DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE INVESTIGATION OF THE ANTITUMOR AGENT CISPLATIN ON ELECTRON TRANSPORT SYSTEM ENZYMES BY EUKORYOTIC MODEL

by
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İZMİR

THE INVESTIGATION OF THE ANTITUMOR AGENT CISPLATIN ON ELECTRON TRANSPORT SYSTEM ENZYMES BY EUKORYOTIC MODEL

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by
Gizem KURŞUNLUOĞLU

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İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"THE INVESTIGATION OF THE ANTITUMOR AGENT CISPLATIN ON ELECTRON TRANSPORT SYSTEM ENZYMES BY EUKORYOTIC MODEL"** completed by **GİZEM KURŞUNLUOĞLU** under supervision of **ASSOC. PROF. HÜLYA AYAR KAYALI** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

In order to determination of the antitumor agent cisplatin toxicity effect on electron transport chain (ETC) enzymes; succinate dehidrogenase (SDH), adenine nucleotide levels as well as catalase (CAT) and lipid peroxidation (LPO) levels of five different tissues, three of which are composed of post mitotic cells (brain, heart) and two of slowly dividing cells (liver and kidney) as well as lung tissues of Male Sprague Dawley adult rats, were investigated with respect to various days. Cisplatin levels reached to maximum in liver as zero point thirty eight ppm/gr tissue and the levels were ordered as kidney, brain, lung and heart as zero point thirty six, zero point twenty four, zero point twenty four and zero point fifteen ppm/gr tissue, respectively. The results shows that cisplatin transported all studied tissues.

In the present study, the SDH activities of liver, lung, heart, brain and kidney decreased sixty six, fourty nine, fourty four, fourty seven, sixty three percent compared to control at first day. These decreases were accompanied by ATP levels as fifty three, eighty three, sixty two, thirty, twenty four percent at first day. The results may suggest that cisplatin agent induce the inhibition of SDH enzyme activity in addition ATP levels. Nevertheless, as an antioxidant enzyme CAT activity showed different trends depending on the tissue. LPO levels increased depending on cisplatin toxicity zero point four-fold, zero point four-fold, zero point five-fold, zero point two-fold and zero point five-fold compared with control groups of liver, lung, heart, brain and kidney, at first day respectively. According to the our results, cisplatin induced toxicity in each tissue especially for first day were determined with higher LPO levels, the results can be explained by insufficiency in enzymatic and non-enzymatic antioxidant systems against to cisplatin toxicity therefore in the second last step of the thesis, we added cisplatin with capsaicin which is non-enzymatic antioxidant to determine capsaicin effect on these system.

In general, SDH activity in cisplatin with capsaicin treated tissue increased while CAT activity and LPO levels decreased compared to only cisplatin groups. The results suggest that capsaicin have antioxidant capacity to scavenge ROS to prevent membrane damage.

Keyword: Cisplatin, capsaicin, Sprague Dawley, ETC enzymes, adenine nucleotides, CAT, LPO.

ÖKARYOTİK MODELDE CİSPLATİN ANTİTÜMÖR AJANININ ELEKTRON TRANSPORT SİSTEMİ ÜZERİNE ETKİSİNİN ARAŞTIRILMASI

ÖZ

Erkek Sprague Dawley yetişkin sıçanlarının dokularından postmitotik hücrelerden oluşan beyin ve kalp ve bu dokularla birlikte yavaş hücre bölünmesine sahip karaciğer ve böbrek dokularının yanı sıra akciğer dokusunda, antitümör ajanı sisplatin, elektron transport sistemi enzimi; süksinat dehidrogenaz (SDH), adenine nükleotid seviyeleri, katalaz aktivitesi ve lipid peroksidasyon düzeylerine olan toksik etkisileri çalışılmıştır. Sisplatin seviyeleri karaciğerde yüzde otuz sekiz ppm/gr olarak maksimum seviyeye ulaşmıştır ve böbrek, beyin, akciğer ve kalpte sırasıyla yüzde otuz altı, yüzde yirmi dört, yüzde yirmi dört ve yüzde onbeş ppm/gr olarak belirlenmiştir. Bu sonuçlar sisplatinin tüm dokulara transportunun olduğunu göstermektedir.

Karaciğer, akciğer, kalp, beyin ve böbrekteki SDH aktiviteleri sırasıyla, yüzde altmış altı, yüzde kırk dokuz, yüzde kırk dört, yüzde kırk yedi, yüzde altmış üç oranlarında birinci günlerde kontrol gruplarına kıyasla azalış göstermiştir. Bu azalış birinci günün ATP seviyelerindeki yüzde elli üç, yüzde seksen üç, yüzde altmış iki, yüzde otuz ve yüzde yirmi dört azalışı ile ilişkilidir. Bu sonuçlar ışığında sisplatinin SDH enzim aktivitesi ve ATP düzeylerinin inhibisyonunu indüklediği önerilmektedir. Bununla birlikte, bir antioksidant enzim olarak CAT aktivitesi dokulara bağımlı olarak farklılıklar göstermektedir. LPO seviyeleri birinci günde sisplatin toksisitesine bağlı olarak sırasıyla karaciğer, akciğer, kalp, beyin ve böbrek dokularında kontrole kıyasla bir onda dört, bir onda dört, bir onda beş, bir onda iki ve bir onda beş kat artış göstermiştir. Sisplatin uygulanan dokularda birinci gündeki LPO seviyelerinin kontrole kıyasla yüksek olması sisplatin indüklü toksisiteyi göstermektedir. Bu sonuçlar sisplatin toksisitesine karşı antioksidant sistemin yeterli olmaması ile açıklanabilir. Bu yüzden çalışmanın ikinci aşamasında enzimatik

olmayan bir antioksidant olan kapsaisin sisplatin ile birlikte enjekte edilerek kapsaisinin bu sistem üzerine etkisi araştırılmıştır.

Anahtar sözcükler: Sisplatin, kapsaisin, Sprague Dawley, ETS enzimleri, adenin nükleotidleri, CAT, LPO.

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CHAPTER ONE
INTRODUCTION

1.1 Cancer

Cancer which is known to be malignant neoplasm is the common name for a group of more than 100 diseases (Cancer, 2012). In cancer, abnormal cells divide and grow by out of control cell growth than these cells form malignant tumors and are able to invade other tissues (Wikipedia, 2012). Usually cancer cells have divided faster than healty cells, and they just keep on growing and dividing (Silverstein & Nunn, 2006). Different factors involved in genetic changes during lifetime may give rise to cancer. In generally cancer cells proceed to different parts of the body, and they begin to grow and form new tumors which displace normal tissue and this process is reffered to metastasis.

Cancer is a multistep progression of changes or phases that occur in the genes (Kowski, 2011, chap. 2). The genotypic changes are characterized by the loss of normal cellular differentiation and an alteration in tissue morphology due to an increase of unrepaired DNA damage and the formation of abnormal genomic variants (Schottenfeld & Fraumeni, 2006).

The progressive changes involved in tumor that occur on the cellular level are variable from individual to individual, and not all neoplasms follow the same progress such as metaplasia, to atypia and dysplasia (Collins, Haines, Perkel & Enck, 2007). Metaplasia, the first phase of cancer development, is the transformation of a mature differentiated cell type into a different mature differentiated cell type (Collins et al, 2007). This transformation is in response to an injury or insult at a cellular level which can make the tissues more susceptible to a malignant alteration. Atypia is defined as an abnormality associated with a precancerous process. An atypical cell (atypia) can also be an indication of an infection or irritation (Collins et al, 2007; Price & McCarthy-Wilson, 1992).Atypia can be caused by a chronic irritation and this has been shown increases the probability of premalignant lesions (Rivera,

Detterbeck & Mehta, 2003). Dysplasia is typically an irreversible condition or change in the cell that is a precursor of invasive epithelial tumors. There levels or grades of dysplasia and high grade dysplasia can be difficult to distinguish from carcinoma in situ during histologic examination (Collins et al, 2007; Price & McCarthy-Wilson, 1992).

In oncologic history, Sir Pervical Pott's first descriptions in 1775 of environmental association with human cancer is belied by John Hill'a report in 1761 of an association between nasal cancer and snuff, and by Paracelsus' report in 1531 of one between lung cancer and mining dust.

1.1.1 Classification of Cancer

Cancer types has been grouped as main categories of cancer include (Cancerlibrary, 2012; Farrell, (n.d.)).

