

ENCLOSURE - I

INTRODUCTION

Chemotherapy or 'Chemical treatment' has been around since the days of the ancient Greeks. Sometimes referred to simply as "chemo", chemotherapy is used most often to describe drugs that kill cancer cells directly. However, chemotherapy for the treatment of cancer began in the 1940s with the use of nitrogen mustard. Since then many new anti-cancer drugs or antineoplastics have been developed and tried. Today's therapy uses more than 100 drugs to treat cancer. There are even more chemo drugs still under development and investigation.

Since cancer is a word used to describe many different diseases, there isn't any one type of treatment that is used universally. Chemotherapy is used for a variety of purposes.

- a) To cure a specific cancer
- b) To control tumour growth when cure is not possible
- c) To shrink tumours before surgery or radiation therapy
- d) To relieve symptoms (such as pain) and
- e) To destroy microscopic cancer cells that may be present after the known tumour is removed by surgery (called adjuvant therapy).

+Reactive oxygen species (ROS) are the substances such as O₂-derived free radicals including hydroxyl (HO·), superoxide anion (O₂·-), peroxy (RO₂·), and alkoxy (RO·) radicals, or O₂-derived nonradical species including hydrogen peroxide (H₂O₂) (Circu and Aw, 2010; Ray et al., 2012). Over production or insufficient elimination of ROS will lead to oxidative stress. As ROS are able to induce damage of nucleic acids, proteins, and lipids, oxidative stress may be implicated in numerous diseases such as cancer (Kim et al., 2014), neurodegenerative (Curro et al., 2014) and cardiovascular diseases (Chan and Chan., 2014). The use of anticancer drugs following surgery is known as adjuvant therapy. It is a systematic therapy, meaning that drugs travel through the blood stream and affect normal as well as cancer cells all over the body. The body effects depend largely on the specific drugs and the dose. The drugs commonly employed can be classified according to the mode of action as depicted in Table 1.

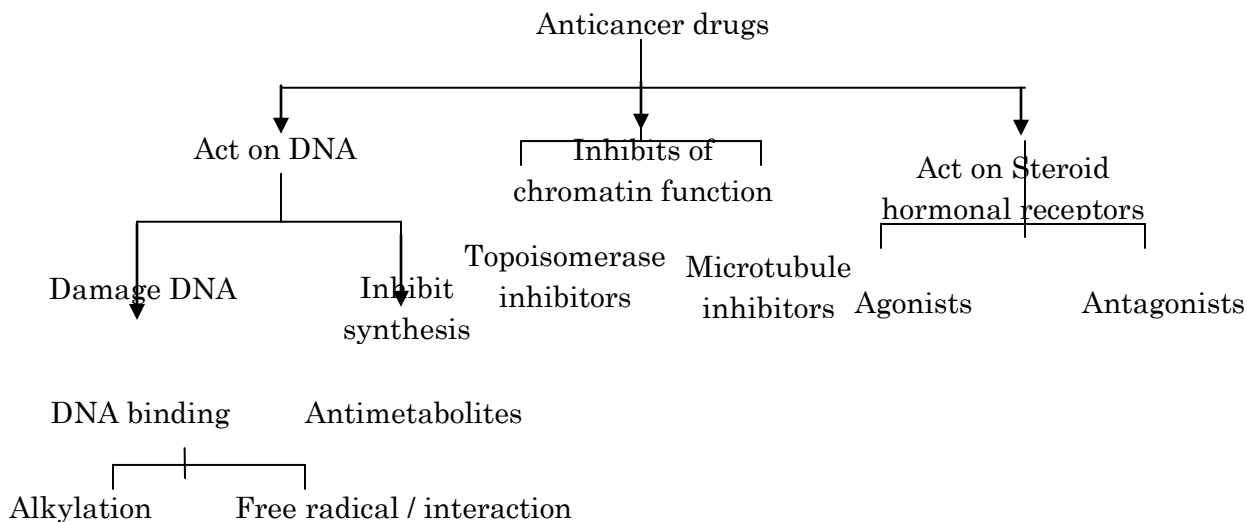


Table 1: Classification of anticancer drugs

1. Covalent DNA binding drugs :

Alkylating agents

- a) Mustard gas derivatives: cyclophosphamide, chloramburil, mechlorethamine melphalan and ifosfamide.

- b) Hydrazine's and triazines : altretamine, procarbazine, dacarbazine and temozolomide
- c) Nitrosureas : carmustine, lomustine and streptozocin
- d) Metal composition: carboplatin, cisplatin and oxaliplatin
- e) Alkyl sulfonates : busulfan
- f) Plant alkaloids : vinka alkaloids

2) Non-covalent DNA binding drugs :

- a) Anthracyclines : daxorubicin, daunorubicin
- b) Chromomycins : dactinomycin and plicomycin
- c) Miscellaneous : bleomycin

3) Antimetabolites :

- a) Folate antagonists : methotrexate
- b) Pyrimidine antagonists : cytarabine, gemcitabine and 5- fluorouracil
- c) Purine anatognists : 6-mercaptopurine and 6-thioguanine
- d) Sugar-modified analog: hydroxy urea

4) Inhibitors of chromatin function:

- a) Topoisomerase inhibitors : ironotecan, topotecan
- b) Drugs affecting endocrine function: tamoxiferon, prednitone

Antimetabolites are types of chemotherapy treatments that are very similar to normal substances within the cell. When the cells incorporate these substances into the cellular metabolism, they are unable to divide. Antimetabolites are cell-cycle specific. They attack cells at very specific phases in the cycle. With the increase in success of cancer treatment due to the aggressive use of high-dose drug therapies, there has been a growing concern about the long-term side effects of alkylating agents and other anti neoplastic drugs (Rodjer *et al.*, 1990; Sultan *et al.*, 1990).

CYCLOPHOSPHAMIDE

Cyclophosphamide (CP) (trade names Endoxan, Cytosan, Neosar, procytox, Reviimmune) also known as cytophosphate is a nitrogen mustard alkylating agent from the oxazophorines group. It is used to treat various types of cancer and some autoimmune disorders. It is a prodrug; it is converted in the liver to active forms that have chemotherapeutic activity. The main use of CP is together with other chemotherapy agents in the treatment of lymphomas, some forms of leukaemia (e) (Shanafelt *et al.*, 2007) and some solid tumours (Young *et al.*, 2006). It is a chemotherapy drug that works by showing or stopping cell growth.

CP is used to treat cancers and autoimmune disorders. As a prodrug, it is converted by liver cytochrome P450 (CYP) enzymes to form the metabolite 4-hydroxy cyclophosphamide that has chemotherapeutic activity (Kasper *et al.*, 2005). CP has severe and life-threatening adverse effects, including acute myeloid leukaemia, bladder cancer, hemorrhagic cystitis, and permanent infertility, especially at higher doses. For autoimmune diseases, doctors often substitute less-toxic methotrexate or azathioprine after an acute crisis (WHO, 2014). It is on the World Health Organization's List of Essential Medicines, a list of the most important medication needed in a basic health system (Kirshon, 1988). CP is used for the treatment of numerous malignant processes and certain autoimmune diseases. Goals of therapy are prompt control of the underlying pathological process and discontinuation or replacement of cyclophosphamide with less toxic, alternative medication as soon as possible in order to minimize associated morbidity. Regular and frequent laboratory evaluations are required to monitor renal function, avoid drug-induced bladder complications, and screen for bone marrow toxicity like other alkylating agents, cyclophosphamide is teratogenic and contradicted in pregnant women (Pregnancy Category D) except for life-threatening circumstances in the mother (Clowse, *et al.*, 2005) and (Shanafelt *et al.*, 2007). Additional relative contradictions to the use of CP include lactation, active infection, neutropenia, or bladder toxicity. The main use of CP is with other chemotherapy agents in the treatment of lymphomas, some forms of brain cancer, leukaemia, (Young *et al.*, 2006) and some solid tumours (Nelius *et al.*, 2010).

It is a chemotherapy drug that works by inducing the death of certain T cells. A 2004 study (Nelius *et al.*, 2010) showed the biological actions of cyclophosphamide are dose-dependent. At higher doses, it is associated with increased cytotoxicity and immunosuppression, while at low, continuous doses, it shows immunostimulatory and antiangiogenic properties. A 2009 study of 17 patients with docetaxel-resistant metastatic hormone refractory prostate cancer showed a prostate-specific antigen (PSA) decrease in 9 of the 17 patients. Median survival was 24 months for the entire group, and 60 months for those with a PSA response. The study concluded low-dose cyclophosphamide "might be a viable alternative" treatment for docetaxel-resistant MHRPC and "is an interesting candidate for combination therapies, e.g. immunotherapy, tyrosine kinase inhibitors, and antiangiogenesis" (Steinberg *et al.*, 1971).

CP decreases the immune system's response, and although concerns about toxicity restrict its use to patients with severe disease, it remains an important treatment for life-threatening manifestations of autoimmune diseases where disease-modifying anti-rheumatic drugs (DMARDs) have been ineffective. For example, systemic lupus erythematosus with severe lupus nephritis (Townes *et al.*, 1971) may respond to pulsed cyclophosphamide. CP is also used to treat minimal change disease, (Townes *et al.*, 1971) severe rheumatoid arthritis, (Novack *et al.*, 1971) granulomatosis with polyangiitis (Makhani *et al.*, 2009) and multiple sclerosis. CP also decreases the immune system's response to various diseases and conditions.

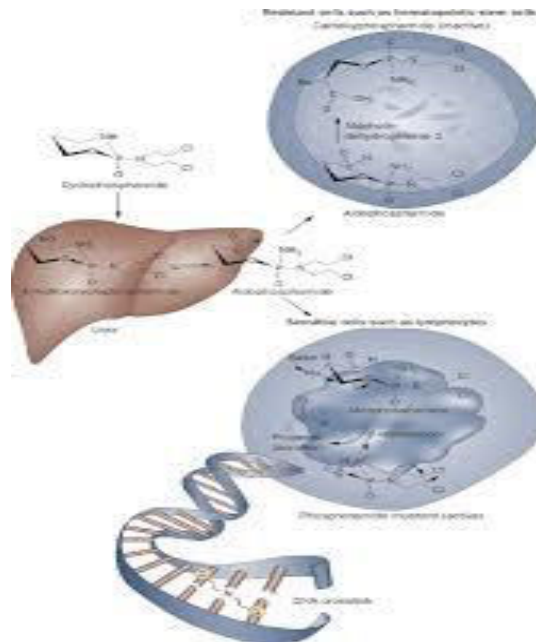
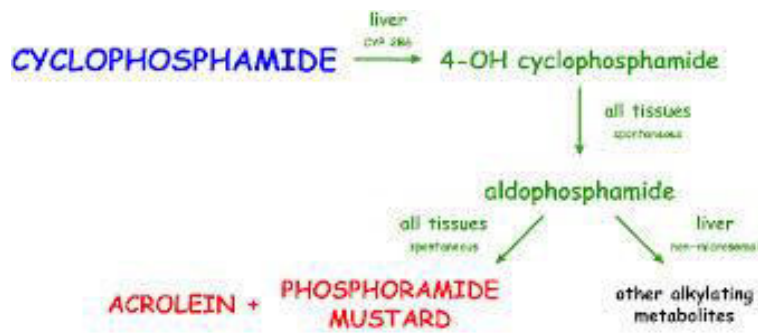
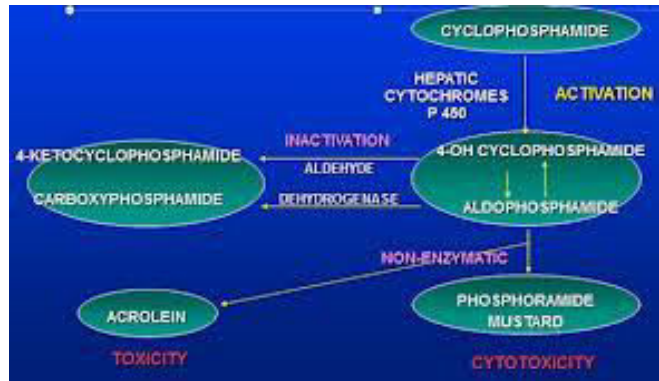
Mechanism of Action

The main effect of cyclophosphamide is due to its metabolite phosphoramidate mustard. This metabolite is only formed in cells that have low levels of ALDH. Phosphoramidate mustard forms DNA crosslinks both between and within DNA strands at guanine N-7 positions (known as interstrand and intrastrand crosslinkages, respectively). This is irreversible and leads to cell apoptosis. CP has relatively little typical chemotherapy toxicity as ALDHs are present in relatively large concentrations in bone marrow stem cells, liver and intestinal epithelium.

ALDHs protect these actively proliferating tissues against toxic effects of phosphoramidate mustard and acrolein by converting aldophosphamide to carboxyphosphamide that does not give rise to the toxic metabolites phosphoramidate mustard and acrolein. CP induces beneficial immunomodulatory effects in adoptive immunotherapy. Suggested mechanisms include (Hall *et al.*, 1992).

- 1) Elimination of T regulatory cells (CD4+CD25+ T cells) in naive and tumour-bearing hosts.
- 2) Induction of T cell growth factors, such as type I IFNs, and/or
- 3) Enhanced grafting of adoptively transferred tumour-reactive effector T cells by the creation of an immunologic space niche.

Thus, cyclophosphamide preconditioning of recipient hosts (for donor T cells) has been used to enhance immunity in native hosts, and to enhance adoptive T cell immunotherapy regimens, as well as active vaccination strategies, inducing objective antitumor immunity (Hall *et al.*, 1992).



In humans the total length of fetal development (gestation period) is about 270 days which is divided into three trimesters. In avian, the total gestation period (21 days) is divided into three trimesters, which is shown in Table-2.

Table-2: The length of each trimester during gestation period in avian and human systems

S.No.	Trimester	No. of days of gestation period	
		In avians	In human
1	First trimester	1-7	1-90
2	Second trimester	7-14	90-180
3	Third trimester	14-21	180-270

Embryonic development in chick system

The chick embryo system permits a well-controlled administration of substances, and straight forward observation of the embryo development and moreover the effect of the substances is not modified by pharmacokinetic properties of the mother (as in other embryonic system) and the chick embryo is capable of metabolic activation of xenobiotics as easy as the initial organogenetic period (Hamilton *et al.*, 1983; Gamett and Klein, 1984; Brunstrom, 1986).

The chick embryo was selected as the suitable system in toxicological studies in developmental biology because of:

- 1) The eggs are easily available in all seasons.
- 2) The development of embryo is known in detail (Ramanoff, 1967).
- 3) The development takes place in 21 days outside the maternal organism and thus easily accessible for experimentation and observation of the subsequent development (Hamburger and Hamilton, 1951).
- 4) The studies of toxicity and teratogenicity done in the chick embryo can often be directly applied to mammalian and human embryo in spite of some differences in morphology and physiology.
- 5) The chick embryo has proved to be one of the most valuable systems for studies in developmental biology, since the chick embryo is encased in an egg shell, receiving nutrition from resources entirely within the egg.

In chick embryo, most of the organogenesis occurs during the third and fourth day of incubation (Pattern, 1971). Furthermore, during the period of day 7 to day 11 of incubation there is significant increase in body weight and development of cerebral hemispheres (Nurhberger, 1958). Another interesting aspect of chick embryonic development is that it assimilates large amount of calcium into the bones from the egg shell at the stage of day 10-12 of embryonic life (Abbas *et al.*, 1985).

In fact, the day of 14, secondary stage of organogenesis starts in chick embryo correspond to neuronal proliferation and calcium assimilation. The chick embryo and its surrounding constituents inside shown in Fig.3 and stages in chick embryo are shown in Fig.4.

The formation and absorption of amniotic fluid

The amniotic sac is closely applied to the embryo in the beginning but it is soon carried away as a thin, clear fluid which accumulates in the amniotic cavity in increasing amounts.

The origin of this fluid, although not definitely established, seems to be extra-embryonic, since amnions that develop in the absence of the embryo are nevertheless fluid-filled. The fluid, if not a secretion of the amniotic wall, may be a transudate from the blood vessels of the area pellucida. The biological fluid surrounding the embryo has a very complex and dynamic nature (Cherry, 1967; Lind *et al.*, 1971). The major functions of the amniotic fluid are:

1. To protect the embryo from external shocks.
2. To maintain the internal temperature of the foetus.
3. To participate in foetal biochemical homeostasis.
4. To permit foetal mobility and proper growth of the embryo (Carl *et al.*, 1956; Ermalinda and Fierick, 1976).

On fifth day of development of chick, the amount of liquid within the amnion is sufficient to keep the sac well away from the embryo. The volume of the fluid continues to increase until it attains a maximum of 3-4 ml about the 13th day of the incubation. The quantity represents 8-9 percent of the original weight of the egg.

The amount of fluid usually declines slightly until the 15th day, then rises again and finally decreases to zero rapidly during the last few days of the incubation.

The increase in the fluid of the amniotic sac after 10th day may be attributed chiefly to the influx of albumin through the ruptured sero-amniotic connection. Conditions that retard or accelerate the perforation of the sero-amniotic plate therefore affect the fluctuations in the volume of amniotic fluid. The final phase of decrease is throughout to be mostly due to the ingestion of the fluid by the embryo. The solid content of the fluid is enormously high between the 13th and 18th day, the time at which albumin flows through the sero-amniotic connection into the amniotic fluid.

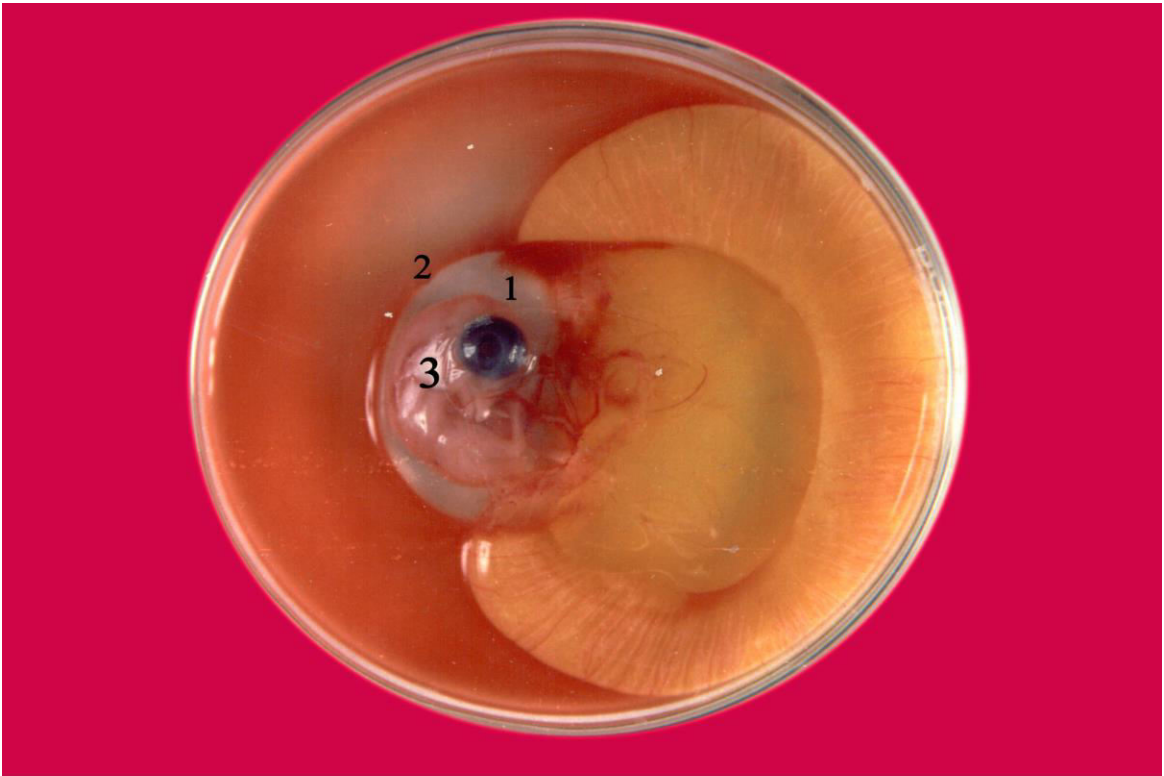


Fig.3 :The Chick embryo and its surrounding constituents inside the egg
1) Amniotic fluid
2) Amniotic membrane
3) Embryo

According to Lind *et al.* (1971), the composition of the amniotic fluid in early pregnancy is more closely related to foetal plasma than maternal serum. Amniotic fluid studies are having a wide use in clinical diagnosis and management. The analysis of amniotic fluid is an index of foetal status in uterus (Cherry, 1967).

Generation of reactive oxygen species (ROS) and free radical

The biological combustion produces harmful intermediates called free radicals. A free-radical is simply defined as any species capable of independent existence that contains one or more unpaired electrons. It may be superoxide (O_2^-), thiyl ($Rs\bullet$); Trichloro methyl (CCl_3) or nitric oxide ($N\bar{O}$) in which the unpaired electrons is decolorized between both atoms. They are very transient species due to their high chemical reactivity that leads to lipid peroxidation and oxidation of some enzymes and a massive protein oxidation and degradation (Mates *et al.*, 1999a). The O_2^- , hydroxyl radicals (OH) and other reactive oxygen species (ROS) such as H_2O_2 are continuously produced *in vivo*.

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. These free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air pollutants, pesticides etc. (Tiwari, 2001). The living creatures have evolved a highly complicated defence system with antioxidants composed of enzymes and vitamins against oxidative stress in the course of their evolution. These defence system are mainly classified (Noguchi *et al.*, 2000) as (i) suppression of generation of ROS, (ii) scavenging of ROS, (iii) clearance, repairing and reconstitution of damage and (iv) induction of antioxidant proteins and enzymes. The oxidative stress, defined as the imbalance between oxidants and antioxidants in favour of the former potentially leading to damage has been suggested to be the where of aging and various human disease (Sies, 1982).

Radicals can react with the other molecules in a number of ways (Slater, 1984). Thus if two radicals meet, they can combine their unpaired electrons and join to form a covalent bond (as a shared pair of electrons).

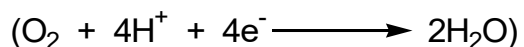
Free radical formation takes place in the following three ways by the haemolytic cleavage of a covalent bond of a normal molecule, with each fragment retaining one of the electrons.

- By the loss of a single electron from a normal molecule.
- By the addition of a single electron to a normal molecule (Suresh and Tiwary, 1999).

Hence a radical can donate its unpaired electron to another molecule, or it might take an electron from another molecule in order to pair. However, if a radical gives an electron to, or takes from, a non-radical, that non-radical becomes a radical. Thus, a feature of the reactions of free radicals is that they tend to proceed as chain reactions. One radical gets another and so on (Halliwell, 1989).

One of the peroxides of life on this planet is that the one molecule that sustains aerobic life, oxygen, is not only fundamentally essential for energy metabolism and respiration but it can also be dangerous for long time existence (Bandopadhyay *et al.*, 1999).

Although O₂ can behave like a radical (a di radical) owing to the presence of two unpaired electrons of parallel spin, it does not exhibit extreme reactivity due to quantum mechanical restrictions. More than 90 per cent of the O₂ taken up by the human body is used by mitochondrial cytochrome oxidase, which adds four electrons and four protons onto each O₂ molecule to form water.



In the sequential univalent process, O_2 undergoes reduction and forms several reactive intermediates, called reactive oxygen species (ROS) oxygen, by accepting one electron generates a superoxide (O_2^- radical. Subsequent reactions from hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$) and water (Aruoma, 1994).

At the mitochondrial level due to electron leakage about 1-4 per cent of consumed oxygen is converted to and H_2O_2 (Loschen *et al.*, 1974; Chance *et al.*, 1979). Leakage of electrons is normally restricted by cytochrome C oxidase but occasional leakage of electrons occurs (Bast *et al.*, 1991). Superoxide and hydrogen peroxide are relatively underactive and long-lived in biological systems but their properties readily give rise to highly reactive hydroxyl radicals, which are involved in numerous forms of damage to cellular macromolecules.

Physiological Functions of Reactive Oxygen Species and Free Radicals:

Active oxygen and related species play an important physiological role, at the same time, may exert toxic effects as well. The active oxygen species are essential for production of energy, synthesis of biologically essential compounds and phagocytosis, a critical process of our immune system. They also play a vital role in signal transduction, which is important for cell communication and function (Noriko Noguchi and Niki, 1999). Nitric oxide is well identified as signalling molecule (Furchgott, 1995; Palmer *et al.*, 1987) and is now well known as regulator of transcription factor activities and other determinations of gene expression (Bogdan, 2001). Hydrogen peroxide and superoxide have similar intracellular effect (Sunderesan *et al.*, 1995; Finkel, 1998; Kamata and Hirata, 1999; Rhee, 1999; Patel *et al.*, 2000). On the other hand, they appear to have broader significance in the production of tissue injury under conditions of oxidative stress (Farber *et al.*, 1990).

Several cytokines, growth factors, hormones and neurotransmitters use ROS as secondary messengers in the intracellular signal transduction (Thannical and Fanberg, 2000). For several transcription factors, ROS function as physiological mediator of transcription control.

Because of high reactivity ROS, they prone to cause cellular damage, and are thereby potentially toxic, mutagenic or carcinogenic. Polyunsaturated fatty acids (PUFA), protein, DNA and carbohydrates are all susceptible to free radical attack which may result in oxidative damage such as membrane dysfunction, protein modification, enzyme inactivation and breaks in DNA strands and modification of DNA bases ultimately resulting in cell death (Sies, 1993; Stadtman and Levine, 2000).

The effect of ROS on DNA

ROS have been shown to be mutagenic (Mates *et al.*, 1999; Marnett 2000), an effect that is likely to result from chemical modification of DNA. In addition a number of alterations e.g., cleavage of DNA, DNA-protein cross-links, oxidation of purines, etc., takes place. If the DNA-repair systems are not able to immediately regenerate intact DNA, a mutation will result from erroneous base pairing during replication. This mechanism may partly explain the high prevalence of cancer in an individual exposed to oxidative stress (Mates *et al.*, 1999 a; Marnett, 2000).

The effect of ROS on lipids

Lipid peroxidation is probably the most explored area of research when it comes to ROS (Steinberg, 1997; Yla-Hertuala, 1999). It is commonly considered as a deleterious process (Benzie, 1996; Sevanian and Ursini, 2000) leading to structural modification of lipid/protein-assemblies, such as bio membranes and lipoproteins, and is usually associated with cellular modification. Cell membranes, which are structurally made up of large number of polyunsaturated fatty acids (PUFA), are highly susceptible to oxidative damage, resulting in membrane fluidity, permeability, and cellular metabolic functions (Bandhopadhyaya *et al.*, 1999). All biological material contains a variety of PUFA, which are predominantly located in the membrane lipids. The spatial arrangement and interrelationships of these amphipathic lipids is thought to contribute to the overall susceptibility of cell membranes to free radical attack.

PUFA have a characteristic methylene interrupted double bond structure. Because of double bond PUFA is susceptible to abstraction of hydrogen atoms mediated by •OH. The process of lipid peroxidation is a free radical chain reaction and involves three distinct steps; initiation, propagation and termination. PUFA reacts with a free radical to form a carbon centred radical, which further reacts with the molecular oxygen to form a lipid peroxy radical. This is the initiation stage of lipid peroxidation process shown in Fig.5.

In the propagation step the peroxy radical reacts with another PUFA moiety, resulting in the formation of lipid hydro peroxides and lipid radicals. Lipid hydroperoxide is the first comparatively stable, product of the lipid peroxidation chain reaction (Halliwell and Gutteridge, 1985; Fuller *et al.*, 1988).

In the termination stage, these lipid or lipid peroxy radicals are either consumed or they interact with each other to form stable compound. Thus, lipid peroxidation products include lipid peroxides, hydro peroxides, epoxy alcohols, Malondialdehyde, ethane, pentane and 4-hydroxy alkenes (Bendetti *et al.*, 1980; Mead *et al.*, 1980; De Zwart, *et al.*, 1998).

Some of the aliphatic products are documented as cytotoxic that can destructively interact with the other molecules. Disrupted tissues are known to undergo lipid peroxidation at a faster rate than the normal ones.

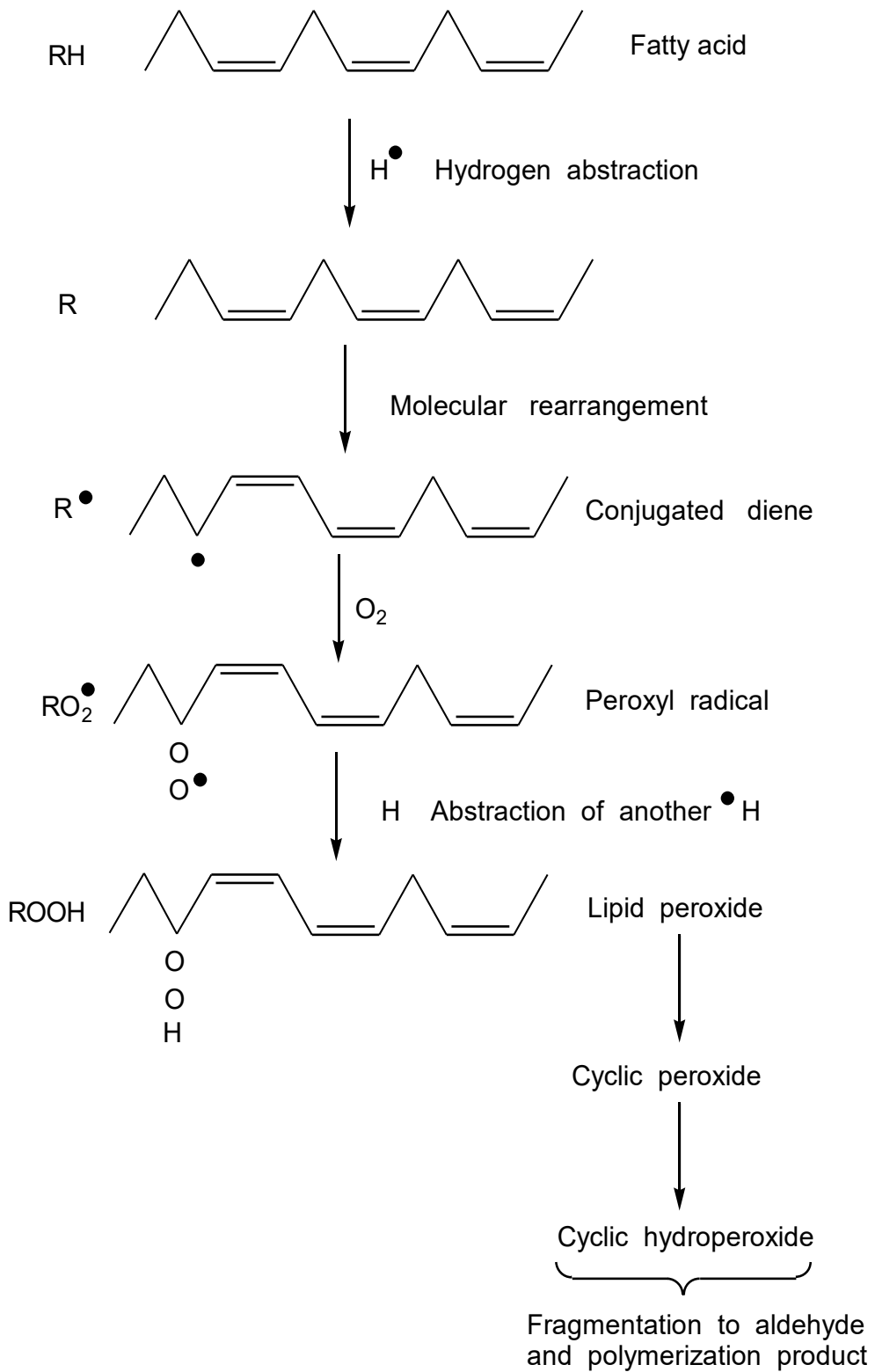


Fig.5 : Lipid peroxidation

Lipid peroxidation results in structural and functional changes in membranes, swelling and lysis of mitochondria and changes in ion pumps (Hunter *et al.*, 1963). Such alterations of membrane phospholipids would disrupt the hydrophobic core of the membrane (Halliwell, 1992).

Antioxidant enzymes

Antioxidants are substances that either directly or indirectly protects cells against adverse effects of xenobiotics, drugs, carcinogens and toxic radical reactions (Halliwell, 1995). Evolution has generated a variety of enzymatic and non-enzymatic defence systems. These include glutathione peroxidase (GPX), superoxide dismutase (SOD), Catalase (CAT), glutathione transferase (GST), nitricoxide synthase (NOS), eosinophil peroxidase (EPO), vitamin C (ascorbic acid), vitamin E (λ -tocopherol), vitamin A, β -carotene, metallothionin, polyamines, melatonin, NADPH, polyphenols, Flavonoids and phytosterol etc., (Diplowk, 1994; Krishna *et al.*, 1996; Chanvitayapongs *et al.*, 1997; Evans *et al.*, 1997; Beyer *et al.*, 1998; Devamanoharan *et al.*, 1998; Halliwell, 1999; Nohl *et al.*, 1998; Fremont, 2000). Antioxidants are now well defined as “any substrate that when present at low concentration compared to those of an oxidizable substrate significantly delays or inhibits the oxidation of that substrate”. In other words “an antioxidant is defined as any substrate that delays or inhibits oxidative damage to a target molecule” (Halliwell and Gutteridge, 1990).

Evolution has generated a variety of enzymatic and non-enzymatic antioxidative defence systems, protecting living organisms from the deleterious effects of oxidative compounds (Diplowk, 1994; Halliwell, 1995). Antioxidants are now well defined as “any substrate that, when present at low concentration, compared to those of an oxidizable substrate, significantly delays or inhibits the oxidation of the substrate”. In other worlds “an antioxidant is defined as any substrate that delays or inhibits oxidative damage to a target cell” (Halliwell and Gutteridge, 1990). The role of antioxidants has received, increased attention with the increasing experimental, clinical and epidemiological evidence showing the involvement of free radicals and active oxygen species in a variety of diseases.

Enzymatic defences system involves molecules that are capable of removing, neutralizing or scavenging ROS, Reactive nitrogen species (RNS) and their intermediates. Antioxidant defence mechanisms may also include the inhibition of ROS and RNS formation, the binding of metal ions needed for catalysis of ROS generation, and the up-regulation of antioxidant defence activity, suggesting that the removal of free radicals by cells is not likely accomplished by a single pathway but composed of several cascades of intricately related events (Mruk *et al.*, 2002).

The non-enzymatic antioxidants are low molecular weight compounds, mostly considered to be chain-breaking antioxidants that interrupt the auto-catalytic spread of radical reactions (Cadenos, 1989). This includes reduced glutathione (GSH), vitamin C, vitamin E, and β -carotene.

Selenium is indirectly an antioxidant it is required for the production of the major antioxidant enzyme glutathione peroxidase (Tripathi *et al.*, 2001).

There are two lines of antioxidant defence within the cell. The first line, found in the fat, soluble cellular membrane consists of Vit. E, beta carotene and coenzyme Q₁₀. Inside the cell water, soluble antioxidant scavengers are present. These include vitamin C, glutathione peroxidase, superoxide dismutase and Catalase (Jain *et al.*, 2004). It is well known that SOD, Catalase and glutathione peroxidase play an important role as protective enzymes against lipid peroxidation in tissues (Shahjahan *et al.*, 2004).

Antioxidants are either nutrients or enzymes which mop up damaging free radicals in our bodies (Karanth *et al.*, 2004).

The antioxidants might protect a target at many different stages in an oxidative sequence such as:

1. decreasing the localized oxygen concentration by combining with it or displacing it
2. Destroying badly damaged target molecules and replacing them with new ones.
3. Removing key reactive species like single O₂[·] or NOO⁻.

4. Removing peroxides by converting them into non-radical products such as alcohols.
5. Scavenging the initiating radicals such as $\bullet\text{OH}$, RO , RO_2 .
6. Removing catalytic metal ions.

