GSH level in the DL cells with tumor growth may suggest its involvement to facilitate proliferation and metabolism of tumor cells in the host and agrees with the earlier report that elevation of intracellular GSH in tumor cells is associated with mitogenic stimulation (Shaw and Chou, 1986), and that GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH (Terradez *et al.*,1993). The increase in DL cells GSH may reflect an elevated uptake of essential amino acids by DL cells from the plasma and render the blood cells insufficient precursors for GSH synthesis. Glutathione efflux from tumor cells and interorgan flow of glutathione into plasma has also been reported (Estrela, 1992; Meister and Anderson, 1983). The increase in the TGSH concentration observed in the tissues of tumor-bearing mice may suggest the utilization of plasma glutathione by these tissues. The involvement of glutathione to decrease the level of thiobarbituric reacting substances (TBARS) in the mice treated with CP, methotrexate and 5-flourouracil combinations has been also reported (Muralikrishnan and Shyamaladevi, 1996). Reduced form of glutathione (GSH) plays an important role in cell defense mechanisms by acting as an antioxidant or by reacting with electrophiles. It can react with many toxic agents to form conjugates, which are eliminated from the cells (Deleve and Kaplowitz, 1991). As compared to the control (group-I), after the treatment of mice with CP alone for 24-96h (group-III) or with combined therapy of AA and CP (group-IV), total GSH (TGSH) decreased significantly (ANOVA $P \leq 0.01$) in spleen and DL cells (Table 6; Figure 12 & 13) which may lead to

alteration in the cellular antioxidant machinery, less protective mechanisms and increasing CP-induced cytotoxic effect on DL cells. However, treatment of mice with CP alone for 24-96h (group- III) did not show significant change in TGSH in liver while it increased in kidney. As compared to the group of mice treated with CP alone (group-III), the combined therapy of mice with AA plus CP (group-IV) caused a significant decrease in TGSH in liver, kidney (ANOVA; $P \leq 0.01$) and spleen (ANOVA; $P \leq 0.05$). A gradual fall in the serum GSH level was noted with the progression of tumor growth. Although after 24h of CP treatment, a decrease of ~19% in the TGSH level in the serum was noted. However, there was recovery of TGSH in the later period of treatment (48-96h). The combined therapy of AA and CP further showed a significant increase in the serum TGSH almost bringing the concentration to the normal level, suggesting the protective role of ascorbic acid, thereby preventing the possible involvement of CP-glutathione complex formation which would lead to less cellular mechanisms.

Glutathione acts by scavenging the reactive form of carcinogens, acting with electrophilic sites produced on the carcinogenic molecule by cytochrome P450 hydroxylase thereby blocking the neutrophilic attack on DNA. The tripeptide glutathione is extremely important in the antioxidant defenses of the cell and has multiple roles: as a substrate for antioxidant enzymes, as an independent scavenger of hydroxyl and singlet oxygen, a function in the reactivation of enzymes inhibited under oxidizing conditions, and a role in vitamin E regeneration (Halliwell & Gutteridge, 1989; Meister & Anderson, 1983). Indeed, the ratio of reduced to oxidized glutathione (GSH/GSSG) in the cell is a good indicator of the level of oxidative stress. Enzymes involved in antioxidant defenses exist as a coordinated system and include superoxide dismutase (SOD), which catabolizes superoxide

radicals, and catalase (CAT) and glutathione peroxidase (GPOX), which degrades hydrogen peroxide and hydroperoxides, respectively. Oxidative stress may be initiated by a decline in the antioxidative defense system or oxidative stress caused by other factors may decrease the concentrations of antioxidants. The most robust and significant alteration in the antioxidant defense is a decrease in GSH concentration. Initially, a complete absence of GSH in the presence of high GSSG concentrations was reported (Perry *et al.*, 1982; Perry and Yong., 1986; Perry *et al.*, 1988). The decrease in TGSH level by CP seems to play a significant role in the antitumor activity of CP against Dalton's lymphoma. Elevation of GSH levels has been shown to increase the resistance of cancer cells to cisplatin (Russo *et al.*, 1986), while a depletion of GSH levels could potentiate the cytotoxicity of a variety of antitumor agents (Arrick *et al.,* 1984).In the present study also, the GSH depletion caused by CP may play a role in enhancing cell mortality by increasing the susceptibility to oxidative stress thereby increasing hosts survival. The protective role of Vitamin C against cisplatin-induced mutagenic and nephrotoxic effects have also been noted with possible cooperative involvement of GSH in its protective function (Giri *et al.*, 1998a, b). Inspite of a decrease in GSH in the tissues, the availability of AA in combined treatments should be helpful to protect these tissues. GSH depletion may be the ultimate factor determining vulnerability to oxidant attack. However, oral ascorbate helps conserve GSH, ascorbate and GSH are known to have actions in common and can spare each other under appropriate experimental conditions (Meister, 1994). The pretreatment of mice with AA enhanced the CP-mediated decrease of TGSH in DL cells, which might occur through the potentiating effect of AA on CP or its metabolite to enhance its effect in lowering TGSH levels as compared to CP alone, rather than direct effect of AA on GSH level. Infact, the

direct effect of AA leading to changes in cellular GSH levels is not definitely known. Some findings showed that ascorbate to GSH-deficient animals led to increased GSH levels (Meister, 1994). Therefore, the decreased GSH titre in DL cells may be one of the important factors in providing better antitumor activity in combined therapy of AA and CP as compared to CP administration alone. The AA exposure of tumor cells may also increase their susceptibility to lysis by DNA damaging /interacting agents including CP, similar to the reports that the treatment of cells with Vitamin C positively modulated Mut L homologue-1 (MLH1) and p73 genes and its improved cellular susceptibility to apoptosis triggered by the DNA damaging agent cisplatin (Catani *et al.*, 2002)