- **Carcinoma** – cancer are the most common cancer that originate in the skin or in epithelial tissues that line or cover internal organs. Breast, lung and colon cancer are types of carcinomas.
- **Sarcoma** - cancer that begins in connective tissue. Some examples of sacromas are bone, cartilage, fat, muscle, blood vessels or supportive tissue.
- **Leukemia** - cancer that devolops in blood-forming tissue such as the bone marrow, lymph systems and lead to much more abnormal blood cells to be produced and enter the blood.
- **Lymphoma and myeloma** - cancers that start in the immune system cells.
- **Central nervous system cancers** - cancers that begin in the brain and spinal cord tissues.

1.1.2 Origins of Cancer

Whole cancer types start in the cells and to eveluated the cancer mechanism it is beneficial to recognize what happens when normal cells transform cancer cells because of damage to DNA, but this process is so complex because of the body is

made up of several types cells (Cancerlibrary, 2012). In healty cells, when the genetic material DNA becomes damaged, the cells repair the destruction or programmed cell-death has been served, however in cancer cells, the damaged DNA has not been repaired, but contrary to expectations (unlike) the cells don't die due to apoptosis process breaks down. And cancer cells continue to produce new cells that is not necessary for the body and the new cells have the same damaged DNA properties (Figure 1.1).

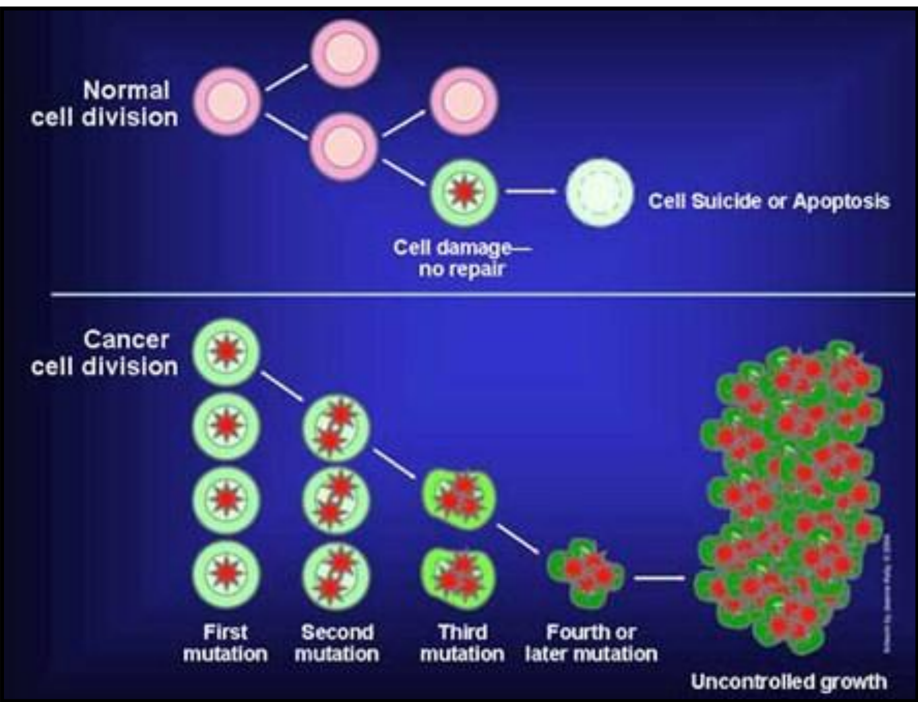


Figure 1.1 Loss of normal growth control.

All tumors have not been supposed the cancer by reason of tumors can be benign or malignant. Bening tumors can give rise to some promblems such as healty tissue/organs may have been pressed because of bening tumors could develop very large and speedy however this tumors are not able to invade other tissue/organs and are never life threatening (Cancerbasics, 2012).

1.1.3 Cancer Symptoms

Cancer symptoms are involved in both type and location of the tumor such as lung cancer bring about coughing, shortness of breath, or chest pain, and blood in the

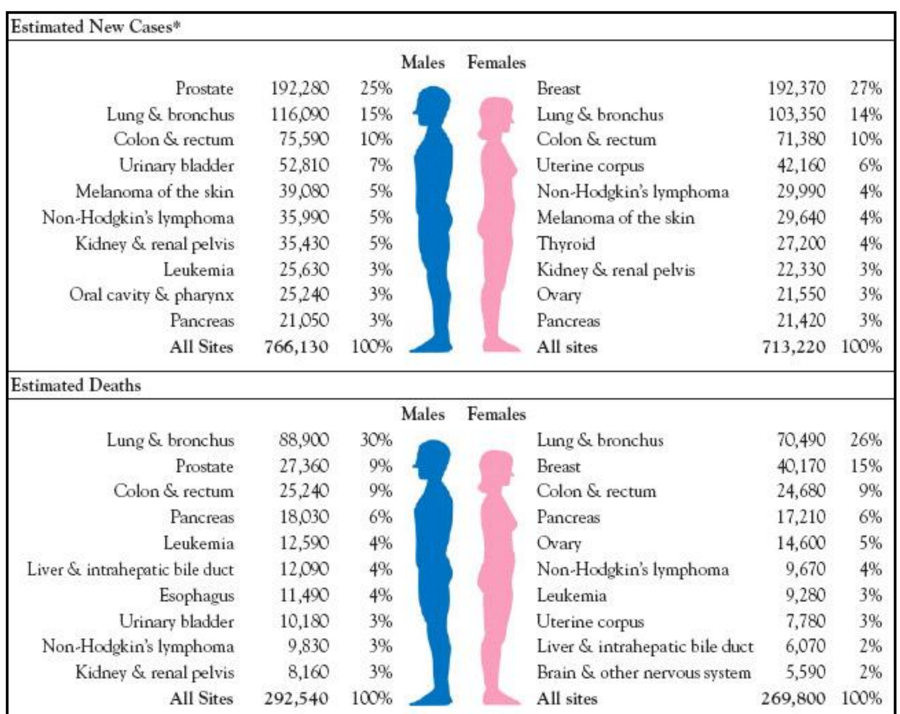
stool, diarrheal, constipation can originate from colon cancer. In addition, any symptoms may not form in some cancer the least little bit.

Generally in definite cancers, symptoms has not originated until the illness has reached an advanced stage, such as gallbladder cancer. Some symptoms may take place with most cancers: chills, fatigue, fever, loss of appetite, malaise, night sweats, weight loss (Health Guide, (n.d.)).

1.1.4 Effectiveness of Cancer on Human

Half of all men and one-third of all women in the US will develop cancer during their lifetimes. Today, millions of people are living with cancer or have had cancer. The risk of developing many types of cancer can be reduced by changes in a person’s lifestyle, for example, by staying away from tobacco, limiting time in the sun, being physically active and healthy eating. There are also screening tests that can be done for some types of cancers so they can be found as early as possible while they are small and before they have spread. In general, the earlier a cancer is found and treated, the better the chances are for living for many years (Cancerbasics, (n.d.))

Cancer rates are variety for men and women (Figure 1.2). The five most prevalent cancers for men, in descending order, are prostate, lung and bronchus, colorectal, urinary bladder and skin cancer. The cancers such as breast, lung and bronchus, colorectal, non-Hodgkin’s lymphoma, and skin cancer are most commonly diagnosed cancers in women. Lung and bronchus, colorectal and pancreatic cancers are among the top five most fatal forms of the disease in both men and women. For both men and women the number one cancer killer lung and bronchus is the most preventable as well since smoking, the greatest risk factor for this disease, is a life style choice (Almeida & Barry, 2010).



Estimated New Cases*

Males			Females		
Prostate	192,280	25%	Breast	192,370	27%
Lung & bronchus	116,090	15%	Lung & bronchus	103,350	14%
Colon & rectum	75,590	10%	Colon & rectum	71,380	10%
Urinary bladder	52,810	7%	Uterine corpus	42,160	6%
Melanoma of the skin	39,080	5%	Non-Hodgkin's lymphoma	29,990	4%
Non-Hodgkin's lymphoma	35,990	5%	Melanoma of the skin	29,640	4%
Kidney & renal pelvis	35,430	5%	Thyroid	27,200	4%
Leukemia	25,630	3%	Kidney & renal pelvis	22,330	3%
Oral cavity & pharynx	25,240	3%	Ovary	21,550	3%
Pancreas	21,050	3%	Pancreas	21,420	3%
All Sites	766,130	100%	All sites	713,220	100%

Estimated Deaths

Males			Females		
Lung & bronchus	88,900	30%	Lung & bronchus	70,490	26%
Prostate	27,360	9%	Breast	40,170	15%
Colon & rectum	25,240	9%	Colon & rectum	24,680	9%
Pancreas	18,030	6%	Pancreas	17,210	6%
Leukemia	12,590	4%	Ovary	14,600	5%
Liver & intrahepatic bile duct	12,090	4%	Non-Hodgkin's lymphoma	9,670	4%
Esophagus	11,490	4%	Leukemia	9,280	3%
Urinary bladder	10,180	3%	Uterine corpus	7,780	3%
Non-Hodgkin's lymphoma	9,830	3%	Liver & intrahepatic bile duct	6,070	2%
Kidney & renal pelvis	8,160	3%	Brain & other nervous system	5,590	2%
All Sites	292,540	100%	All sites	269,800	100%

Figure 1.2 Comparison of the estimated new cases and cancer-related esitmated deaths in men and women.