Many antioxidants have more than one mechanism of action. The preventive antioxidants acting in the first line of defence, suppress the formation of free radical and oxygen species. The radical-scavenging antioxidant enzymes are responsible for the second line of defence and inhibit chain initiation and / or break the chain propagation. The enzymes that repair the damaged molecules act as the third line of defence.

The most active and universal components among the body's free radical systems are the antioxidants and antioxidant enzymes. SOD, CAT, GPx is enzymic antioxidants (Granot and Kohen, 2004).

Superoxide dismutase (SOD)

The first enzyme involved in the antioxidant defence system (McCord and Fridovich, 1969) is superoxide dismutase: a metalloprotein found in both prokaryotes and eukaryotic cells. Since SOD is present in all aerobic organisms and in most (if not all) sub-cellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defence against oxidative stress. There are three distinct types of SOD, classified on the basis of the metal cofactor: the copper / zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) isozymes.

The sub-cellular distribution of these isozymes is also distinctive. The Mn-SOD is found in the mitochondria of eukaryotic cells; some Cu/Zn-SOD isozymes are found in the cytosol, others in the chloroplasts of higher plants. Bacteria contain both Mn-SOD and Fe-SOD isozymes (Yost and Fridovich, 1973) and in some case Cu, Zn-SOD (Fridovich, 1997).

SOD catalyses the dismutation of the highly reactive superoxide anion to oxygen and to less reactive hydrogen peroxide species, which is further destroyed by Catalase and glutathione peroxidase (Sandlio *et al.*, 1999) as shown in Fig.6.

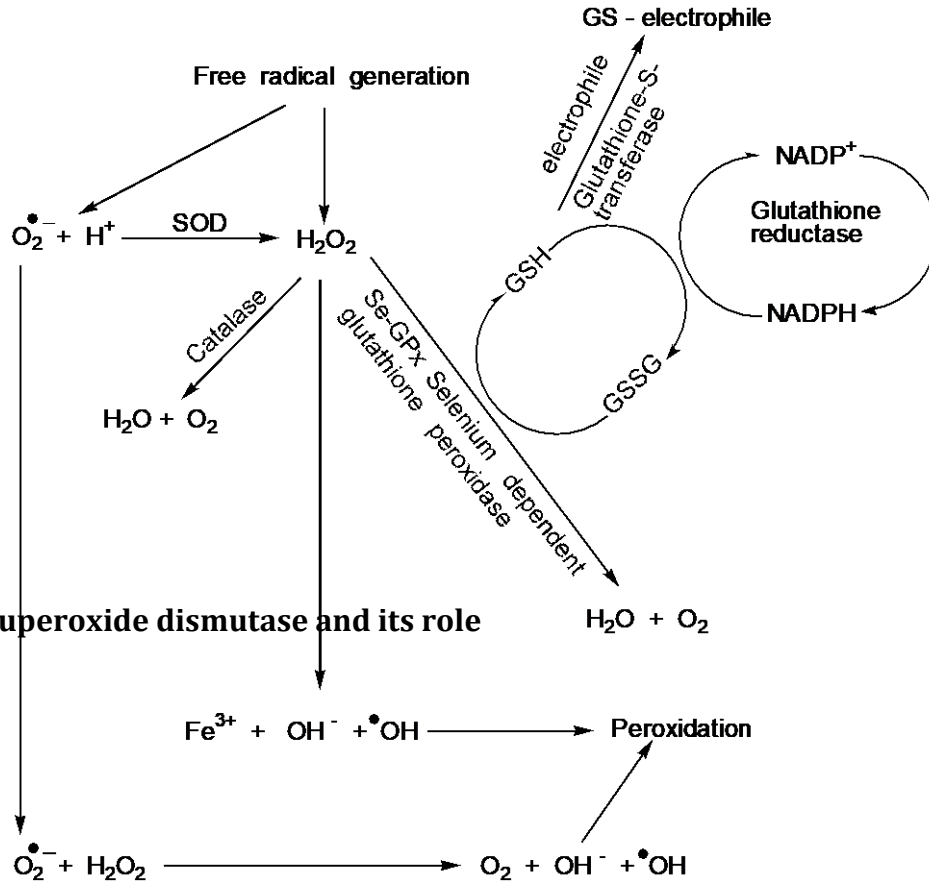


Fig.6: Superoxide dismutase and its role

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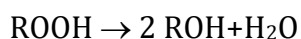
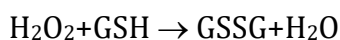
Catalase (CAT)

Catalase is a haeme-containing enzyme that catalyses the dismutation of hydrogen peroxide into water and oxygen (Aebi, 1974; Criag, 1997). The enzyme is found in all aerobic eukaryotes and is important in the removal of hydrogen peroxide generated in peroxisomes (micro bodies) by oxidises involved in β -oxidation of fatty acids, the glyoxylate cycle (photorespiration) and purines catabolism.

Catalase also functions in detoxifying different substrates, e.g., phenols and alcohols via the coupled reduction of hydrogen peroxide. One antioxidative role of Catalase is to lower the risk of hydroxyl radical formation from H₂O₂ via the Fenton reaction catalyzed by Cu⁺ or Fe⁺⁺ (Fridovich, 1999; Halliwell, 1999). Catalase binds NADPH, which protects the enzymes from inactivation and increases its efficiency (Kirkman and Gaetani, 1984; Kirkman *et al.*, 1999). It is a haeme protein catalyzing the decomposition of H₂O₂ to H₂O and O₂ and this protects the cell from oxidative damage by H₂O₂ and OH (Devasagayam and Sainis, 2002).

Glutathione peroxidase (GPx)

Glutathione peroxidase catalyses the reaction of hydroperoxide with reduced glutathione (GSH) to form glutathione disulfide (GSSG) and the reduction product of hydroperoxide (Meister and Anderson, 1983; Flagg *et al.*, 1995; Gladyshev and Hatfield, 1999; Brigelius-Flohe, 1999; Arthur, 2000). It can also reduce other peroxides (e.g., lipid peroxides) to alcohol and this in turn is dependent upon the availability of NADPH and glutathione Reductase (GR), which are seleno-enzymes of 85 kDa. A seleno-cysteine amino acid has been identified at the active site of this enzyme and this amino acid is involved in the catalytic cycle (Zachara, 1992). This enzyme is specific for its hydrogen donor, GSH, and nonspecific for the hydro peroxides ranging from H₂O₂ to organic hydro peroxides (Grozioli *et al.*, 1998). There are at least four different GPx in mammals and are predominantly present in erythrocyte and kidney with high expression seen in renal epithelial cells (DeHaan *et al.*, 1998; Sigalov and Stern, 1998). GPx catalyses the oxidation of GSH to GSSG at the presence of H₂O₂ (Manonmani *et al.*, 2002).



Jones *et al.*, 1981, indicated that GPx plays an important role during physiological conditions while Kelner and Bagnell, 1990 stated it to play an important role only during the oxidative stress.

Glutathione-S-transferase (GST)

This is a multifunctional enzyme, which plays a significant role in the biotransformation of xenobiotics. It is widely distributed in all species from bacteria to human tissues, the highest concentration being found in the aqueous extract of the supernatant of the liver (Tredger and Sherwood, 1997). It catalyses the detoxification by conjugation of reduced form of glutathione (GSH) with an electrophile (Habig *et al.*, 1974). The electrophile may include many ranges of compounds as carcinogens, chemotherapeutic derivatives and xenobiotics (Beckett and Hayes, 1993). Apart from the catalysis of glutathione conjugation to electrophilic compounds, these enzymes appear to play other detoxification functions by conjugating GSH with xenobiotics to protect cellular membranes from oxidative stress by expressing non-Se-GPx activity and this may aid in removing the toxic substances before damaging the membrane (Chang *et al.*, 1990).

Mammalian GSTs have been intensively studied. Even though these isoenzymes have overlapping substrate activities; they can be distinguished according to their physical, chemical, immunological and structural properties. The cytosol GSTs are grouped into six classes: alpha, mu, pi (Mannervik *et al.*, 1985), theta (Meyer *et al.*, 1991), sigma (Meyer and Thomas, 1995) and zeta (Board *et al.*, 1997). In addition, a class kappa GST from mitochondria (Pemble *et al.*, 1996) a membrane bound form of the isoenzymes (Morgenstern *et al.*, 1985) has also been reported. The GST subunits are designated according to the class-based subunit nomenclature (Mannervik *et al.*, 1992).

Comparatively, very sparse data are available on avian GSTs. Avian species are exposed to a wide spectrum of xenobiotics. The use of feed additives represents a necessary requirement in poultry farming. Surprisingly, GSTs of the phase II biotransformation system have received attention only recently.

Different homo and heterodimeric combinations of various subunits constitute the GST isoenzymes; they have different but overlapping substrate specificities against a wide array of substrates like CDNB, EPNP, p-NBC, p-NPA and BSP (Habig *et al.*, 1974). Subunit composition of each GST dimer CL₂, CL₃, CL₅ conform homodimers, CL₁ and CL₄ exist only as CL₁₋₂ and CL₃₋₄ heterodimers (Chang *et al.*, 1990). The characterization of individual GST isozymes in each organ require a battery of tests involving specific activities with several substrates, sensitivities to several inhibitors, molecular weight of subunits, Isoelectric points and precipitation with specific antibodies, determination of amino acid and nucleic acid sequences.

Glutathione Reductase (GR)

This enzyme catalyses the conversion of oxidized, glutathione (GSSG) to reduced GSH by using reduced NADPH (Kanzok *et al.*, 2001). This enzyme is found in cytosol and mitochondria, which matches GPx distribution (Becker, 2001).

GR maintains high concentration of the reduced form of glutathione and low levels of oxidized glutathione, thus maintaining the redox state.



The GR synergistically acts with the ascorbate, vitamin E and GSH and scavenges free radicals (Mutacich and Powis, 2000).

Non-enzymatic antioxidants

Glutathione

Glutathione (GSH) is a tripeptide (Glu-Cys-Gly) and is considered to be one of the most important components of the antioxidant defence of living cells.

GSH is the reducing agent that recycles ascorbic acid from its oxidized to its reduced form by the enzyme dehydroascorbate reductase.

The reduced tripeptide GSH is a hydroxyl radical and singlet oxygen scavenger, and participates in a wide range of cellular functions such as protein and DNA synthesis, intermediary metabolism, and transport (Meister and Anderson, 1983; Halliwell and Gutteridge, 1989; Deneke and Fanburg, 1989).

On oxidation, the sulphur forms a thiyl radical that reacts with a second oxidized glutathione forming a disulphide bond (GSSG) (Van Bladeron, 2000).

GSH can function as an antioxidant in many ways. It can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger (Sen, 1997). GSH may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions.

GSH also participates in the detoxification of xenobiotics as a substrate for the enzyme glutathione-S-transferase (Armstrong, 1997). GSH also act as a substrate for glutathione peroxidase to scavenge peroxides. GSH / GSSG form the major intracellular redox system whose concentration is maintained by glutathione reductase and NADP (Williams, 1992; Dahl *et al.*, 1997). Hence, GSH / GSSG ratio is frequently used as an indicator of the level of oxidative stress in cells.

Vitamin A

Vitamin A or retinol is fat soluble vitamin, which is essential for growth and maintenance of visual function. Vit A reported to play a vital role in suppressing carcinogenesis by increasing immunity to tumours through several mechanisms (Tits *et al.*, 2000).

Vitamin E

Vitamin E a hydrophobic dietary antioxidant is an integral component of bio membranes and renders protection against free radical initiated injury both independently and through the interaction with the glutathione (GSH). Vit.E occurs in nature as and tocotrienols. The biological activity of each of the forms is dependent upon its absorption lipoprotein transport, delivery to tissues and metabolism .Vit. E protects cells from the cancerous effects of X- rays, chemicals; air pollutants and UV light (Rekha *et al.*, 2001).

Vitamin C (Ascorbic acid)

Vitamin C is a micronutrient essential for various metabolic functions of the body. It cannot be synthesized by humans and other primates and so it must be absorbed from the diet.

Ascorbic acid (ASA) acts as a potent reducing agent and acts as an antioxidant both *in vivo* and *in vitro* (Bendetti *et al.*, 1980; Tolbert, 1985; Levine, 1986; Burns *et al.*, 1987; Niki, 1990). Its antioxidant property is due to its ability to react with a variety of free radicals and active oxygen species. Thus it reduces oxygen, nitrogen and sulphur centred radicals in the plasma and it complements the reductive ability of glutathione towards free radicals.

Herbal medicines as antioxidants

Ancient literature mentions herbal medicines for age-related diseases namely memory loss, osteoporosis, diabetic wounds, cardiovascular diseases, immune and liver disorders etc, which are free radical-mediated and have no modern medicine or only palliative therapy is available. Since oxidative damage to crucial bio molecules due to excess generation of active oxygen species has been implicated as a major cause of organ damage, compounds capable of negating such damage have potential benefits (Cullen *et al.*, 1997).

In the recent years there has been considerable interest in natural products with antioxidant property in human diet. One of the areas which had attracted a great deal of attention is the possible use of antioxidant supplements in the prevention of diseases caused by oxidative damage (Sreepriya *et al.*, 2001). Several antioxidants of plant origin have been identified and used as effective protective agents against oxidative stress (Nishigaki *et al.*, 1992).

Each plant contains a number of phytochemical whose presence is indicated by hereditary factors. A well designed research can determine, whether any of these chemicals would be useful for preventing any disease produced by the damaging reactions of free radicals and active oxygen species.

Immunomodulators from plant sources

In recent years, there has been an upsurge in the clinical use of indigenous drugs, Ayurveda; a science of life has a great potency to face this challenge. Ayurvedic drugs enhance the immune power of the body which not only help to cure the disease but also avoids the recurrence. Adaptogenic agents produce complex of biochemical, neural and immunological mechanism and plays a role in the restoration of normal physiological condition and generalized increase in the resistance against infection. Rasayanas are a group of drug preparations made of several plant products used in Ayurvedic system of

medicine to improve body's immune system (body's immune system, memory, intelligence, youthfulness and efficiency (Singh S, 1971).

The biological products obtained from plant sources such as polysaccharides, lectins, peptides etc. have been shown to stimulate the immune system (Kuttan *et al*, 1992). The mixture of phytochemical, as they are naturally in plants, might be more effective in cancer chemoprevention than single compounds. This is known as "combination chemoprevention" that explains why low doses of chemo preventive agents differing in their mode of action may increase efficacy and minimize toxicity, through synergistic mechanisms (Russo GL 2007). Immunomodulatory and immunorestorative properties of Rasayanas find their use in diseases like cancer, AIDS, tuberculosis etc. Chemoprotective effects of Rasayanas have been shown to stimulate stem cell proliferation and possibly its differentiation (Praveen kumar *et al*, 1994). Amalki Rasayanas is a non-toxic polyherbal drug preparation and one of main ingredients is *Emblica officinalis* which has high antioxidant potency. According to Ayurvedic texts, Rasayanas therapy arrests aging, increases intelligence, vigour and resistance to disease.

Collection of Plant material

The medicinal plant material was collected. The plant material was *Andrographis paniculata* (Burm.f) Nees.

Common name : Nelavemu, King of Bitters.

Family : Acanthaceae.



Fig.7. Morphology of *Andrographis paniculata* (field trip conducted for the collection of plant material)

Overview

Andrographis is a shrub that is found throughout India and other Asian countries. It is sometimes referred to as “Indian Echinacea”. It contains as its primary chemical constituents, diterpenoid lactones (andrographolides), paniculides, farnesols and flavonoids. It was used historically in the Indian flu epidemic in 1919 during which it was credited with reversing the spread of the disease. Over the last decade, *Andrographis* has become popular, particularly in Scandinavia, as a treatment for colds, and is now available in the United States. Herb leaves and roots are used in different additional applications. *In vitro* and *in vivo* studies suggest that *andrographis* has anti-infective, antiviral, anti-diarrheal, antipyretic and analgesic activities. In addition, immunostimulant and anti-inflammatory activities have been observed in chick embryo.

Preparation of *Andrographis paniculata* extract

Powdered plant material was soaked in 95% ethanol for 48 h. The extract was distilled and a dark green residue was obtained.

The remaining ethanol was removed by evaporating the residue under reduced pressure.

Toxicity studies of *Andrographis paniculata*

AP extracts at a dose of 1.0, 2.0, 3.0, 4.0, 5.0 mg / egg was given to chick embryo on 12th day. The changes in body weight, liver, heart and brain weight were recorded. Levels of glucose, urea, protein, cholesterol and the activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured to ensure the non-toxic nature of the extract.

Fixation of optimum dosage for the plant extract

AP extract was given different doses 1.0, 2.0, 3.0, 4.0, and 5.0 mg / egg wt / day on 12th day of embryonic development. The dosage which gives maximum protection (as elicited by the activity of ALT and AST) against CP induced hepatic damage was fixed as the optimum dosages.

Collection of embryonic tissues

After collection of chick embryonic tissues of liver, heart and brain, the tissues were immediately stored in chilled cold saline at -200C.

Preparation of embryonic tissue 10% homogenate (pH 7.4)

10% homogenate of the embryonic liver, brain and heart was prepared by grinding it in Tris HCl buffer, pH 7.4. The homogenate was centrifuged at 3000 rpm Remi refrigerated centrifuge and the supernatant was used for the measurement of glutathione and antioxidant enzyme activities.

Objectives

Cyclophosphamide is a potent cytotoxic and immunosuppressive drug. However, this develops numerous side effects both in animal and human studies. The role of CP on Lipid peroxidation and antioxidant activities in embryonic development is very limited. The

embryonic tissue injury could be due to the increased lipid peroxide during CP treatment in pregnancy.

Mechanism of Action

The main effect of cyclophosphamide is due to its metabolite phosphoramidate mustard. This metabolite is only formed in cells that have low levels of ALDH. Phosphoramidate mustard forms DNA crosslinks both between and within DNA strands at guanine N-7 positions (known as interstrand and intrastrand crosslinkages, respectively). This is irreversible and leads to cell apoptosis.

Specific Objectives:

The objective of the present study is:

1. To study the effect of CP : Mortality, LD₅₀ and Embryo toxic effect of chick embryo
2. To evaluate the cyclophosphamide induced Biochemical changes in 15th day – old chick embryo
3. To evaluate the Chemoprotective effect of *Andrographis paniculata* on cyclophosphamide induced biochemical changes in chick embryo
4. To study the cyclophosphamide induced oxidative damage in 15th day - old chick embryo
5. To study the Chemoprotective effect of *Andrographis paniculata* against cyclophosphamide induced oxidative damage in 15th day - old chick embryo
6. Histopathological studies with cyclophosphamide and pre-treated antioxidant in chick embryo
7. To study the purification and partial characterization of induced glutathione-s-transferase isozyme from 15 day-old chick embryonic liver treated with cyclophosphamide.

Work plan (including detailed methodology):

- 1). Effect of cyclophosphamide on biochemical system.
- 2). Effect of cyclophosphamide on antioxidant system.
- 3). To study the protective effect of biochemical system.
- 4). To study the protective effect of antioxidant systems.
- 5). Histopathological studies of liver, kidney, brain and testes of cyclophosphamide and pre-treated antioxidant treated chick embryo.

The following equipment is required to run effectively.

- 1). Rota vapour
- 2). Cooling centrifuge
- 3). Deep-freezer – 40°C.

Year wise plan of work and targets to be achieved.

First year:

- 1). Procurement of chemicals and equipment
- 2). Recruitment of research personnel
- 3). Collection of literature
- 4). Plant extracts preparation

Second year:

- 1). Effect of cyclophosphamide on biochemical systems
- 2). Effect of cyclophosphamide on antioxidant systems
- 3). Protective effect of *Andrographis paniculata* on cyclophosphamide induced biochemical toxicity in chick embryo.

Third year:

- 1). Protective effect of *Andrographis paniculata* on cyclophosphamide induced oxidative damage in chick embryo.
- 2). Histopathological studies in cyclophosphamide and pre-treated antioxidant chick embryo.

Antioxidant Activity

DPPH is used as a main substrate to evaluate antioxidant activity. DPPH assay is based on a change in purple colored ethanol solution of DPPH in presence of hydrogen donating antioxidants, by formation of yellow colored non radical form. This absorbance was read yellow 517nm. The scavenging ability of ethane was umpired with ascorbic acid. The DPPH free radical scavenging activity was calculated using the following formula

$$\text{Inhibition (\%)} = (A_0 - A_t / A_0) \times 100.$$

A_0 is the absorbance of control

A_t is the absorbance of control

(a) Preparation of Ethanolic extract of *Andrographis paniculata*

Powdered plant material was soaked in 95% ethanol for 48 h. The extract was distilled and dark green residue was obtained. The remaining ethanol was removed by evaporating the residue under reduced pressure.

(b) Preparation of Methanolic extract of *Andrographis paniculata*

Powdered plant material was soaked in 80% methanol for 48 h. The extract was distilled and dark green residue was obtained. The remaining methanol was removed by evaporating the residue under reduced pressure.

Screening of Phytochemicals

Phytochemical screening of the reconstituted extracts thus obtained was done qualitatively for the presence of various phytochemical constituents like Alkaloids, Terpenoids,

flavonoids, tannins, sterols, and phenols by using standard phytochemical methods of (Swadhini et al, 2011 and Pathaketal, 2011).

Detection of Steroids

One ml of the extract is dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids (Gibbs, R.D., 1974).

Detection of Terpenoids

2 ml of extract is added to 2 ml of acetic anhydride and concentration of H_2SO_4 . Formation of blue 2, 4 green rings indicate the presence of Terpenoids (Ayoola, G.A., *et al.*, 2008).

Test for Alkaloids

0.5ml of leaf extract was dissolved in 5ml of 1% HCL in steam bath. To 1ml of this, 6 drops of dragenoff's reagent was added; Precipitate or turbidity indicated the presence of alkaloids.

Test for Flavanoids

To 1ml of leaf extract, 5ml of diluted ammonia was added followed by concentrated H_2SO_4 . Appearance of yellow colour indicates the presence of Flavanoids.

Test for Tannins

2 ml of extract is added to few drops of 1% lead acetate. A yellowish precipitate indicated the presence of tannins (Treare, G.E. and W.C. Evans, 1985).

Detection of Coumarins

3 ml of 10% NaOH was added to 2 ml of ethanolic extract formation of yellow colour indicates the presence of Coumarins (Rizk, A.M., 1982).

Test for Saponins

5 ml of extract is mixed with 20 ml of distilled water and then agitated in a graduated cylinder for 15 minutes. Formation of foam indicates the presence of Saponins. (Kumar A. *et. al* 2009).

Test for Phenols

The total phenolic content was determined using the Folin-Ciocalteu method. Supernatant of EE, ME, AE extracts was diluted to the concentration of 1 mg/ml, and aliquots of 0.5ml were mixed with 2.5ml of FC reagent (10-fold dilution with distilled water) and 2ml of NaHCO₃ (7.5%). After 15 min of staying at the 45°C the absorbance was measured at 765nm on spectrophotometer versus blank sample. Total phenols were determined as Gallic acid equivalents (mg GA/g extract), and the values are presented as means of triplicate analyses.

Preparation of extracts:

The plants were collected and shade dried for two weeks and ground in to a powder. About 50g powder extracted with of 250ml ethanol using soxhlet apparatus. The extracts were concentrated to dryness to yield crude residue. The residues were used preliminary phytochemical and antimicrobial activity.

Maintenance of Eggs

Freshly laid Bobcock strain zero day old fertilized eggs procured from Govt. Veterinary University, Tirupati, Chittoor District, and Andhra Pradesh. Immediately after bringing the eggs to the laboratory, they were cleaned with distilled water and then alcohol. They were placed in an egg incubator maintained at 37°C with 65 per cent relative humidity. The humidity of the incubator is maintained by keeping the tray full of water inside. The water is replaced every alternate day and the water level is maintained to keep the same percentage of humidity throughout the incubation. The eggs were rotated manually and were examined through the Candler every day for the proper growth and viability. The dead eggs were removed immediately from the incubator. During all experiments, the embryos were maintained at 37°C except for brief intervals (60-120 seconds) required during the different treatment conditions. During this interval, embryos experienced ambient room temperature (29°C – 30°C).

The eggs were injected with different doses of cyclophosphamide and *Andrographis paniculata* extract ascorbic acid into the chick embryo. The drug was injected into the air sac. The air sac was marked with a pencil by placing the egg on the Candler. The marked part of the egg shell was scraped and made thinner with the help of a scalpel. At this site a small hole was made for injecting the drug. Immediately after the injection of the drug, scraped area was covered with the wax to prevent contamination. Every day the eggs were examined for mortality.

Chemicals

Bovine serum albumin (BSA), glutathione reductase (GR), glutathione (GSH), Nicotinamide Adenine Dinucleotide Phosphate reduced (NADPH), Nicotinamide Adenine Dinucleotide (NAD), Tetraethoxypropane (TEP), Thiobarbituric acid (TBA) were purchased from Sigma Chemicals Company St. Louis, U.S.A., Cyclophosphamide was purchased from Dabur India Limited, Baddi, Solan District, Himachal Pradesh-173255, India.

Acids, bases, solvents and salts used for the investigation were of analytical grade and were obtained from Qualigens, Merck, Mumbai, India; Fischer Inorganic and Aromatics, Chennai, India.

Determination of LD₅₀

Several groups of eggs with each group containing 6 numbers were exposed to different doses (10 µg to 500 µg) of CP. The number of dead embryos on 18th day of development was tabulated. Survival time of each embryo was recorded. LD₅₀ was calculated by comparison of dose exposed and observed survival time (David *et al.*, 1966).

Preparation of *Andrographis paniculata* extract

Powdered plant material was soaked in 95% ethanol for 48 h. The extract was distilled and dark green residue was obtained. The remaining ethanol was removed by evaporating the residue under reduced pressure.

Toxicity studies of *Andrographis paniculata*

Andrographis paniculata (AP) extract at a dose of 2.0, 4.0, 6.0, 8.0 and 10.0 mg / egg was given to chick embryo on 12th day. The changes in body weight, liver, heart and brain weight were recorded. Levels of glucose, urea, protein, cholesterol and the activities of serum Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured to ensure the non-toxic nature of the extract.

Fixation of optimum dosage for the plant extract

Ap extract was given different doses 1.0, 2.0, 3.0, 4.0, 5.0 and 6 mg / egg wt / day on 12th day of embryonic development. The dosage which gives maximum protection (as elicited by the activity of ALT and AST) against cyclophosphamide induced hepatic damage was fixed as the optimum dosages.

Collection of embryonic tissues

After collection of chick embryonic tissues of liver, heart and brain, the tissues were immediately stored in chilled cold saline at -20°C.

Preparation of embryonic tissue 10% homogenate (pH 7.4)

10% homogenate of the embryonic liver, brain and heart was prepared by grinding it in Tris HCl buffer, pH 7.4. The homogenate was centrifuged at 3000 rpm Remi refrigerated centrifuge and the supernatant was used for the measurement of glutathione and antioxidant enzyme activities.

Estimation of Protein

Protein content in the sample was estimated by the method of Bradford, 1976. This method is based on the binding of Commassive Brilliant Blue G-250 to protein. The binding of the dye to a protein causes a shift in the absorption maximum of the dye from 465 to 495 nm, and it is increase in absorption at 595 nm, which is monitored.

Reagents

1. Commassive Brilliant Blue G-250
2. 85% phosphoric acid
3. Ethanol 95%

Procedure

Preparation of Protein reagent: Commassive brilliant blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this, 100 ml 85% phosphoric acid was added. The resulting solution was further diluted to a final volume of 1 litre.

Protein assay

Protein solution containing 10-100 µg protein as BSA in a volume up to 0.1 ml was pipetted out into 12 test tubes. The volume in the test tubes is well adjusted with appropriate buffer. 5.0 ml of protein reagent was added to the tubes and the contents mixed by vortexing. The absorbance was read at 595 nm against the reagent blank. The weight of the protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein of the unknown sample.

Estimation of lipid peroxidation

Lipid peroxide concentration was determined by thiobarbutric acid reaction as described by Ohkawa *et al.*, 1979.

The total amount of lipid peroxidation products present in the biological samples were estimated by using the thiobarbutric acid (TBA) method. Since this method measures the Malondialdehyde (MDA) reactive products, the final result obtained is referred as MDA-equivalents (MDA-Equiv).

Reagents

1. 10% of Trichloro acetic acid (TCA)
2. 0.33% TBA (dissolved in water and acetic acid in 1:1)
3. n-Butanol

PROCEDURE

Embryonic tissues

Determination of lipid peroxides in tissues (liver, brain and heart) was carried out and it is on the reduction of thiobarbutric acid with Malondialdehyde (MDA) to give a pink coloured complex and read at absorbance at 532 nm.

Reagents

1. 1.15% Potassium chloride
2. 8.1% Sodium dodecyl sulphate (SDS)
3. 0.8% TBA
4. n-Butanol-pyridine mixture (15:1 v/v)

Procedure

Chick embryo was removed quickly, weighed, chilled in cold saline and the tissues were perfused. The tissue homogenate were prepared in a ratio of 1 gm of wet tissue in 9.0 ml (10% homogenate) of 1.15% KCl by using Potter Elvehjem homogenizer. The homogenate was used for estimation of LPO.

The assay mixture contained 0.1 ml of the tissue homogenate, 0.2 ml of the 8.1% SDS and 1.5 ml of the 0.8% TBA. The mixture was finally made up to 4 ml with distilled water and boiled at 95°C for 60 min. After cooling, 1.0 ml of distilled water, 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added and shaken vigorously and then centrifuged at 4000 rpm for 10 min. Then the absorbance of the organic layer was measured at 532 nm. Amount of LPO were expressed as n moles of MDA formed / mg protein. A standard curve of TEP (1, 1, 2, 2-tetraethoxypropane) was prepared in a similar condition and used in calculation.

Determination of antioxidant enzymes in embryonic tissues

10% homogenate of the embryonic liver, brain and heart was prepared by grinding it in Tris HCl buffer, pH 7.4. The homogenate was centrifuged at 3000 rpm in a refrigerated centrifuge and the supernatant was used for the measurement of glutathione and antioxidant enzyme activities.

Assay of superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase (SOD) was measured according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine transition by the enzyme.

Reagents

1. 50 mM carbonate-bicarbonate buffer, pH 10.2 containing 0.1 mM of ethylene diamine tetra acetic acid (EDTA).
2. 0.6 mM epinephrine (Adrenaline)

Procedure

To the 0.5 ml of supernatant 2.0 ml of carbonate buffer and 0.5 ml of the 0.6 mM of adrenaline was added. Adrenaline was the last component to be added and the adrenochrome formed in the next four min was recorded at 470 nm in a Shimadzu UV spectrophotometer. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of adrenaline auto-oxidation.

Assay of Catalase (EC 1.11.1.6)

Catalase assay was carried out by the method of Aebi (1984). The decomposition of hydrogen peroxide was followed directly by measuring the decrease in absorbance at 240 nm.

Reagents

1. 50 mM Phosphate buffer, pH 7.0
2. 30 mM Hydrogen peroxide: 340 μ l of 30% (v/v) H₂O₂ was dissolved in 100 ml of phosphate buffer (pH 7.0).

Procedure

1.0 ml of the tissue homogenate was made up to 2.0 ml with buffer in a 3.0 ml quartz cuvette and 1.0 ml of H₂O₂ was added to this and change in absorbance was recorded after every 15 sec up to 60 sec. The activity of Catalase was expressed as Units / mg protein / min.

Assay of glutathione-S-transferase (EC 2.5.1.18)

Glutathione-S-transferase activity was determined by measuring the increase in absorbance at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate (Habig *et al.*, 1974).

Reagents

1. 0.5 mM Phosphate buffer, pH 6.5
2. 30 mM CDNB
3. 30 mM Reduced glutathione (GSH)

Procedure

To a 3.0 ml of quartz cuvette, 1.0 ml phosphate buffer, 0.1 ml CDNB and 0.1 ml of sample were added and volume of the reaction mixture was adjusted to 2.9 ml with distilled water. The reaction mixture was pre-incubated at 37°C for 10 minutes. Reaction was initiated by adding 0.1 ml of 30 mM glutathione. The reaction was monitored spectrophotometrically for increase in absorbance at 340 nm. Measuring and subtracting the rate in the absence of enzyme made correction for the spontaneous reaction. The enzyme activity was measured as μ moles of CDNB-GSH conjugate formed / mg protein.

Assay of glutathione peroxidase (EC 1.11.1.9)

Assay of glutathione peroxidase (GPx) was carried out by continuous monitoring of NADPH oxidation in a recycling assay as described by Wendell (1981). Total GPx was measured using cumen hydroperoxide as a substrate.

Reagents

1. 0.25 M Phosphate buffer, pH 7.0 containing 2.5 mM Disodium ethylenediaminetetracetic acid and 2.5 mM sodium azide.
2. Glutathione reductase : 0.3 U/ml
3. 10 mM Glutathione
4. 2.5 mM NADPH in 0.1% Sodium bicarbonate
5. 12.5 mM Cumen hydroperoxide

Procedure

0.1 ml each of phosphate buffer, glutathione reductase, reduced glutathione and 2.5 mM NADPH were transferred into a 1.0 ml quartz cuvette containing 0.5 ml of sample and incubated at 37°C for 10 minutes. The reaction was started by the addition of 100 µl of 12.5 mM cumen hydroperoxide. The linear decrease in NADPH absorption was recorded at 340 nm. The Spontaneous reaction was assayed without enzyme and was subtracted from the samples. Amount of NADPH oxidized was calculated using molar extinction coefficient 6.22×10^3 . Activity of GPx was expressed as µmoles of NADPH oxidized / mg protein / min at 25°C.

Assay of glutathione reductase (EC 1.6.4.2)

Glutathione reductase was assayed by the method of (Staal *et al.*, 1969).

Reagents

1. 0.3 M Sodium phosphate buffer, pH 6.8
2. 250 mM EDTA
3. 12.5 mM Glutathione oxidized
4. 3 mM Nicotinamide adenine dinucleotide phosphate reduced (NADPH)

Procedure

The reaction mixture containing 1 ml of phosphate buffer, 0.5 ml EDTA, 0.5 ml GSSG and 0.2 ml of NADPH was made up to 3 ml with distilled water. After the addition of 0.1 ml of tissue homogenate, the change in optical density at 340 nm was monitored for 2 min. The enzyme activity was expressed as μ moles of NADPH consumed / mg protein under incubation conditions.

Assay of reduced glutathione

Total reduced glutathione was estimated according to the modified method of Moron *et al.*, 1979 using 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB).

Reagents

1. 5% Trichloroacetic acid (TCA)
2. 0.2 M Phosphate buffer, pH 8.0
3. 0.6 mM DTNB

Procedure

To the samples of embryonic tissues, 5.0 ml of 5% TCA was added for deproteination. To an aliquot of deproteinised solution, 5.0 ml of distilled water 2.0 ml of 0.2 M phosphate buffer pH 8.0 and 0.05 ml of 0.6 mM DTNB were added. The colour developed rapidly in 2 min was read at 412 nm.

To 0.5 ml of AF 4.5 ml of distilled water and 1.0 ml of 0.2 M phosphate buffer pH 8.0 were added. 3.0 ml of this solution was placed in each of 1 cm cuvette using one to adjust the absorbance to zero and to the other 0.01 ml of DTNB was added. The absorbance was read at 412 nm after 1 h. Results were expressed as μg of GSH oxidized / mg protein using reduced glutathione as standards.

Estimation of Ascorbic acid

Ascorbic acid was estimated by the method of (Omaye *et al.*, 1979).

Reagents

1. 5% Trichloro acetic acid
2. 2,4-Dinitrophenyl hydrazine – thiourea – copper sulphate reagent (DTC) : 0.4 g thiourea 0.05 g copper sulphate and 3.0 g of 2,4-dinitrophenyl hydrazine were dissolved in 100 ml of 9 N H_2SO_4 .
3. 65% Sulphuric acid (v/v)
4. Standard ascorbic acid: 50 mg of ascorbic acid was dissolved in 100 ml of 4% TCA.