Ascorbic acid has been reported to be an effective protectant against a variety of toxic chemical agents including heavy metals (Holloway and Peterson, 1984). One of the main functions of ascorbic acid is the maintenance of biochemical homeostasis under stress. Ascorbic acid at a nontoxic concentration, in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition. However, the extent of modification of the effects of agents by vitamin C depends upon the tumor cells and the type of pharmacological agents (Prasad and Rama, 1983).Vitamin C has been reported as an immunostimulatory agent involving various components of the immune system in the host (Anderson, 1984).The tumor-bearing mice have a lower tissue and serum ascorbic acid concentration than the normal mice. Following CP treatment there is a slight increase in the concentration of ascorbic acid. Vitamin C administration further increased the ascorbic acid level in the tissues and serum (Table 7; Figure 14 & 15). Dehydroascorbic acid is considered the major form for the cellular uptake of Vitamin C by the blood cells. Taper *et al.*, (1987) reported that Vitamin C may increase the

effectiveness of CP without producing new side effects. It is interesting to note that the combined treatment of AA and CP increased the concentration significantly almost bringing the concentration to normal level. Das *et al.*, (2002) reported that AA treatment caused a protective effect on the CP-induced testicular gametogenic and androgenic disorders. Decreased level of serum ascorbic acid in tumor-bearing hosts has been reported (Ghosh and Das, 1984,). Antioxidants represent a first line body defence against oxidative stress produced by the generation of free radicals and reactive oxygen species (ROS). Vitamin C is utilized by the animals for the maintenance of their defense mechanism, which include immunocompetence and phagocytosis. It has been reported that Vitamin C status of the host is often low in malignant conditions as reflected by the plasma and tissue ascorbate levels. This explains to a certain extent the generally low immuno-incompetence of the host bearing malignant tumors. Vitamin C is the most important water soluble biological antioxidant which can scavenge both reactive oxygen species (ROS) and reactive nitrogen species. It is well known that antioxidants are almost universal antimutagenic agents (Gonzales de Mejia *et al.*, 1977; Odin, 1997; Giri *et al.*, 1998; McCall *et al.*, 1999).Vitamin C's possible anticarcinogenic effects may be accounted for by its ability to detoxify carcinogens as well as its ability to block carcinogenic processes through its antioxidant activity. Cameron and his colleagues (1975) reported that administration of high doses of vitamin C to terminal cancer patients produced regression of tumor and prolonged their life expectancy. Bishnu *et al.*, (1978) reported that Vitamin C action was mediated by its inhibitory effect on the DNA synthesis in tumor cell lines in culture. Cameron and Pauling (1973) had proposed a different mechanism for the action of Vitamin C on tumor growth. According to them, the vitamin has an inhibitory effect on the action of

hyaluronidase, which is responsible for invasiveness of malignant tumors. Clinical trials indicate that ascorbic acid may confer protection on various normal tissues without attenuating anti-tumor response. The mechanism of protection is base on physiological differences between the tissue types and on differential uptake of ascorbic acid and normal tissue. Pauling *et al.,* (1985) reported that large quantities of dietary vitamin C decreased the incidence of and delayed the first appearance of spontaneous mammary tumors in RIII/Imr in mice. Vitamin C can inhibit tumors produced by nitrosamine in animals through inhibition of nitrosamine formation (Chen *et al.*, 1988). It is thus suggested that depletion of the ascorbic acid could be an early and critical event during CP induce toxicity. Epidemiologic reviews have also linked lower dietary ascorbic acid consumption with an increased risk for gastric cancer (Block, 1991; Cohen and Bhagavan, 1995). On the basis of the release of ascorbic acid after CP treatment of tumor cell, a similar mechanism is suggested in the regression of Dalton's lymphoma after CP treatment.

Lipid peroxidation has been reported as a major contributor to the loss of cell function under oxidative stress situations. Oxidative stress is expressed as a disturbance of the stable equilibrium between pro-and antioxidant processes in a direction where prooxidant processes prevail. This disturbance leads to various types of damage at the molecular and cell level (Loft and Poulsen, 1996). Lipid peroxidation can alter vital membrane protein structure and function. The presence of lipid hydro peroxides in a membrane disrupts its function by altering fluidity and allowing ions such as Ca++ to leak across the membrane, the consequences of which include activation of phospholysis, membrane blebbing and eventual membrane rupture. The antioxidants in such cases can act as stabilizers of homeostasis. Cancer cells can generate large amounts of hydrogen peroxides, which may contribute to