1.1.5 Cancer Types

Some cancer types are simply and shortly explained as follows;

- **Cervical cancer** is caused by infection with oncogenic subtypes of genital human papillomavirus (HPV) (Wall, 2008).
- **Lymphoma cancer** is a lymphocytes cancer, a type of cell which forms part of the immune system and generally, lymphoma is present as a solid tumor of lymphoid cells (Wikipedia, 2012).
- **Prostate caner** is a form of cancer which develops in the prostate, a gland in the men reproductive system. Most prostate cancers are slow growing; however, there are cases of aggressive prostate cancers (Wikipedia, 2012).
- **Colorectal cancer** is a cancer arising from uncontrolled cell growth and division in the colon or rectum or appendix. This cancer results from complex interactions between inherited susceptibility and environmental factors (Islam, 2005).

- **Skin cancer** is the uncontrolled growth of abnormal skin cells. There are two types of skin cancer; basal cell carcinoma caused by dividing basal layer of the epidermis and squamous cell carcinoma appear in the epidermal keratinocytes (Williams, 2010).
- **Breast cancer** that form in breast tissues, generally in the inner lining of milk ducts and in the lobules (glands that make milk). It occurs in both men and women, however male breast cancer is rarely diagnosed (Breast cancer, 2012).
- **Ovarian cancer** is a type of cancer forming from the ovary. Typically ovarian cancers are either ovarian epithelial carcinomas (cancer which originate in the cells on the surface of the ovary) or malignant germ cell tumors (cancer which initiate in egg cells) (Ovarian cancer, 2012).
- **Lung cancer** is described by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue and, eventually, into other parts of the body (Wikipedia, 2012).

1.2 Cemotherapeutics

Cemotherapeutics drugs as a chemical agents have been used for the treatment of different diseases. This drugs aims to occur toxic effect on cause of disease microorganism but not damage the body cells. Human cell and microorganism cell is different with regards to biochemistry and structures there by this selective effect occurs (Farrell, (n.d.)). There is a great number of chemotherapeutic drugs commonly classified into various categories;

- Antimalarial agents
- Antihelmintic agents
- Antibacterial agents
- Antiamebic agents
- Antirickettsial agents
- Antiviral agents
- Antineoplastic agents

1.2.1 Antineoplastic Agents

Antineoplastic drugs as a chemotherapy agents influence the cell division or DNA function and synthesis with different way (Figure 1.3). Some drugs interfered directly with the DNA. Some of antineoplastic agents involve the antibodies which are monoclonal and there are the kinase inhibitors that result in abnormality in some types of cancer like gastrointestinal cancer or in myelogenous leukemia. Some chemotherapy drugs can be used for modulating the cell behavior without attacking the cells directly (Chemotherapy agents, (n.d.)). Antitumor agents has been divided in different type;

- Alkylating drugs
- Antimetabolites
- Antitumour antibiotics
- Anthracyclines
- Topoisomerase inhibitors
- Herbal agents
- Anthracyclines
- Hormone and hormone antagonists
- Other agents.

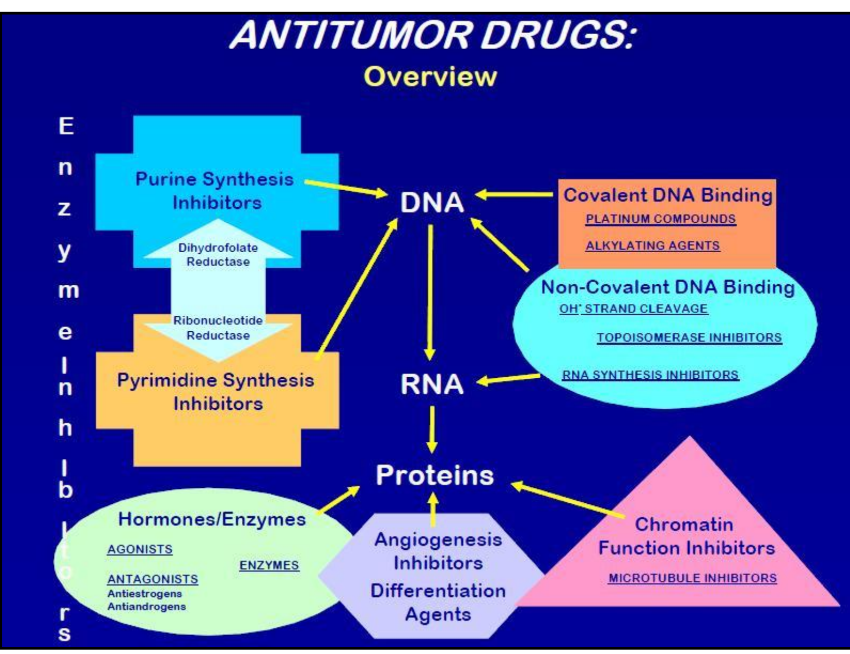


Figure 1.3 Schema of antitumor drug involved in effect mechanisms.

1.2.1.1 Cisplatin

cis-Diammineplatinum(II) dichloride is the linear name of cisplatin (Figure 1.4).

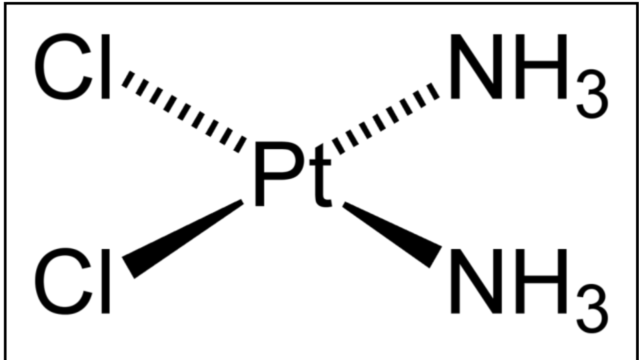


Figure 1.4 The chemical structure of cisplatin

1.2.1.1.1 Properties of Cisplatin. The characteristic properties of cisplatin were shown in Table 1.1.

Table 1.1 The characteristic properties of cisplatin

IUPAC name	(*SP*-4-2)-diamminedichloridoplatinum
Molecule formule	$H_6C_{l2}N_2Pt$
Moleculer weight	301.1 g/mol
Melting point	270°C
Colour	Yellow to orange powder
Protein binding	>95%
Solubility	in water at 25°C (0,253g/100g) and soluble in alcohol and in NaCl (0,5x10^{-2} M) and in DMF (16,6mg/ml)
Half life	30-100 hours
Stability	Cisplatin stability in aqueous solutions is improved by increasing NaCl concentrations (for 24 hours) and this stability is affected negatively in alkaline solutions.

1.2.1.1.2 General Use of Cisplatin. Cisplatin (*cis*-dichlorodiamine platinum II), which is known to be inorganic complex, is made up of central platinum atom, ammonia molecule and chlorine atom which is in the *cis*-position (Minoru et al., 2003). This molecule is a neutral platinum complex with simple structure due to 2+ charge of the original platinum(II) ion is definitely cancelled by the two negative charges supplied by the chloride ions (Clark, 2003). Cisplatin is an inorganic complex compared to other antitumor drugs whit organic structure and has been used as the gold standard against the new medicine (Trzaska, 2005).

Cisplatin is a more effective anticancer agent that is widely used in the treatment of a variety of many solid tumours and is currently one of the most significant cytostatic drug (Kopelman, Budnik, Sessions, Kramer & Wong 1988; Gandara, Perez, Philips, Lawrence & Degregorio, 1989; Chirino, Hernandez-Pando, & Pedraza-Chaverri, 2004). Cisplatin has exhibited important anticancer activity against tumor that is include squamous cell carcinomas of the head and neck, some tumors of the lung, and ovarian and testicular cancers (Joseph, Feghali, Wei Liu, Thomas & Van De, 2001).