Procedure

0.5 ml of homogenate, 0.5 ml of distilled water and 1.0 ml of 5% of TCA were added, mixed thoroughly and centrifuged for 20 minutes. To 1 ml of the supernatant 0.2 ml of the DTC reagent was mixed and incubated at 37°C for 3 hrs. Then 1.5 ml of 65% H₂SO₄ was added, mixed well and the solutions were allowed to stand at room temperature for another 30 minutes. The colour developed was read at 540 nm. Standards were also treated in a similar fashion.

Ascorbic acid contents were expressed as mg/dL in AF and µg/100 mg dry weight in tissues.

Estimation of glucose

Glucose was estimated in amniotic fluid and other tissues by the O-toluidine method Sasaki and (Matsui 1972).

Reagents

1. 10% Trichloro acetic acid
2. O-Toluidine reagent: 12.5 g of thiourea and 12 g of boric acid were dissolved in 50 ml of water by heating over a mild flame. 75 ml of redistilled O-toluidine and 375 ml of acetic acid were mixed with thiourea boric acid mixture and the total volume was made up to 500 ml with water. The reagent was left in a refrigerator overnight and filtered before storing in the brown bottle.
3. 10% TCA.

The proteins in amniotic fluid and tissue homogenates were precipitated with 3.0 ml of 10% TCA.

Standard glucose solution: 10 mg of dextrose was dissolved in 100 ml of 0.2% of benzoic acid in water.

Procedure

1.0 ml of the supernatant was mixed with 4.0 ml of O-toluidine reagent and was kept in a boiling water bath for about 15 minutes and the green colour developed was read at 640 nm in Shimadzu spectrophotometer. The calibration curve of standard glucose was determined in similar lines.

Blood glucose was expressed as mg/dL.

Assay of Lactate dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase (LDH) activity was determined by the method of King (1965).

Reagents

1. 0.1 M glycine buffer: pH (7.6), 7.5 g of glycine and 5.85 g of sodium chloride was dissolved in one litre of distilled water.
2. Buffered substrate: 2.76 g of lithium lactate was dissolved in 1.25 ml of glycine buffer containing 75 ml of 0.1 N NaOH solutions. This was prepared just before use.
3. 0.4 N NaOH solution
4. 5.0 mg of NAD⁺ was dissolved in 10 ml of distilled water (Prepared freshly every time).
5. 2, 4-dinitrophenylhydrazine (DNPH) : 200 mg of DNPH was dissolved in one liter of 1.0 N HCl.
6. Standard pyruvate solution: 12.5 g of sodium pyruvate was dissolved in 100 ml of buffered substrate solution.

Procedure

To 1.0 ml of the buffered substrate, 0.1 ml of the sample was added and the tubes were incubated at 37°C for 15 min. After adding 0.2 ml of NAD⁺ solution, the incubation was continued for another 15 minutes. The reaction was arrested by adding 1.0 ml of DNPH reagent. The tubes were then incubated for a further period of 15 min after which 7.0 ml of 0.4 N NaOH was added and colour developed was measured at 420 nm in a Shimadzu UV spectrophotometer. The calibration curve was constructed simultaneously with the test sample. LDH activity was expressed as μ moles of pyruvate formed / mg protein.

Assay of Succinate dehydrogenase (EC 1.3.99.1)

The Succinate dehydrogenase (SDH) activity was assayed according to the method of Slater and Bonner (1952).

Reagents

1. 0.3 M Phosphate buffer, pH 7.6
2. 0.3 M EDTA, pH 7.6
3. 0.03 M Potassium cyanide
4. 0.4 M Sodium Succinate
5. 3% BSA
6. 3% Potassium ferricyanide

Procedure

To, 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 0.1 ml of BSA, 0.3 ml of sodium Succinate and 0.2 ml of potassium ferricyanide were added and the total volume was made up to 2.8 ml with distilled water. The reaction was initiated by the addition of 200 μ l of mitochondrial suspension. The changes in optical density were recorded at 15 sec time interval for 15 min. The enzyme activity was expressed as μ moles of Succinate oxidized/min/mg protein.

Assay of Malate dehydrogenase (EC 1.1.1.37)

The Malate dehydrogenase (MDH) activity was measured using the method of Mehler *et al.*, (1948).

Reagents

1. 0.25 M Tris HCl buffer, pH 7.4
2. 15 μ M NADPH
3. 760 μ M Oxaloacetic acid

Procedure

To, 3.0 ml buffer, 0.1 ml of NADPH and 0.1 ml of Oxaloacetic acid were added and the total volumes were made up to 2.9 ml with distilled water. The reaction was started by the addition of 0.1 ml of mitochondrial suspension. The change in optical density was measured at 350 nm in an interval of 15 sec for 5 min. The enzyme activity was expressed as n moles of NADPH oxidized/min/mg protein.

Estimation of Urea

Urea was estimated in amniotic fluid by the method of Natelson (1956) by measuring the coloured complex formed with diacetyl monoxime in acidic medium.

Reagents

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. Diacetyl monoxime (DAM) in 2% acetic acid
4. Sulphuric acid and phosphoric acid reagent: 140 ml of water was mixed with 150 ml of 85% phosphoric acid and then 50 ml of concentrated sulphuric acid was added slowly.

5. Standard urea: 250 mg of urea was dissolved in 100 ml of water. This solution was diluted 1 to 100 to give a solution containing 25 µg/ml, which was used as a working standard.

Procedure

0.1 ml of amniotic fluid was mixed with 3.3 ml of water, 0.3 ml of sodium tungstate and sulphuric acid respectively and mixed well followed by centrifugation. To this 1 ml of supernatant was mixed with 1 ml of water, 0.4 ml of DAM and 1.6 ml of sulphuric acid-phosphoric acid reagent. The tubes were placed in a boiling water bath for 30 minutes and cooled. The colour developed was read at 480 nm against water blank. A series of standards were treated in a similar manner. Blood urea was expressed as mg/dL.

Estimation of uric acid

Uric acid was estimated by the method of Caraway, 1963.

Reagents

1. Phosphotungstic acid

To prepare a stock, 50 g of sodium tungstate was dissolved in 400 ml of water and 40 ml of 85% phosphoric acid was refluxed gently for two hours, cooled and transferred to a 500 ml flask and made up to the mark with distilled water. The reagent was stored in a brown bottle diluted 1 to 10 for use.

2. 15% Sodium carbonate

3. Standard uric acid

100 mg of uric acid and 60 mg of lithium carbonate was dissolved in 60 ml of distilled water. This was then heated to about 60°C to dissolve the uric acid completely. After cooling the solution was made up to 100 ml with distilled water i.e., 1 mg/ml.

4. **Working standard**

1 ml of stock standard was diluted to 10 ml with double distilled water. This solution contains 0.1 mg / ml of uric acid.

Procedure

To 0.5 ml of the sample 2.5 ml of distilled water and added followed by 0.6 ml of Phosphotungstic acid and 0.6 ml of sodium carbonate. Measured the colour at 640 nm against reagent blank. Uric acid was expressed as mg/dL.

Estimation of Creatinine

Serum Creatinine was estimated by the method of Broad and Sirota, 1948.

Reagents

1. 10% Sodium tungstate
2. 2/3 N Sulphuric acid
3. Saturated Picric acid
4. 0.75 N Sodium hydroxide
5. Standard Creatinine: 20 mg of Creatinine in 100 ml of distilled water (200 µg/ml).

Procedure

1.0 ml of Amniotic fluid was mixed with 7 ml of water 1.0 ml of sodium tungstate and 1.0 ml of sulphuric acid was added and centrifuged. From this 4.0 ml of supernatant was mixed with 1.0 ml of 0.75 N NaOH and 1.0 ml of 0.4 M picric acid. The tubes were kept in a boiling water bath of 15 minutes. The colour developed was read at 470 nm using a photochemical colorimeter. Serum Creatinine was expressed as mg/dL.

Estimation of Inorganic phosphorous

Phosphorous was estimated by the method of Fiske and (Subbarow, 1952).

Reagents

1. 2.5% Ammonium molybdate solution
2. 1, 2, 4-aminonaphtho sulphonic acid (ANSA) : 500 mg of ANSA was dissolved in 195 ml of 15% sodium bisulphate and 5.0 ml of 20% sodium sulphite was added to it. The solution was filtered and stored in brown bottle.
3. Procedure: The supernatant along with the aliquot of standard in the range of 8-40 μ g was made up to 4.3 ml with distilled water. 0.5 ml of ammonium molybdate and 0.2 ml ANSA and mixed well. The colour developed was read at 620 nm after 20 min against the reagent blank using a photochem colorimeter.
The values are expressed in mg / dL.

Estimation of sodium and potassium

Sodium and potassium were determined on a diluted aliquot sample solution by using flame photometry (Raghuramulu *et al.*, 2003).

Standard: 2.90 g of Na_2SO_4 and 1.85 g of K_2SO_4 was weighed accurately and dissolved in 200 ml standard flask using double distilled water.

Working standard: The above stock solutions were diluted to give the concentration of 10-50 ppm.

Procedure

A known concentration of sodium and potassium solutions was used as a standard. Double distilled water was used to set a zero. The fluid analysis is sprayed as a fine mist into a non-luminous flame, which becomes coloured according to the characteristic emission of the (Na⁺ and K⁺) present in the fluid. The flame is simultaneously monitored for the sodium and potassium channels. Sodium and potassium concentrations were expressed as mEq/dL.

Estimation of Marker enzymes

Acid Phosphatase (EC 3.1.3.2)

Acid Phosphatase was assayed by the method of King (1965a).

Reagents

1. 0.1 M Citrate buffer, pH 4.3
2. 0.1 M Disodium phenyl phosphate (DPP)
3. 15% Anhydrous Sodium carbonate
4. Folin-Ciocalteus (commercial sample) reagent
5. Standard: 100 mg of phenol was dissolved in 100 ml of double distilled water.

Procedure

The incubation mixture containing 1.5 ml of buffer, 1.0 ml of substrate was pre incubated at 37°C for 10 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of FC reagent. Control without enzyme was incubated and the homogenate was added after the addition of FC reagent. The 1.0 ml of Na₂CO₃ was added at the end. After 10 minutes

blue colour developed was read at 640 nm. The enzyme activity was expressed as μ moles of Phenol liberated/min/mg protein under incubation conditions.

Alkaline Phosphatase (EC 3.1.3.1)

Alkaline Phosphatase was assayed by the method of King, 1965a.

1. **0.1 M Carbonate** – bicarbonate buffer, pH 10.0: 6.36 g of Sodium carbonate and 3.36 g of sodium bicarbonate were dissolved in one liter of water.
2. **0.1 M Substrate:** 246 g of DPP was dissolved in 100 ml of distilled water.
3. **0.1 M Magnesium chloride:** 406 g of $MgCl_2$ was dissolved in 20 ml of distilled water.
4. **15% Sodium carbonate:** 15 g of Na_2CO_3 was dissolved in 100 ml of distilled water.
5. Folin-Ciocalteu reagent
6. 10% TCA
7. **Standard:** 100 mg of phenol was dissolved in 100 ml of double distilled water.

Procedure

The incubation mixture containing 1.5 ml of buffer, 1.0 ml of substrate and 0.1 ml of magnesium chloride were pre-incubated at 37°C for 10 min.

Then 0.1 ml of enzymes was added and incubated at 37°C for 15 minutes. The reaction was arrested by 1.0 ml of 10% TCA. Control without enzyme was incubated and the homogenate was added after the addition of TCA solution. Then 1.0 ml of Na_2CO_3 and 0.5 ml of FC reagent were added. After 10 minutes blue colour developed was read at 640 nm.

The enzyme activity was expressed as μ moles of phenol liberated /min/mg protein under incubation conditions.

Alanine aminotransferase (EC 2.6.1.2)

Alanine aminotransferase (ALT) was assayed by the method of King, 1965b.

Reagents

1. **0.1 M Phosphate buffer:** pH 7.5
2. **Substrate:** 1.78 g of DL – Alanine and 30 g of 2-oxoglutarate were dissolved in 20 ml of buffer. 0.5 ml of NaOH was added and made up to 100 ml with distilled water.
3. **2, 4-Dinitrophenyl hydrazine (DNPH):** 0.02% of DNPH in 1 N HCl.
4. 0.4 N Sodium hydroxide
5. **Standard:** 11 mg of sodium pyruvate was dissolved in 100 ml phosphate buffer. This contained 1moles of pyruvate/ml.

Procedure

1.0 ml of the substrate was incubated at 37°C for 10 minutes. Then 0.2 ml of enzyme was added followed by incubation of tubes at 37°C for half an hour. To the control tubes enzyme was added after the reaction was arrested with 1.0 ml of DNPH reagent. The tubes were kept at room temperature for 20 minutes. Then 5.0 ml of 0.4 N NaOH was added and colour developed was read at 540 nm using a photochemical colorimeter.

The enzyme activity was expressed as moles pyruvate liberated/min/mg protein.

Aspartate aminotransferase (EC 2.6.1.1)

Aspartate aminotransferase (AST) was assayed by the method of King, 1965b.

Reagents

1. 0.1 M phosphate buffer, pH 7.5
2. **Substrate:** 1.33 g of aspartic acid and 1.5 mg of 2-oxoglutarate were dissolved in 20 ml of buffer. 0.5 ml of sodium hydroxide was added and made up to 100 ml with distilled water.
3. **2, 4-dinitrophenyl hydrazine (DNPH):** 0.02% of DNPH in 1 N HCl.
4. 0.4 N NaOH
5. **Standard:** 11 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer.

Procedure

1.0 ml of the substrate was incubated at 37°C for 10 minutes. Then 0.2 ml of enzyme was added followed by incubation of tubes at 37°C for half an hour. To the control tubes enzyme was added after the reaction was arrested with 1.0 ml of DNPH reagents. The tubes were kept at room temperature for 20 minutes. Then 5.0 ml of 0.4 N NaOH was added and colour developed was read at 540 nm using photochemical colorimeter. The enzyme activity was expressed as μ moles pyruvate liberated/min/mg protein.

Histological Studies

The Tissues (liver, brain and heart) kept in neutral buffered formalin were used for the study of Histopathological changes. Tissues were sectioned and stained with Haematoxylin-Eosin stain (H & E) and observed under light microscope for pathological changes.

Statistical Methods

The results were expressed as \pm standard deviation, differences between groups were considered significant when the P value determined by unpaired student's 't' value was less than 0.05.

RESULTS ACHIEVED

OBJECTIVE - I

1.1. Mortality, LD₅₀ and Embryo toxic effect of chick embryo

Chick embryos were exposed to different concentration of cyclophosphamide (ranging from 10µg–500µg) at different stages of embryonic development. Table.2.1 represents a dose-dependent effect of CP at various stages of development.

The embryo toxic effect at every stage showed a dose-dependent increase in the death of the embryos. The percentage of mortality with 10, 20, 40,80 and 100 µg at 3rd, 4th, 5th, 7th day exposure, resulted in 27%, 39%, 51%, 67%, and 100% at 3rd day 30%, 35%, 49%, 79% and 100%, at 4th day 32%, 46%, 55%, 77%, and 100%, at 5th day 22%, 36%, 41%, 58% and 100% at 7th day respectively.

The percentage of mortality with 50, 100, 150, 200, and 250 µg at 9th day exposure resulted in 16%, 28%, 36%, 52% and 100% respectively. The percentage of mortality with 100, 200, 300, 400 and 500 µg at 12th day exposure resulted in 14%, 24%, 42%, 56% and 100% at 15th day with 100, 200, 300, 400 and 500µg exposure it was 26%, 34%, 44%, 62% and 100% respectively.

The percentage of mortality was increased with proportionate of the drug concentration in all the stages of embryonic development. The lethal dose (LD₅₀) was calculated directly from the graph drawn between percentage of mortality and concentration of CP in µg.

The embryos treated on 3rd day, LD₅₀ was assessed as a dose close to 30 µg while dose of 32µg corresponded to LD₅₀ value of 4th day, while the values 5th , 7th and 12th and 15th day were 44, 79, 172, 320, 376 µg respectively. (Fig. 2.4-2.10).

Table. 1.1: Effect of CP on Mortality of chick embryo

Day	CP Concentration in μg	Mortality	LD ₅₀ (μg)
3 day	10	32	30
	20	44	
	40	56	
	80	84	
	100	100	
4 day	10	30	32
	20	40	
	40	52	
	80	76	
	100	100	
5 day	10	28	44
	20	38	
	40	48	
	80	56	
	100	100	
7 day	10	18	79
	20	32	
	40	46	
	80	50	
	100	100	
9 day	50	16	172
	100	28	
	150	36	
	200	52	
	250	100	
12 day	100	14	320
	200	24	
	300	42	
	400	56	
	500	100	
15 day	100	26	376
	200	34	
	300	44	
	400	62	
	500	100	

Table 1.2 shows significant decrease in the weight of the embryo with increase in the concentration of CP at every stage of embryonic development. Table also shows significant decrease in the volume of amniotic fluid with increase in the concentration of CP at every stage of embryonic development compared to control.

Table.1.2. Volume of Amniotic fluid and weight of the 15th day chick embryo after 48 hrs CP treatment

Sl.No		Day of Exposure	CP concentration in µg				
			Control	100	200	300	CP 300µg+6.0mg AP
1	Volume of AF	12 th day	2.03±0.21	1.82±0.04	1.84±0.02	1.84±0.04	1.99±0.06
2		15 th day	1.96±0.04	1.80±0.02*	1.76±0.02*	1.74±0.02*	1.92±0.03*
3	Weight of Embryo	12 th day	10.6±0.3	10.4±0.09	9.66±0.16*	9.25±0.06*	10.2±0.28*
4		15 th day	10.27±0.42	10	9.48±0.1*	9.14±0.14*	9.98±0.06*

* p < 0.001 **p <0.05

The values are average of sets of experiments

1.2. Morphological changes

Anomalies noted were multiple and never single in any foetus. The malformations observed were, hemorrhagic brain and hydrocephalous, thickening neck, displaced limbs and defects in the development of the beak. Growth retardation was the major defect observed with teratogenic dose Fig.2.1 and 2.2.

Embryos treated on 3rd day showed hemorrhagic brain, on day 4, 5, 7 and 9th develop a short lower beak. There is thus an abrupt change in the severity of the facial abnormalities in embryos treated on day 4, 5, 7 and 9th when compared to those treated latter. The defeat in feathers occurs in treated at any stage through day 15. The embryos injected on 15th day showed weight loss feather inhibition and hemorrhagic Fig.2.3.

1.3. Discussion

Cyclophosphamide inhibits the development of the chick embryo in vivo and causes a variety of developmental defeats, which are related to the stage of development at the time of treatment. The dose of CP required causes growth inhibition or death of the chick embryo progressively from 10 µg/ embryo at 3 days to 5000 µg at 15 days, Which parallels the increase in mass of the embryo 3rd day to 15 the day. Thus there is a direct correlation between the increasing weight of the embryo and the LD50 dose of CP.

Growth retardation and internal hemorrhage represent the most frequent malformations. Altered proportions of yolk sac or extra embryonic vascular network may reduce nutrient transfer and hence responsible for growth retardation of the embryo. In comparison to controls, growth retardation was less apparent at the later stages of embryonic development suggesting partial compensation of cell loss during the later stages of development.

Fig.1.1. Morphological changes in 9th exposed chick embryo



- A) Control**
- B) 100 μ g CP exposed**
- C) 200 μ g CP exposed**
- D) 300 μ g CP exposed**

Fig.1.2. Morphological changes in 15th day exposed chick embryo



- A) Control**
- B) 100 μ g CP exposed**
- C) 200 μ g CP exposed**

D) 300µg CP exposed

E) 400µg CP exposed

Fig. 1.3. Morphological changes in 18th day exposed chick embryo



(1) Control. (2) 300µg of CP treated.(hemorrhagic)

Fig.1.4. Determination of LD₅₀ on 3rd day chick embryo with CP treatment

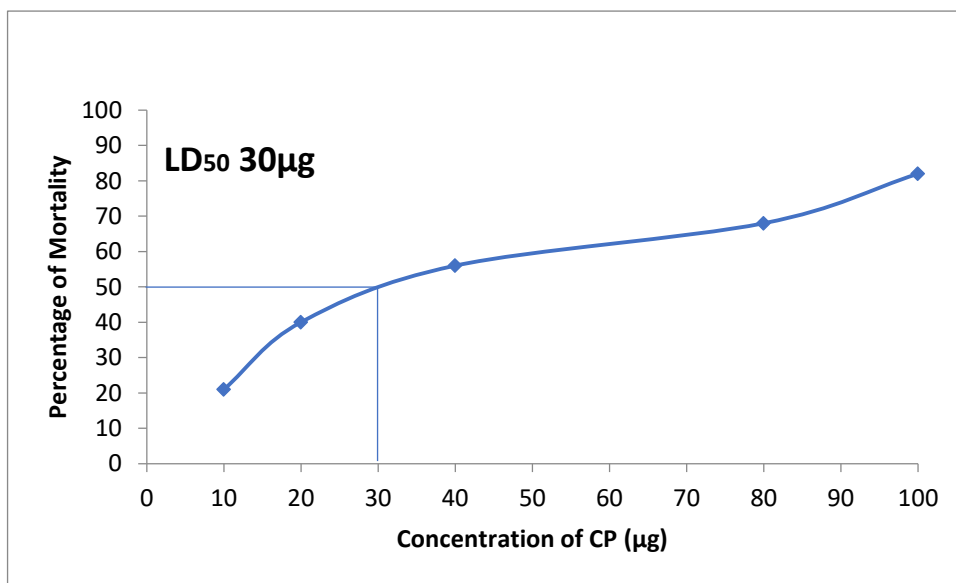


Fig.1.5. Determination of LD₅₀ on 4th day of chick embryo with CP treatment

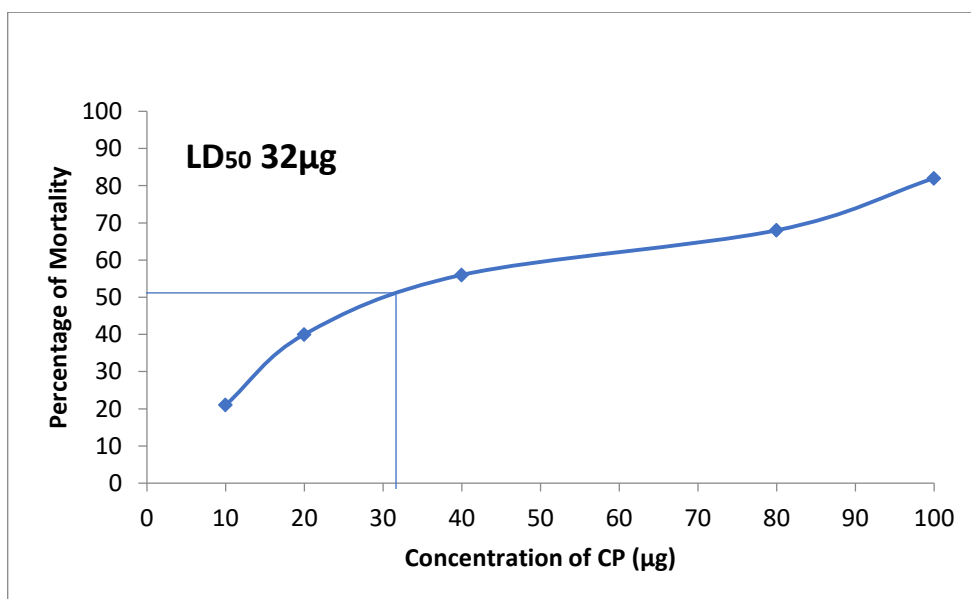


Fig.1.6. Determination of LD₅₀ on 5th day of chick embryo with CP treatment

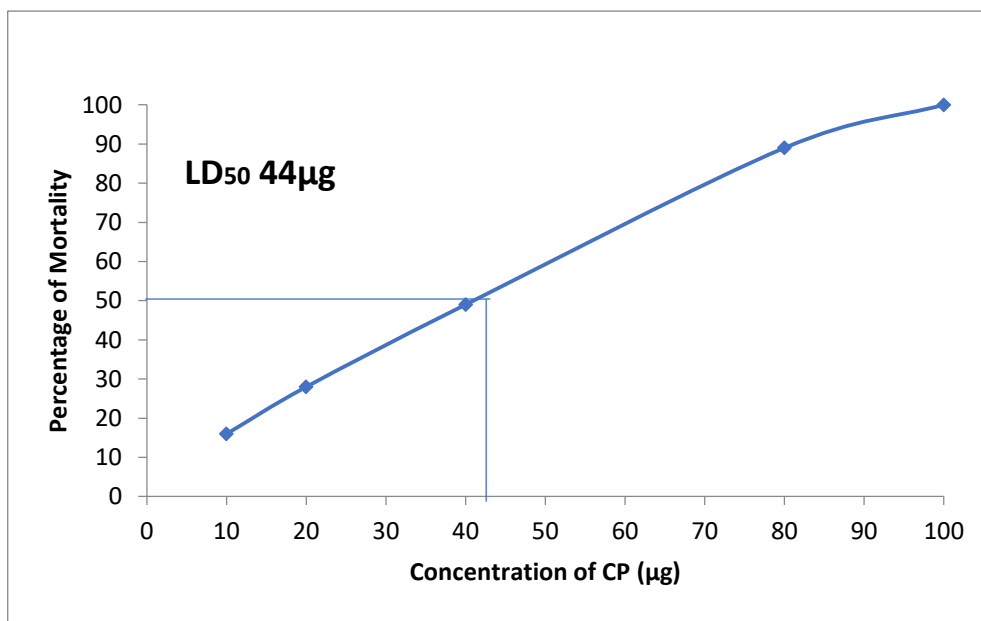


Fig.1.7. Determination of LD₅₀ on 7th day of chick embryo with CP treatment

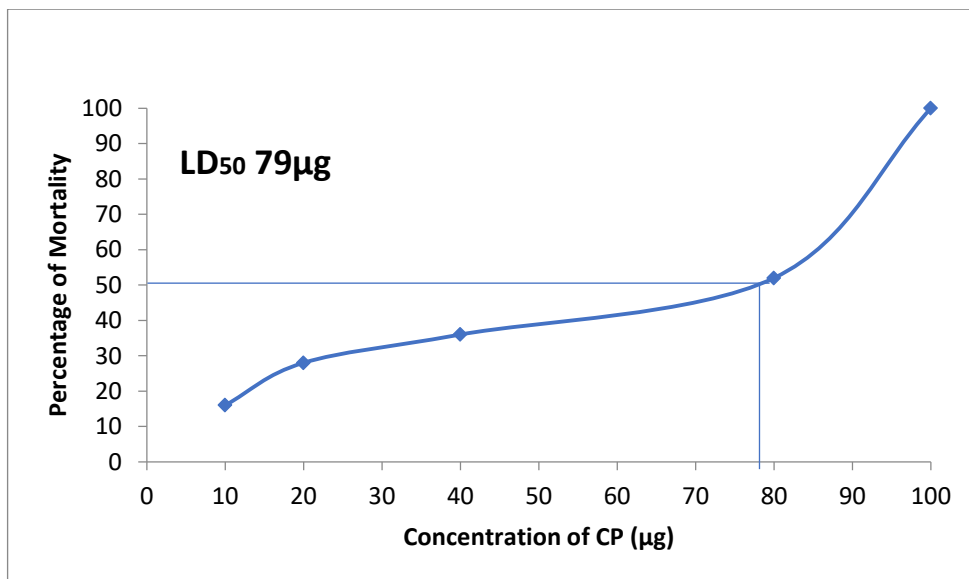


Fig.1.8. Determination of LD₅₀ on 9th day of chick embryo with CP treatment

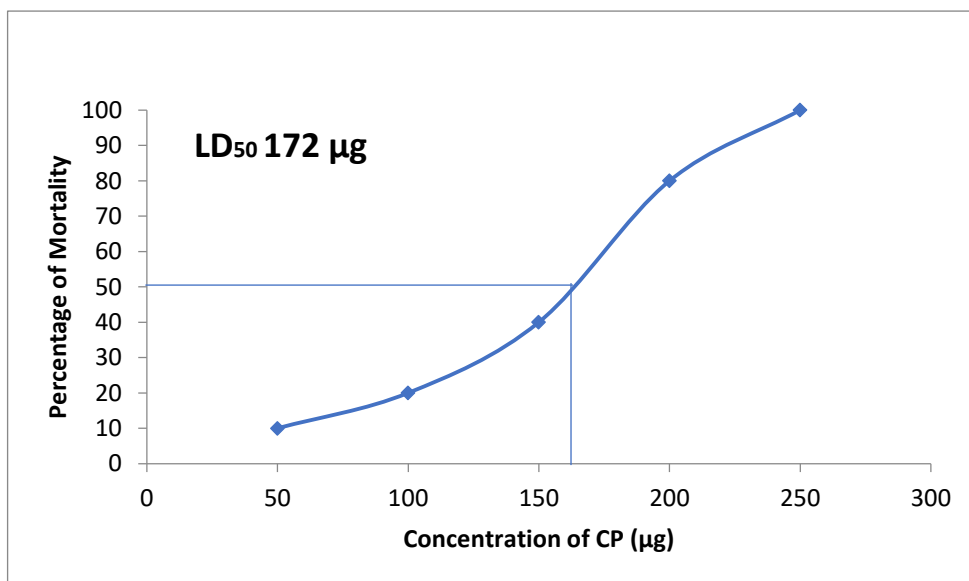


Fig.1.9. Determination of LD₅₀ on 12th day of Chick embryo with CP treatment

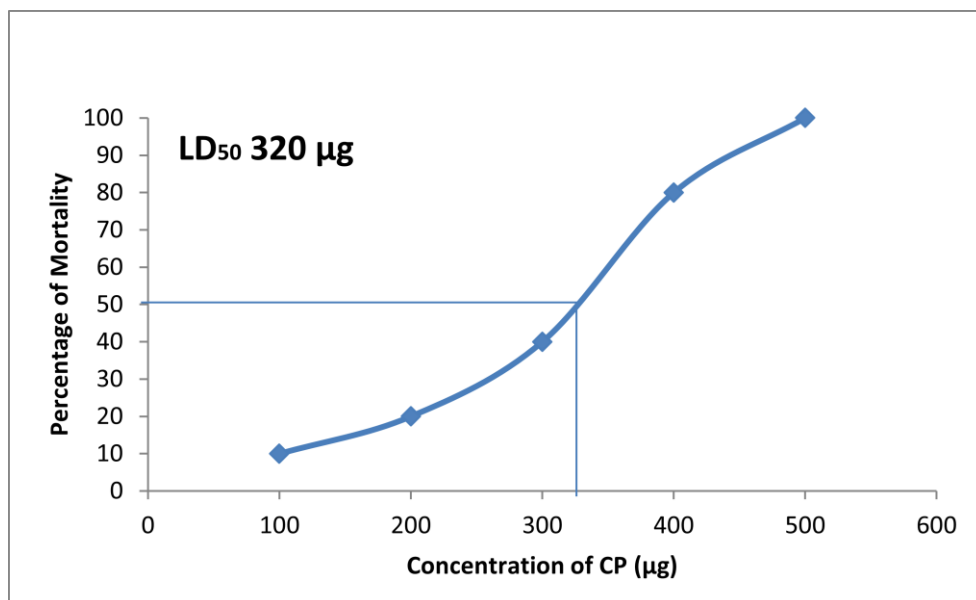
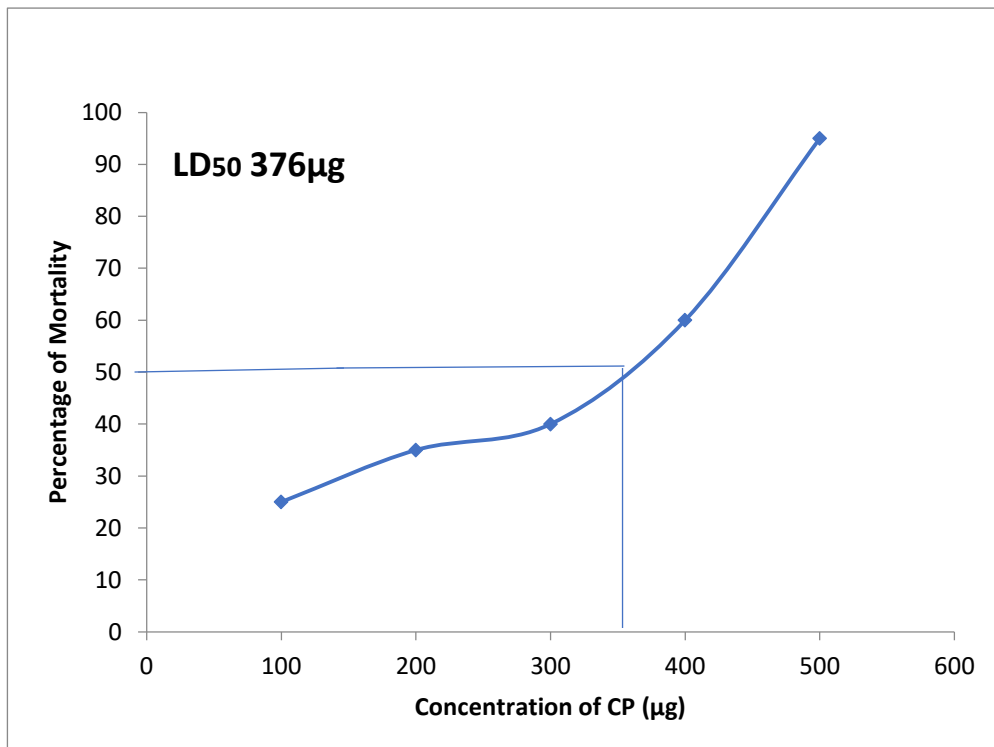


Fig.1.10. Determination of LD₅₀ on 15th day of chick embryo with CP treatment



2.1. CYCLOPHOSPHAMIDE INDUCED BIOCHEMICAL CHANGES IN 15 DAY - OLD CHICK EMBRYO

Table 2.1 represents the biochemical parameters in chick amniotic fluid in controls and CP treated embryos. CP treatment was given on the 15th day of incubation and biochemical analysis was done after 24hrs of treatment.

Significant increase ($p < 0.001$) in the levels of glucose, uric acid, creatinine, inorganic phosphorous levels were observed with 100 μg , 200 μg and 300 μg of CP treatment, where as CP, 200 μg caused no significant increase in urea which showed an increased trend ($p < 0.001$) with 300 μg compared with control. Protein levels were significantly increased with 100 μg ($p < 0.05$) and with 200 and 300 μg ($p < 0.001$) of CP treatment.

Ascorbic acid, calcium and cholesterol levels were significantly decreased ($p < 0.001$) in a dose dependent manner with CP treatment in AF.

The enzymatic activity of alkaline phosphatase (ALP), serum Alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were increased considerably ($p < 0.001$) with CP treatment in AF compared with to controls.

The iconic balance was significantly decreased with CP treatment compared with to Controls.

Table 2.2 shows the effect of CP biochemical parameters in chick embryonic liver.

Liver protein, inorganic phosphorous and ascorbic acid levels were found to significant decrease ($p < 0.001$) with CP treatment.

Increased activity of ALP, ALT, AST, LDH and MDH was observed in chick embryonic liver with CP treatment when compared to control.

Table 2.3 represents the effect of CP on chick embryonic heart.

CP declined protein, inorganic phosphorous and cholesterol contents significantly ($p < 0.001$) in heart.

The ascorbic acid levels were significantly reduced ($p < 0.001$) with CP treatment when compared to controls.

LDH and MDH levels were significantly increased ($p < 0.001$) in heart with CP treatment when compared to controls.)