their ability to damage normal tissues (Szatrowski and Nathan, 1991). CP treatment is associated with induction of oxidative stress by generation of free radicals and reactive oxygen species (McDermott and Powell, 1996; Manda and Bhatia, 2003) and the potential role of dietary antioxidants, as ascorbic acid, tocopherol, β-carotene etc to reduce the activity of free radical-induced reactions has drawn increasing attention (McCall and Balz, 1999). Ascorbic acid is an excellent antioxidant and free radical scavenging nutrient, protecting cells from damage by oxidants. Co-administration of AA in CP treated mice resulted in a significant restoration of the parameters to the normal level. The results of our experiment suggest CP treatment is associated with induction of oxidative stress that can be ameliorated significantly by AA administration. So, our data (Table 8; Figure 16a, 16b & 17) indicate some potential implications. The combined effect of CP and AA on plasma lipids and lipoprotein profiles are important since ascorbic acid encumbers the lipid abnormalities initiated by CP during cancer chemotherapy. The results suggest that some clinical entanglement of CP was refrained by co-administration of ascorbic acid in tumor stress condition, thereby minimizing the damage caused by oxidative processes. Increased levels of lipid peroxidation and decreased levels of glutathione and ascorbic acid were seen in serum; lung tissue and lavage cells in pathogen free male Wistar rats of cyclophosphamide groups (Venkatesan *et al.*, 1995). Numerous studies have shown that toxicity of superoxide and hydrogen peroxide is highly dependent on the presence of iron or copper and that the nature and extent of damage initiated by these species is related to the sub cellular location of these metals (Halliwell (1992). All cellular components are susceptible to attack by ROS, particularly by OH. Attack on proteins can lead to the modification of amino acids, oxidation of sulfhydryl groups leading to conformational changes, altered enzymatic

activity, crosslinking, peptide bond cleavage as well as carbohydrate modification in glycoproteins, loss of metal in metalloproteins, altered antigenicity, and increased proteolytic susceptibility (Stadtman, 1992, Sies, 1993). ROS attack also causes DNA strand breaks and base modifications (leading to point mutations) Sies (1993). Because of the damaging effects of ROS, all cells maintain antioxidant defenses. Three levels of protection have been considered: 1) prevention of ROS formation, 2) termination of the ROS using free radical scavengers or antioxidant enzymes, and 3) repair of damaged cellular components. An important aspect of prevention is the segregation or chelation of metals that can catalyze OH formation, such as by iron binding to ferritin (Balla *et al.*, 1992). Nonenzymatic antioxidants include glutathione, alpha-tocopherol (vitamin E), ascorbic acid, beta-carotene, and uric acid. LDH is the cytosolic enzyme, which plays a significant role in the cells and tissues under anaerobic conditions. It has been reported that cancer cells have diminished oxidative phosphorylation in mitochondria. Enzymatic changes may reflect the overall changes in metabolism that occur in malignancy (Stefanini, 1985). Increased activity of LDH in most malignant tumors, 5′-nucleotidase in cancers of the stomach, glucose-6-phosphate dehydrogenase in breast, prostate and lung cancers, while decreased activity of adenylate kinase in lung cancer and 5′-nucleotidase in ovarian carcinoma have also been noticed. Rogers *et al.,* (1981) demonstrated that the activity of LDH seems higher in the malignant tissue .The changes triggering biochemical processes aiding cancerous cells over normal surrounding tissue cells may be related to the aggressiveness of the tumor. In the present study, as compared to the normal animals, the LDH activity in the tumor-bearing hosts increases ~ 62% in the serum, ~ 70% in liver, ~ 38% in spleen with a slight decrease in the kidney (~26%) (Table 9; Figure 18 & 19). The increase in the LDH activity may suggest the

release of LDH in the tissues of tumor-bearing hosts. The rapid turnover of malignant cells releases ecto-and endo-enzymes into the blood stream (Stefanini, 1985). The highest LDH activity in tumor bearing mice was observed in the liver, the main site of glycolysis. The studies of LDH should have the significant to understand a correlation between the cytological and metabolic changes in the host after CP treatment. Hill and Levi (1954) were among the first investigators to study serum LDH, who reported marked elevations in LDH activity in many patients with leukemia. Elevation of serum LDH activities has also been reported in cases of hepatocellular necrosis and metastatic carcinoma (Mac Donald *et al.*, 1957). Normal cells do not generally produce lactic acid in the presence of glucose and oxygen. Under, the same conditions a large amount of lactic acid was formed in malignant cell populations. Increased LDH activity was also observed in cancerous breast (Stefanini, 1985). CP treatment of the tumor-bearing hosts resulted in an overall decrease in the kidney and in Dalton's lymphoma cells. In the liver, there is a significant increase of ~56% after 48h CP treatment which gradually decreases in the later hours of treatment (72h-96h). The LDH activity in the kidney and spleen shows an overall decrease at 96h of treatment. The Dalton's lymphoma cells and ascites supernatant show a different pattern in the LDH activity. At 24h-96h of CP treatment, the LDH activity in the supernatant was found to be more than 2 fold higher over the control value, while a significant decrease (~45%) was noted in the Dalton's lymphoma cells. The administration of both AA and CP (group IV) also resulted in an enhanced decrease in the LDH activity in the tissues as well as in the serum (Table 9; Figure 18 & 19). The decrease in the LDH activity may indicate decreased synthesis and/or increased leakage from the cells due to injury, which implicates increased leakage from the cells due to injury. The second proposition