1.2.1.1.3 Mechanism of Cisplatin. Cisplatin enters the cells and its chloride ligands have been replaced by water forming aquated species that react with nucleophilic sites in cellular macromolecules to form protein, RNA and DNA adducts (Kartalou & Essigmann, 2001) (Fig2). This reaction, water replaces one of chloride atoms, lead to the formation of monohyrated cisplatin that is highly reactive with nitrogen as compared to main medicine (Kelland & Farrell, (Eds.). 2000). In addition, it is supposed that cisplatin enters the cells by passive diffusion, however some evidence display that cisplatin uptake is mediated by membrane proteins (Binks & Dobrota, 1990; Mann, Andrews & Howell, 1991; Hromas, North & Burns, 1987; Andrews, Velury, Mann & Howell, 1988). Cisplatin treatment results in inhibition of DNA replication, RNA transcription, arrest at the G2 phase of the cell cycle and/or programmed cell death (Kartalou et al., 2001; Desoize & Madoulet 2002; Wang, Lu & Li, 1996). Any factors that may influence the formation of

cisplatin adducts or the downstream effects initiated due to the presence of these adducts in DNA would affect survival (Kartalou et al., 2001).

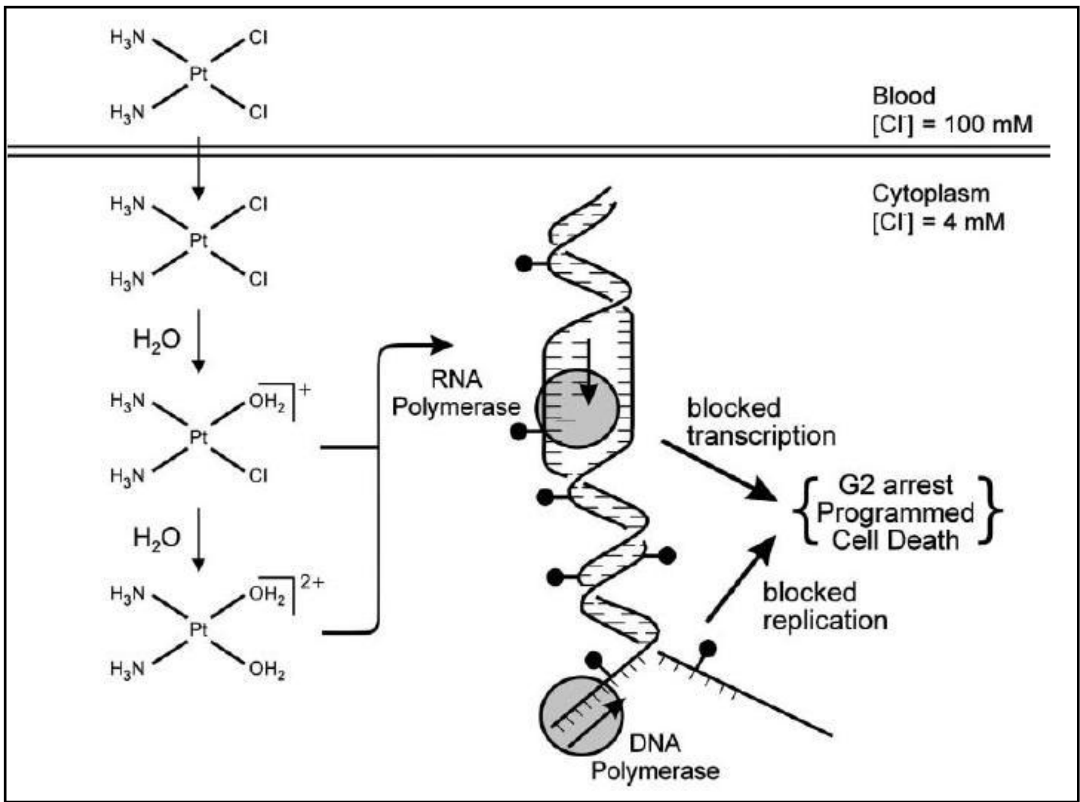


Figure 1.5 Mechanism of cisplatin

Oxidative stress, endoplasmic reticulum stress, DNA-damaging stres, that give rise to the activation of several signal transduction pathways, may have been induced by cisplatin (Kohno et al., 2005). Cisplatin, that is remarkable crosslinking agent, react indirectly with nitrogen atoms on DNA to produce: intra- and interstrand DNA crosslinks, DNA–protein crosslinks, cisplatin DNA glutathione crosslinks and DNA monoadducts (Fichtinger-Schepman et al., 1985; Wang & Lippard, 2005).

Parameters of cytotoxicity is indicated as the following (Farrell, (n.d.));

- Platinum uptake and efflux: cytotoxicity is involved in total Pt uptake.
- DNA binding: Pt-DNA adducts concerned with major biolojical effects.
- Methabolism and interaction with thiols and thioethers: Glutathione deactivation, plasma protein binding referring to this toxicity.

1.2.1.1.4 Side effects of Cisplatin. The clinical usefulness of cisplatin has been limited by various side effect;

- Nephrotoxicity
- Ototoxicity
- Renal toxicity
- Peripheral neuropathy
- Renal dysfunction- renal proximal tubular cell apoptosis
- Nausea and vomiting
- Diarrhoea

Although its effectiveness, cisplatin is associated with significant side effect and nephrotoxicity and ototoxiciy have been common recognized adverse effect which is involved in dose-limiting factor for cisplatin therapy (Schweitzer, 1993; Chirino et al., 2004). Different methods such as hydration were used for the prevention of nephrotoxicity, but these methods have not been succeed for reducing the adverse effect of cisplatin (Arts, 1998). Reactive oxygen species (ROS) and nitric oxide (NO) are concerned with as significant mediators of the toxic agent-induced acute kidney injury and nephrotoxicity, respectively (Baliga, Ueda, Walker & Shah, 1999; Srivastava et al., 1996; Li, Bowmer & Yates, 1994a, 1994b). In addition to this toxicity, cisplatin bring about apoptosis which is known to be an important mechanism of cell death (Kroning, Katz, Lichtenstein & Nagami, 1999; Lieberthal, Triaca & Levine, 1996; Okuda, Masaki, Fukatsu, Hashimoto & Inui, 2000; Takeda, Fukuoka & Endou, 1996; Zhou et al., 1999). The influence of hydroxyl radicals, which is most reactive among oxygen radicals, and other free radical species in cisplatin-induced nephrotoxicity and cell death have not been exactly elucidated (Kim, Jung, Lee & Kim, 1997; Minoru et al., 2003).

Some anorganic complex as a drug are used instead of cisplatin. Carboplatin has been used a cisplatin analogue. This analogue has less nephrotoxicity and ototoxicity compared with cisplatin. However, carboplatin has not displaced cisplatin because of the fact that cisplatin more effective as compared with its analogue (Joseph et al., 2001).

1.3 Metabolism

Metabolism is essentially a linked series of chemical reactions that begins with a particular molecule and converts it into some other molecule or molecules in a carefully defined fashion. There are many such defined pathways in the cell.

Metabolic pathways can be divided into two broad classes:

- Convert energy into biologically useful forms,
- Require inputs of energy to proceed.

Although this division is often imprecise, it is nonetheless a useful distinction in an examination of metabolism. Those reactions that transform fuels into cellular energy are called catabolic reactions or, more generally, catabolism.

Fuels (carbohydrates, fats) $\xrightarrow{\text{Catabolism}}$ CO_2 + H_2O + useful energy

Those reactions that require energy; such as the synthesis of glucose, fats, or DNA are called anabolic reactions or anabolism. The useful forms of energy that are produced in catabolism are employed in anabolism to generate complex structures from simple ones, or energy-rich states from energy-poor ones.

Useful energy +small molecules $\xrightarrow{\text{Anabolism}}$ complex molecules

The processes of energy conversion in higher organisms has taken in three main steps. In the first stage, large molecules in food are broken down into smaller units. Proteins are hydrolyzed to their 20 kinds of constituent amino acids, poly saccharides are hydrolyzed to simple sugars such as glucose, and fats are hydrolyzed to glycerol and fatty acids. This stage is strictly a preparation stage; no useful energy is captured in this phase. In the second stage, these numerous small molecules are degraded to a few simple units that play a central role in metabolism. In fact, most of them sugars, fatty acids, glycerol, and several amino acids are converted into the acetyl unit of acetyl CoA. Some ATP is generated in this stage, but the amount is small compared with that obtained in the third stage (Figure1.6).

In the third stage, ATP is produced from the complete oxidation of the acetyl unit of acetyl CoA. The third stage consists of the citric acid cycle and oxidative phosphorylation, which are the final common pathways in the oxidation of fuel molecules. Acetyl CoA brings acetyl units into the citric acid cycle [also called the tricarboxylic acid (TCA) cycle or Krebs cycle], where they are completely oxidized to CO_2. Four pairs of electrons are transferred (three to NAD and one to FAD) for each acetyl group that is oxidized. Then, a proton gradient is generated as electrons flow from the reduced forms of these carriers to O_2, and this gradient is used to synthesize ATP (Berg, Tymoczko & Stryer, 2002).