Table. 2.1. Biochemical components of Amniotic fluid in 15th day old chick embryo

Sl.No.	Biochemical Parameter	Control	Cyclophosphamide		
			100 µg	200 µg	300 µg
1.	Glucose (mg/dL)	52.5 ± 1.4	54.9 ± 1.1*	57.8 ± 0.5*	60.7 ± 0.1*
2.	Protein (mg/dL)	16.4 ± 0.1	17.4 ± 0.4**	20.2 ± 0.1*	22.4 ± 0.4*
3.	Uric acid (mg/dL)	8.6 ± 0.07	13.4 ± 0.3*	14 ± 0.4*	14.3 ± 0.3*
4.	Urea (mg/dL)	8.2 ± 0.2	14.1 ± 0.3*	12.1 ± 0.2*	16 ± 0.3*
5.	Creatinine (mg/dL)	0.3 ± 0.1	0.4 ± 0.04**	0.56 ± 0.04*	0.9 ± 0.02*
6.	Sodium (mEq/L)	135.2 ± 2.1	109 ± 1.7*	112.3 ± 0.5**	121 ± 1.4*
7.	Potassium (mEq/L)	2.5 ± 0.4	4.1 ± 0.1*	7.8 ± 0.5**	5.5 ± 0.4*
8.	Inorganic Phosphorous (mg/dL)	0.35 ± 0.02	0.48 ± 0.05*	0.77 ± 0.04*	0.8 ± 0.05*
9.	Ascorbic Acid (mg/dL)	3.6 ± 0.1	2.7 ± 0.1*	1.8 ± 0.05*	0.6 ± 0.1*
10.	Cholesterol (mg/dL)	39.6 ± 1	28.1 ± 1*	23.5 ± 1.6*	21.2 ± 0.8*
11.	Calcium (mg/dL)	10.6 ± 0.1	8.5 ± 0.2*	5.5 ± 0.02*	4.2 ± 0.2*
12.	ALP #	4 ± 0.1	5.5 ± 0.2*	6.7 ± 0.2**	15.4 ± 0.9*
13.	Alanine amino Transferase## (µmole/min/mg protein)	12.2 ± 0.5	14.7 ± 1.3**	17 ± 1.7*	26.5 ± 1.5*
14.	Aspartate amino transferase## (µmole/min/mg protein)	1.9 ± 0.08	3.1 ± 0.07*	6.1 ± 0.1*	7.2 ± 0.1*
15.	LDH##	13.4 ± 0.2	16.8 ± 0.2*	18.5 ± 0.08	21.2 ± 0.2*
16.	MDH###	16.5 ± 0.1	18.6 ± 0.2*	20.9 ± 0.2**	22.5 ± 0.2*

* $p < 0.001$ ** $p < 0.05$

Units: μmol phenol liberated/min/mg protein

Units: μmol pyruvate formed/min/mg protein

Units: nmol NADPH oxidized/min/mg protein

Table. 1.2. Biochemical components of Liver tissue in 15th day old chick embryo

Sl.No.	Biochemical Parameter	Control	Cyclophosphamide		
			100 μg	200 μg	300 μg
1.	Protein (mg/100mg dry weight)	29.2 \pm 0.07	25.5 \pm 0.09*	22.4 \pm 0.08*	19.5 \pm 0.1*
2.	Inorganic Phosphorous (mg/100mg dry weight)	1.7 \pm 0.01	0.8 \pm 0.01*	0.6 \pm 0.03*	0.2 \pm 0.04*
3.	Ascorbic Acid (mg/100mg dry weight)	3.5 \pm 1.4	3.1 \pm 1.4*	2.8 \pm 1.4*	2.1 \pm 1.4*
4.	ALP #	8.4 \pm 0.1	8.7 \pm 0.1**	9.8 \pm 0.1*	13.2 \pm 0.1*
5.	Alanine Amino Transferase ($\mu\text{mole}/\text{min}/\text{mg}$ protein)	9.8 \pm 0.3	11.5 \pm 0.1*	15.7 \pm 0.2*	18.4 \pm 0.1*
6.	Aspartate amino transferase ($\mu\text{mole}/\text{min}/\text{mg}$ protein)	6.8 \pm 0.08	7.2 \pm 0.1*	8.1 \pm 0.1*	9.4 \pm 0.1*
7.	LDH##	65.6 \pm 0.1	71.5 \pm 0.2*	75.4 \pm 0.2*	78.4 \pm 0.2*
8.	MDH###	82.1 \pm 0.1	86.2 \pm 0.2*	88.5 \pm 0.3*	90.5 \pm 1.3*

*p<0.001 **p<0.05

Units: μmol phenol liberated/min/mg protein

Units: μmol pyruvate formed/min/mg protein

Units: nmol NADPH oxidized/min/mg protein

Table. 1.3. Biochemical components of Heart tissue in 15th day old chick embryo

Sl.No.	Biochemical Parameter	Control	Cyclophosphamide		
			100 µg	200 µg	300 µg
1.	Protein (mg/100mg dry weight)	18.6 ± 0.1	17.8 ± 0.2*	16.5 ± 0.1*	15.2 ± 0.1*
2.	Inorganic Phosphorous (mg/100mg dry weight)	0.9±0.07	0.7±0.08*	0.55±0.1*	0.33 ± 0.1*
3.	Cholesterol (mg/100mg dry weight)	26.5±2.1	21.6±1.2*	16.8±0.8**	14.2 ± 0.4**
4	ALP #	6.2±0.1	7.1±0.1*	7.8±0.07**	8.6 ± 0.1*
5.	Ascorbic acid (mg/100mg dry weight)	1.12±2.2	1.07 ± 0.1*	1.0±0.3**	0.85 ± 0.1*
6.	Alanine amino transferase (µmole/min/mg protein)	4.6±0.1	5.2 ± 0.1	5.8 ±0.1**	6.8±0.3*
7.	Aspartate amino transferase (µmole/min/mg protein)	5.2±0.2	6.4 ± 0.2*	7.1±0.2	7.9±0.2*
8.	LDH##	21.2±0.1	22.4±0.2*	25.4 ±0.4*	29.2 ± 0.2*
9.	MDH###	38.2±0.1	40.4 ± 0.1	44.1±0.02**	46.1±0.1*

*p<0.001 **p<0.05

Units: µmol phenol liberated/min/mg protein

Units: µmol pyruvate formed/min/mg protein

Units: nmol NADPH oxidized/min/mg protein

3.1. CHEMOPROTECTIVE EFFECT OF *ANDROGRAPHIS PANICULATA* ON CYCLOPHOSPHAMIDE INDUCED BIOCHEMICAL CHANGES IN CHICK EMBRYO

Group I Controls treated with normal saline

Group II Treated with cyclophosphamide 300µg/15 day's old chick embryo

Group III Treated with ethanolic extract of *Andrographis paniculata* 6.0 mg/12 day prior to cyclophosphamide (300µg) treatment.

Administration of CP to chick embryo revealed a significant increase in biochemical parameters in AF, Liver and Heart tissues (Table. 3.1, 3.2 and 3.3). Treatment with *Andrographis paniculata* afforded a significant protection against CP induction.

Table.3.1 represents the biochemical parameters in chick amniotic fluid in controls and CP (CP + AP) treated embryos. AP was given on 12th day and CP treatment was given on the 15th day of incubation and biochemical analysis was done after 24hrs of treatment.

Significant increase ($p < 0.001$) in the levels of glucose, uric acid, Creatinine, inorganic phosphorous levels were observed with 100, 200, 300µg of CP treatment, whereas CP, 200µg caused no significant increase in urea which showed an increased trend ($p < 0.001$) with 300µg compared with control. Protein levels were significantly increased with 100 µg ($p < 0.05$) and with 200 µg and 300 µg ($p < 0.001$) of CP treatment.

Ascorbic acid, calcium and cholesterol levels were significantly decreased ($p < 0.00$) in a dose dependant manner with CP treatment in AF. Whereas with CP + AP treatment a significant decrease ($p < 0.001$) in glucose, protein, urea, uric acid, Creatinine and inorganic phosphorous were observed. Glucose levels were significantly decreased ($p < 0.05$) with 6.0mg AP treatment.

Ascorbic acid levels were significantly decreased ($p < 0.001$) with CP treatment. Whereas CP+AP caused significant ($p < 0.001$) increase in ascorbic acid levels. The calcium levels were significantly decreased ($p < 0.001$) with CP treatment. The cholesterol, sodium and potassium significantly decreased with CP treatment, whereas increased AP treated embryos.

The enzymatic activity of alkaline Phosphatase (ALP), serum Alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were increased considerably ($p < 0.001$) with CP treatment in AF compared with to controls, whereas decreased with CP + AP treatment. The ionic balance was significantly decreased with CP treatment compared with to controls.

Table 3.2 shows the effect of CP biochemical parameters in chick embryonic liver. Liver protein, inorganic phosphorous and ascorbic acid levels were found to significant decrease ($p < 0.001$) with CP treatment. Increased activity of ALP, ALT, AST, LDH and MDH was observed in chick embryonic liver with CP treatment when compared to control. The altered biochemical features were significantly brought towards normalisation treatment with AP extract in Liver.

Table 3.3 represents the effects of CP on Chick embryonic heart. CP declined protein, inorganic phosphorous and cholesterol contents significantly ($p < 0.001$) in heart. The ascorbic acid levels were significantly reduced ($p < 0.001$) with CP treatment when compared to controls. LDH and MDH levels were significantly increased ($p < 0.001$) in heart with CP treatment when compared to controls. The altered protein, inorganic phosphorous, cholesterol and ascorbic acid levels observed AP treated embryos. The enzymatic levels LDH and MDH levels were also altered AP treated embryos compared to CP treated ones.

Table. 3.1. Biochemical components of Amniotic fluid in 15th day old chick embryo

Sl.No.	Biochemical Parameter	Control	CP (300µg/egg)	CP +AP
				300µg+ 6.0mg
1.	Glucose (mg/dL)	52.5 ± 1.4	62.1 ± 0.1*	53.1 ± 1.1**
2.	Protein (mg/dL)	16.4 ± 0.1	23.4 ± 0.3*	16.4 ± 0.1*
3.	Uric acid (mg/dL)	8.6 ± 0.07	14.6 ± 0.3*	8.8 ± 0.1*
4.	Urea (mg/dL)	8.2 ± 0.2	15.1 ± 0.3*	8.1 ± 0.2*
5.	Creatinine (mg/dL)	0.32 ± 0.1	0.92± 0.02*	0.36 ± 0.02*
6.	Sodium (mEq/L)	135.2 ±2.1	120 ± 1.3*	132.3 ±0.1**
7.	Potassium (mEq/L)	2.5 ± 0.4	4.1± 0.4*	2.68± 0.07**
8.	Inorganic Phosphorous (mg/dL)	0.35 ±0.05	0.76 ±0.05*	0.28±0.01*
9.	Ascorbic Acid (mg/dL)	3.6 ± 0.1	0.4 ± 0.1*	2.7±0.05*
10.	Cholesterol (mg/dL)	39.6 ± 1	20.1 ± 0.7*	36.9±0.3*
11.	Calcium (mg/dL)	10.6 ±0.1	4.9 ± 0.2*	8.9± 0.02*
12.	ALP #	4.2 ± 0.1	14.5 ±0.8*	5.1± 0.3**
13.	Alanine amino Transferase (µmole/min/mg protein	13.2 ± 0.5	28.2 ±1.3*	14.4 ± 0.4
14.	Aspartate amino transferase (µmole/min/mg protein)	2.1 ±0.08	6.5 ± 0.15*	2.4 ± 0.3*
15.	LDH##	13.4±0.2	16.8 ± 0.2*	12.9 ± 0.08
16.	MDH###	16.5±0.1	18.6 ±0.2*	15.9 ± 0.2**

*p<0.001 **p<0.05

Units: µmol phenol liberated/min/mg protein

Units: µmol pyruvate formed/min/mg protein

Units: nmol NADPH oxidized/min/mg protein

Table. 3.2. Biochemical components of Liver tissue in 15th day old chick embryo

Sl.No.	Biochemical Parameter	Control	CP (300µg/egg)	CP +AP
				300µg+ 6.0mg
1.	Protein (mg/100mg dry weight)	29.2±0.07	18.6±0.01	27.4±0.8*
2.	Inorganic Phosphorous (mg/100mg dry weight)	1.7±0.01	0.2±0.04	1.72±0.1*
3.	Ascorbic Acid (mg/100mg dry weight)	2.74±1.4	1.72±1.4*	2.5±0.1*
4.	ALP #	8.4±0.1	13.6±0.1*	8.9±0.08*
5.	Alanine amino Transferase (µmole/min/mg protein)	9.8±0.3	17.6±0.1*	10.1±0.2**
6.	Aspartate amino transferase (µmole/min/mg protein)	6.8±0.08	9.4±0.1*	6.3±0.1*
7.	LDH##	65.6±0.1	84.5±0.2*	68.2±1.05*
	MDH###	82.1±0.1	96.2±1.2*	84.9 ± 0.3*

*p<0.001 **p<0.05

Units: µmol phenol liberated/min/mg protein

Units: µmol pyruvate formed/min/mg protein

Units: nmol NADPH oxidized/min/mg protein

Table. 3.3. Biochemical components of Heart tissue in 15th day old chick embryo

Sl.No.	Biochemical Parameter	Control	CP (300µg/egg)	CP +AP
				300µg+6.0mg
1.	Protein (mg/100mg dry weight)	18.6±0.1	15.2±0.1*	17.82±0.1*
2.	Inorganic Phosphorous (mg/100mg dry weight)	0.9±0.07	0.7±0.08*	0.88±0.1*
3.	Cholesterol (mg/100mg dry weight)	26.5±2.1	14.6±1.2*	24.8±0.8**
4.	ALP #	6.2±0.1	8.6±0.1*	6.07±0.07**
5.	Ascorbic acid (mg/100mg dry weight)	1.12±2.2	0.85±0.1*	1.0±0.3**
6.	Alanine amino transferase (µmole/min/mg protein)	4.6±0.1	6.8±0.3*	4.4±0.1**
7.	Aspartate amino transferase (µmole/min/mg protein)	5.2±0.2	7.9±0.2*	5.5±0.2
8.	LDH##	21.2±0.1	29.2±0.2*	22.2±0.4*
9.	MDH###	38.2±0.1	46.1±0.1*	37.1±0.02**

*p<0.001 **p<0.05

Units: µmol phenol liberated/min/mg protein

Units: µmol pyruvate formed/min/mg protein

Units: nmol NADPH oxidized/min/mg protein

3.2. Discussion

In the present study the administration of three doses of CP (100µg, 200µg, and 300 µg) resulted in significant biochemical changes in AF and other embryonic tissues. The biochemical changes in amniotic fluid and tissues would be well used to extrapolate the findings similar to the CP induced changes in higher model system and even with humans, provided the mechanism by which the damage induced is clearly understood. The levels of glucose were significantly increased with CP treatment in AF compared to controls. The change in glucose levels in indication of alterations on Carbohydrate metabolism. It also major source of energy for the nervous system and erythrocytes. The reversal of increased glucose return to normal by AP supplementation. Elevated levels of urea, uric acid and Creatinine may be due to the damage caused by CP on the function of kidneys. Uric acid, the metabolic end product of purines metabolism has been proven to be selective antioxidant, capable of reacting with free radicals and hypochlorous acid. The increased parameters return to normal by AP administration.

The result, which we observed following different doses of CP administration in chick embryo, reveals alterations in several marker enzymes. The levels of glucose were significantly increased with higher concentration in CP in amniotic fluid compared to controls. The change in glucose level is an indication of alterations in carbohydrate metabolism. It is also major source of energy for the nervous system and erythrocytes. The increased levels of glucose in amniotic fluid could be due to alteration in the membrane permeability and diffusion of embryonic glucose into amniotic fluid or may have a direct effect on glucose metabolism in AF.

Increased levels of urea, uric acid and Creatinine may be due to the damages incurred by high dose of CP on the function of embryonic kidneys. Uric acid, the metabolic and product of purines metabolism has been proven to be a selective antioxidant. Capable of reacting with free radicals and hypochlorous acid.

CP administration caused significant elevation in marker enzymes of amniotic fluid as well as in embryonic tissues. The significant increase in the activity of enzymes in liver and AF are due to the effect of CP on hepatocytes and possible transfer of the enzyme to AF.

ALP is the prototype of hepatic marker enzyme that reflects the pathological alterations in bile flow. ALP and bilirubin concentration have been used to evaluate chemically induced hepatic injury.

There are important class of enzymes linking carbohydrate and amino acid metabolism and establish a relationship between the intermediate of TCA cycle and amino acids. Acid Phosphatase is the marker enzyme to assess the lysosomal changes in vivo because it is localized almost exclusively in the particle and it is released parallels that of lysosomal hydrolase. A significant higher levels of these enzymes with CP treatment, indicates the damage of liver cell lysosomes.

The increased protein content in AF may be due to the leakage of RBC cells into amniotic fluid. It is clearly suggest that there is a decline in protein Content, which could affect the metabolic activity of the liver. A Protein being involved in the architecture and also in the physiology of the cell seems to occupy a key role in the cell metabolism. The fall in protein content during stress may be due to increased proteolytic activity decreased anabolic activity of protein. The decreased protein content might also be due to tissue destruction by necrosis or disturbance of cellular fraction and consequent impairment in protein synthetic machinery. The reversal protein levels were observed with AP administration.

LDH is a cytosolic enzyme that catalyzes the reversible oxidation of L-lactate to pyruvate. In CP mediated acute toxicity increased activity of LDH. In the present study enzymatic activity of MDH also increased with higher dose of CP in embryonic tissues. AP extract ensured a rapid protection and maintained the levels of LDH.

4.1. CYCLOPHOSPHAMIDE INDUCED OXIDATIVE DAMAGE IN 15th DAY OLD CHICK EMBRYO

The chick embryo was treated with cyclophosphamide 300 µg. The fertilised eggs were classified into four groups.

Group-I Controls treated with normal saline

Group-II Treated with cyclophosphamide 100µg/15 day's old chick embryo

Group-III Treated with cyclophosphamide 200µg/15 day's old chick embryo

Group-IV Treated with cyclophosphamide 300µg/15 day's old chick embryo

After 24, 48 and 72 hrs of injection of CP. The embryos were sacrificed; liver, heart and brain tissues were collected for the determination of the following parameters.

Fig. 4.1, 4.2 and 4.3 represents the effects of ethanolic extract of AP on LPO levels in CP treated liver, brain and heart in chick embryo. In control LPO levels were decrease with duration. LPO levels were significantly induced in chick embryo in a time-dependent manner. AP treated to normal embryos decreased LPO levels significantly at 24, 48 and 72 h duration in liver, brain and heart tissues. AP treated in normal embryos decreased the LPO levels significantly in liver, brain and heart more effectively after 48 h compared to controls. Treatment of AP 72 h prior to CP administration (Pre-treatment significantly decreased the LPO levels at 24, 48 and 72 h compared to CP.

The effect of AP on CP treated embryonic liver, brain and heart tissues caused significant decline in the levels of lipid peroxides compared with CP.

The Fig. 4.4, 4.5 and 4.6 illustrate the effect of AP on SOD activity in liver, brain and heart of CP treated chick embryos. The decreased activity of enzyme with CP treatment was significantly altered with AP pre treatment in liver, brain and heart.

Fig. 4.7, 4.8 and 4.9 corresponds to the effect of AP on CAT activity in liver, brain and heart of CP treated chick embryos. A significant suppression of CAT activity was seen in Liver, brain and heart with CP treatment. AP treated normal embryos increased the enzymatic activity at 24, 48 and 72 h compared to controls. AP pre-treatment elevated the enzyme levels significantly in liver, brain and heart tissues.

The effect of AP on GST activity in liver, brain and heart of CP treated embryo was seen in Fig. 4.10, 4.11 and 4.12. CP significantly increased the enzymatic activity in liver and brain.

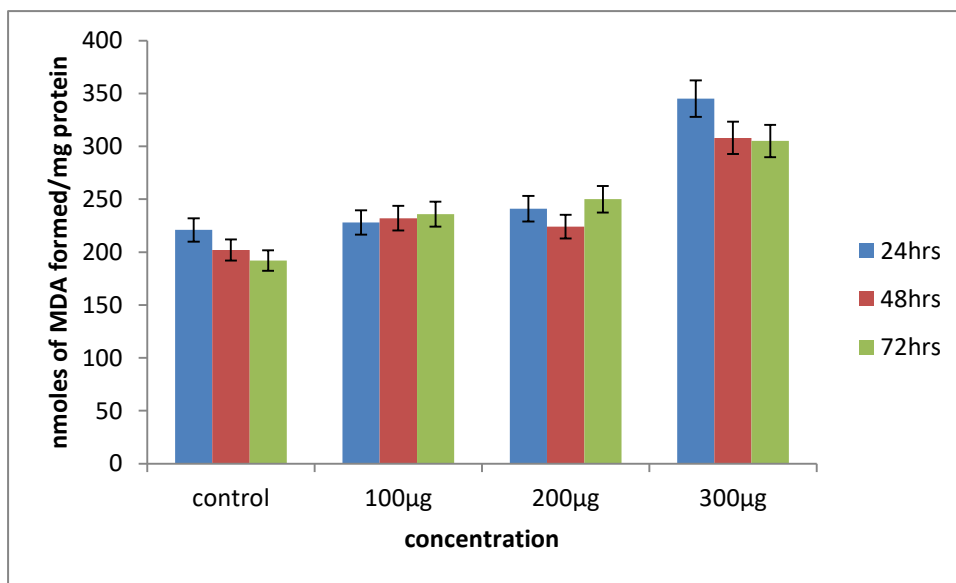
A significant suppression of GST activity was seen in heart with CP treatment. AP treatment alone enhanced the enzyme levels compared to controls. AP treatment to exposed embryos has significantly decreased the GST activity and reaches to never controls in liver and brain. But in heart the enzyme levels were significantly increased with AP treatment and reaches to hear control level.

The effect of AP on GPX activity in liver, brain and heart is shown in Fig. 4.13, 4.14 and 4.15. The suppressed activity of enzyme with CP treatment was significantly increased with AP pre-treatment in liver, brain and heart.

Fig. 4.16, 4.17 and 4.18 represent the effect of AP on GSH in liver, brain and heart of CP treated chick embryos. Significant decrease in GSH activity was observed with CP treatment in liver, brain and heart. AP treated normal elevated the enzyme levels more significantly at 72 h than at 24 and 48 h in liver brain and heart. AP pre-treatment significantly elevated the GSH activity in liver, brain and heart with development compared to CP treated ones.

Fig. 4.19, 4.20 and 4.21 corresponds to the effect of AP extract on GR activity in liver, brain and heart of CP treated chick embryos. A significant suppression of GR activity was seen in liver, brain and heart with CP treatment. AP treatment alone did not cause any significant increase in the GR activity in brain and heart but in liver increased enzyme levels were observed. AP pre-treatment elevated the enzyme levels significantly in liver, brain and heart tissues.

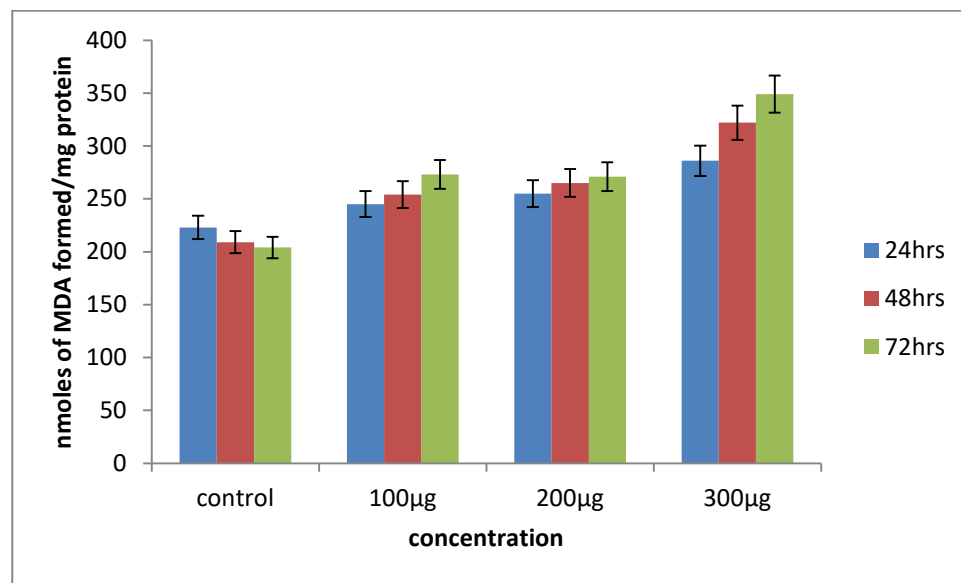
Fig. 4.1: Effect of CP on Lipid peroxidation in Liver of 15-day-old Chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	221.4 ± 1.95	204.5 ± 5.37	192 ± 3.46
2.	CP 100 µg	228 ± 1.87	232 ± 2.45	236.8 ± 2.28
3.	CP 200 µg	242.6 ± 1.67	244 ± 2.71	250.2 ± 3.86
4.	CP 300 µg	345.2 ± 3.27	308 ± 5.02	305 ± 2.5

Values are average of six sets of separate experiments (Mean ± SD)

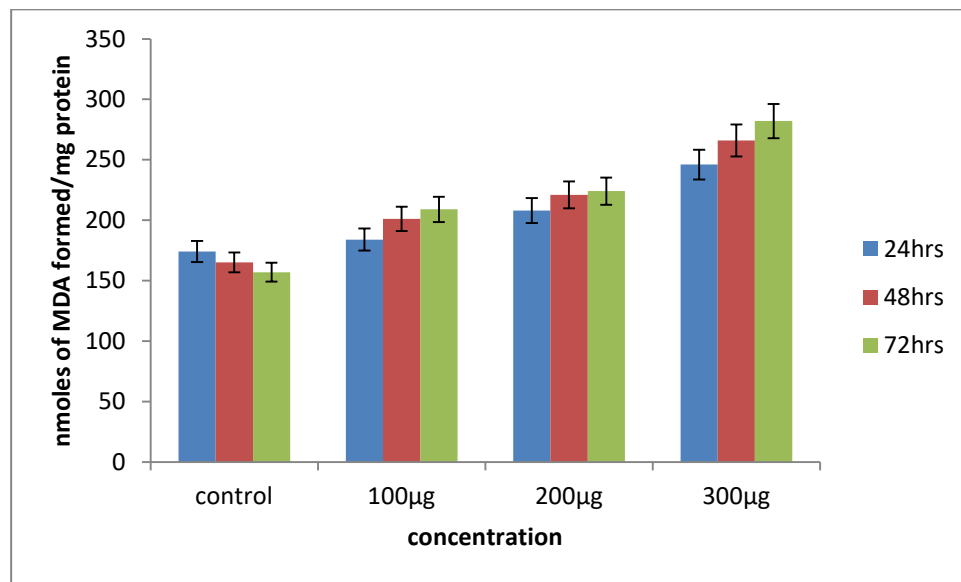
Fig. 4.2: Effect of CP on Lipid peroxidation in Heart of 15-day-old Chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	223.4 ± 1.52	209.5 ± 5.37	204.4 ± 2.6
2.	CP 100 µg	229.2 ± 1.58	239 ± 2.16	239.8 ± 2.25
3.	CP 200 µg	247.5 ± 1.60	248 ± 2.65	253.2 ± 3.82
4.	CP 300 µg	277.5 ± 1.65	308 ± 5.02	305 ± 2.5

Values are average of six sets of separate experiments (Mean ± SD)

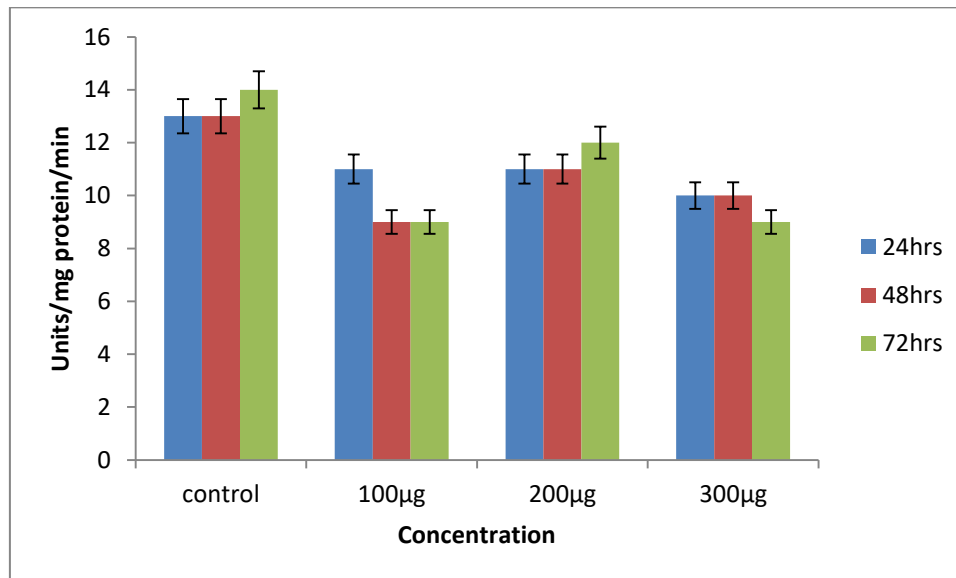
Fig. 4.3: Effect of CP on Lipid peroxidation in Brain of 15-day-old Chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	223.4 ± 1.52	209.5 ± 5.37	204.4 ± 2.6
2.	CP 100 µg	229.2 ± 1.58	232 ± 2.16	236.8 ± 2.25
3.	CP 200 µg	242.6 ± 1.67	244 ± 2.71	250.2 ± 3.86
4.	CP 300 µg	345.2 ± 3.27	308 ± 5.02	305 ± 2.5

Values are average of six sets of separate experiments (Mean ± SD)

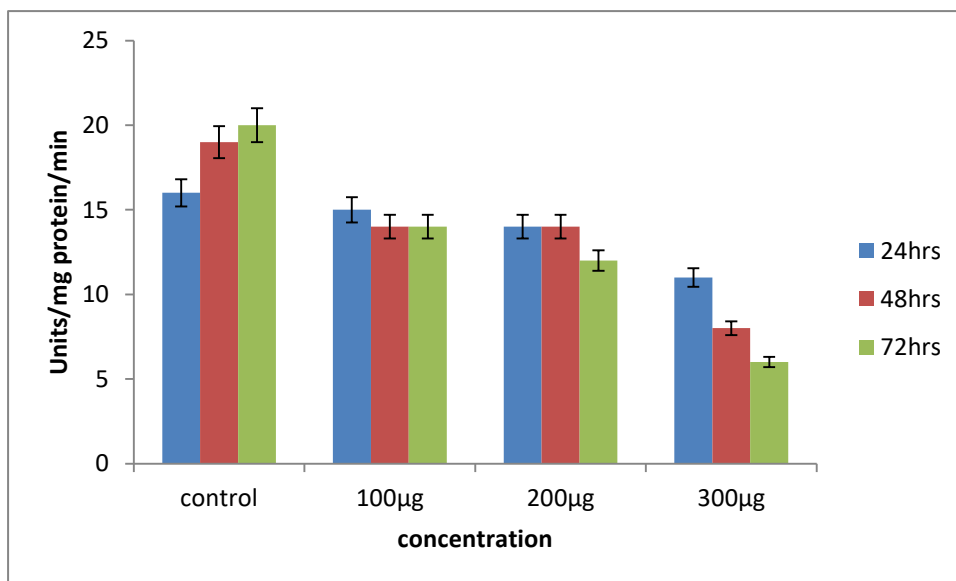
Fig.4.4: Effect of CP on SOD activity in liver of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	CP 100 µg	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	CP 200 µg	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

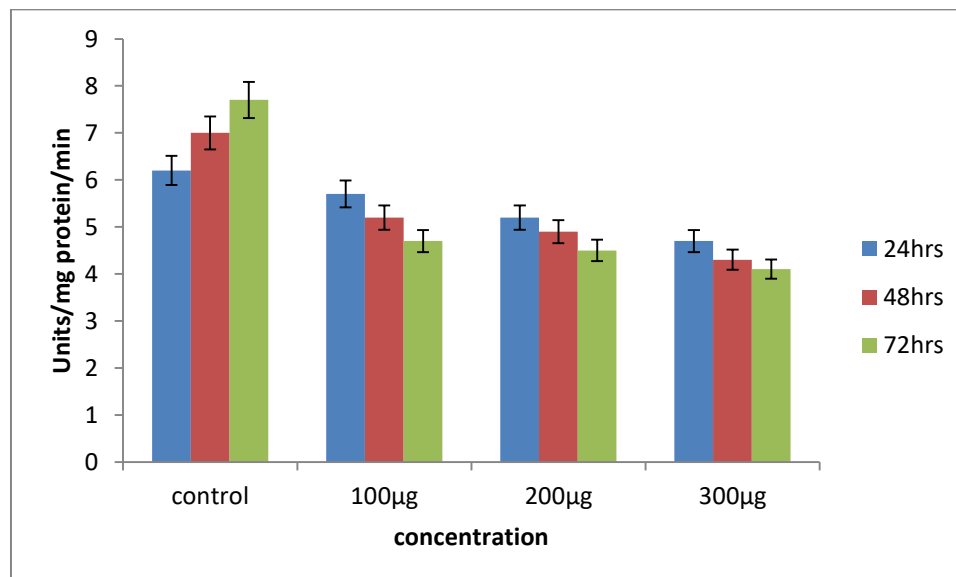
Fig.4.5: Effect of CP on SOD activity in Heart of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	CP 100 µg	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	CP 200 µg	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

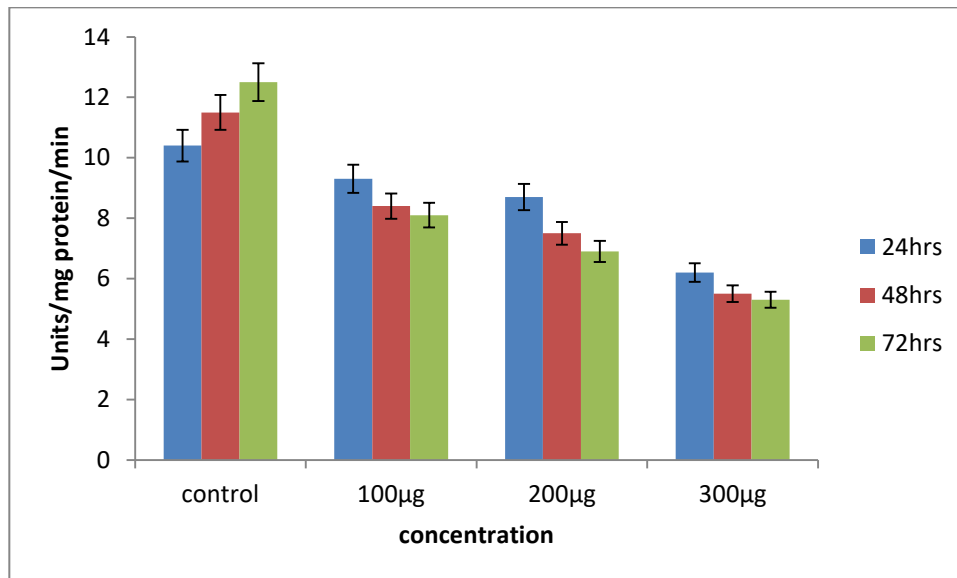
Fig.4.6: Effect of CP on SOD activity in Brain of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	CP 100 µg	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	CP 200 µg	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