may be supported as a sustained increase in the LDH activity was noted in the ascites SN after CP treatment. alone as well as an overall increase in the LDH activity in the ascites SN after combined treatment of AA plus CP. Enzymes entering the blood after cell necrosis of certain organs have been used to indicate the degree of tissue damage. The effect of some pesticides (copper sulphate, paraquat and methidation) on fish causing tissue necrosis has been demonstrated by increased levels of LDH in blood sera (Asztalos and Nemcsok, 1985). However, the combined application of both AA and CP lowers the LDH activity in the serum also depicting a decreased synthesis. Simultaneously, the continuous decrease in the enzyme activity in the tumor cells with a parallel increase in the tumor supernatant is an evident indication of altered membrane permeability of tumor cells for LDH and cell injury of Dalton's lymphoma cells. Takema *et al.*, 1991 have shown a correlation between cytotoxicity and LDH release. It has been observed that cisplatin treatment of cultured renal epithelial cells and renal cortical slices in vitro causes the leakage of LDH from the cells into the medium and it may indicate the cell injury (Gemba and Fukuishi 1991, Kim *et al.*, 1997).

The use of LDH isozyme as a diagnostic aid has been amply documented (Wilkinson, 1970; Dito, 1973). LDH isozyme analysis has also been proved valuable in determining the effect of carcinostatic agents upon certain tumors (Starweather *et al.*, 1966). Alterations in the pattern can be useful in evaluating tissue disease entities such as haemolytic anaemia, leukemia and neoplasia. It has been reported that human malignant cells, both lymphoid (Rambotti and Davis, 1981) and from other organs (Goldman *et al.*, 1964; Fleischner *et al.*, 1981) possess LDH isozyme patterns distinguished by lower H: M ratios (i.e. predominance of LDH-5). The present study also showed predominance of LDH-5 (Figure 20 a, b & c), which goes in accordance

with earlier reports. Tumor cells have higher glycolytic activity (Warburg, 1956, Yesher, 1978) and it is known that LDH-5 is highly active in anaerobic glycolysis (Dawson *et al.*, 1964). Thus, the predominance of LDH-5 may reflect the anaerobic metabolism of the tissues, since hypoxic conditions have been known to exist in the tumors (Kallman, 1972). The LDH-5 intensity appeared very dense towards the cathodic region of the gel in the ascites supernatant, suggesting the presence of increased release of LDH-5 from the tumor cells and /the appearance of an additional isozyme band. It has been postulated that anaplasia is accompanied by molecular adjustments, which precede morphological changes in malignancy and may manifest themselves through changes in the enzyme patterns, thus there is predominance of LDH-1 in normal colonic mucosa and LDH-5 in colonic cancer tissue (Carda-Abella *et al.*, 1982). An important notable feature was the presence of an additional band near the LDH-5 isozyme towards the cathode in the serum of tumor bearing hosts which is also faintly visible in the tumor cells and supernatant, but absent in the kidney, liver and spleen. This phenomenon may indicate the presence of a new isozyme variant and it has been tentatively designated as LDH-T for being present in the tumorous condition. Though the exact physiological significance and mechanism of this new isozyme variant cannot be clearly understood at present, but it may reflect the consequence of a physiological adaptation of the tumor cells to the general hypoxic condition and this in turn, might serve as an important biomarker for Dalton's lymphoma *in vivo*. It has also been suggested that oncogenes may play a role in the production of variants by malignant cells (Stefanini, 1985). LDH has been suggested to be a fairly sensitive marker for most solid tumors with the presence of some isozyme variants of LDH in the serum of these patients (Lippert *et al.*, 1981). Thus, the present biochemical studies on the LDH activity indicates that the LDH

enzyme activity is altered in the tumor condition and during the tumor regression phase after treatment, suggesting that LDH activity could play a vital parameter in malignancy and cyclophosphamide-mediated chemotherapy. The LDH isozyme patterns also revealed the presence of tissue specificity of different LDH isozyme forms.

The structural and functional properties of malignant cells are related to changes in the cell surface-cell membrane.(Hynes, 1979; Gallagher, 1985).The presence of cell–cell contacts has been described in solid tumors (Hoshino, 1963) and a few ascitic tumors (Hayashi and Ishimaru,1981;Gupta *et al.*, 1985). Cell association in malignant cells has been suggested to regulate the pattern of growth and malignancy in tumors (Curtis, 1973). The leukocytes surrounding the tumor cells were noticed to increase in number after treatment with CP alone and combined application of AA and CP (Figure 21 a, b, c & d). Ascorbic acid is known to enhance the phagocytic functions of leukocytes (Shilotri, 1977). Ascites Dalton's lymphoma cells showed the presence of numerous cytoplasmic blebs and ruffles all over the cells and cellular processes. An almost similar trend in the distribution of Zajdela ascetic hepatoma cells has been reported upto 5days (Gupta *et al.*, 1985). The aggregation of 2-3 or even more cells in the ascitic fluid may be helpful in the acquisition of the characteristic growth properties of tumor cells in the host. Cell to cell adhesion in malignant cells has been suggested to be a multifunctional process and it regulates the pattern of growth and behavior of malignancy in tumors (Curtis, 1973). The cell-to-cell connections have been reported to contain different types of junctions, which are arranged in a manner such that a direct channel is formed through the plasma membrane of both the apposing cells. These channels probably act as regulators of the hydrophilic pathway between the adjacent cells and thus