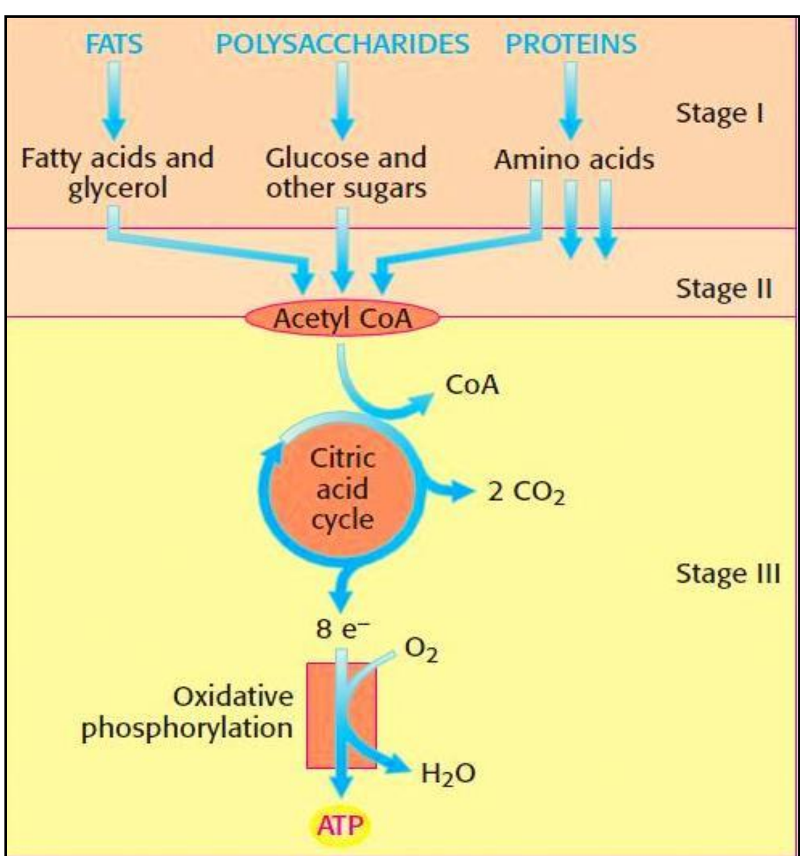


Figure1.6 The extraction of energy from fuels as three stages.

1.3.1 Electron Transport Chain

Electron transport consists of a series of reactions in which electrons and protons are passed through a cascade of these electron carriers. Natural electron transport involves consecutive reduction and oxidation of a series of electron carriers, which

must include electron transfer, but does not always include transfer of the protons. Eventually, the electrons are passed to molecular oxygen, and protons are reclaimed from the aqueous medium through the formation of water. (Switzer & Garrity, 1999).

The cascade of redox reactions that couples the oxidation of organic substrates to reduction of molecular oxygen in biological systems is called electron transport, and is often presented schematically as shown bellow reaction;

$CH_3COOH + 2\ O_2 \longrightarrow 2\ CO_2 + 2\ H_2O$

The general features of electron transport are similar throughout nature. The processes take place in highly structured environments within the cell membranes of bacteria and within specialized subcellular particles the mitochondria of higher plants and animals. The electron carrier molecules are also similar throughout nature: the diphosphopyridine nucleotide NAD^+, proteins that contain flavin (FMN and/or FAD) and iron–sulfur (Fe-S) clusters, quinones that are soluble in the lipid component of membranes, and several heme-containing proteins called cytochromes (Switzer & Garrity, 1999).

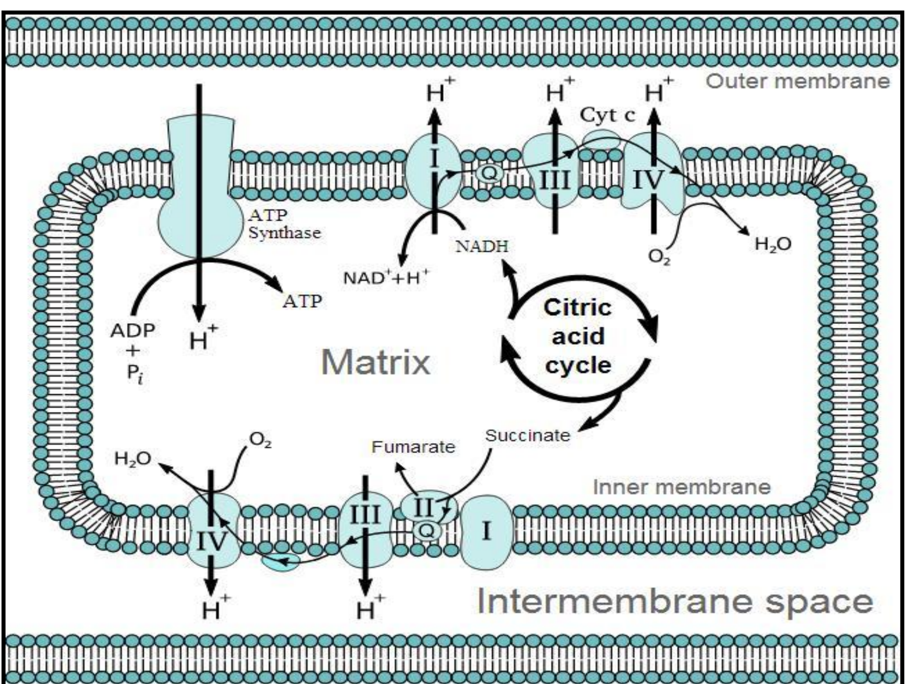


Figure 1.7 General schema of electrone transport chain. I- NADH reductase, II- succinate dehydrogenase, III- cytochrome reductase, IV- cytochrome oxidase.

The electron transport chain consists of three protein complexes (complexes I, III, and IV), which are integrated into the inner mitochondrial membrane, and two mobile carrier molecules; ubiquinone (coenzyme Q) and cytochrome c (Figure1.7) (Koolman et al., 2005). Coenzyme Q (CoQ) and cytochrome C are responsible for electron transfer between certain complexes of the ETC. Other carriers include heme groups and iron-sulfur clusters within complexes. CoQ transfers electrons from either complex I or complex II to complex III. CoQ also works in membranes and outside the mitochondria as an antioxidant, removing excess reducing power formed by glycolysis. CytC is responsible for the transfer of electrons from complex III to complex IV. During these transfers, the electrons move to lower energy states providing enough free energy removed from substrates for protons to be transferred across the inner mitochondrial membrane, generating a proton gradient to power ATP formation (Jones, 2002, p. 15).

1.3.1.1 The Mitochondria

Mitochondria are traditionally recognized for their ability to efficiently produce large amounts of energy for cells to carry out vital functions. Only in the past several years has our view of mitochondria as the “powerhouse of the cell” evolved into the knowledge that mitochondria are intimately involved in cellular life, death and function. Mitochondria are highly complex and compartmentalized organelles that are able to change shape and move throughout the cell in response to varying cellular conditions. Mitochondria now rely on cellular machinery for transcription and translation to make the majority of proteins needed for oxidative phosphorylation and ATP synthesis (Mans, 2010, p. 11-12).

Mitochondria are complex, double membrane organelles made up of five major constituents: the outer membrane (OM), inner membrane (IM), the inter-membrane space (IMS), cristae, and the matrix. The OM contains docking sites for signaling proteins such as those involved in cell fate, as well as major receptors for protein trafficking into the organelle. The IMS is the fluidfilled space between the OM and IM, while the IM contains all of the complexes of the electron transport chain (ETC)

involved in respiration and oxidative phosphorylation. Complexes I, III, and IV of the ETC serve as proton pumps which fuel the flow of electrons and continually pump protons from the matrix into the IMS. Protons flow back into the mitochondrial matrix to fuel Complex V, or ATP Synthase, which phosphorylates ADP into ATP, the energy currency of the cell (Nicholls, & Budd, 2000). The inner membrane is continuous with multiple invaginations that completely fill the space, or matrix, of the organelle. These folds are referred to as cristae, and are able to change shape as the mitochondrion adapts to cell signaling events (Mans, 2010, p. 13).