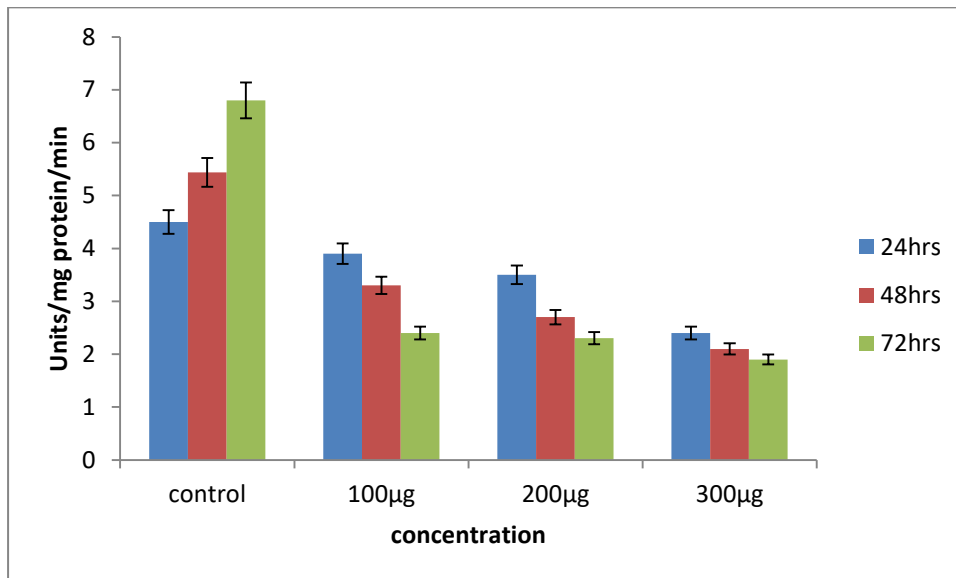
Fig.4.7: Effect of CP on CAT activity in liver of 15-day-old chick embryo



I. No.		24 hrs	48 hrs	72 hrs
1.	Control	10.45 ± 0.25	11.5 ± 0.17	12.5 ± 0.15
2.	CP 100 µg	6.25 ± 0.14	5.75 ± 0.35	5.3 ± 0.33
3.	CP 200 µg	11.4 ± 0.22	12.5 ± 0.12	13.2 ± 0.14
4.	CP 300 µg	13.4 ± 0.24	13.2 ± 0.14	13.5 ± 0.24

Values are average of six sets of separate experiments (Mean ± SD)

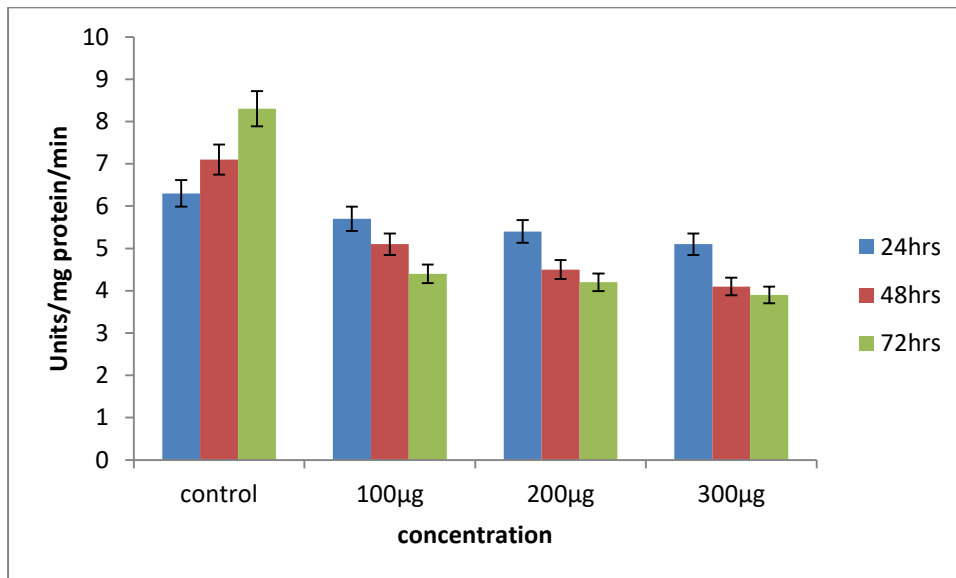
Fig.4.8: Effect of CP on CAT activity in heart of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	4.6 ± 0.22	5.5 ± 0.17	6.5 ± 0.25
2.	CP 100 µg	4.5 ± 0.22	3.75 ± 0.14	1.85 ± 0.07
3.	CP 200 µg	5.4 ± 0.19	5.8 ± 0.02	6.2 ± 0.22
4.	CP 300 µg	15.5 ± 0.25	6.5 ± 0.11	8.8 ± 0.32

Values are average of six sets of separate experiments (Mean ± SD)

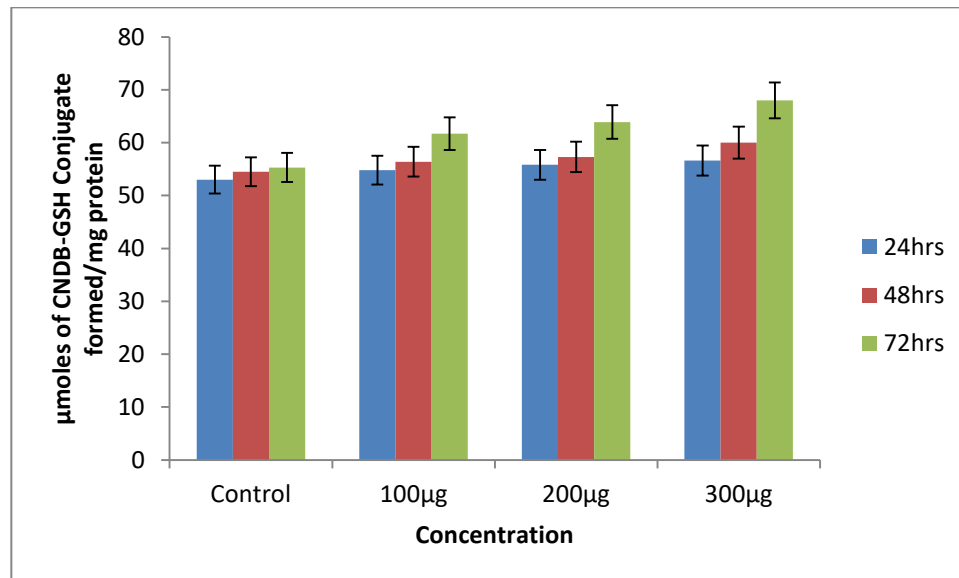
Fig.4.9: Effect of CP on CAT activity in brain of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	6.61 ± 0.18	7.5 ± 0.55	8.5 ± 0.35
2.	CP 100 µg	5.5 ± 0.22	4.75 ± 0.04	3.5 ± 0.07
3.	CP 200 µg	7.5 ± 0.24	7.65 ± 0.06	8.9 ± 0.14
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

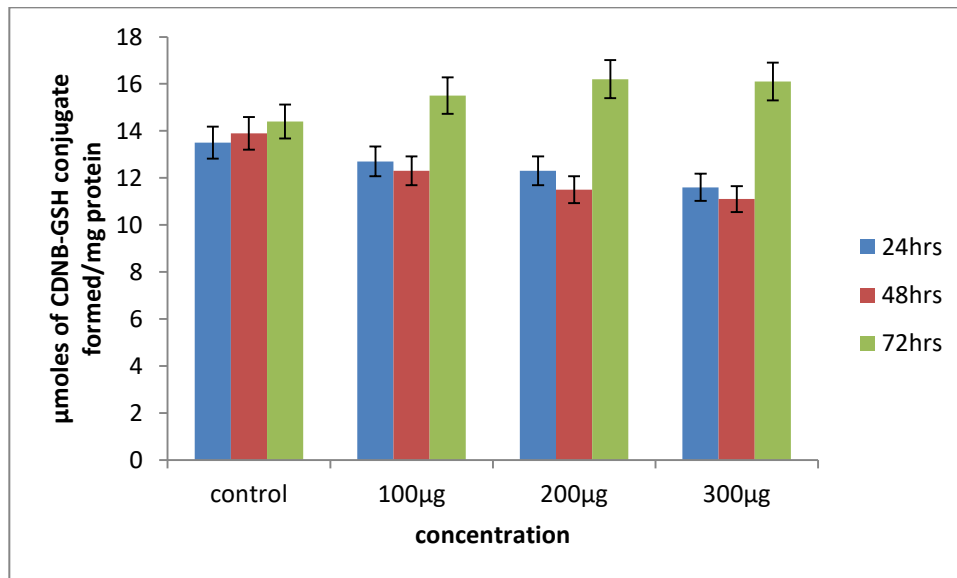
Fig.4.10: Effect of CP on GST activity in Liver of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	51.6 ± 0.61	53.5 ± 0.75	55.5 ± 0.54
2.	CP 100 µg	56.6 ± 0.35	60 ± 0.75	65.5 ± 1.41
3.	CP 200 µg	60.5 ± 0.62	62.5 ± 0.75	65.2 ± 0.15
4.	CP 300 µg	62.4 ± 0.75	64.5 ± 0.76	62.2 ± 0.18

Values are average of six sets of separate experiments (Mean ± SD)

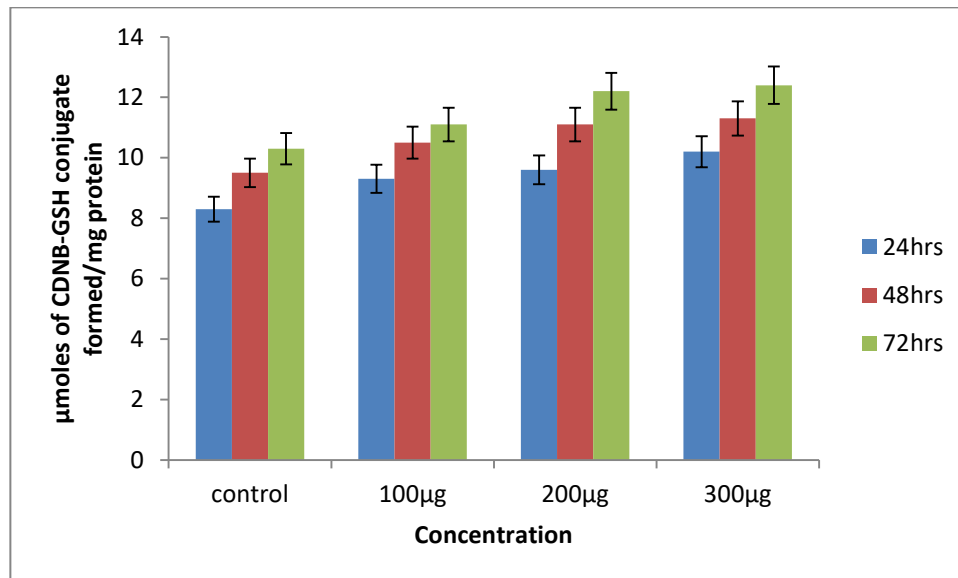
Fig.4.11: Effect of CP on GST activity in Heart of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	CP 100 µg	13.5 ± 0.25	13.75 ± 0.26	14.4 ± 0.19
3.	CP 200 µg	14.5 ± 0.22	14.5 ± 0.17	14.8 ± 0.22
4.	CP 300 µg	15.4 ± 0.26	15.7 ± 0.24	16.2 ± 0.24

Values are average of six sets of separate experiments (Mean ± SD)

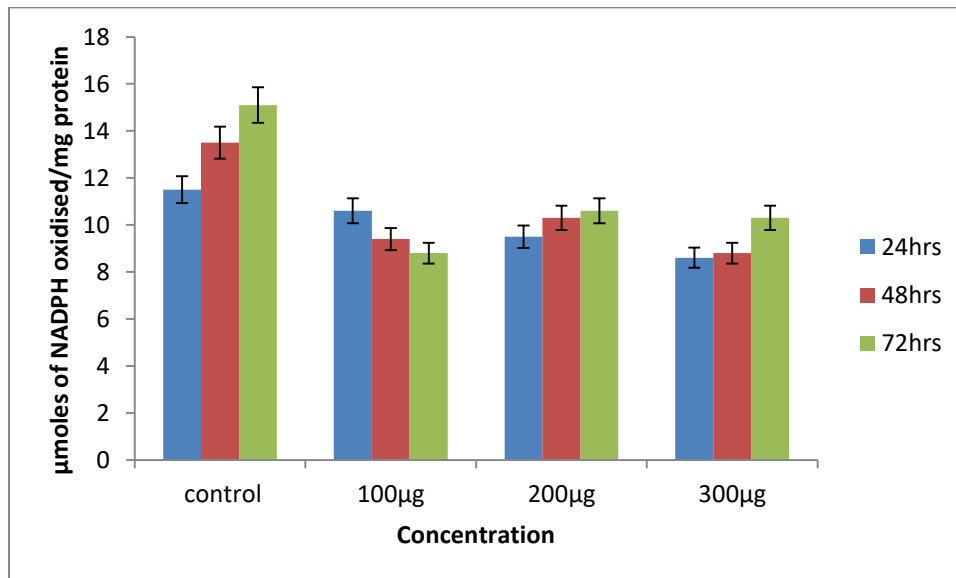
Fig.4.12: Effect of CP on GST activity in Brain of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	8.6 ± 0.1	9.5 ± 0.25	16.1 ± 0.35
2.	CP 100 µg	10.25 ± 0.35	11.7 ± 0.31	12.4 ± 0.32
3.	CP 200 µg	12.5 ± 0.22	12.5 ± 0.18	13.2 ± 0.12
4.	CP 300 µg	13.4 ± 0.24	13.35 ± 0.21	13.7 ± 0.24

Values are average of six sets of separate experiments (Mean ± SD)

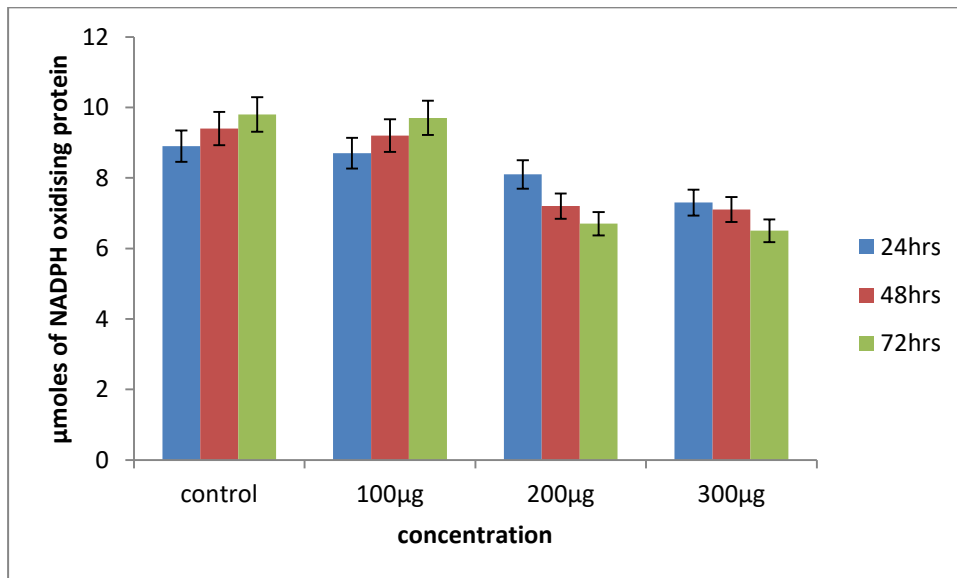
Fig.4.13: Effect of CP on GPx activity in Liver of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	11.5 ± 0.25	13.5 ± 0.32	15.4 ± 0.15
2.	CP 100 µg	8.6 ± 0.12	8.8 ± 0.15	10.5 ± 0.21
3.	CP 200 µg	10.5 ± 0.22	11.5 ± 0.12	12.2 ± 0.24
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

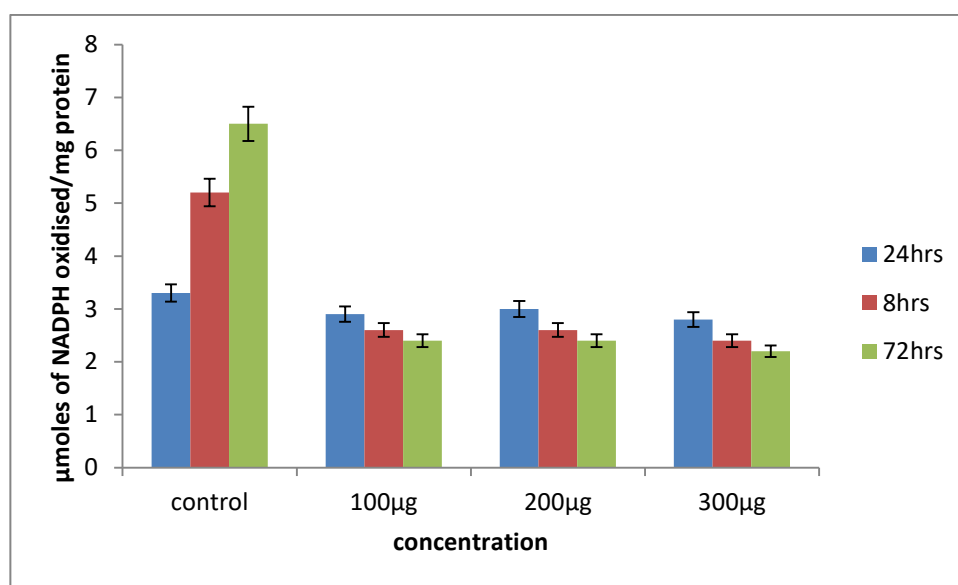
Fig.4.14: Effect of CP on GPx activity in Heart of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	8.6 ± 0.26	9.5 ± 0.16	9.5 ± 0.15
2.	CP 100 µg	7.5 ± 0.11	7.75 ± 0.18	6.5 ± 0.25
3.	CP 200 µg	9.5 ± 0.1	10.5 ± 0.12	10.2 ± 0.18
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

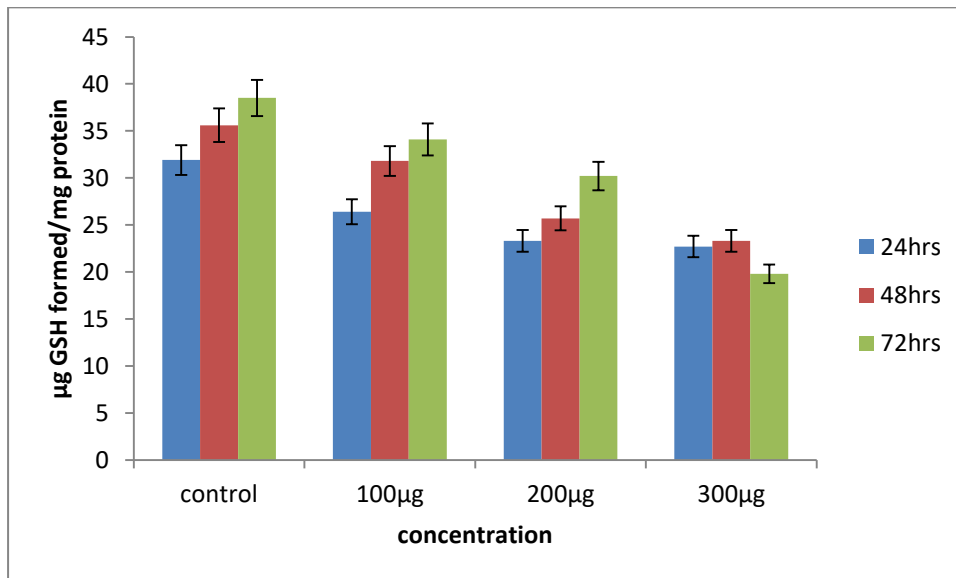
Fig.4.15: Effect of CP on GPx activity in Brain of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	3.6 ± 0.26	5.5 ± 0.28	6.5 ± 0.31
2.	CP 100 µg	2.5 ± 0.1	2.75 ± 0.33	2.5 ± 0.25
3.	CP 200 µg	4.5 ± 0.12	6.5 ± 0.22	7.2 ± 0.34
4.	CP 300 µg	7.4 ± 0.24	8.2 ± 0.11	9.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

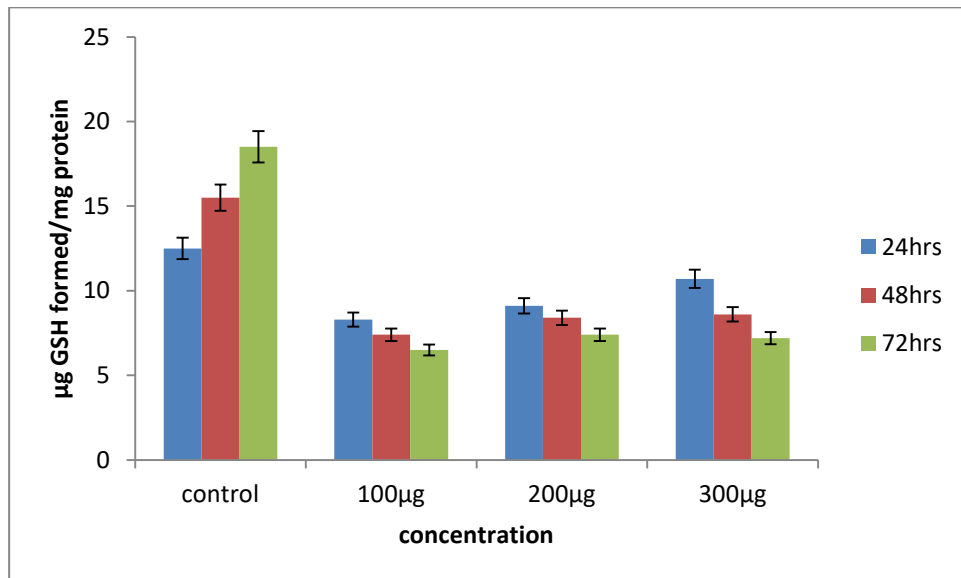
Fig.4.16: Effect of CP on GSH activity in Liver of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	31.6 ± 1.02	35.5 ± 0.7	38.5 ± 0.19
2.	CP 100 µg	22.5 ± 0.21	24.75 ± 0.11	19.5 ± 0.67
3.	CP 200 µg	24.5 ± 0.26	25.5 ± 0.22	21.2 ± 0.24
4.	CP 300 µg	25.4 ± 0.2	27.5 ± 0.17	23.2 ± 0.18

Values are average of six sets of separate experiments (Mean ± SD)

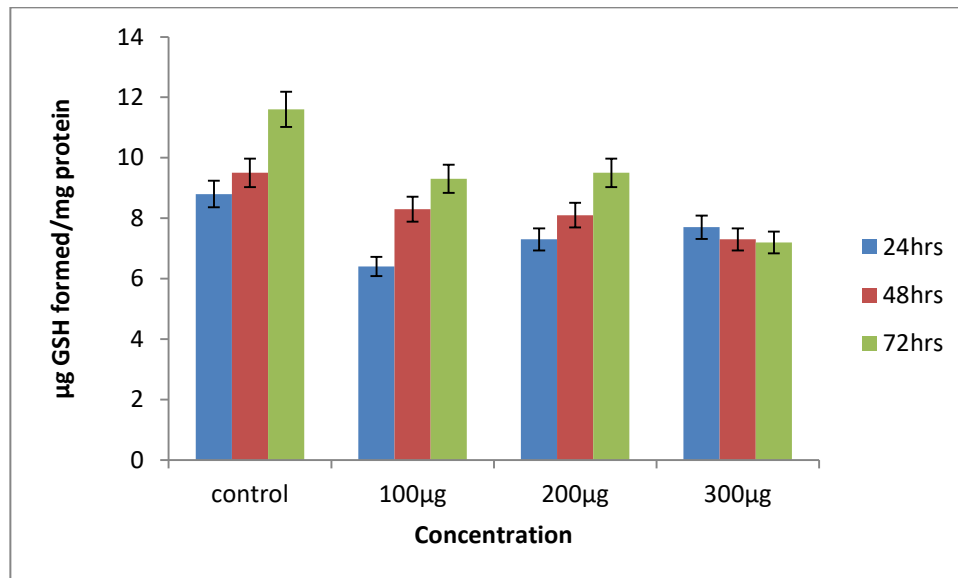
Fig.4.17: Effect of CP on GSH activity in Heart of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	12.6 ± 0.2	15.5 ± 0.15	18.5 ± 0.25
2.	CP 100 µg	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	CP 200 µg	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

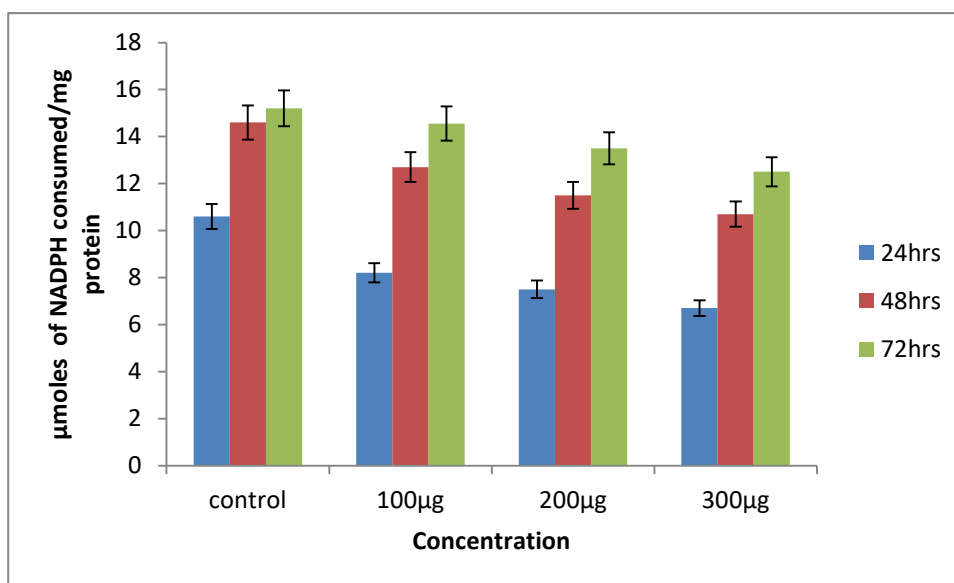
Fig.4.18: Effect of CP on GSH activity in Brain of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	8.6 ± 0.28	9.5 ± 0.12	11.5 ± 0.15
2.	CP 100 µg	7.5 ± 0.15	7.3 ± 0.11	7.5 ± 0.2
3.	CP 200 µg	9.5 ± 0.17	10.5 ± 0.16	12.2 ± 0.18
4.	CP 300 µg	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

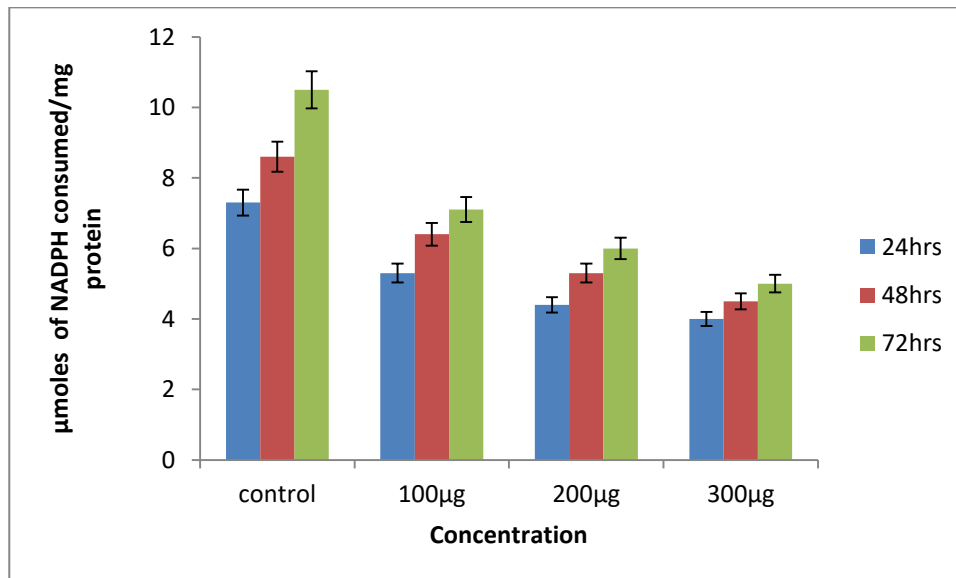
Fig.4.19: Effect of CP on GR activity in Liver of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	10.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	CP 100 µg	6.5 ± 0.15	9.75 ± 0.11	12.5 ± 0.17
3.	CP 200 µg	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	CP 300 µg	14.4 ± 0.2	13.8 ± 0.11	14.8 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

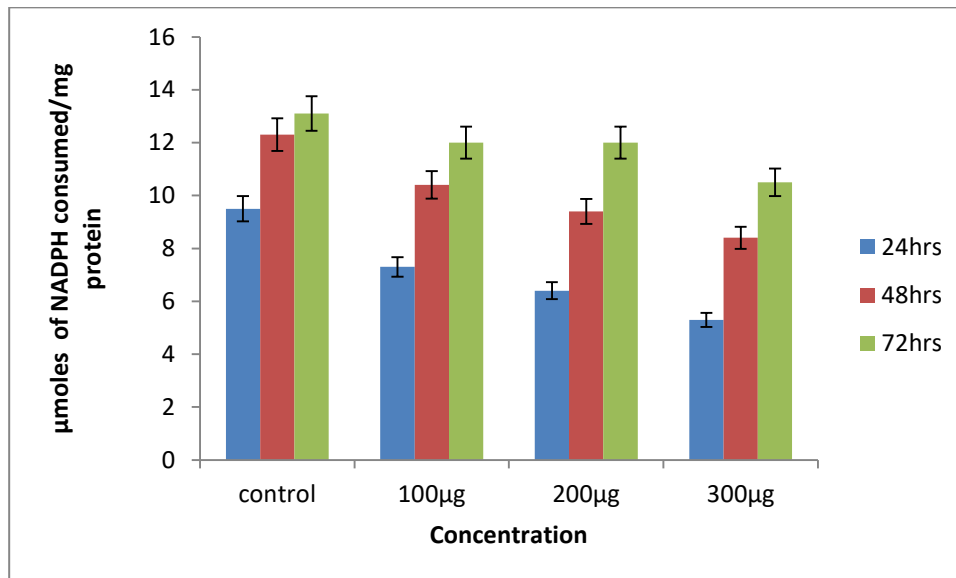
Fig.4.20: Effect of CP on GR activity in Heart of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	7.6 ± 0.12	8.5 ± 0.19	10.5 ± 0.25
2.	CP 100 µg	5.5 ± 0.11	6.75 ± 0.16	7.5 ± 0.13
3.	CP 200 µg	4.5 ± 0.12	5.5 ± 0.11	6.2 ± 0.3
4.	CP 300 µg	4.4 ± 0.2	4.5 ± 0.11	6.2 ± 0.14

Values are average of six sets of separate experiments (Mean ± SD)

Fig.4.21: Effect of CP on GR activity in Brain of 15-day-old chick embryo



Sl. No.		24 hrs	48 hrs	72 hrs
1.	Control	9.5 ± 0.21	12.3 ± 0.1	13.1 ± 0.25
2.	CP 100 μg	7.5 ± 0.05	10.75 ± 0.21	12 ± 0.22
3.	CP 200 μg	6.8	9.5	12
4.	CP 300 μg	5.4 ± 0.11	9.5 ± 0.16	10.2 ± 0.25

Values are average of six sets of separate experiments (Mean ± SD)

5.1. CHEMOPROTECTIVE ROLE OF *ANDROGRAPHIS PANICULATA* AGAINST CYCLOPHOSPHAMIDE INDUCED OXIDATIVE DAMAGE IN CHICK EMBRYO

The chick embryo was treated with cyclophosphamide 300 µg. The fertilised eggs were classified into four groups.

Group I Controls treated with normal saline

Group II Treated with cyclophosphamide 100, 200 and 300µg/15 days old chick embryo

Group III Treated with ethanolic extract of *Andrographis paniculata* 6.0 mg/12th day prior to cyclophosphamide (300µg) treatment.

After 24, 48 and 72 hrs of injection of CP. The embryos were sacrificed; liver, heart and brain tissues were collected for the determination of the following parameters.

Fig. 5.1, 5.2 and 5.3 represents the effects of ethanolic extract of AP on LPO levels in CP treated liver, brain and heart in chick embryo. In control LPO levels were decrease with duration. LPO levels were significantly induced in chick embryo in a time-dependent manner. AP treated to normal embryos decreased LPO levels significantly at 24, 48 and 72h duration in liver, brain and heart tissues. AP treated in normal embryos decreased the LPO levels significantly in liver, brain and heart more effectively after 48 h compared to controls. Treatment of AP 72 h prior to CP administration (Pre-treatment significantly decreased the LPO levels at 24, 48 and 72 h compared to CP.

The effect of AP on CP treated embryonic liver, brain and heart tissues caused significant decline in the levels of lipid peroxides compared with CP.

The Fig. 5.4, 5.5 and 5.6 illustrate the effect of AP on SOD activity in liver, brain and heart of CP treated chick embryos. The decreased activity of enzyme with CP treatment was significantly altered with AP pre treatment in liver, brain and heart.

Fig. 5.7, 5.8 and 5.9 corresponds to the effect of AP on CAT activity in liver, brain and heart of CP treated chick embryos. A significant suppression of CAT activity was seen in Liver, brain and heart with CP treatment. AP treated normal embryos increased the enzymatic activity at 24, 48 and 72 h compared to controls. AP pre-treatment elevated the enzyme levels significantly in liver, brain and heart tissues.

The effect of AP on GST activity in liver, brain and heart of CP treated embryo was seen in Fig. 5.10, 5.11 and 5.12. CP significantly increased the enzymatic activity in liver and brain.

A significant suppression of GST activity was seen in heart with CP treatment. AP treatment alone enhanced the enzyme levels compared to controls. AP treatment to exposed embryos has significantly decreased the GST activity and reaches to never controls in liver and brain. But in heart the enzyme levels were significantly increased with AP treatment and reaches to hear control level.

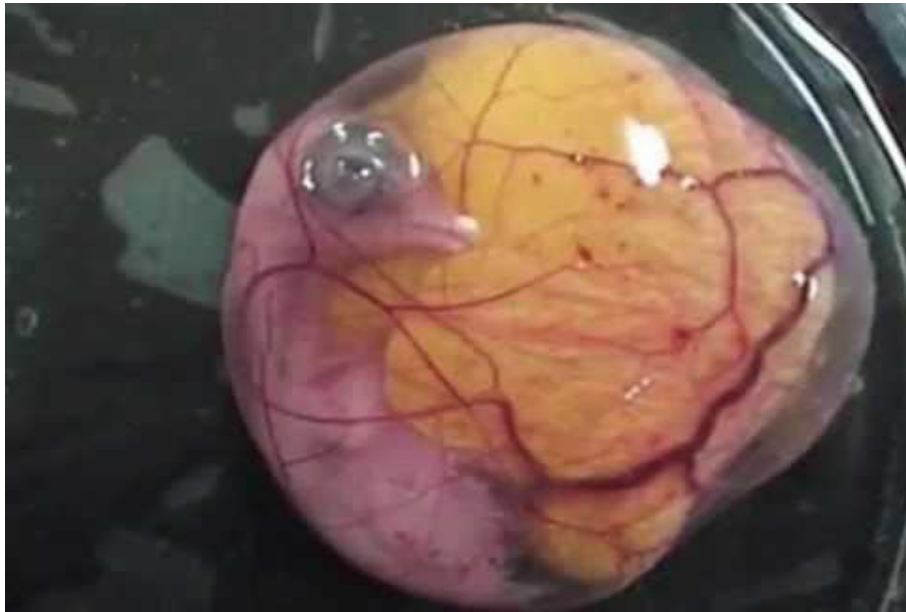
The effect of AP on GPX activity in liver, brain and heart is shown in Fig. 5.13, 5.14 and 5.15. The suppressed activity of enzyme with CP treatment was significantly increased with AP pre-treatment in liver, brain and heart.

Fig. 5.16, 5.17 and 5.18 represent the effect of AP on GSH in liver, brain and heart of CP treated chick embryos. Significant decrease in GSH activity was observed with CP treatment in liver, brain and heart. AP treated normal elevated the enzyme levels more significantly at 72 h than at 24 and 48 h in liver brain and heart. AP pre-treatment significantly elevated the GSH activity in liver, brain and heart with development compared to CP treated ones.