aiding transport of ions and small molecules from one cell to another (Loewenstein, 1975; Good enough, 1976). The fusion of 2-3 or more cells resulting in the formation of a multinucleated tumor Dalton's lymphoma cell probably acquires a more stable metabolic existence. At certain points infoldings of the plasma membrane were also observed. These membrane infoldings have been well marked in ascitic cells (Gupta *et al.*, 1985). Porter *et al.*, (1973) reported the presence of unusual ruffles on several virally and spontaneously transformed Balb/C3T3 cells and showed that ruffles appeared around the cell margins and occupied a significant part of the top surface of the cells, a feature probably related to the known capacity of tumor cells to phagocytose their environment. Blebs have also been reported to appear in unusual numbers on many transformed cell lines e.g. Adenovirus-type-5-transformed hamster embryo cells and human carcinoma A 549 and mouse embryo cells transformed chemically (Gonda *et al.*, 1976). It is likely that blebs result from alterations of the cortical microfilament network (Allred and Porter, 1979). Cyclophosphamide treatment of Dalton's lymphoma cells *in vivo* did not show significant changes in the pattern of cell-to-cell association in groups or as single cells, which in turn suggest that does not change cell-to-cell connection. On the other hand, cyclophosphamide treatment shows significant changes in the arrangement-movement of ruffles and blebs over the cells. The disintegration of the plasma membrane observed at 96 h of the treatment could lead to the lysis of tumor cells. Ribereau-Gayon *et al.*, (1986) reported that bacterially fermented mistletoe preparation (BFMP) treatment brings significant modifications of cell surface of rat hepatoma cells and disintegration of the plasma membrane takes place in the antitumor effect of BFMP. The reorientation on the arrangement of cell surface ruffles-blebs and the disintegration in the plasma membrane resulting from with CP alone and also CP in combination with AA

appears to be the direct cause of tumor cell lysis. The formation and shedding of membrane vesicles has been reported to occur during T-lymphocyte mediated cytolysis (Liepens and de Harven, 1982; Liepens, 1983). Hence, taking into consideration the present data and the earlier reports, it is clear that the formation of membrane vesicles/blebs is an important event occurring during tumor cell lysis following CP treatment which might arise as a consequence of induced oxidative stress during drug metabolism (Lemasters *et al.*, 1987) and / or immune cell mediated killing (Liepins, 1983).Ruffles are plasma membrane bound sheets of cytoplasm which contain microtrabecular lattice (MLT) usually found at the leading stage of migrating cells while blebs are seen on the top surface of cells resulting from the alterations of the microfilament network (Liepins and de Harven, 1982). The movement of surface membrane ruffles /blebs from the top surface to the marginal areas and the appearance of leukocytes around the vicinity of tumor cells and forming connections with the latter were observed after 24h of CP treatment. The increase in the number of leukocytes in tumor cell population after CP treatment *in vivo* suggests the infiltration of many leukocytes towards tumor cells. Infiltration of many lymphocytes and macrophages to murine fibro-sarcoma tumor cells has been noted after *in vivo* cisplatin treatment with different degrees of tumor cell degeneration adjacent to lymphocytes and macrophages (Sodhi and Sarna, 1979). In the later period of treatment (48h-72h), tumor cell-leukocyte connections become wider with deep membrane folds and more thin cellular processes arise from the tumor cells towards the adjacent leukocytes . Singh and Sodhi (1988) reported that murine peritoneal macrophages treated *in vitro* with cisplatin showed increased binding to Dalton's lymphoma cells through distinct cytoplasmic extensions, which transfer the lysosomes from the cytoplasm of macrophages to the tumor cell

cytoplasm. Lymphocytes attack cancer cells and deep folds on the surface membrane indicate the death of the cell. It has been suggested that lymphocytes kill tumor cells by the release of toxic factors that disrupt the cell membrane (Old, 1977; Young and Cohn, 1991). The present study also revealed the disintegration in the plasma membrane of tumor cells surrounded / connected by leukocytes which could be due to the release of some toxic factors from the leukocytes. The combined treatment with ascorbic acid and cyclophosphamide showed enhanced irregularities in the shape of the mitochondria, thickening of cristae and formation of vacuoles in the later periods of treatment with complete disappearance in the number of cell processes (Figure 22 A-K). These irregularities in the structure of mitochondria and the cellular deformation might ultimately lead to cell death. Electron microscopic studies revealing fewer and structurally altered mitochondria also support the respiratory impairment in cancer cells (White *et al.*, 1974). Various recent findings have indicated that mitochondria may be involved in mutagenesis, maintenance of the malignant phenotype, and control of apoptosis (Cavalli and Liang, 1998; Murphy and Smith, 2000). Mitochondria undergo various structural changes concomitant with changes in their functions under a variety of pathological conditions. Cisplatin treatment also caused deformations in the mitochondria different tissues of Dalton's lymphoma bearing-mice leading to the enlargement and/or irregularities in the mitochondrial cristae (Kharbangar, 2002) I 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; Zamzani *et al.*, 1995), suggesting that mitochondria may play a pivotal role in the process. Recently, it has been reported that mitochondrial dysfunction could be a major mechanism of drug-induced liver diseases such as nonalcoholic