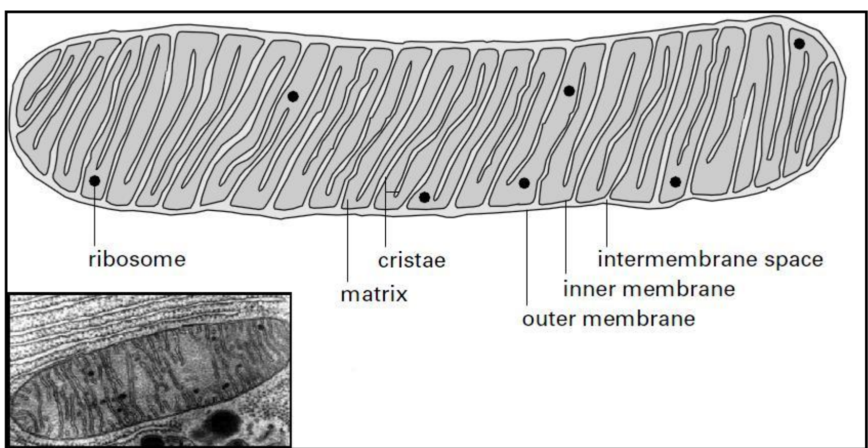


Figure 1.8 Structure of mitochondria

Mitochondria are central to several of these vital processes, chief among them being energy production, programmed cell death, cell cycle regulation, calcium signaling, and synaptic transmission in the case of nerve cells (Figure 1.8). Understandably, the critical nature of these cellular activities means that dysfunction or deregulation in any of them has severe pathological ramifications that result in diseases, aging, and/ or lethality. Thus, a comprehensive understanding of mitochondrial biology is paramount to a human health and longevity (Baqri, 2011, chap. 1). The mitochondria is organelle that has its critical physiology and functions and it is a very important drug target (Ke, 2011, p. 18).

Mitochondria, the main source of energy generated in the cell, are large sources and targets of damage caused by ROS. Enzymatic complexes responsible for energy

metabolism suffer attacks from ROS from the pre-transcriptional to post-translational (Jones, 2002, p. 9).

1.3.1.2 Electron Carriers in All Living Cells

Metabolism is the interconnected, integrated ensemble of chemical reactions cells use to extract energy and reducing power from their environments, synthesize the building blocks of their macromolecules, and carry out all the other processes that are required to sustain life. The most important molecules for storing and carrying energy in metabolic processes, including ATP, the universal currency of energy in biological systems, are described next section. The energy for ATP synthesis comes from the oxidation of carbon compounds, and the pathways that perform these oxidations can be classified into three stages.

All living cells draw on a spectrum of a few activated carriers to help run these reactions, including the electron carriers;

- Nicotinamide adenine dinucleotide (NAD^+ and NADH)
- Flavoproteins (FAD and FMN)
- Ubiquinone or coenzyme Q
- Cytochromes (heme coenzymes)
- Iron-sulfur clusters (Berg, Tymoczko, & Stryer, 2002).

Nicotinamide adenine dinucleotide **NAD^+** and **NADH** (its reduced form), generally considered a key component involved in redox reactions, has been found to participate in an increasingly diverse range of cellular processes, including signal transduction, DNA repair, and post-translational protein modifications (Figure 1.9) (Chen, 2008). NAD^+ and NADH are widely distributed as coenzymes of dehydrogenases. They transport hydride ions ($2e^-$ and 1 H^+) and always act in soluble form Figure. NAD^+ transfers reducing equivalents from catabolic pathways to the respiratory chain and thus contributes to energy metabolism (Koolman & Roehm, 2005).

Figure 1.9 The oxidized and reduced form of NADH structure.

The flavin coenzymes **FMN** and **FAD** contain flavin (isoalloxazine) as a redox-active group (Figure1.10). This is a three-membered, N-containing ring systemthat can accept amaximumof two electrons and two protons during reduction. FMN carries the phosphorylated sugar alcohol ribitol at the flavin ring.

Figure 1.10 The oxidized and reduced form of FMN structure.

FAD arises from FMN through bonding with AMP and the two coenzymes are functionally similar. They are found in dehydrogenases, oxidases, and monooxygenases. In contrast to the pyridine nucleotides, flavin reactions give rise to radical intermediates. To prevent damage to cell components, the flavins always remain bound as prosthetic groups in the enzyme protein (Koolman et al., 2005).

Figure 1.11 The oxidized and reduced form of FAD structure

FMN (like FAD) can accept 2 e^- + 2 H^+ to yield $FMNH_2$ (Figure 1.11). When bound at the active site of some enzymes, FMN can accept 1 e^-, converting it to the half-reduced semiquinone radical. The semiquinone can accept a second e^- to yield $FMNH_2$ (Figure 1.12) (Diwan, 2007).

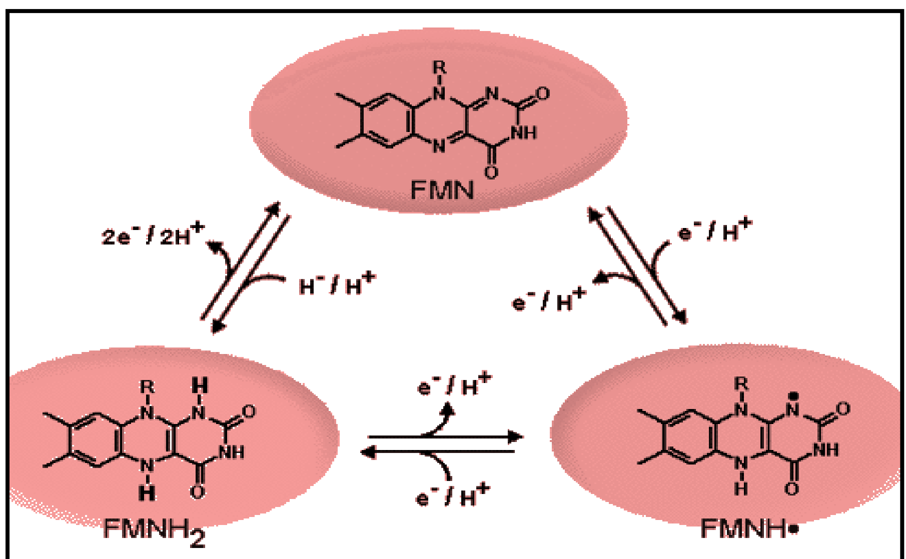


Figure 1.12 Recharging reaction between FMN, $FMNH^{\bullet}$ and $FMNH_2$.

The role of **ubiquinone** (coenzyme Q) in transferring reducing equivalents in the respiratory chain (Figure 1.13). During reduction, the quinone is converted into the hydroquinone (ubiquinol). The isoprenoid side chain of ubiquinone can have various lengths. It holds themolecule in themembrane, where it is freely mobile. Similar

coenzymes are also found in photosynthesis (plastoquinone). Vitamin E and vitamin K also belong to the quinone/hydroquinone systems.

Figure 1.13 The oxidized and reduced form of ubiquinone structure.

Iron–sulfur clusters (Fe-S) occur as prosthetic groups in oxidoreductases, but they are also found in lyases e. g., aconitase and other enzymes. Iron–sulfur clusters consist of 2–4 iron ions that are coordinated with cysteine residues of the protein (–SR) and with anorganic sulfide ions (S). Structures of this type are only stable in the interior of proteins. Depending on the number of iron and sulfide ions, distinctions are made between [Fe_2S_2], [Fe_3S_4], and [Fe_4S_4] clusters as shown in Figure 1.14. These structures are particularly numerous in the respiratory chain and they are found in all complexes except complex IV (Koolman et al., 2005).

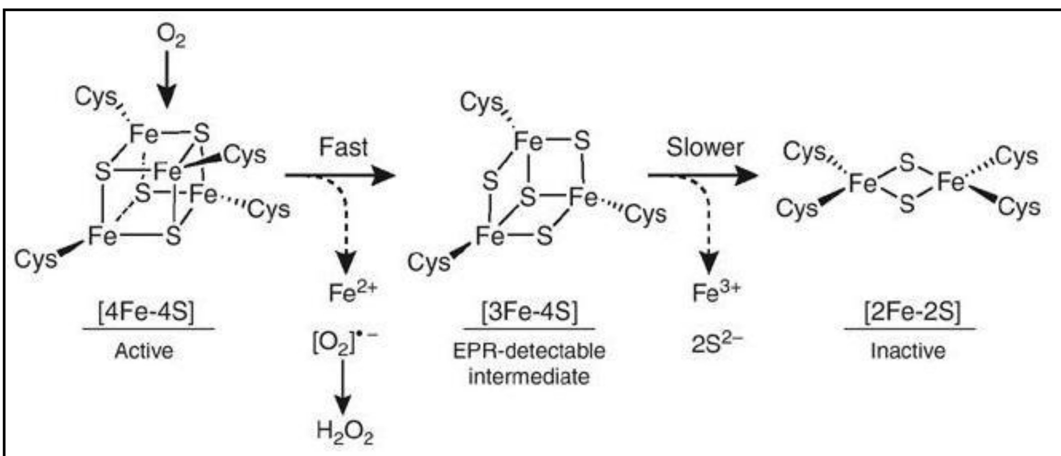


Figure 1.14 Mechanism of iron–sulfur cluster oxidation and conversion.