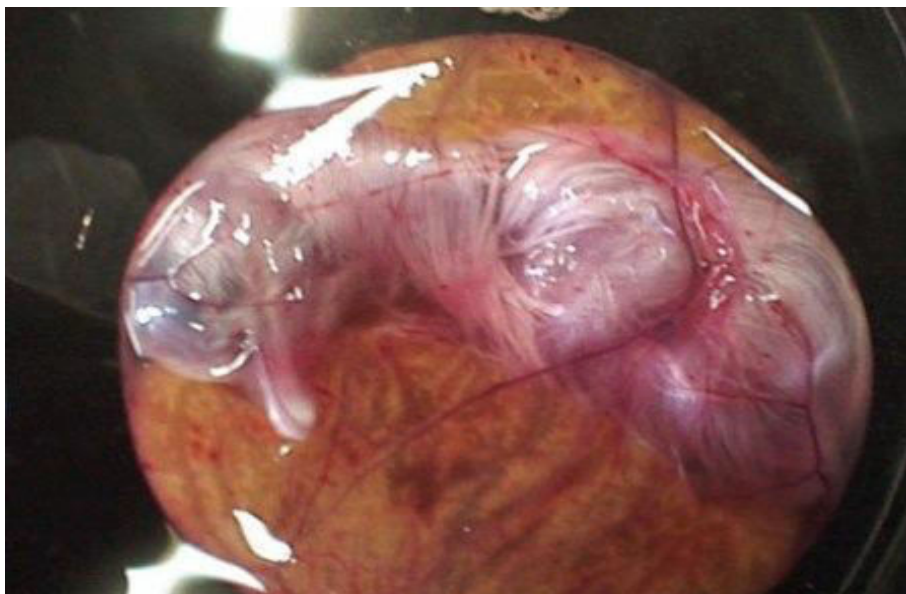
Fig. 5.19, 5.20 and 5.21 corresponds to the effect of AP extract on GR activity in liver, brain and heart of CP treated chick embryos. A significant suppression of GR activity was seen in liver, brain and heart with CP treatment. AP treatment alone did not cause any significant increase in the GR activity in brain and heart but in liver increased enzyme levels were observed. AP pre-treatment elevated the enzyme levels significantly in liver, brain and heart tissues.

Fig. 5.1. Developmental Stages of Chick embryo

1. 15th day old chick embryo



2. 16th day old chick embryo



3. 17th day old chick embryo



4. 18th day old chick embryo



5.2. Discussion

The present study deals with the evaluation of the protective effect of AP extract on cyclophosphamide induced oxidative damage in chick embryo. Many natural products are reported to influence the antioxidant system and are good cyto protective agents (Dragsted *et al.*, 1997). Hence AP extract was subjected to preliminary investigation to identify its influencing role on antioxidant system in normal chick embryo at various concentrations. The concentration in which there was maximum elevation in GSH and GST was taken as the optimum dosage. *A.paniculata* has been identified to exhibit significant anti oxidative and anti lipid peroxidative property (Tirupati *et al.*, 1996). The antioxidative and anti-lipid peroxidative property was observed in AP treated fibro sarcoma rats (Rohini *et al.*, 2004).

The antioxidant potential of the *Andrographis paniculata* extract in rat brain by increasing the activities of SOD, CA and GPx was demonstrated (Bhattacharya *et al.*, 2000). The alcoholic extract of *Andrographis paniculata* showed greater protection with FeSO₄ and Cumene hydro peroxide induced lipid peroxidation (Tirupati *et al.*, 1996). The alcoholic extract of *Andrographis paniculata* induced a significant increase of lipid peroxidation and a significant decrease in liver antioxidant enzymes in the morphine treated rats for hepatoprotection (Sumathy *et al.*, 2001). Its role as a free radical scavenger and the redox interrelationship between as a free radical scavenger and the redox interrelationship between *Andrographis paniculata* and other antioxidants are in the focus of continuous interest.

Previous studies on the *in vitro* anti-lipid peroxidative activity of *Andrographis paniculata* extract suggested that it may act as a chain breaking antioxidant, presumably as a free radical scavenger (Yamini *et al.*, 1996). *Andrographis paniculata* exhibits neuro protective and cognitive enhancing effects, in part due to its, capacity to modulate the cholinergic system (Bhattacharya *et al.*, 1999) and to control oxidative stress (Bhattacharya *et al.*, 2000, Russo *et al.*, 2003 a, b). *Andrographis paniculata* is able to directly inhibit the superoxide anion formulation (Russo and Borelli, 2005).

Andrographis paniculata prevent variety of neurodegenerative diseases associated with reduced the intracellular oxidants, consequently preventing DNA damage (Colasanti and Suzuki 2000; Russo *et al.*, 2003b).

Free radicals and active oxygen species appear to have broader significance in the production of tissue injury under conditions of oxidative stress (Atlri *et al.*, 2001). They are now well recognized in the pathogenesis of various diseases such as cancer, atherosclerosis, hepatitis, inflammation, diabetes, arthritis and ageing process. (Moko Aniya *et al.*, 2003).

There is an increased lipid peroxide levels with cyclophosphamide treatment which were later decreased significantly by *Andrographis paniculata* pre-treatment in liver, brain and heart tissues. This indicates that *Andrographis paniculata* is able to ameliorate the toxic effect of cyclophosphamide. *Andrographis paniculata* scavenges the ROS by rapid electron transfer that inhibits lipid peroxidation.

Peroxidation of unsaturated lipid membranes by free radicals is known to contribute to the toxic side effects observed following administration of cyclophosphamide.

Cyclophosphamide treatment to 15-day -old chick embryo significantly decreased SOD and CAT activity within 24 h in liver, brain and heart. The decreased enzyme levels were enhanced on pre-treatment with *Andrographis paniculata* extract. The function of SOD is to protect the cells against the high chemical reactivates of various oxygen derived radicals (Fridovich, 1986). SOD catalyzes to scavenging excess superoxide anions and converts them to hydrogen peroxide, which in turn either detoxified by CAT or GSH dependant reactions.

Decreased SOD, CAT and GPx increases the oxidative stress already induced by cyclophosphamide treatment. This is reflected with the increased lipid peroxides produced by CP treatment. The elevation in embryonic tissue free radicals was further supported the diminution of peroxides and Catalase, an important scavenger enzyme of free radicals.

Our findings in this chapter show significant depletion of GSH levels after cyclophosphamide administration in chick embryonic liver, brain and heart.

The GSH antioxidant system consists of an array of non-enzymic and enzymic reaction pathways involving the neutralization of free radical species (Sreepriya *et al.*, 2001). Moreover, GSH reflects the summation of a number of processes GR maintains GSH in a reduced form, GPx utilises it for the decomposition of lipid hydro peroxides and other ROS and GST maximizes the conjugation of free radicals and various lipid hydro peroxides to GSH to form water soluble products that can be easily excreted out (Ahmed *et al.*, 2000).

GSH and vitamin C are water soluble antioxidants that serve as the first line of defence in combating free radicals (Martens son and Meister, 1991) and GSH can also facilitate the generation of vitamin C from its oxidation (Well *et al.*, 1990).

Cyclophosphamide treatment significantly decreased GPx activity in liver, brain and heart. Treatment with *Andrographis paniculata* significantly increased the GPx activity in liver, brain and heart tissues. CP pre-treatment was able to increase the GPx activity in liver, brain and heart.

Our findings in this chapter show significant elevation of GST levels after cyclophosphamide administration liver and brain whereas in heart GST levels were decreased.

Treatment with *Andrographis paniculata* significantly increased the GST activity in liver, brain and heart. *Andrographis paniculata* pre-treatment was decreased the GST activity in liver, brain and heart.

The GST, in *Andrographis paniculata* extract treated animals showed significant increase in its activity, which further accounts for the antioxidant property of the plant extract (Vijayalakshmi, 2001).

Andrographis paniculata induced a dose – related increase in hippocampus SOD, CAT and GPx activities (Bhattacharya *et al.*, 2000).

Cyclophosphamide treatment significantly decreased GR activity in liver, brain and heart. Treatment with *Andrographis paniculata* significantly increased in liver and brain but in heart. *Andrographis paniculata* pre-treatment was able to increase the GR activity in liver, brain and heart tissues.

The decrease in enzyme activities may be due to inactivation of the enzymes by the free radicals. But the treatment with ethanolic extract of *Andrographis paniculata* significantly enhanced the activities of antioxidant enzymes. SOD and CAT, GPX and GST acts as protective enzymes against lipid peroxidation in embryonic tissues. The above results showed that the plant extract has activated the enzyme for defence mechanism.

On the whole, this plant extract exhibited a powerful antioxidant effect by interacting with the free radical metabolites produced by cyclophosphamide in the following ways.

- By enhancing hepatic GSH and GST levels which aid in maintaining the antioxidant armoury.
- By decreasing lipid peroxidation by its direct free radical scavenging action.
- By modulating the key enzymes associated with the enzymatic antioxidant defence.

This elaborates that the plant extract has a potent and powerful efficacy as a good inhibitor of drug induced oxidative stress. Thus our results show a chemo protective role of *Andrographis paniculata* extract against cyclophosphamide.

Possibly ours is a first report on the effect of *Andrographis paniculata* extract on cyclophosphamide in chick embryos, further studies are needed to explore the mechanism

of action of *Andrographis paniculata* in ameliorating the toxic effects of cyclophosphamide induced oxidative damage.

Fig. 5.1: Effect of CP + AP on Lipid peroxidation in liver of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	221.4 ± 1.95	204.5 ± 5.37	192 ± 3.46
2.	Group II	228 ± 1.87	232 ± 2.45	236.8 ± 2.28
3.	Group III	242.6 ± 1.67	244 ± 2.71	250.2 ± 3.86
4.	Group IV	345.2 ± 3.27	308 ± 5.02	305 ± 2.5
5.	Group V	222 ± 1.95	204 ± 5.35	192.5 ± 3.45

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

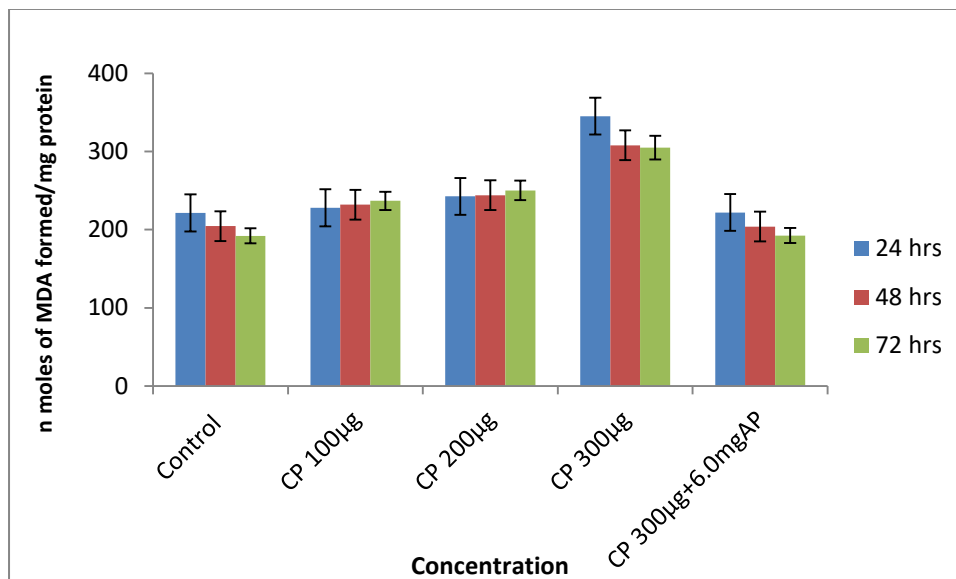


Fig. 5.2. Effect of CP + AP on lipid peroxidation in heart of 15-day old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	223.4 ± 1.52	209.5 ± 5.37	204.4 ± 2.6
2.	Group II	229.2 ± 1.58	232 ± 2.16	236.8 ± 2.25
3.	Group III	242.6 ± 1.67	244 ± 2.71	250.2 ± 3.86
4.	Group IV	345.2 ± 3.27	308 ± 5.02	305 ± 2.5
5.	Group V	223 ± 1.55	209 ± 5.34	204 ± 2.55

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

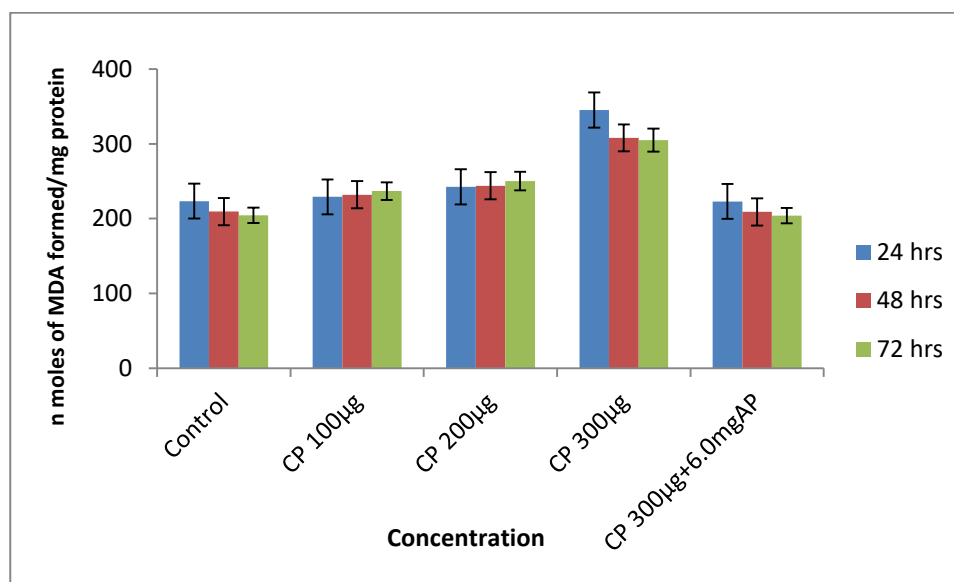


Fig.5.3. Effect of CP + AP on Lipid peroxidation in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	223.4 ± 1.52	209.5 ± 5.37	204.4 ± 2.6
2.	Group II	229.2 ± 1.58	232 ± 2.16	236.8 ± 2.25
3.	Group III	242.6 ± 1.67	244 ± 2.71	250.2 ± 3.86
4.	Group IV	345.2 ± 3.27	308 ± 5.02	305 ± 2.5
5.	Group V	224.6 ± 1.54	211.5 ± 5.3	205.4 ± 2.64

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

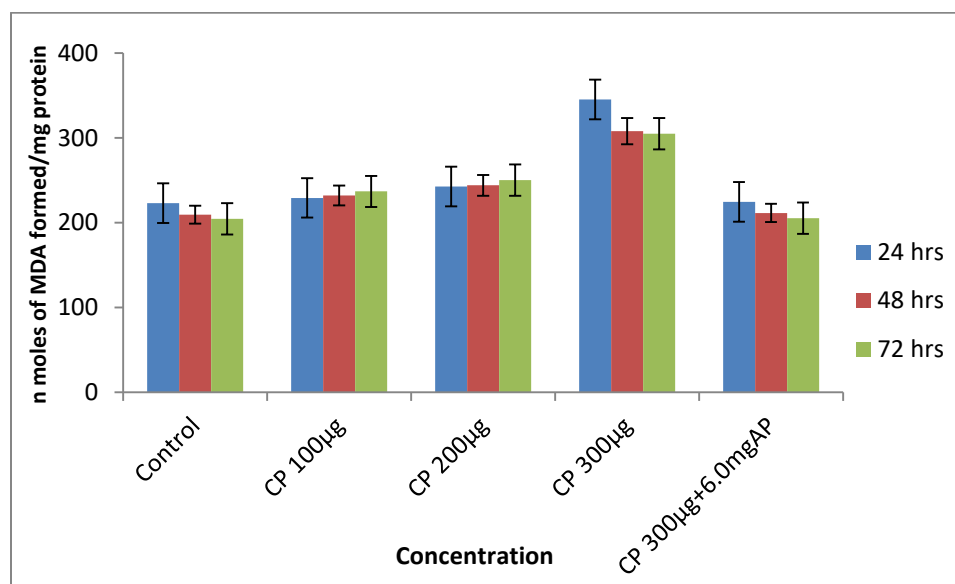


Fig 5.1, 5.2 and 5.3 represent the effects of ethanolic extracts of cyclophosphamide + *Andrographis paniculata* in the LPO levels in cyclophosphamide treated with liver, brain and heart in chick embryo.

In control LPO levels were decreased with duration. Cyclophosphamide treated to normal embryos increased LPO levels significantly at 24hrs, 48hrs, and 72hrs duration in liver, heart and brain. Treatment of *Andrographis paniculata* 72hrs prior to cyclophosphamide administration significantly decreased the LPO levels at 24, 48 and 72 hrs compare to cyclophosphamide treatment. LPO levels come down to more are less to normal levels.

Fig. 5.4. Effect of Cyclophosphamide + *Andrographis paniculata* on SOD (Super Oxide Dismutase) activity in liver of 15-day-old chick embryo.

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	Group II	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	Group III	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	13.1 ± 0.25	13.8 ± 0.17	14.4 ± 0.32

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

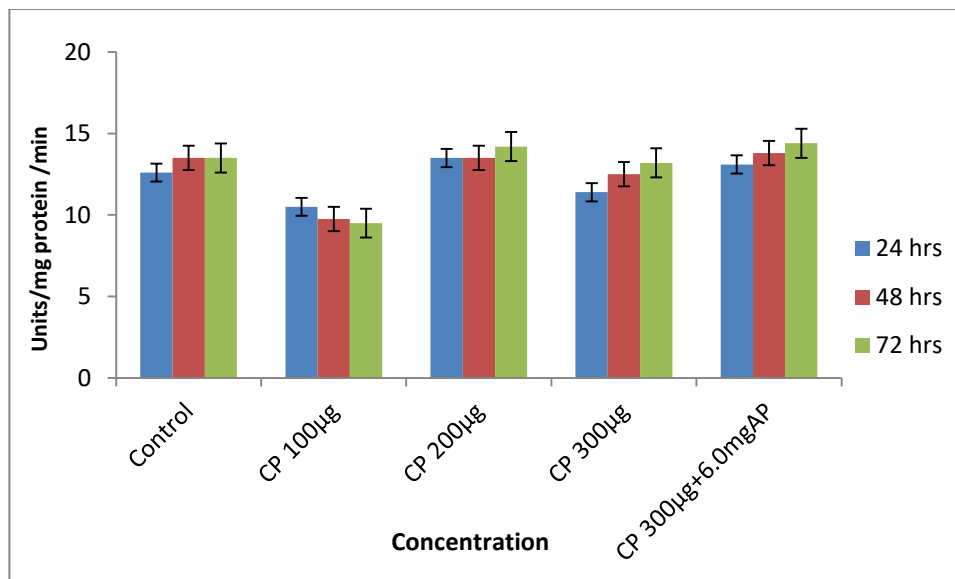


Fig. 5.5. Effect of cyclophosphamide + *Andrographis paniculata* on SOD activity in heart of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	Group II	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	Group III	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	13.2 ± 0.24	13.7 ± 0.15	14.2 ± 0.31

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

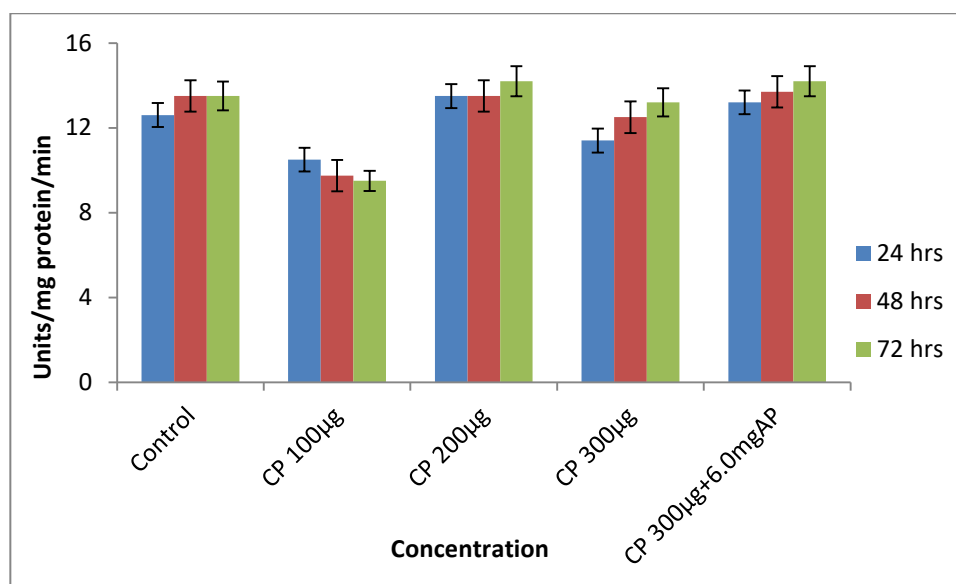


Fig. 5.6. Effect of cyclophosphamide + *Andrographis paniculata* on SOD activity in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	Group II	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	Group III	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	12.5 ± 0.27	13.5 ± 0.15	14.8 ± 0.33

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

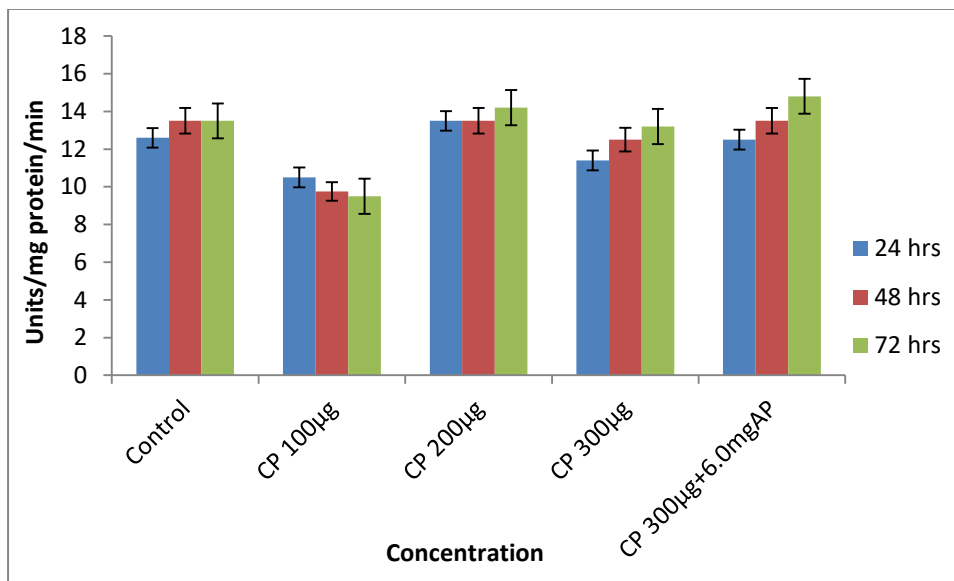


Fig. 5.4, 5.5 and 5.6 illustrate the Protective effect of AP on SOD activity in liver, brain and heart with CP treated embryo.

Fig. 5.7. Effect of CP + AP on CAT (Catalase) activity in liver of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	10.45 ± 0.25	11.5 ± 0.17	12.5 ± 0.15
2.	Group II	6.25 ± 0.14	5.75 ± 0.35	5.3 ± 0.33
3.	Group III	11.4 ± 0.22	12.5 ± 0.12	13.2 ± 0.14
4.	Group IV	13.4 ± 0.24	13.2 ± 0.14	13.5 ± 0.24
5.	Group V	11.5 ± 0.22	12.2 ± 0.15	13.5 ± 0.17

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

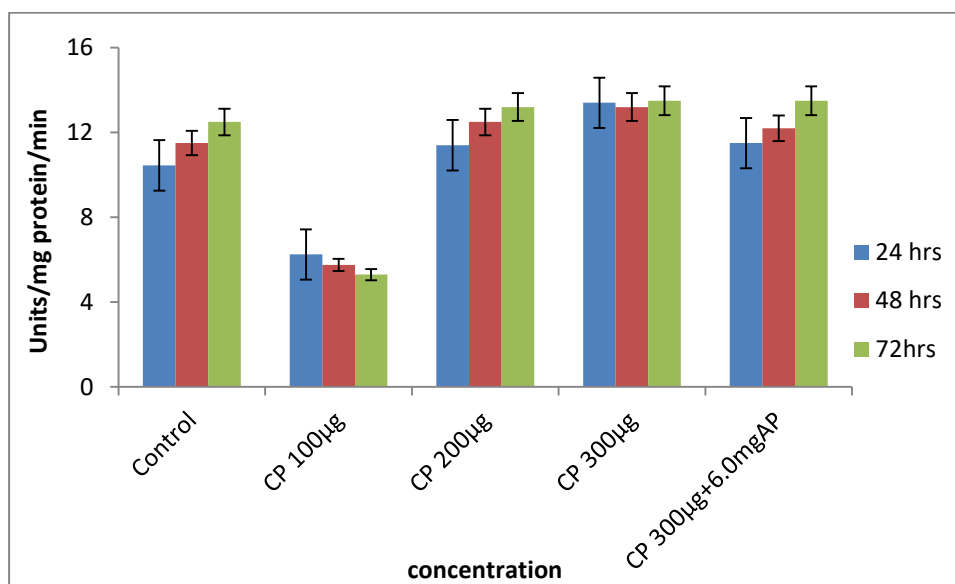


Fig. 5.8. Effect of CP + AP on CAT activity in heart of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	4.6 ± 0.22	5.5 ± 0.17	6.5 ± 0.25
2.	Group II	4.5 ± 0.22	3.75 ± 0.14	1.85 ± 0.07
3.	Group III	5.4 ± 0.19	5.8 ± 0.02	6.2 ± 0.22
4.	Group IV	15.5 ± 0.25	6.5 ± 0.11	8.8 ± 0.32
5.	Group V	4.5 ± 0.27	5.7 ± 0.15	6.8 ± 0.26

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

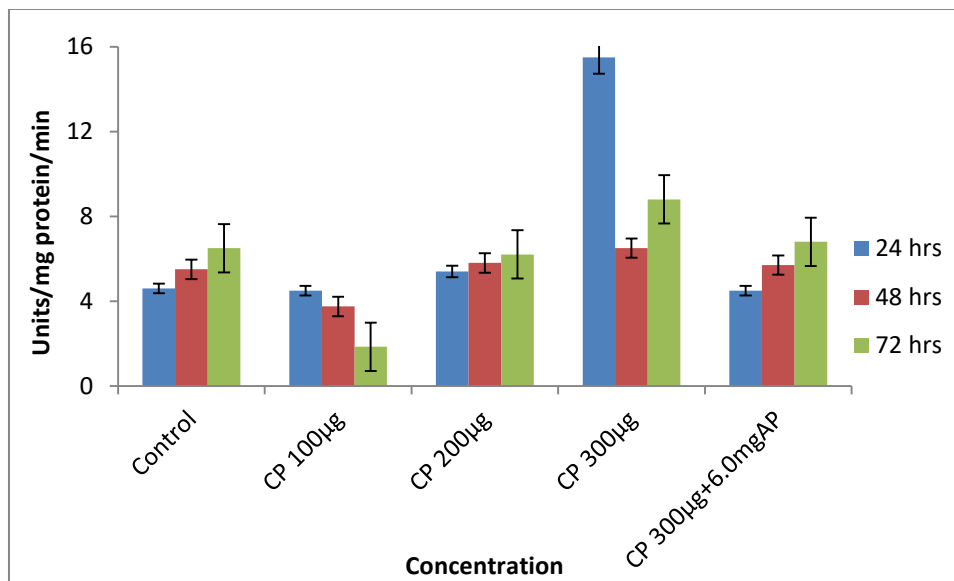


Fig. 5.9. Effect of CP + AP on CAT activity in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	6.61 ± 0.18	7.5 ± 0.55	8.5 ± 0.35
2.	Group II	5.5 ± 0.22	4.75 ± 0.04	3.5 ± 0.07
3.	Group III	7.5 ± 0.24	7.65 ± 0.06	8.9 ± 0.14
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	6.62 ± 0.2	7.75 ± 0.35	9.8 ± 0.33

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

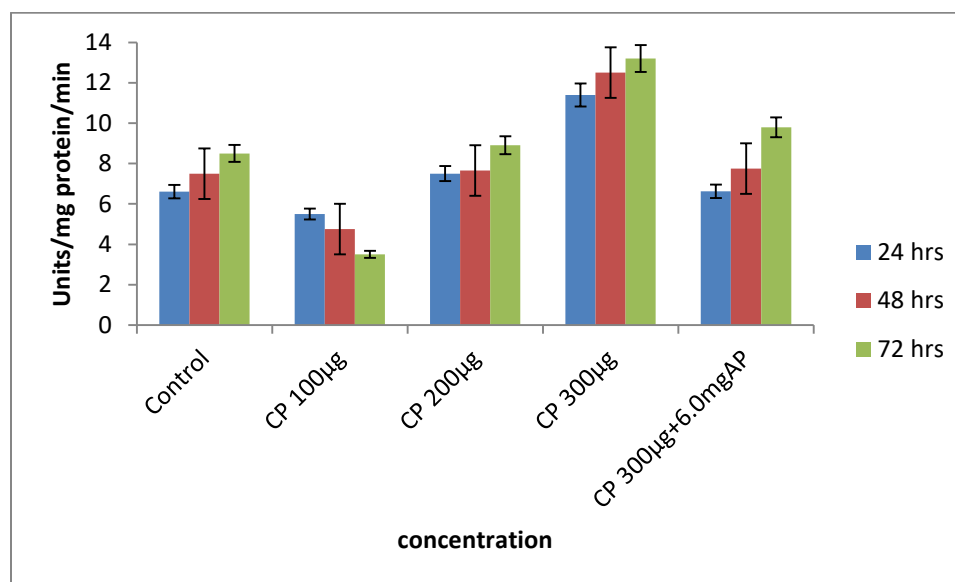


Fig 5.7, 5.8 and 5.9 corresponds to the effect of on CAT activity.

Fig. 5.10. Effect of CP + AP on GST (Glutathione-S-transferase) activity in liver of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	51.6 ± 0.61	53.5 ± 0.75	55.5 ± 0.54
2.	Group II	56.6 ± 0.35	60 ± 0.75	65.5 ± 1.41
3.	Group III	60.5 ± 0.62	62.5 ± 0.75	65.2 ± 0.15
4.	Group IV	62.4 ± 0.75	64.5 ± 0.76	16.2 ± 0.18
5.	Group V	51.5 ± 0.65	53.3 ± 0.73	55.8 ± 0.57

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

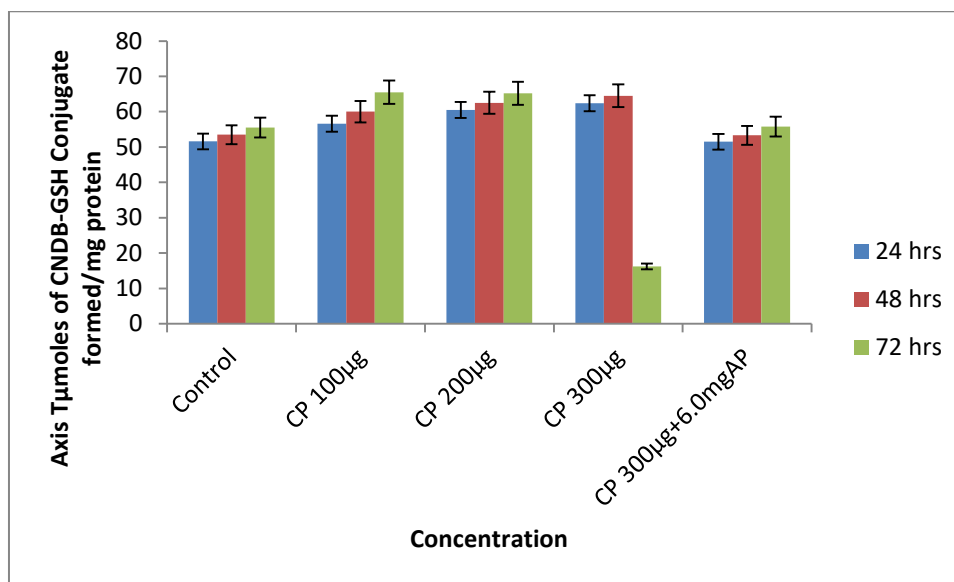


Fig. 5.11. Effect of CP + AP on GST activity in heart of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	Group II	13.5 ± 0.25	13.75 ± 0.26	14.4 ± 0.19
3.	Group III	14.5 ± 0.22	14.5 ± 0.17	14.8 ± 0.22
4.	Group IV	15.4 ± 0.26	15.7 ± 0.24	16.2 ± 0.24
5.	Group V	13.5 ± 0.27	13.7 ± 0.15	14.1 ± 0.23

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

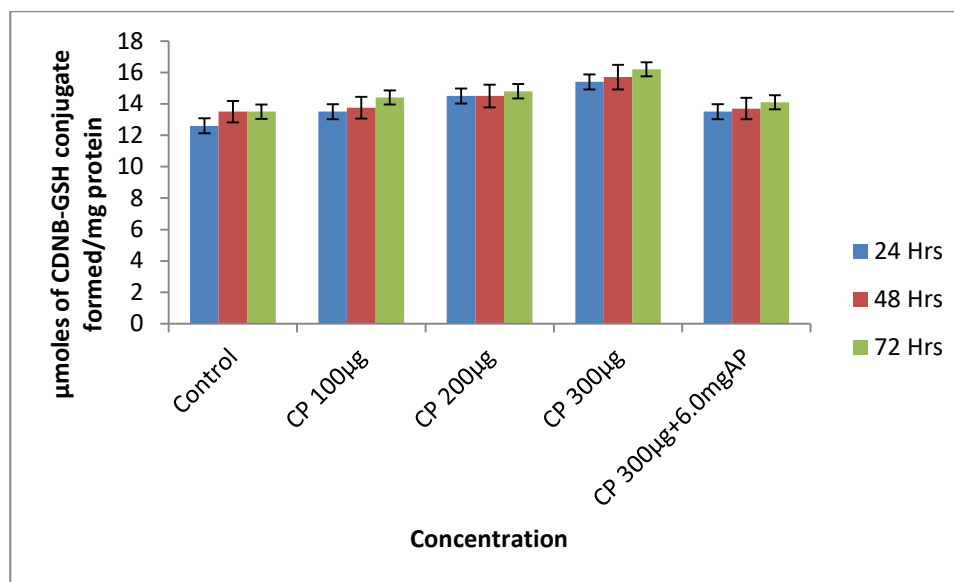


Fig.5.12. Effect of CP + AP on GST activity in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	8.6 ± 0.1	9.5 ± 0.25	16.1 ± 0.35
2.	Group II	10.25 ± 0.35	11.7 ± 0.31	12.4 ± 0.32
3.	Group III	12.5 ± 0.22	12.5 ± 0.18	13.2 ± 0.12
4.	Group IV	13.4 ± 0.24	13.35 ± 0.21	13.7 ± 0.24
5.	Group V	8.8 ± 0.27	10.2 ± 0.15	16.8 ± 0.33

Group I: Control (Saline);

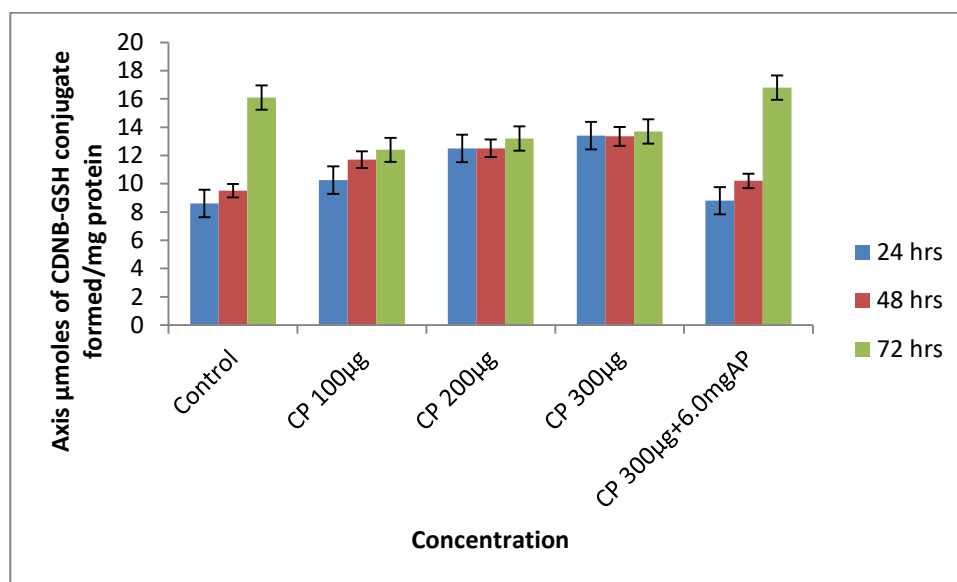
Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)



The effect of CP + AP on GST activity was seen Fig 5.10, 5.11, 5.12.