steatohepatitis, cytolytic hepatitis (Pessayre *et al.*, 2000) and other disorders (Murphy and Smith, 2000). 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). A characteristic feature in the cell surface morphology consisting of the appearance of protrusions at discrete regions of the plasma region have been considered as a fairly general cell response during toxic oxidative and anoxic injury (Jewel *et al.*, 1982; Lemasters *et al.*, 1987).The formation of membrane vesicles and vacuoles on the tumor cells following CP treatment alone and combined treatment of AA and CP could be an indication of tumor cell lysis which eventually leads to cell death. Prasad and Sodhi (1982) reported that cisplatin treatment removes the cell surface sialic acid moieties and acid mucopolysaccharides which may in turn enhance the antigenecity of tumor cells by exposing new cryptic epitopes, thus permitting immunological recognition and tumor cell lysis.

Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosomes. The *in vivo* micronucleus test detects the effect of mutagenic agents on chromosomes by the identification of acentric fragments and lagging chromosomes (Schmid, 1975; Heddle *et al.,* 1978). The frequency of micronuclei reflects the level of genetic damage induced in the erythopoietic system. Chemicals that cause chromosome aberrations are known to induce micronuclei in a variety of proliferating cell systems, both *in vivo* and *in vitro*. These micronuclei are irregular in their cell cycle progression and divide asynchronously with the main body nucleus. When the parent nucleus enters the division phase the macronuclei may remain in the interphase. This results in micronuclei premature chromosome condensation MNPCC). Depending on the cell cycle stage of micronuclei MNPCC may be G1, S or G2 type (Obe and Beek, 1982).

It is generally agreed that micronuclei and MNPCC are seen only in proliferating cells subjected to treatment with potent clastogens (Rao, 1982; Tommerup, 1992; Zur Hausen, 1996). Detrimental chemical agents accept the occurrence of micronuclei to be a sensitive biological indicator of genotoxicity. (Mavournin *et al.*, 1990; Krishna *et al.*, 1992; Grawe *et al.*, 1993; Ribeiro *et al.*, 1993). The bone marrow suppression and depletion of circulating leukocytes are major side effects of cancer chemotherapy (Hoagland, 1982). Bone marrow micronucleated erythrocytes provide a simple and rapid method for detection of chromosomal damage by chemical and physical agents. Since micronuclei arise from disturbed genetic materials such as acentric chromosomal fragments (Heddle and Carrano, 1977) or even whole chromosomes (Weissenborn and Streffer, 1991), the cell containing micronucleus are potentially dead. Cyclophosphamide is an anticancer drug having profound haemotoxicity (Yeager *et al.,* 1982), inducing myelosuppression in mice. The cellular toxicity caused by cyclophosphamide is connected primarily with DNA damage (Brookes, 1990; Ferguson and Pearson, 1996). In comparison to the untreated control, the micronuclei increased in PCEs and NCEs in the bone marrow cells as well as in RETs and NCEs in the peripheral blood after cyclophosphamide treatment (Table 10 & 11; Figure 24). However, the frequency of micronuclei was observed to decrease significantly in the group of animals pre-treated with ascorbic acid as compared to CP treatment alone in the bone marrow cells ($p < 0.001$) and peripheral blood cells ($p<0.01$). The results of the present investigation have shown the antimutagenic protective effect of ascorbic acid against the genotoxicity caused by cyclophosphamide in the mouse erythropoietic system. In the erythropoietic system, micronuclei are formed in the erythroblasts during mitosis. Erythropoiesis is an ongoing process, so there is continual progression of cells from erythroblasts through

the PCE stage to the NCEs. Time-dependent changes in the micronucleus incidence were observed in the bone marrow cells of tumor mice exposed to cyclophosphamide. Cyclophosphamide appeared to affect 'delayed apoptosis' in the mouse erythropoietic system. It is known that apoptotic cell death is induced by DNA- damaging agents (Darzynkiewicz *et al.*, 1997; Mazur and Darzynkiewicz *et al.*, 1998). After exposure of cells to the toxic agents, in contrast to the interphase apoptotic cell death, the post-mitotic apoptosis occurs as a result of irrepairable damages. The post-mitotic apoptosis may represent 'delayed apoptosis', thus the cells containing micronuclei may die by 'delayed apoptosis'. The incidence of higher percentage of micronuclei over the controls following CP treatment clearly indicates the effect of CP thereby suggesting its mutagenic potentials in the hosts. The significant decrease in the frequency of micronuclei in ascorbic acid plus CP treated group than the group receiving CP alone suggests the chemopreventive effect of ascorbic acid against CP induced mutagenecity. These findings propose that combination chemotherapy of CP with ascorbic acid could be very useful in enhancing CP-mediated therapeutic efficacy and decreasing its mutagenic effects in the host.