Heme coenzymes with redox functions exist in the respiratory chain, in photosynthesis, and in monooxygenases and peroxidases. Hemecontaining proteins

with redox functions are also referred to as **cytochromes** (Figure 1.15). In cytochromes, in contrast to hemoglobin and myoglobin, the iron changes its valence (usually between +2 and +3). There are several classes of cytochrome (a, b, and c), which have different types of substituent – R1 to – R3. Hemoglobin, myoglobin, and the heme enzymes contain heme b. Two types of heme a are found in cytochrome c oxidase, while heme c mainly occurs in cytochrome c, where it is covalently bound with cysteine residues of the protein part via thioester bonds (Koolman et al., 2005).

Figure 1.15 The structure of cytochromes; cytochrome a, also called heme a (cytochrome a), heme b (cytochrome b), heme c (cytochrome c).

Table 1.2. Type of electron carriers involved in mitochondrial respiratory chain.

Multienzyme Complexes of the Electron-Transport Chain in Mammalian Mitochondria			
Complex	**Mass (kDa)**	**Number of Subunits**	**Prosthetic Groups**
NADH-ubiquinone reductase	880	34	FMN, Fe-S centers
Succinate-ubiquinone reductase	140	4	FAD, Fe-S centers
Cytochrome *c* reductase	250	10	Heme *b*-562, Heme *b*-566 Heme *c*1 Fe-S centers
Cytochrome oxidase	160	13	Heme *a* Heme *a*3 CuA and CuB

1.3.1.3 Complex I (NADH: Ubiquinone Oxidoreductase)

Complex I, also called NADH dehydrogenase, catalyzes the transfer of electrons from NADH to coenzyme Q through two prosthetic groups, FMN and Fe-S (Figure 1.16). As two electrons are transferred during the oxidation of an NADH molecule to NAD^+, four protons are pumped across the mitochondrial inner membrane. These protons generate the inner mitochondrial membrane potential and are pumped back into the mitochondrial matrix when used to power ATP synthase. Of its 25-30 subunits, 15 are located within the mitochondrial inner membrane and seven of them are encoded by mtDNA (InterPro 3-16-06). The high number of mtDNA-encoded subunits lends a higher probability that complex I will receive a free radical attack. Complex I has been widely shown to be a factor in neurodegeneration related to Leigh Syndrome and Parkinson's Disease (Komaki et al., 2003; Leshinsky-Silver et al., 2005; Duke, 2006; Keeney, 2006; Jones, 2002).

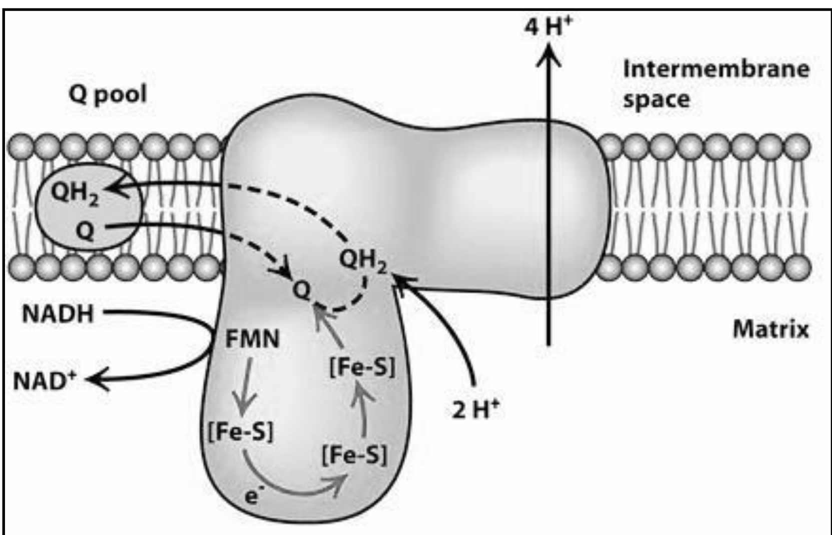


Figure 1.16 The reaction mechanism of Complex I.

Complex I of the electron transport chain is the most affected by ROS because it is susceptible to post-translational attack and 7 of its 13 subunits are encoded by mitochondrial DNA (mtDNA), which itself is a target of ROS (Genova et al., 2004; Jones, 2002).

1.3.1.4 Complex II (Succinate Dehydrogenase)

Succinate dehydrogenase, which actually belongs to the tricarboxylic acid cycle, is also assigned to the respiratory chain as complex II (Koolman et al., 2005). Succinate dehydrogenase is located on the matrix side of the mitochondrial inner membrane, making it the only one of the five main complexes that is not a transmembrane complex (Figure 1.17) (Jones, 2002). The SDH complex is made of four subunits: SdhA, SdhB, SdhC and SdhD. SdhA consists of a flavoprotein subunit containing a covalently-bound FAD moiety. This SdhA subunit is the site of dicarboxylate binding and the catalytic site of succinate oxidation with concomitant reduction of FAD to $FADH_2$. The electrons from $FADH_2$ are sequentially transferred to the iron-sulfur clusters of SdhB (the second catabolic subunit)

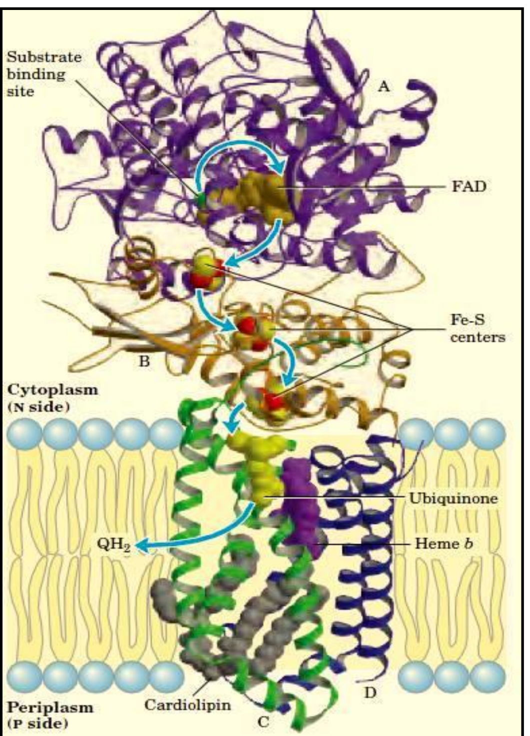


Figure 1.17 Structure of succinate dehydrogenase.

SdhA consists of a flavoprotein subunit containing a covalently-bound FAD moiety. This SdhA subunit is the site of dicarboxylate binding and the catalytic site of succinate oxidation with concomitant reduction of FAD to $FADH_2$. The electrons from $FADH_2$ are sequentially transferred to the iron-sulfur clusters of SdhB (the

second catabolic subunit) (Figure1.18). SdhC and SdhD are heme-containing hydrophobic transmembrane units that anchor the complex in the cytoplasmic membrane. Both SdhA and SdhB are cytoplasmic components of the complex, and they are attached to the inner cytoplasmic wall by subunits SdhC and SdhD. SdhA and B are highly conserved across all species studied, whereas SdhC and SdhD show much more sequence variation (Poilly, 2011, p. 21-22).

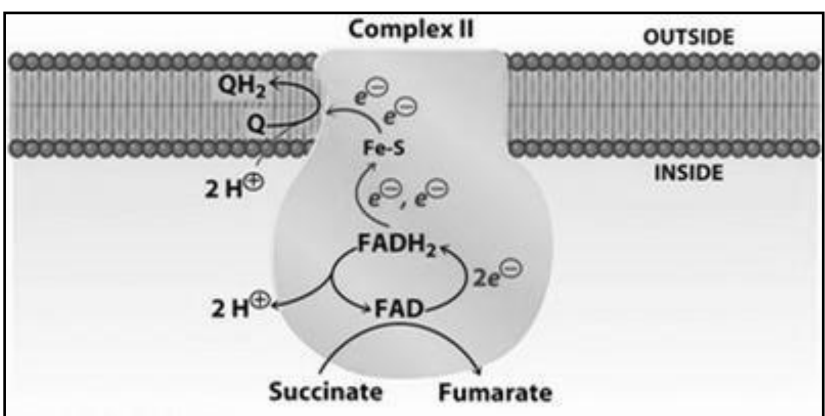


Figure 1.18 The reaction mechanism of Complex II.