Fig.5.13. Effect of CP + AP on GPx (Glutathione Peroxidase) activity in liver of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	11.5 ± 0.25	13.5 ± 0.32	15.4 ± 0.15
2.	Group II	8.6 ± 0.12	8.8 ± 0.15	10.5 ± 0.21
3.	Group III	10.5 ± 0.22	11.5 ± 0.12	12.2 ± 0.24
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	9.5 ± 0.16	12.7 ± 0.18	14.9 ± 0.11

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

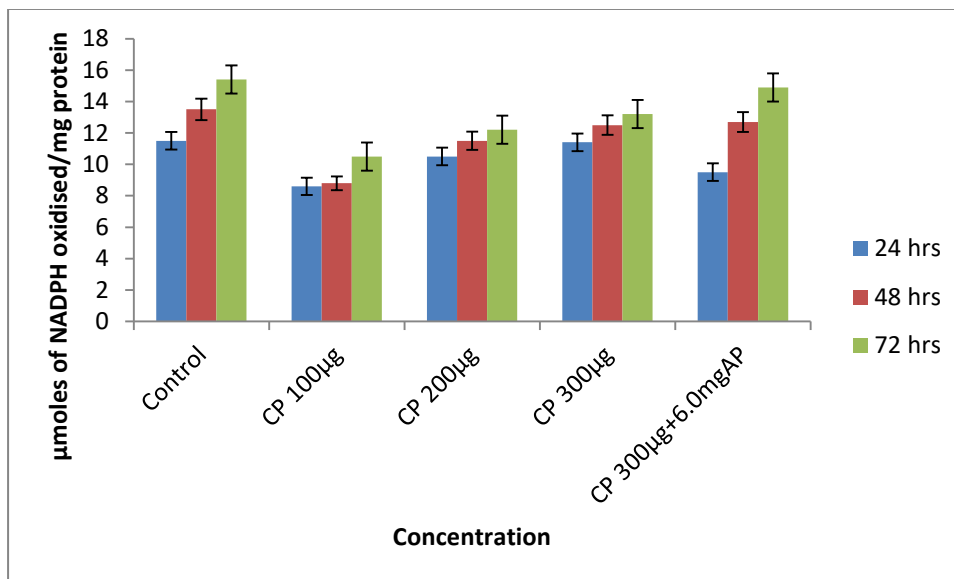


Fig. 5.14. Effect of CP + AP on GPx activity in heart of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	8.6 ± 0.26	9.5 ± 0.16	9.5 ± 0.15
2.	Group II	7.5 ± 0.11	7.75 ± 0.18	6.5 ± 0.25
3.	Group III	9.5 ± 0.1	10.5 ± 0.12	10.2 ± 0.18
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	9.5 ± 0.27	10.5 ± 0.15	12.8 ± 0.33

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

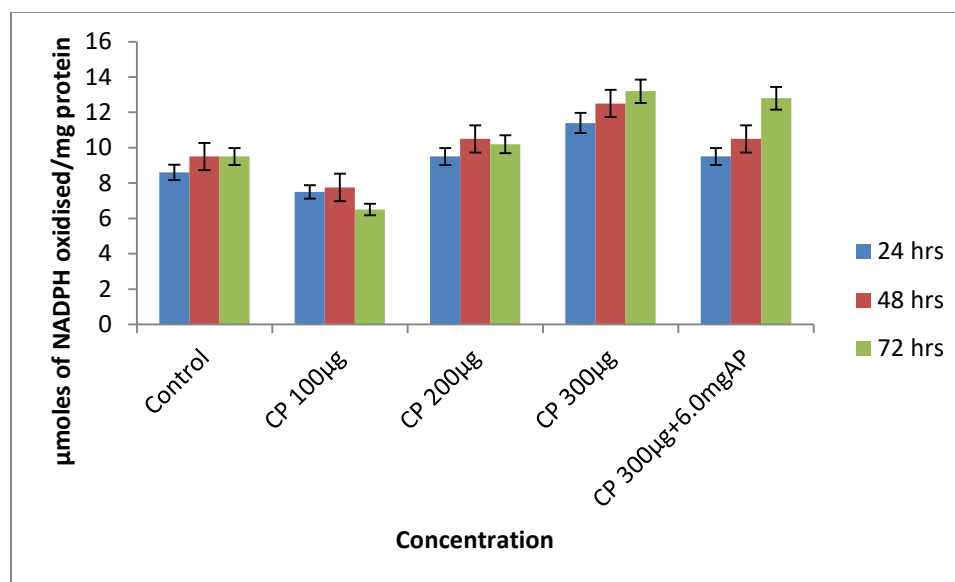


Fig. 5.15. Effect of CP + AP on GPx activity in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	3.6 ± 0.26	5.5 ± 0.28	6.5 ± 0.31
2.	Group II	2.5 ± 0.1	2.75 ± 0.33	2.5 ± 0.25
3.	Group III	4.5 ± 0.12	6.5 ± 0.22	7.2 ± 0.34
4.	Group IV	7.4 ± 0.24	8.2 ± 0.11	9.2 ± 0.14
5.	Group V	3.5 ± 0.22	5.7 ± 0.25	6.8 ± 0.33

Group I: Control (Saline);

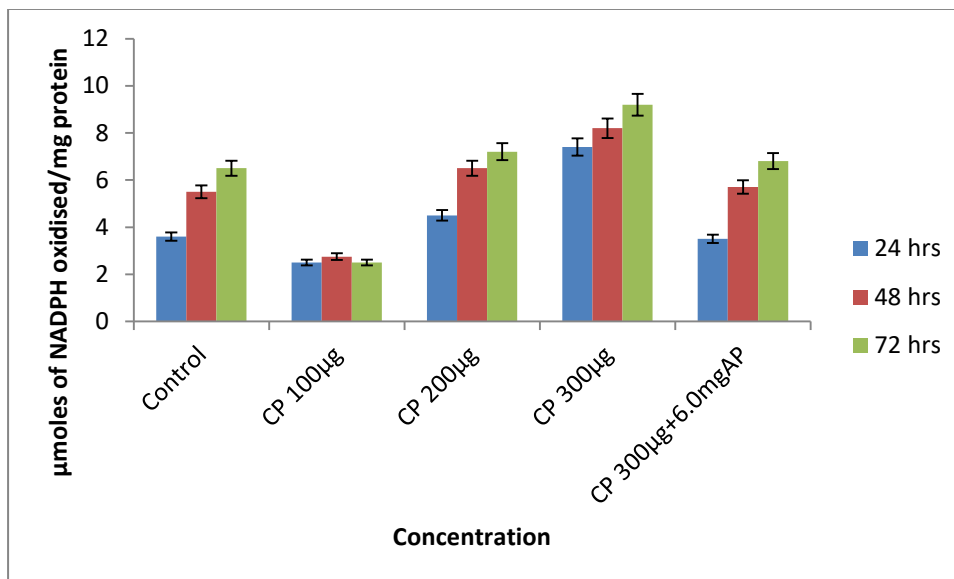
Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)



The effect of CP + AP on GPx activity was shown in Fig 5.13, 5.14, 5.15.

Fig. 5.16. Effect of CP + AP on GSH (Glutathione Reduced) activity in liver or 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	31.6 ± 1.02	35.5 ± 0.7	38.5 ± 0.19
2.	Group II	22.5 ± 0.21	24.75 ± 0.11	19.5 ± 0.67
3.	Group III	24.5 ± 0.26	25.5 ± 0.22	21.2 ± 0.24
4.	Group IV	25.4 ± 0.2	27.5 ± 0.17	23.2 ± 0.18
5.	Group V	31.4 ± 1.07	35.5 ± 0.75	39.8 ± 0.23

Group I: Control (Saline); Group II: CP 100µg Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg; Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

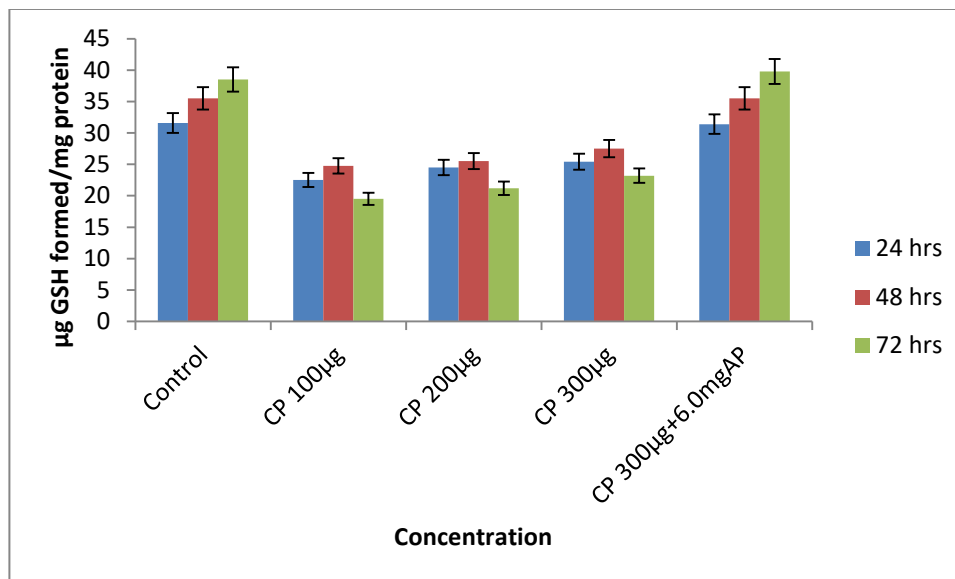


Fig. 5.17. Effect of CP + AP on GSH activity in heart of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	12.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	Group II	10.5 ± 0.15	9.75 ± 0.11	9.5 ± 0.17
3.	Group III	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	12.5 ± 0.27	13.5 ± 0.15	14.8 ± 0.33

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

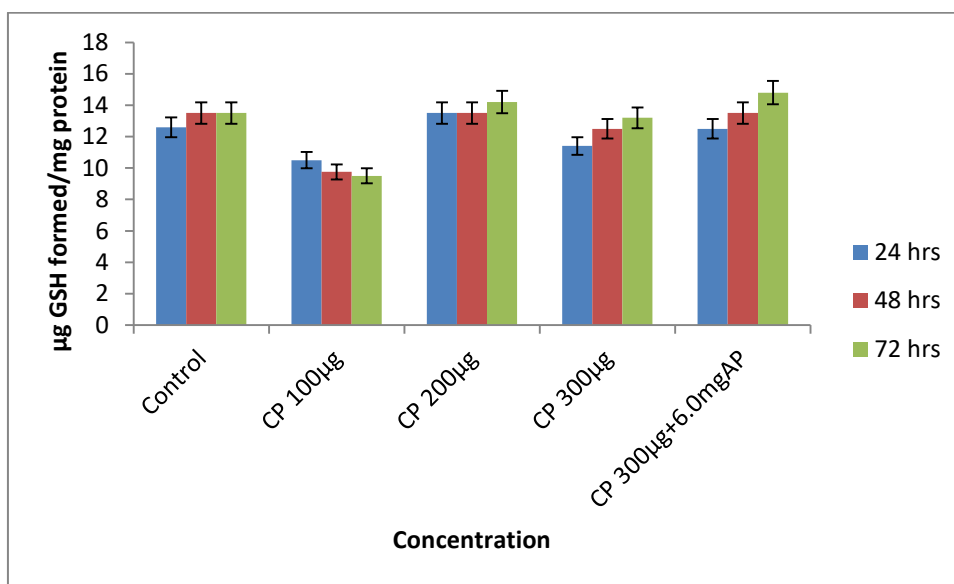


Fig. 5.18. Effect of CP + AP on GSH activity in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	8.6 ± 0.28	9.5 ± 0.12	11.5 ± 0.15
2.	Group II	7.5 ± 0.15	7.3 ± 0.11	7.5 ± 0.2
3.	Group III	9.5 ± 0.17	10.5 ± 0.16	12.2 ± 0.18
4.	Group IV	11.4 ± 0.2	12.5 ± 0.11	13.2 ± 0.14
5.	Group V	9.5 ± 0.24	10.1 ± 0.15	11.8 ± 0.18

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

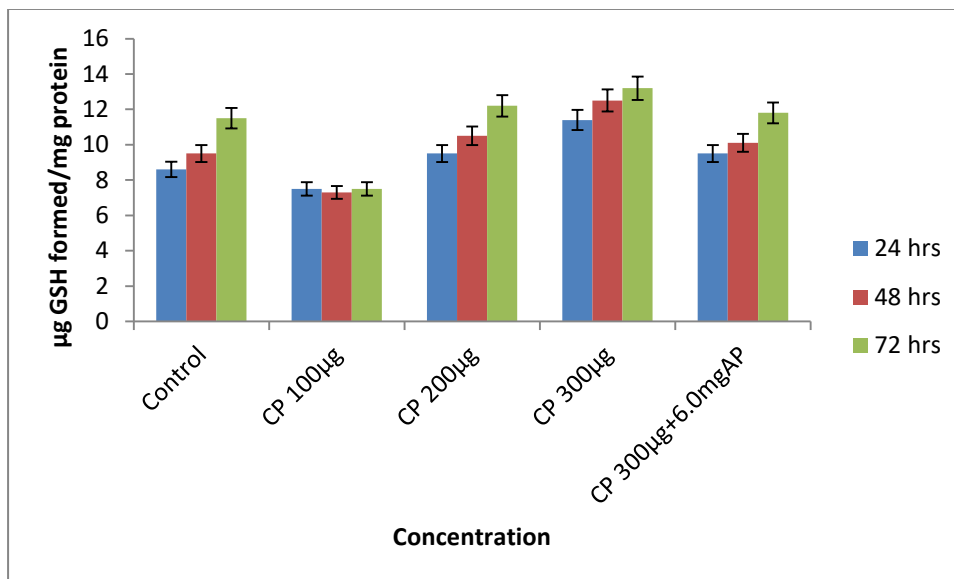


Fig 5.16, 5.17 and 5.18 represent the effect of CP + AP on GSH activity.

Fig. 5.19. Effect of CP + AP on GR (Glutathione Reductase) activity in liver of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	10.6 ± 0.2	13.5 ± 0.15	13.5 ± 0.25
2.	Group II	6.5 ± 0.15	9.75 ± 0.11	12.5 ± 0.17
3.	Group III	13.5 ± 0.22	13.5 ± 0.12	14.2 ± 0.24
4.	Group IV	14.4 ± 0.2	13.8 ± 0.11	14.8 ± 0.14
5.	Group V	11.5 ± 0.27	14.1 ± 0.15	13.8 ± 0.33

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

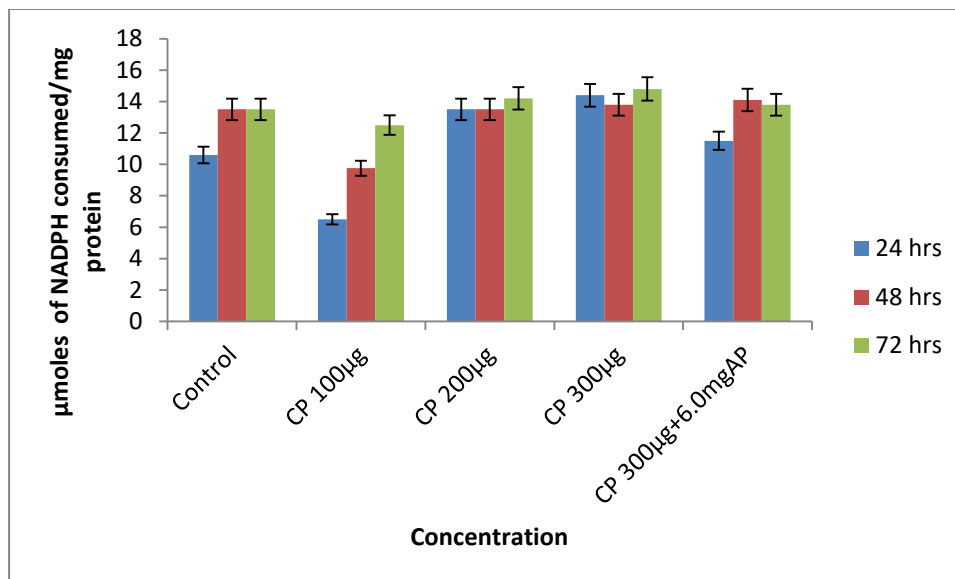


Fig. 5.20. Effect of CP + AP on GR activity in heart of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	7.6 ± 0.12	8.5 ± 0.19	10.5 ± 0.25
2.	Group II	5.5 ± 0.11	6.75 ± 0.16	7.5 ± 0.13
3.	Group III	4.5 ± 0.12	5.5 ± 0.11	6.2 ± 0.3
4.	Group IV	4.4 ± 0.2	4.5 ± 0.11	6.2 ± 0.14
5.	Group V	7 ± 0.1	7.8 ± 0.26	10.8 ± 0.24

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

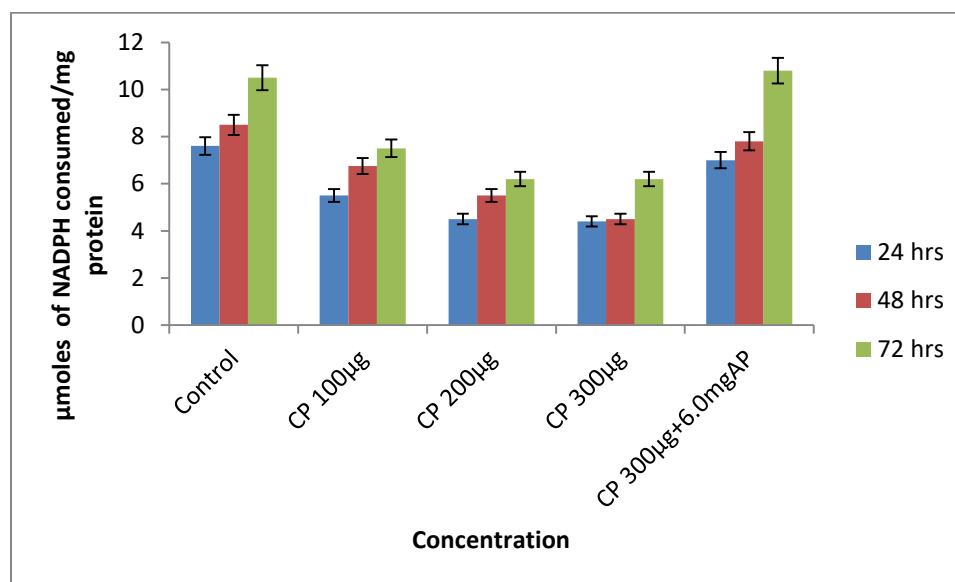


Fig. 5.21. Effect of CP + AP on GR activity in brain of 15-day-old chick embryo

Sl. No.		24 hrs	48 hrs	72 hrs
1.	Group I	9.5 ± 0.21	12.3 ± 0.1	13.1 ± 0.25
2.	Group II	7.5 ± 0.05	10.75 ± 0.21	12 ± 0.22
3.	Group III	6.8	9.5	12
4.	Group IV	5.4 ± 0.11	9.5 ± 0.16	10.2 ± 0.25
5.	Group V	10 ± 0.23	12.8 ± 0.11	13.1 ± 0.24

Group I: Control (Saline);

Group II: CP 100µg

Group V: 300 µg CP + 6.0 mg AP

Group III: CP 200 µg;

Group IV: CP 300 µg

Values are average of six sets of separate experiments (Mean ± SD)

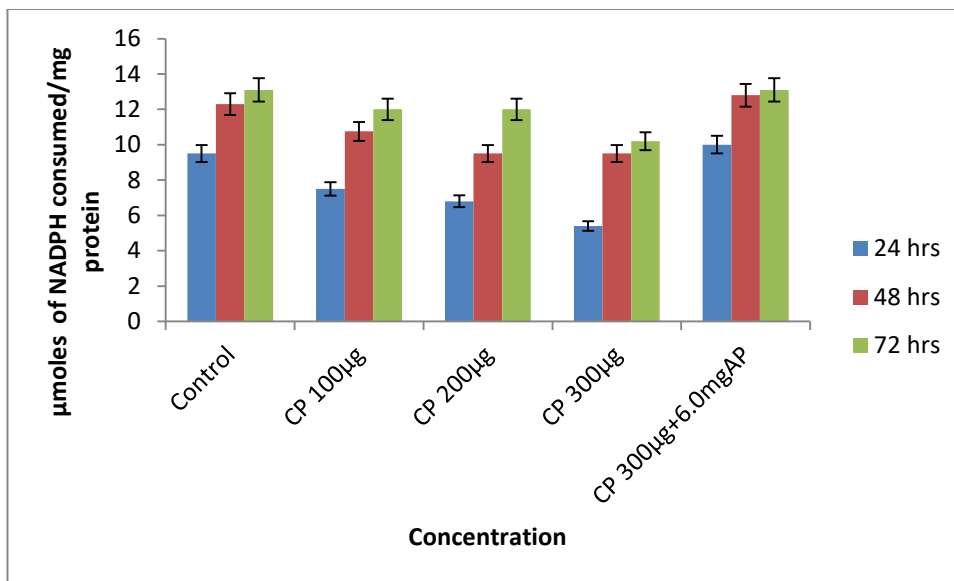


Fig 5.19, 5.20 and 5.21 corresponds to the effect of CP + AP extract on GR activity

HISTOPATHOLOGICAL STUDIES WITH CYCLOPHOSPHAMIDE AND PRE-TREATED ANTIOXIDANT IN CHICK EMBRYO

Histological variations in liver, heart and brain shown in Fig. 6.1 to 6.9. The protective effect of AP on CP induced damage was seen clearly.

Fig.6.1. Liver Control Section showing normal hepatic cells H & EX-280

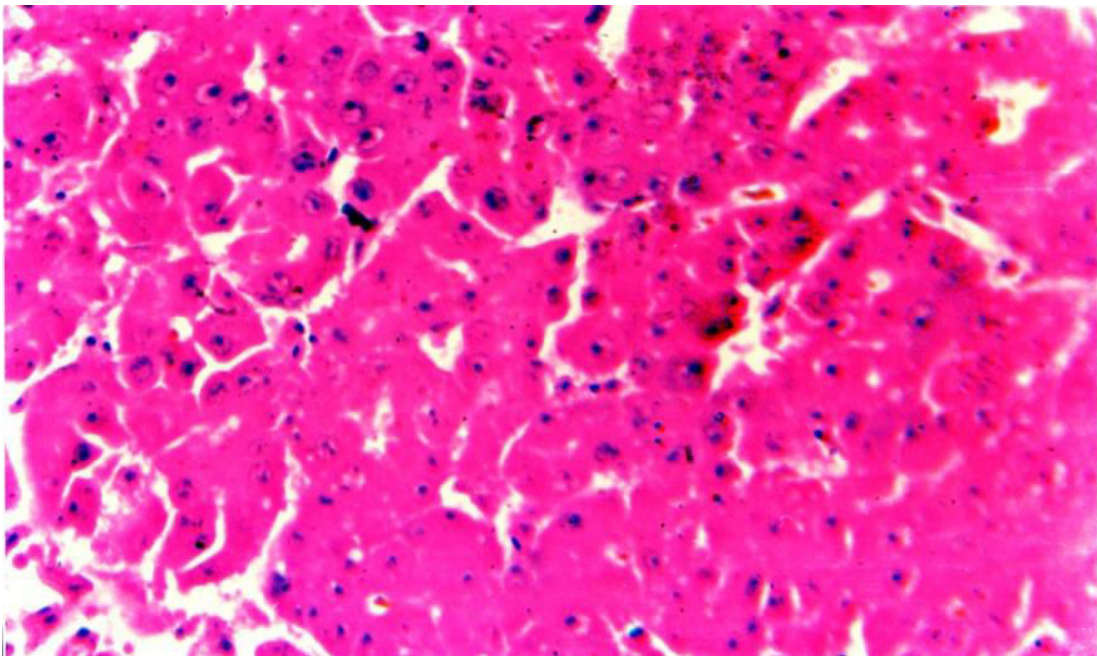


Fig. 6.2. Liver: 300µg of CP. Focal mono nuclear Cell infiltration. H & E.X-280.

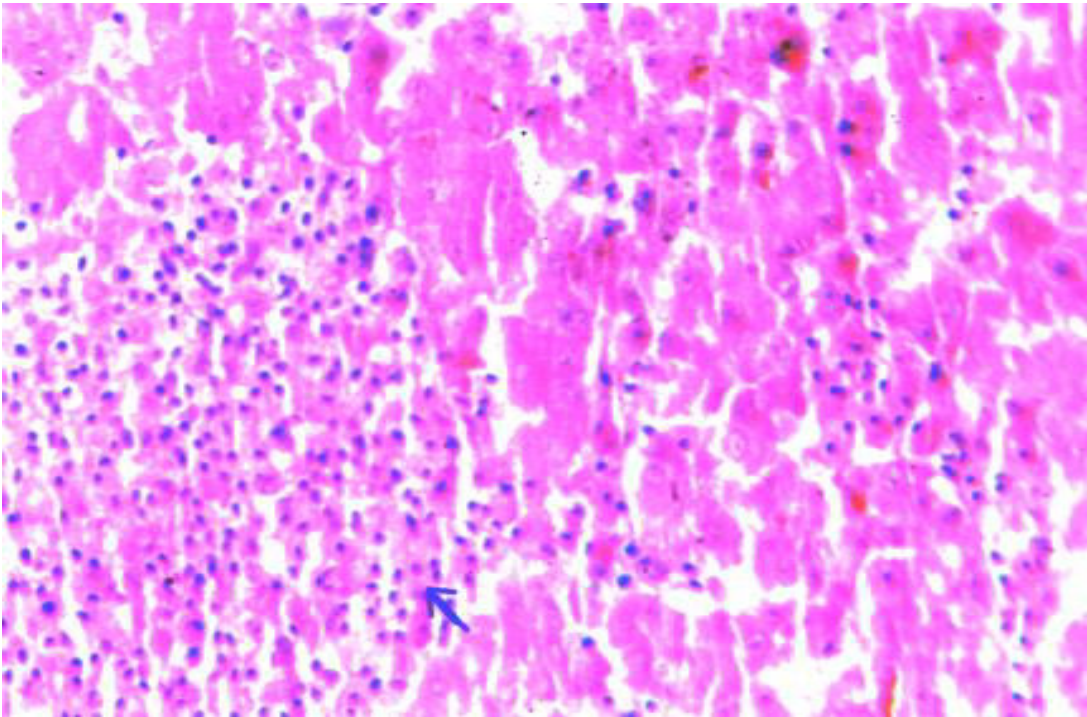


Fig. 6.3. Liver: 300 μ g CP + 6.0mg AP. Section showing Near normal. H & E.X-280

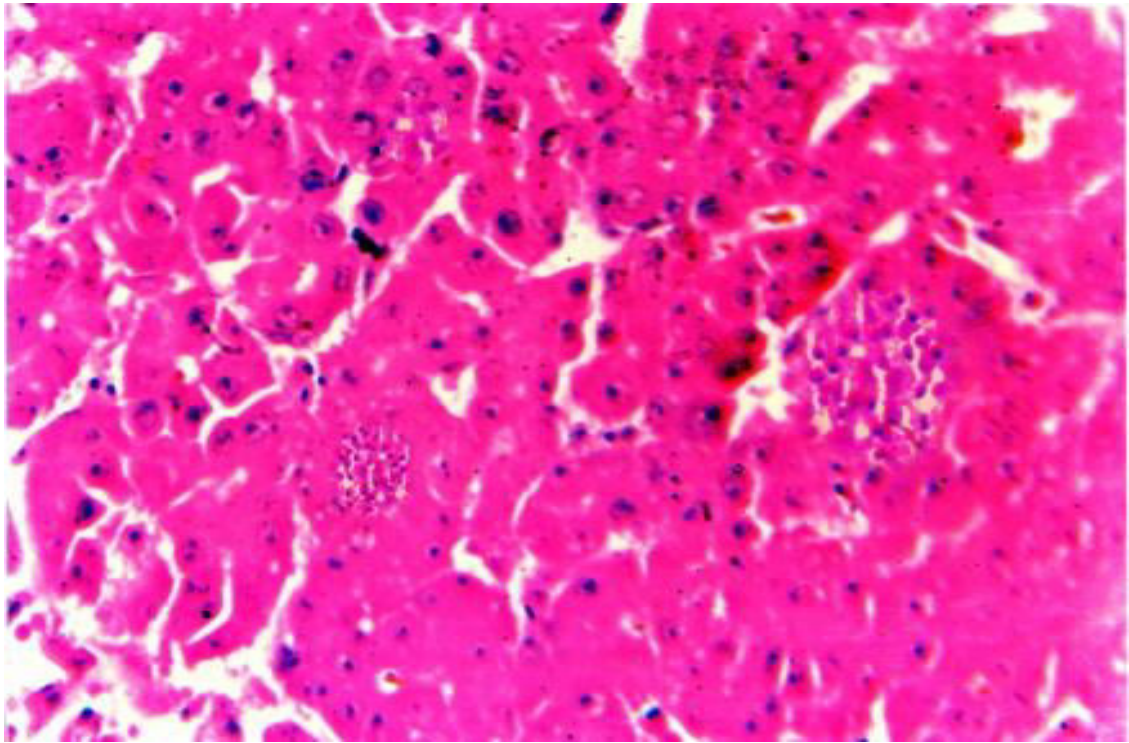


Fig. 6.4. Heart: Control Section showing normal cardiac muscle fibres. H & E.X-280

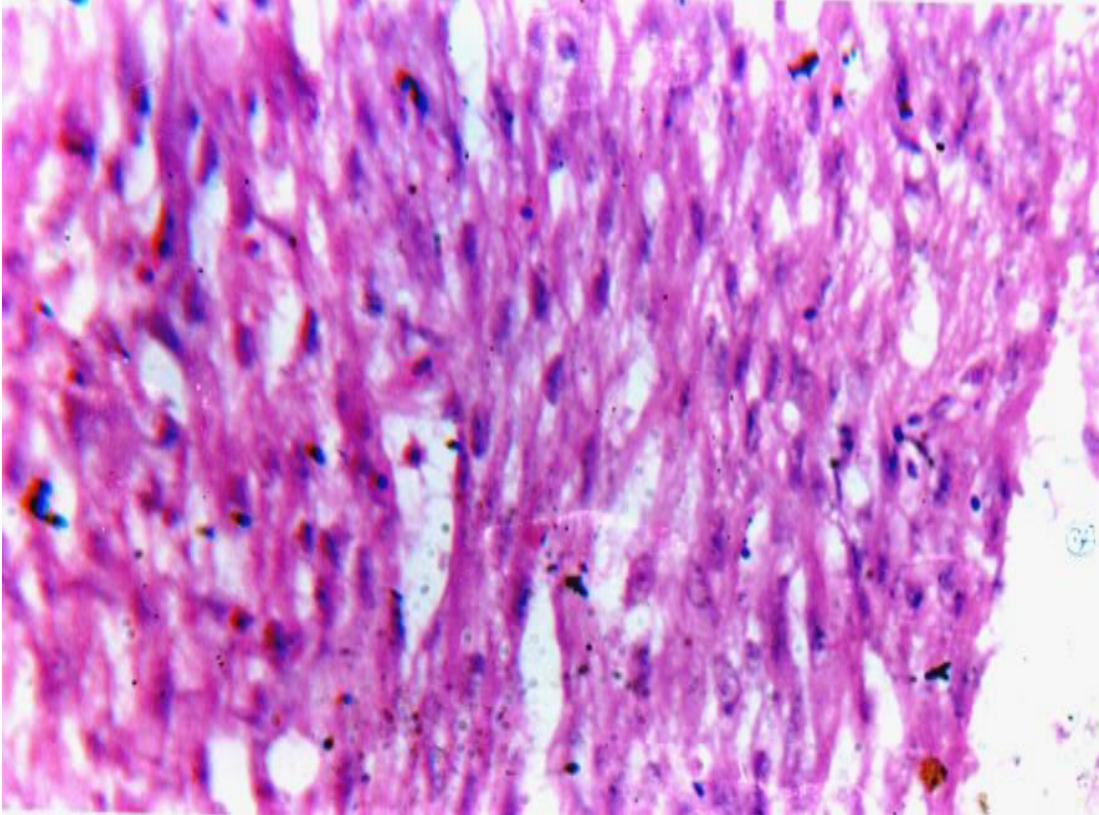


Fig. 6.5. 300µg CP. Section showing severe Sarcolysis and mild infiltration of inflammatory cells, H & E.X-280

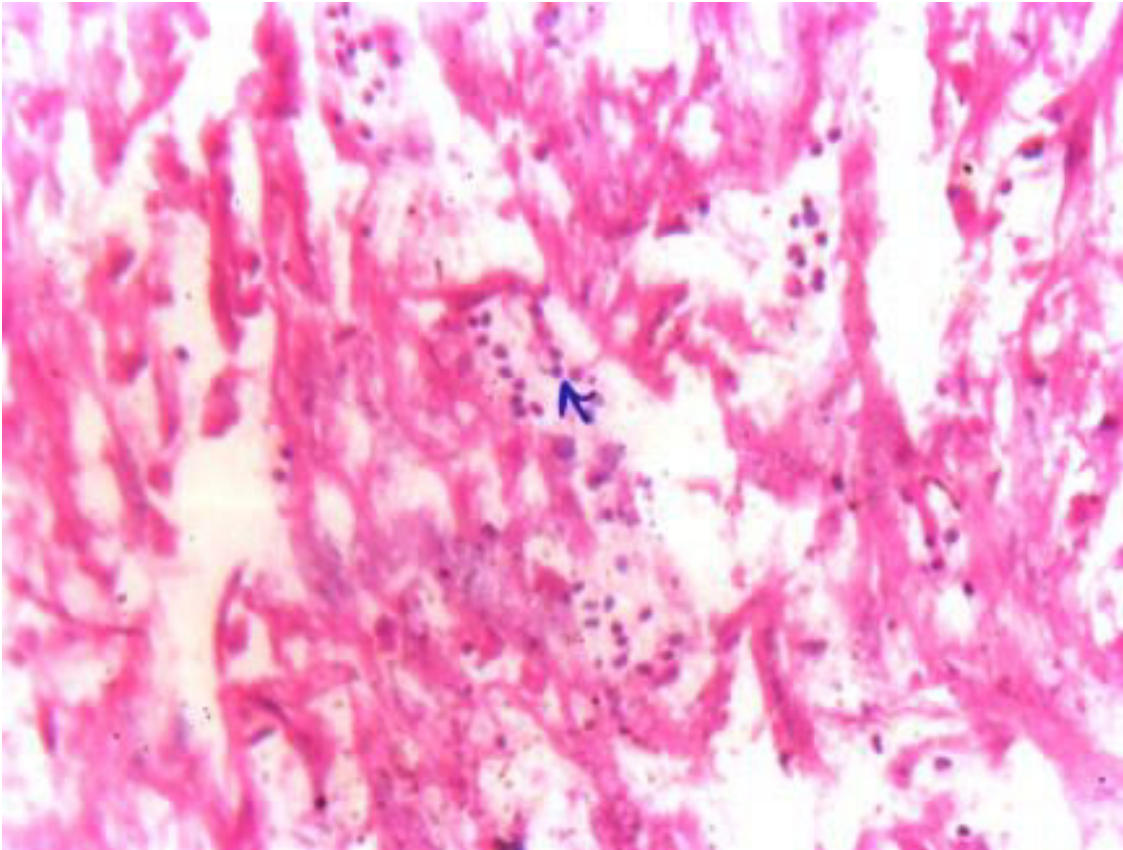


Fig. 6.6. Heart: 300 μ g CP + 6.0mg AP. Section showing near normal appearance.H & E.X-280