The chromosomal aberrations and micronuclei have commonly been used as sensitive biological indicator in the mutagenic bioassay of a drug. In the present study development of all these mutagenic parameters were seen after CP treatment of tumor-bearing mice *in vivo* and supports earlier findings of its genotoxic properties (Overbeck *et al.*, 1996; Pillaire *et al.*, 1994). The pattern of the chromosomal aberrations reveals a higher frequency in the chromatid breaks and gaps. The total number of aberrant metaphase as well as chromosome aberrations was noticed to be highest at 24h of treatment, which decreased gradually during later periods (Table

12; Figure 25). Assessment of chemically induced chromosomal damage has been reported by Gebhart (1977) and suggested that gaps may be associated with chemical mutagenesis. The high frequency of damaged chromosomal plates observed after 24h of CP treatment and simultaneously a decrease in the frequency of chromosome damage in the later hours of treatment (72-96h) suggests that a post-replication repair process might be operating for recovery from the CP induced damage to DNA. The possible involvement of a post-replication repair process has been reported (Sorenson and Eastman., 1988). Rosenberg (1985) proposed that cisplatin lesions on O^6 of guanine in normal cells are repaired before replication, while in cancer cells, the lesions are not so efficiently repaired, because of deficiency in this repair process. The lesions are not removed and the mutation rate increases beyond the limits of survivability. This might thereby explain the fairly high frequency of aberrations observed in the tumor cells following 24h-96h CP treatments. Day *et al.*, 1980 have reported defective repair process of alkylated DNA by human tumor and SV-40 transformed human cell strains.

The results on the present investigation using mutagenic parameters showed the protective role of ascorbic acid on CP- induced mutagenic effects in murine system. The development of chromosome aberrations and micronuclei has been commonly used as sensitive biological indicator in the mutagenic bioassays of a drug. The decrease in frequency of the chromosome aberrations in the ascorbic acid pre-treated group might be due to the protective action of ascorbic acid on the mutagenecity of CP. The frequency and pattern of the chromosomal aberrations as revealed by the present data strongly suggests that ascorbic acid effectively ameliorates the mutagenic effects induced by CP. It is also apparently possible that the observed synergistic antitumor effect of AA and CP may not be at the DNA

level, and an enhancement of the host's immune function could be involved. The enhancement of phagocytic functions of leukocytes (Shilotri, 1977) and the immunostimulatory (Anderson, 1984) action by ascorbic acid have been reported. The protective role of ascorbic acid has also been observed against cisplatin-induced mutagenic effects (Giri and Prasad, 1996; Giri *et al.,* 1998). Therefore, it suggests that the decrease in the frequency of chromosome aberrations after combination treatment with ascorbic acid is due to the protective effect of ascorbic acid from the toxic effect of CP.

The existence of relationships between cancer and metals is known by all oncologists. Some metals are necessary for the functioning of cells; however, amounts exceeding the physiological levels may be highly toxic. A number of metal ions are able to generate free radicals. Usually there is within each cell, a fine balance between antioxidant systems and free radicals. When ROS overwhelm the capacity of the defense, the consequences are mostly ageing and cancer. The interaction of metal ions and the nature of their binding to DNA has been the subject of several studies. The essential /trace elements play an important role in a no of biological process by activating or inhibiting of enzymatic reactions, by affecting the permeability of cell membranes and /or by other mechanisms. It was observed in the present experiment (Table 13) that the total potassium concentration in the tumor cell decreases following CP treatment and this data might therefore support the hypothesis that the fall in intracellular potassium level may result from the inhibition of sodium+ potassium- ATPase activity at the cell membrane (Brady *et al.*, 1990). This fall in sodium-potassium ATPase activity would in turn be expected to result in an inhibition of all the secondary active transport processes causing marked disruption of vectorial solute transport across the tumor cell membrane. Programmed

cell death or apoptosis is a form of cell death in which DNA digestion occurs at an early stage by non-lysosomal endonucleases (Eastman, 1990). Yoshihara *et al.*, (1975) have suggested that the degradation of DNA in the dexramethasone treated thymocytes is mediated by calcium and magnessium dependent endonucleases that are present in an inactive form in the cell nuclei. It has also been reported that the use of calcium ionophores can experimentally increase calcium and induce digestion, whereas intracellular chelation of calcium can inhibit DNA digestion (Mc Conky *et al.*, 1989) and this criterion for a sustained increase in the calcium titre contrasts with the transient increases that are associated with normal intracellular signal transduction pathways (Eastman, 1990). Therefore, the sustained increase in the calcium level after CP treatment (Table 13) in the tumor cells may influence the selective cytotoxicity of CP in the tumor cells. Magnessium acts as an intracellular regulator of the cell cycle and controls apoptosis; it plays a significant role in the stabilization of the tridimensional structure of DNA. It also prevents DNA from alkylation. Mortality due to stomach cancer was correlated to the ratio of Mg2+/Ca2+, rather than to magnesium deficiency itself. In magnesium deficient animals, there is an increased production of free radicals. Magnessium plays an important role in the synthesis and use of energy rich compounds and in the maintenance of the membrane properties and so, its deficiency might affect the bioenergetic processes of efforts. The concentration of elements studied was found to have different concentration at different treatment conditions (Table 13) suggesting that these observed differences may be associated to the anticancer activity of CP. Cancer cells have higher numbers of transferrin receptors (TfR1) probably because they take up iron to a larger degree. TfR2, another transferring receptor has been detected in a wide variety of neoplastic cell lines, and may further increase iron

uptake. A membrane-bound transferring homologue, melanotransferrin, has been reported and is found in larger amounts in tumor cells. The intracellular iron pool serves as a source of iron for both hemoglobin and ferritin synthesis (Jacobs, 1977) however, there is increasing evidence showing that iron can contribute to cancer development either as a cancer initiator or as a cancer promoter. Beginning in the 1980s, some epidemiological reports have associated increased iron exposure with elevated cancer risk in either prospective or retro-prospective studies, by comparing cancer cases with their matched controls (Selby and Friedman, 1988; Stevens *et al.,* 1988). Iron exposure variables in those epidemiological studies included dietary iron intake, iron vitamin supplementation, body iron stores as measured by ferritin, serum iron (also known as transferrin iron), and total iron binding capacity of transferrin or transferrin saturation, and gene status for hereditary hemochromatosis, an iron overload disease. The majority of existing epidemiological data support the role of iron in human cancer along with animal and cellular evidence suggesting that iron may be carcinogenic. In animal studies, results showed that iron supplementation in rats decreased manganese superoxide dismutase activity(Kuratko, 1997), increased lipid peroxidation and free radical generating capacity in the colon and caecum (Lund *et al.*,2001) and elevated colonic aberrant crypt foci (Davis and Feng 1999; Liu *et al.*, 2001) .Indeed, tumor cells in a highly proliferate state have a high density of transferrin receptors, and antisense cDNA for the transferrin receptor was shown to reduce TfR mRNA and expression, resulting in more inhibition of growth of human breast carcinoma cells than normal breast cells (Yang *et al.*, 2001).Monoclonal antibodies against TfR severely restricted the growth of lymphoma tumors in mice (Kemp *et al.,* 1992). Reactive oxygen species (ROS), often under pathological conditions due to oxidative stress, have been shown to be

associated with a wide variety of diseases, such as carcinogenesis, inflammation, radiation, and reperfusion injury (Frenkel, 2002). Depletion of intracellular iron by chelation to induce cell cycle arrest and apoptosis represents one of the clinical approaches for cancer therapy (Buss et al., 2003, Le Richardson 2002).

CONCLUSION

Thus based on the various aspects of studies undertaken and discussed, some of the following important conclusions may be derived:

- As compared to CP treatment alone, combined treatment of dietary AA and CP brings about tumor regression in the host, suggesting an effective anticancer activity of the combined treatment therapy in this murine model.
- During the anticancer activity, this combination treatment, however, did not show definite changes in the cellular protein and carbohydrate concentrations except in some tissues like testes and also in DL cells and ascites supernatant.
- Along with the sialic acid decrease in DL cells this drug-mediated decrease of tissue and serum sialic acid in tumor-bearing mice should also bring about restoration of the functional activity of the tissues and serum to normal in the host, thereby facilitating tumor regression.
- After CP application alone and/or AA and CP administration, the total glutathione concentration decreases significantly. The decrease in GSH level by CP might play a significant role in the antitumor activity of CP against Dalton's lymphoma involving development of unfavourable conditions in the host leading to tumor cell regression.
- Contrary to the pattern of changes in the GSH in the different tissues, lipid peroxidation (LPO) increased in all the tissues after CP treatment. However, a significant decrease was noticed after the combined treatment of AA and CP which may suggest that CP treatment is associated with induction of

oxidative stress that can be ameliorated when it is administered in combination with dietary AA.

- The continuous decrease in the LDH enzyme activity in the tumor cells with a parallel increase in the tumor supernatant after the combined treatment may be used as an indication of the release of LDH from the tumor cells due to cell damage / injury. The LDH isozyme patterns also show the tissue specificity of different LDH isozyme forms.
- The CP treatment causes definite changes in the arrangement of membrane ruffles of the tumor cells and it leads to membrane disintegration which may result in the tumor cell lysis. Electron microscopy observation shows that the treatment with CP alone and combined treatment develops various irregularities in the mitochondria (i.e. cristae and morphology), which indicate towards cell death.
- The studies on mutagenic parameters in terms of micronuclei and chromosome aberrations suggests the protective effect of AA against CP-induced mutagenicity in the host; decrease in the frequency of chromosome aberrations after combination treatment with ascorbic acid may be due to the protective effect of ascorbic acid against the toxic and mutagenic effect of CP.
- The increase in the calcium level in the tumor cells after treatment may be involved in bringing about cell death.
- Lastly, based on these present findings, it may be proposed that combination chemotherapy of CP with dietary ascorbic acid could be very useful in enhancing CP-mediated therapeutic efficacy and at the same time helping the host by decreasing its mutagenic effects.

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Publications

Full Papers:

- Prasad, S. B., Giri, A., Khynriam, D., Kharbangar, A., Nicol, B. M. and C. Lotha (1999). Cisplatin-mediated enzymatic changes in mice bearing ascites Dalton's lymphoma. *Med. Sci. Res;* **27**: pp. 723-730.

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- Nicol, B.M. and Prasad, S.B. (2005). The protective effect of Vitamin C on the development of CP-mediated micronuclei in mice. *Mutation research* (communicated).

Paper Abstracts:

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