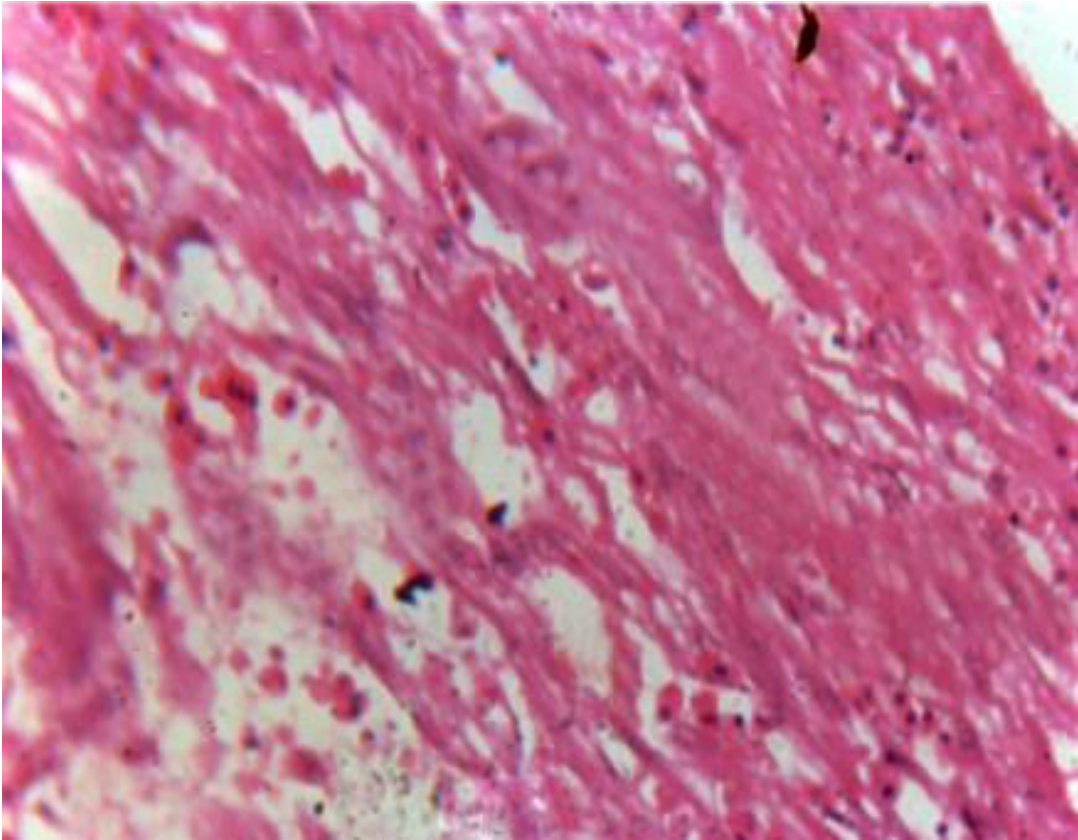


Fig. 6.7. Brain: Control showing normal brain cells, H & E.X-280

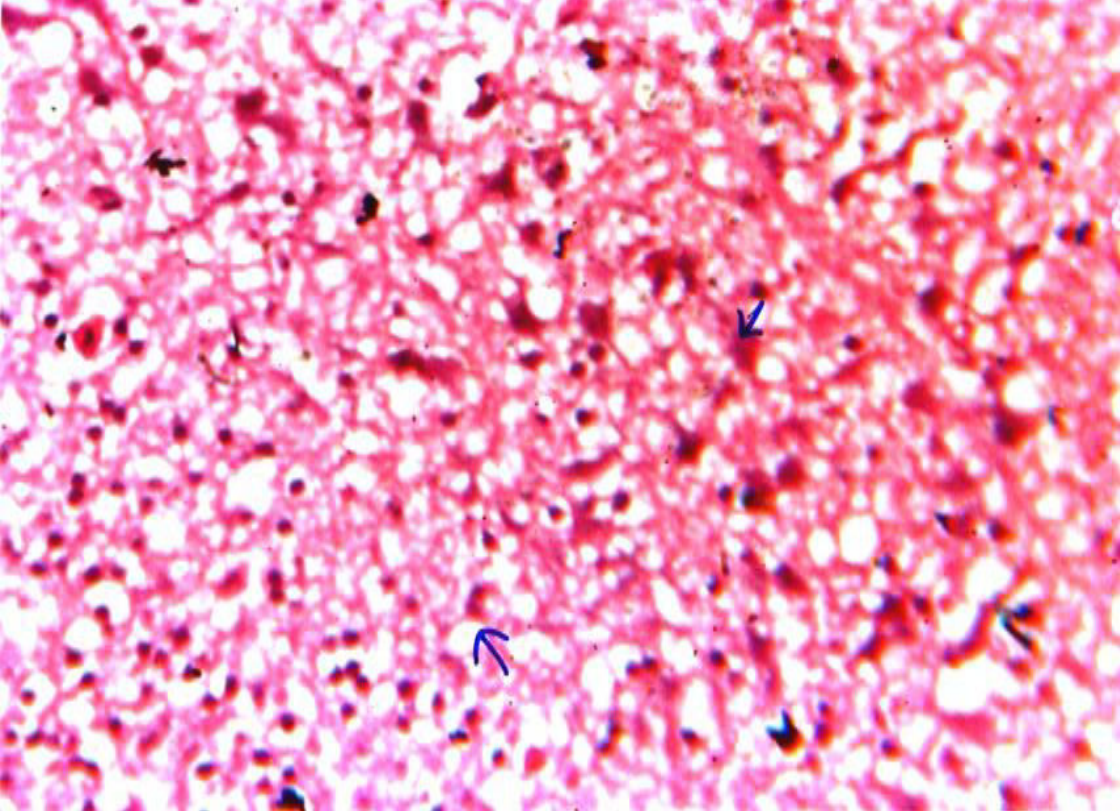


Fig. 6.8. Brain: 3.0 mg CA. Section showing severe congestion, H & E.X-280

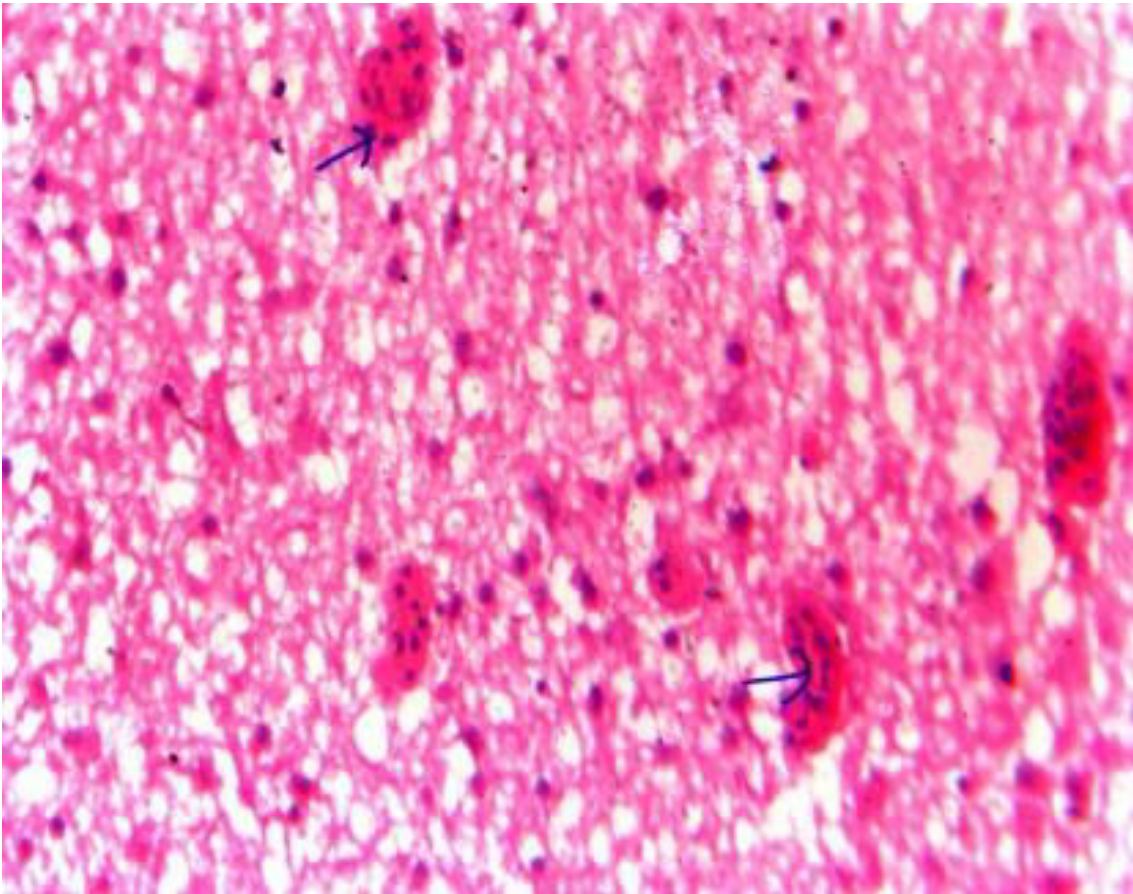
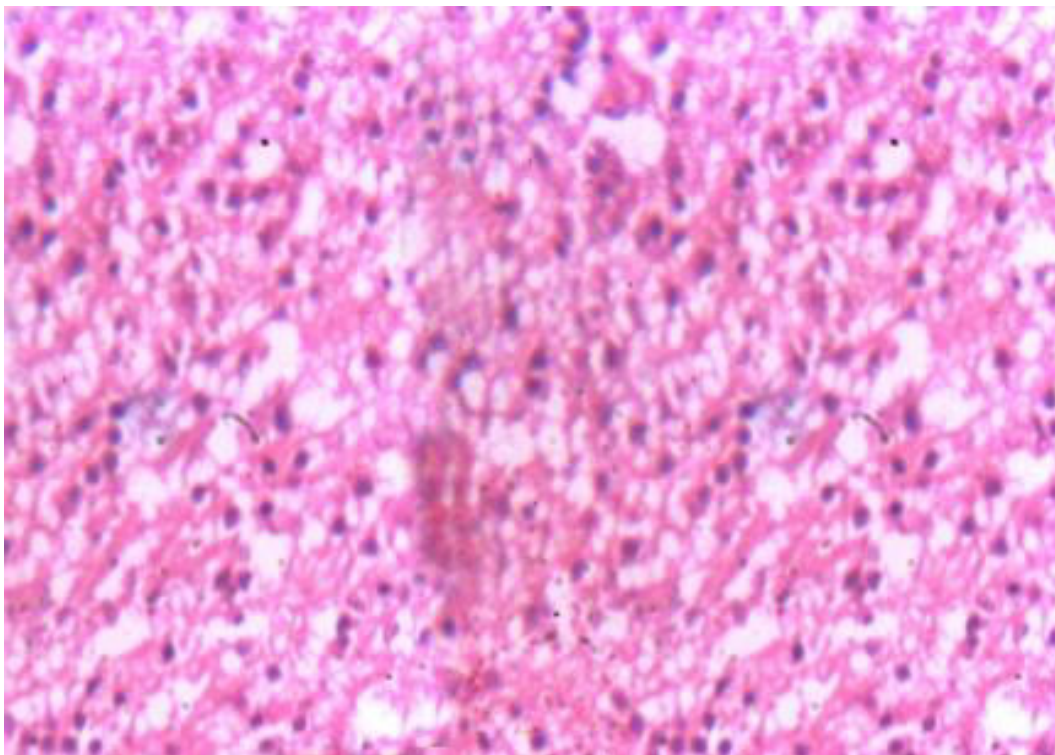


Fig. 6.9. Brain: 6.0 mg BM. Section showing near normal, H & E.X-280



Histological variations in liver, heart and brain shows the protective effect of AP on CP induced damage were seen clearly in fig 6.1- 6.9.

DISCUSSION

The present study deals with evaluation of the protective effect of AP extract on CP induced oxidative damage in chick embryo. Many natural products are reported to influence the antioxidant system and are good Cytoprotective agents (Dragsted et al., 1997). Hence AP extract was subjected to preliminary investigation to identify its influencing role on antioxidant system in normal chick embryo at various concentrations. The concentration in which there was maximum elevation in GSH and GST was taken as the optimum dosage. AP has been identified to exhibit significant anti-oxidative and anti-lipid peroxidative property (Tirupati *et al.*, 1996). The antioxidative and anti lipid peroxidative property was observed in AP treated fibrosarcoma rats (Rohini *et al.*, 2004).

The antioxidant potential of the AP extract in rat brain by increasing the activities of SOD, CA and GPx was demonstrated (Bhattacharya et al., 2000). The alcoholic extract of AP showed greater protection with FeSO₄ and Cumen hydroperoxide induced lipid peroxidation (Tirupati *et al.*, 1996). The alcoholic extract of AP induced a significant increase of lipid peroxidation and a significant decrease in liver antioxidant enzymes in the morphine treated rats for hepatoprotection (Sumathy *et al.*, 2001). Its role as a free radical scavenger and the redox interrelationship between as a free radical scavenger and the redox inter-relationship between *Andrographis paniculata* and other antioxidants are in the focus of continuous interest.

Previous studies on the in vitro anti-lipid peroxidative activity of AP extract suggested that it may act as a chain breaking antioxidant, presumably as a free radical scavenger (Yamini *et al.*, 1996). AP exhibits neuro protective and cognitive enhancing effects, in part due to its capacity to modulate the cholinergic system (Bhattacharya et al., 1999) and to control oxidative stress (Bhattacharya et al., 2000, Russo et al., 2003 a, b). AP is able to directly inhibit the superoxide anion formulation (Russo and Borelli, 2005). AP prevent variety of neurodegenerative diseases associated with reduced the intracellular oxidants, consequently preventing DNA damage (Colasanti and Suzuki 2000; Russo et al., 2003b).

Free radicals and active oxygen species appear to have broader significance in the production of tissue injury under conditions of oxidative stress (Atlri et al., 2001). They are now well recognized in the pathogenesis of various diseases such as cancer, atherosclerosis, hepatitis, inflammation, diabetes, arthritis and ageing process (Moko Aniya et al., 2003).

There is an increased lipid peroxide levels with CP treatment which were later decreased significantly by AP pre-treatment in liver, brain and heart tissues. This indicates that AP is able to ameliorate the toxic effect of CP. AP scavenges the ROS by rapid electron transfer that inhibits lipid peroxidation.

Peroxidation of unsaturated lipid membranes by free radicals is known to contribute to the toxic side effects observed following administration of CP.

CP treatment to 15-day -old chick embryo significantly decreased SOD and CAT activity within 24 h in liver, brain and heart. The decreased enzyme levels were enhanced on pre-treatment with AP extract. The function of SOD is to protect the cells against the high chemical reactivities of various oxygen derived radicals (Fridovich, 1986). SOD catalyzes to scavenging excess superoxide anions and converts them to hydrogen peroxide, which in turn either detoxified by CAT or GSH dependant reactions.

Decreased SOD, CAT and GPx increases the oxidative stress already induced by CP treatment. This is reflected with the increased lipid peroxides produced by CP treatment. The elevation in embryonic tissue free radicals was further supported the diminution of peroxides and Catalase, an important scavenger enzyme of free radicals.

Our findings in this chapter show significant depletion of GSH levels after CP administration in chick embryonic liver, brain and heart.

The GSH antioxidant system consists of an array of non-enzymic and enzymic reaction pathways involving the neutralization of free radical species (Sreepriya et al., 2001). Moreover, GSH reflects the summation of a number of processes GR maintains GSH in a reduced form, GPx utilises it for the decomposition of lipid hydroperoxide and other ROS and GST maximizes the conjugation of free radicals and various lipid hydroperoxide to GSH to form water soluble products that can be easily excreted out (Ahmed et al., 2000).

GSH and vitamin C are water soluble antioxidants that serve as the first line of defense in combating free radicals (Martens son and Meister, 1991) and GSH can also facilitate the generation of vitamin C from its oxidation (Well et al., 1990).

CP treatment significantly decreased GPx activity in liver, brain and heart. Treatment with AP significantly increased the GPx activity in liver, brain and heart tissues. CP pre-treatment was able to increase the GPx activity in liver, brain and heart.

Our findings in this chapter show significant elevation of GST levels after CP administration liver and brain whereas in heart GST levels were decreased.

Treatment with AP significantly increased the GST activity in liver, brain and heart. AP pre-treatment was decreased the GST activity in liver, brain and heart.

The GST, in *Andrographis paniculata* extract treated animals showed significant increase in its activity, which further accounts for the antioxidant property of the plant extract (Vijayalakshmi, 2001).

AP induced a dose – related increase in hippocampal SOD, CAT and GPx activities (Bhattacharya et al., 2000).

CP treatment significantly decreased GR activity in liver, brain and heart. Treatment with AP significantly increased in liver and brain but in heart. AP pre-treatment was able to increase the GR activity in liver, brain and heart tissues.

The decrease in enzyme activities may be due to inactivation of the enzymes by the free radicals. But the treatment with ethanolic extract of AP significantly enhanced the activities of antioxidant enzymes. SOD and CAT, GPX and GST acts as protective enzymes against lipid peroxidation in embryonic tissues. The above results showed that the plant extract has activated the enzyme for defense mechanism.

On the whole, this plant extract exhibited a powerful antioxidant effect by interacting with the free radical metabolites produced by cyclophosphamide in the following ways.

By enhancing hepatic GSH and GST levels which aid in maintaining the antioxidant armoury.

By decreasing lipid peroxidation by its direct free radical scavenging action.

By modulating the key enzymes associated with the enzymatic antioxidant defense.

This elaborates that the plant extract has a potent and powerful efficacy as a good inhibitor of drug induced oxidative stress. Thus our results show a Chemoprotective role of AP extract against CP.

Possibly ours is a first report on the effect of AP extract on cyclophosphamide (CP) in chick embryos, further studies are needed to explore the mechanism of action of AP in ameliorating the toxic effects of Cyclophosphamide induced oxidative damage.

PURIFICATION AND PARTIAL CHARACTERIZATION OF INDUCED GLUTATHIONE-S-TRANSFERASE FROM 15 DAY-OLD CHICK EMBRYONIC LIVER TREATED WITH CYCLOPHOSPHAMIDE

The effect of CP administration as a single dose resulted in increase in the GST activity of chick liver when compared to control. However the GST activity of chick lever when compared to control. However the GST activity increased 3 fold after 72 hrs in CP treated liver compared to control. The specific activity (μ moles of GSH conjugate/min/mg protein) of GST in normal and CP treated 300 μ g chick liver was 51.4 ± 0.31 and 74.2 ± 1.44 respectively.

Table 7.1. Effect of CP on GST activity after 72 h period in chick embryonic liver (Enzyme activity expressed as μ moles of GST conjugate/min/mg protein)

Liver	GST Activity (μ moles of GSH conjugate/min/mg protein)
Control	51.4 ± 0.31
CP 300 μ g exposed	$74.2 \pm 1.44^*$

Values are average of six separate experiments (Mean \pm SD) * $p < 0.001$.

The liver tissue of about 50 gms was collected from chick embryos. The tissue were washed with normal saline to remove blood and fat debris and stored in normal saline at -20°C until further use. The 20% tissue homogenate was prepared in 25 mM Tris HCl buffer pH 8.0, containing 0.2 M sucrose in Potter Elvehjem homogenizer with a Teflon pestle and centrifuged at 20,000 rpm for 60 mins in a Remi cooling centrifuge at 4°C . The supernatant was dialyzed for 24h against 10 volumes of buffer with five changes to remove the endogenous GSH. The dialysate was considered as cytosolic extract. All the purification steps were carried out at 4°C .

The purified isozymes α -GST gave a single band on polyacrylamide gel electrophoresis in denaturing conditions (Protein stain). Isozyme α -GST was purified to 72% yield over the extract. The purification procedure is summarized in Table.

The administration of CP with a time-course effect in a dose dependant manner was studied by using different substrates of GSTs in the tissue specific induction of chick liver. In liver, exposure to CP the GST levels were further increased generally, this was observed using the substrates CDNB, p-NPA, p-NBC, BSP and EPNP (Table.). Though all these substrates are being shown the increased levels of GST with different doses of CP, the maximum activity was found to BSP and p-NBC at the dose of 1 mg CP treatment.

Table. 7.2. Purification of GST from chick embryonic liver

Sl.No	Purification	Total Activity μ mol/min	Total Protein (mg)	Specific activity (μ moles of GSH conjugate/min/mg protein)	% of yield
1.	Crude	37 \pm 0.2	32 \pm 2	1.08 \pm 0.07	
2.	Affinity Purified GST	26 \pm 2	6 \pm 0.5	4.4 \pm 0.01	

The GST activities of control, treated liver tissue was determined by using CDNB, pNPA, pNBC, EPNP and BSP as substrates and the specific activities were expressed in micromoles and nanomoles to respective substrates conjugates formed with GSH per minute per milligram protein.

Table. 7.3. Effect of Cyclophosphamide on liver GST activities with different substrates.

	CDNB	pNPA	pNBC	EPNP	BSP
Control	0.181±0.001	0.144±0.002	0.77±0.002	0.027±0.002	2.7±0.12
CP 100µg	0.433±0.013	0.231±0.001	0.047±0.002	0.029±0.014	0.022±0.002
CP 200µg	0.491±0.001	0.290±0.001	0.022±0.002	0.019±0.002	0.031±0.002
CP 300µg	0.612±0.013	0.057±0.004	0.015±0.004	0.029±0.027	0.055±0.004

Values are average of six separate experiments. Mean ± SD

Protein concentrations in all the samples were determined by the method by Bradford 1976 using BSA as a standard.

IMMUNOLOGICAL STUDIES

The antisera raised against affinity purified GSTs of chick liver α -GSTs on Ouchterlony double diffusion studies showed immunoprecipitin bands with affinity purified GST protein of chick liver.

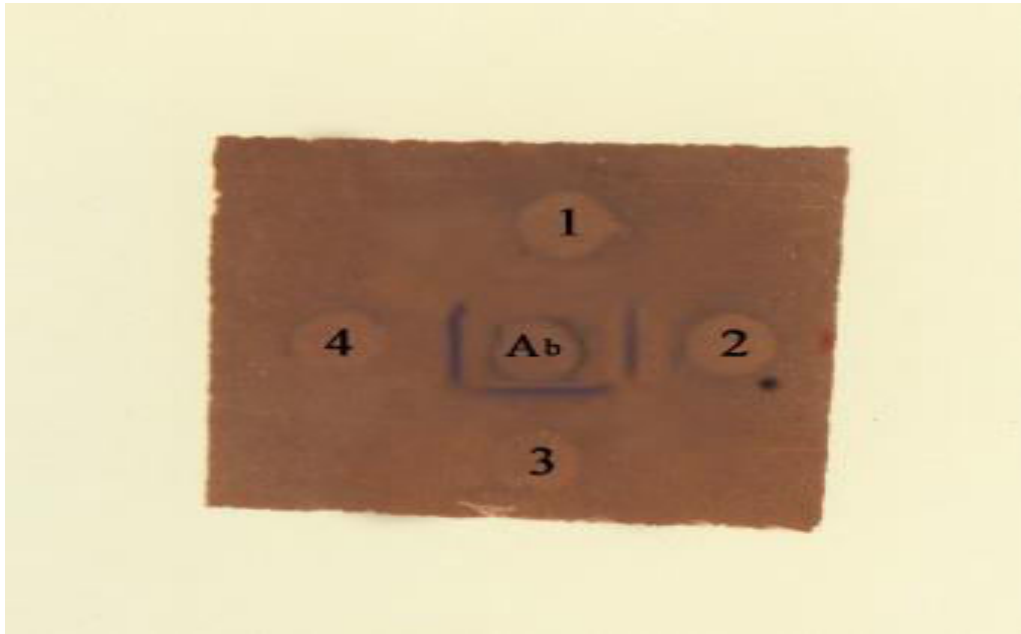


Fig.7.1. Immunodiffusion of α -GST

Central well: Partially purified antibody

Peripheral wells

1. Filled with crude.

2, 3 and 4. Filled with $5\mu\text{g}$ of α -GST

SDS-PAGE ANALYSIS:

SDS-PAGE liver proteins were separated by SDS-PAGE according to Laemmli (1970) in a 15% polyacrylamide gel containing 2M urea. Equal protein concentration was applied in each well. The experiment was done on three different samples. SDS-PAGE analysis of the affinity purified control GST fractions yielded four bands and designated as CL1, CL2, CL3 and CL4. The treated affinity purified GST fraction yielded a single band (CL4) of approximately 25.2 kDa.

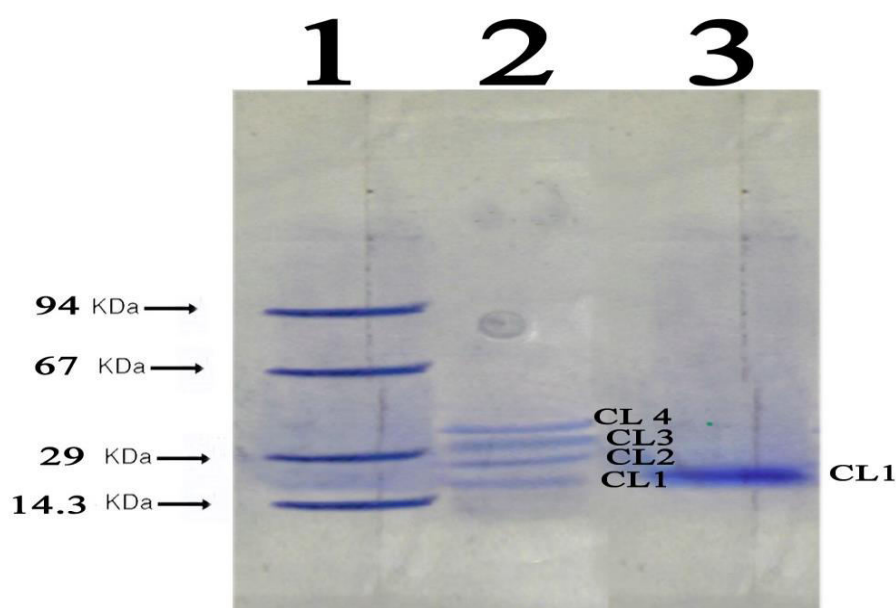


Fig. 7.2. SDS-PAGE (15%) OF PURIFIED α -GST

Lane-1: Standard marker proteins

- (a) Phosphorylase b (94kDa)
- (b) Bovine Serum albumin (66 kDa)
- (c) Carbonic anhydrase (29 kDa)
- (d) Lysozyme (14.3 kDa)

Lane-2: Control

Lane-3: 300 μ g of Cyclophosphamide treated

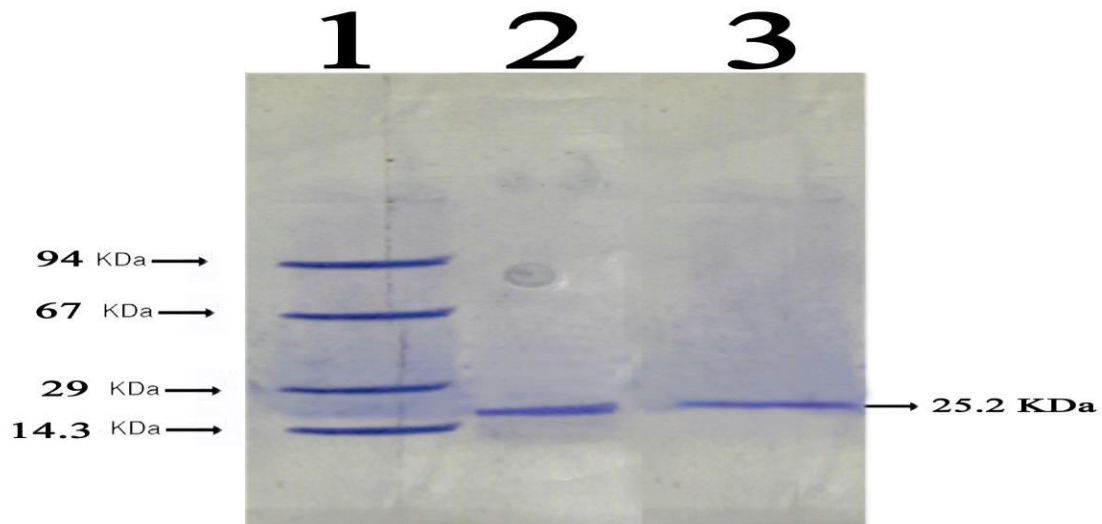


Fig. 7.3. NATIVE-PAGE (15%) of purified α -GST

Lane-1: Standard marker proteins

- (a) Phosphorylase b (94 kDa)
- (b) Bovine serum albumin (66 kDa)
- (c) Carbonic anhydrase (29 kDa)
- (d) Lysozyme (14.3 kDa)

Lane-2: Control

Lane-3: 300 μ g cyclophosphamide treated

SUMMARY OF THE PROJECT

The main objective of the present study was to determine the antioxidant activity of the alcoholic extract of *Andrographis paniculata* and to find out whether its pre-treatment could protect the substantial damages produced by cyclophosphamide in chick embryo.

Cyclophosphamide (CP) is one of the most effective anticancer drugs used in the treatment of cancer. CP shown wide spread activity in lympho hematopoietic malignancies and is used in various neoplastic disorders. This drug destroys not only the cancerous cells but also cause damage to the adjacent normal cells. The course of damage can be brought either by the attack of toxic metabolites of CP on DNA or by the formation of reactive oxygen species. The agents that are capable of accelerating the recovery (or) protect drug induced oxidative damage are considered as chemo protective. The embryo model was used to evaluate the effects of CP – induced oxidative damage to the developing chick embryo.

In the present study, the embryos were subjected to different doses of CP (10 µg to 500 µg) on day 3, 4, 5, 7, 9, 12 and 15 of Embryonic Development (ED).

The effect of CP on growth, visual appearance of toxic symptoms, embryonic development and on mortality rate of the embryos was dose and time dependant. The lethal dose (LD₅₀) values were determined at different stage. Dose of 30 µg correspond to LD₅₀ value for day 3, and 32, 44, 79, 172, 320 and 376 µg LD₅₀ values for ED-4, ED-5, ED-7 ED-9, ED-12 and ED-15 respectively.

In the present investigation, the biochemical constituents of tissues (liver, brain and heart) were determined. The results indicate that the SOD, CAT, GST, GPx and GSH levels play a role in lipid peroxidation during pregnancy.

The effective dose of herbal extract (6.0 mg / 15 day-old embryo) was arrived from the maximum protective effect elicited against the CP induced damages by measuring the activities of AST and ALT in AF and tissues. The versatile medicinal property of AP to serve as a potent antioxidant was revealed by the up regulation of the antioxidant defence as evidenced from the enhancement of GSH and GST in tissues (liver, brain and heart), which also served as criteria for assessing the optimum dosage.

The increased activities of the diagnostic marker enzymes such as AST, ALT, LDH, ACP and ALP by administration of CP, that reflect the loss of structural and functional integrity of cell membrane and liver damage was prevented significantly by the plant drug. This was indicative of the chemo protective nature of AP, which proved very effective in stabilizing the plasma membrane of the cells and hence preserving their structural and functional integrity.

The toxic effects of lipid peroxidation induced by the toxic radicals of CP were observed from an increased production of TBARS and conjugated di-ens with a subsequent decrease in the non-enzymatic and enzymic antioxidant defences. The indication of oxidative stress to the embryonic system. The decrease in herbal extract exhibited a powerful and potent antioxidant role by modulating the antioxidant armoury as follows:

- Enhancing the levels of GSH
- Scavenging free radicals
- Influencing the primary antioxidant enzymes.

Significant increase in GSH, SOD, CAT, GPx and GST in liver, brain and heart by AP extract is very important in reducing the oxidative stress induced by CP treatment. Our results show a chemo protective role of AP extract in chick embryo against CP. CP administration cause significant induction of GST in liver.

Enhance expression of GST isoenzymes in CP exposed liver developing has a definitive role in adaptation against the oxidative damage.

Treatment with *Andrographis paniculata* extract showed protection of the histological lesions and suggest a protective role these vital organs from the ravages caused by the free radicals.

In conclusion, it is becoming increasingly realised that tissue damage due to increased free radical production is the major cause of a large number of patho-physiological processes. Whatever the involvement of free-radical induced cellular oxidative stress, there is much speculation on the role of natural antioxidants in these diverse pathologies. In view of “non toxic” nature and “biological effect” these herbal antioxidant products should be recognised by the modern system of medicine.

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ENCLOSURE – III

ABSTRACTS PRESENTED AT NATIONAL/INTERNATIONAL CONFERENCES

- 1. S. Prabhudas, Dr. M. Mastan.** “Protective role of Cyclophosphamide induced Biochemical changes in chick embryo”. National seminar on Food Processing for Sustainable Food Security and Safety held at Association of Food Scientists and Technologists (India) Hyderabad Chapter, CSIR-IICT, Hyderabad. From 24th – 25th October, 2013.
- 2. Dr. M. Mastan, S. Prabhudas.** “Chemoprotective effect of *Andrographis paniculata* on cyclophosphamide induced Biochemical Changes in chick embryo”. National seminar on Present status and Future Prospects of Modern Biotechnology and their Applications held at Department of Biotechnology, School of Herbal Studies and Naturo sciences, Dravidian University, Kuppam. From March 27th – 29th, 2014.
- 3. S. Prabhudas, Dr. M. Mastan.** “Effect of cyclophosphamide induced Mortality, LD₅₀ and Embryo toxic effect of chick embryo”. National seminar on Present status and Future Prospects of Modern Biotechnology and their Applications held at Department of Biotechnology, School of Herbal Studies and Naturo sciences, Dravidian University, Kuppam. From March 27th – 29th, 2014.
- 4. S. Prabhudas, Dr. M. Mastan.** “Effect of Cyclophosphamide induced Oxidative damage in Chick embryo”. International conference on Applications of Natural Products and Opportunities Ahead – ICAN 2016 held at School of Life Sciences, B. S. Abdur Rahman University, Vandalur, Chennai. From August 2nd – 3rd, 2016.
- 5. S. Prabhudas, Dr. M. Mastan.** “Hepatoprotective effect of *Andrographis paniculata* on cyclophosphamide induced oxidative damage in Chick embryo”.
104th – The Indian Science Congress Association held at Sri Venkateswara University, Tirupati. From 3rd – 7th, 2017.

6. **S. Prabhudas, Dr. M. Mastan.** “Chemoprotective effect of *Andrographis paniculata* against cyclophosphamide induced oxidative damage in chick embryo”. National Seminar on Recent Advances in Biotechnology held at Department of Biotechnology, School of Herbal Studies and Naturo sciences, Dravidian University, Kuppam. From 9th – 10th February, 2017.

ENCLOSURE - IV

CONTRIBUTION TO THE SOCIETY

Natural compounds (plants) are useful to cure various diseases of the human beings by using different isolated or crude extracts. These plant extract control the side effect towards the normal cells from the anticancer drug exposure on cancer cells. Plant extracts are highly useful to protect from the healthy cell damage through this control lipid peroxidation via defence mechanism by enzymatic and non-enzymatic antioxidants which emerged from normal cells.