As complex II is not a transmembrane complex, no protons are transported in this step (Jones, 2002). Electrons are accepted by FAD, passed on through several Fe-S clusturs and eventually used to reduce Q to QH_2. With its highly hydrophobic isoprenoid tail, ubiquinone/ubiquinol can “swim” back and forth in the bi-layer of mitochondrial iner membrane as an electrone shuttle (Ke, 2011).

1.3.1.5 Complex III (Cytochrome c Oxidoreductase)

The ubiquinol:cytochrome *c* oxidoreductase (cytochrome *bc*1 or complex III) is an essential energy transducing electron transfer complex in the mitochondrial electron transfer chain (Figure 1.19) (Hunte, Koepke, Lange, Rossmanith, & Michel, 2000; Sadoski, 2000). The reduced ubiquinol binds to cytochrome *b* in the *bc1* complex (complex III) where it undergoes the so-called Q cycle to become oxidized back to ubiquinone and the electrons are passed on to cytochrome *c* with concomitant pumping protons (2H+/e-) across the membrane, resulted in the generation of transmembrane proton electrochemical gradient (ΔμH) or proton motive force (pmf)

for use in synthesis of ATP by ATP synthase (Vaidya, 2005; Mather, Henry & Vaidya, 2007; Fry & Beesley, 1991; Srivastava, Rottenberg & Vaidya, 1997; Xia, 1997; Hunte, Koepke, Lange, Rossmanith & Michel 2000; Tian, 2000; Stonehuerner, O'Brien, Kendrick, Hall & Millett, 1985; Stonehuerner, 1985; Hunte et al., 2000; Sadoski, 2000).

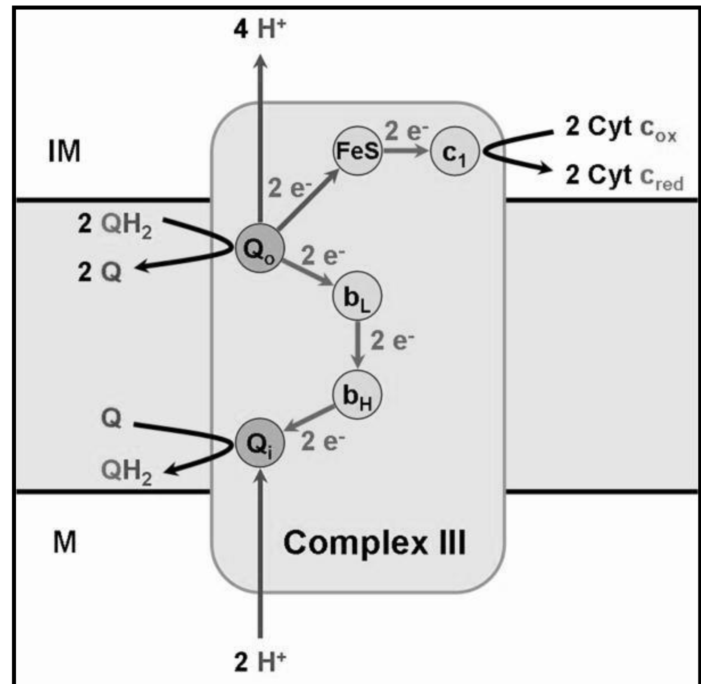


Figure 1.19 The reaction mechanism of Complex III.

The net result of the Q cycle is the transfer of the two electrons from a molecule of reduced coenzyme Q (QH_2) to two molecules of oxidized cytochrome c, forming two molecules of reduced cytochrome c and a molecule of oxidized coenzyme Q (Cronk, 2012) (Figure 1.20).

(i) $QH_2 + \text{cyt } c\ (Fe^{III}) \longrightarrow Q^{\bar{\bullet}} + 2H_P^+ + \text{cyt } c\ (Fe^{II})$
(oxidized) (reduced)

(ii) $QH_2 + Q^{\bar{\bullet}} + 2H_N^+ + \text{cyt } c\ (Fe^{III}) \longrightarrow QH_2 + 2H_P^+ + Q + \text{cyt } c\ (Fe^{II})$
(oxidized) (reduced)

Net: $QH_2 + 2\,H_N^+ + 2\text{ cyt } c\ (Fe^{III}) \longrightarrow Q + 4\,H_P^+ + 2\text{ cyt } c\ (Fe^{II})$
(oxidized) (reduced)

Figure 1.20 The separate and net reaction of Q-cycle.

Three essential subunits are present in all the *bc*1 complexes from different sources These three essential subunits are cytochrome *b* housing two *b*-type hemes (*b*L & *b*H), cytochrome *c*1 containing one *c*-type heme (*c*1), and the "Rieske" iron-sulfur protein having one high-potential [2Fe-2S] cluster. (Hunte et al., 2000; Sadoski, 2000).

1.3.1.6 Complex IV (Cytochrome c Oxidase)

Cytochrome c oxidase (COX) is a membrane protein responsible for the oxidation of cytochrome c, reduction of oxygen to water, and proton pumping to generate an electrochemical gradient across the membrane necessary for the production of ATP (Figure 1.21). The overall cytochrome c oxidase reaction is;

4 Cytochrome c (Fe^{+2}) + O_2 + $8H^+_{in}$ → 4 Cytochrome c (Fe^{+3}) + $2H_2O$ + $4H^+$out.

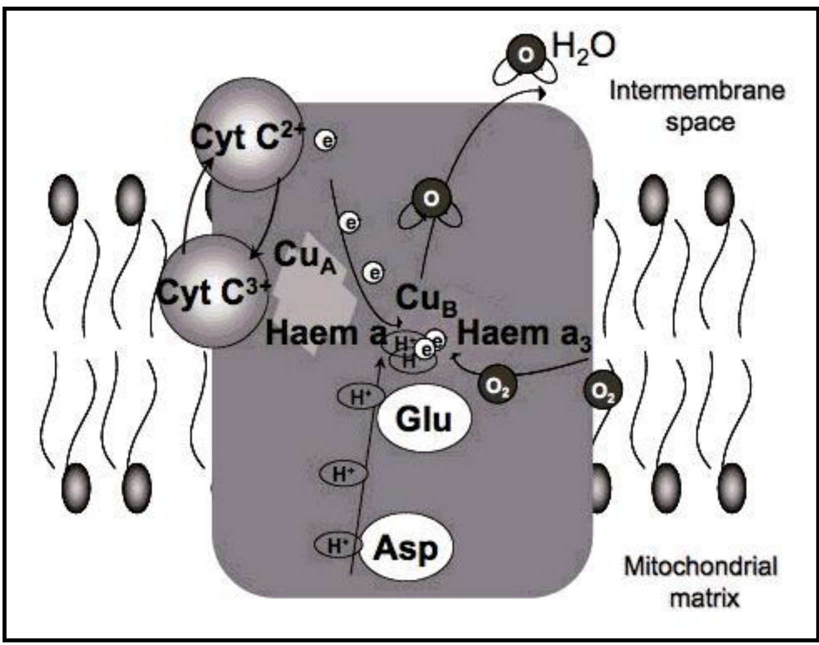


Figure 1.21 The reaction mechanism of Complex III.

The redox pathway for electron transfer stars with ferrocytochrome c docking on to cytochrome c oxidase, then passing electron from the heme c of cytochrome c oxidase to the initial electron acceptor Cu_A, then the electron passes to heme a then to the heme a_3-Cu_B binuclear center where it is reduced to water (Hill, 1994; Gennis & Ferguson-Miller, 1995; Hosler, Ferguson-Miller & Mills, 2006). Oxygenbinds to the

heme a3-CuB binuclear center where it is reduced to water. The theorectical mechanism of oxygen reduciton to water and the timing of proton uptake and release are known as the catalytic cycle. Proton channels provide the pathway for proton movement within cytochrome c oxidase. Currently there are three channels in which protonsa are predicted to be pumped, the K channeli the D channel, and H channel (Hill, 1994; Brändén, Gennis & Brezezinski, 2006). The K channel is necessary to supply protons to the binuclear center during the reductive phase of the catalytic cycle (Gennis, 2004; Fetter et al., 1995). The D channel is necessary for all "pumped protons" and also supplies chemical protons which are incorporated into water (Gennis, 2004; Fetter et al.,1996; Pawate et al., 2002).

1.3.1.7 ATP Synthase or F_0F_1 ATPase

All living organisms need energy to support their lives. For most energyconsuming biological processes, adenosine triphosphate (ATP) is the direct fuel. In eukaryotic cells, more than 95% of ATP is produced in a process called oxidative phosphorylation. Oxidative phosphorylation occurs in the mitochondrial intermembrane and is carried out by the electron transfer chain and ATP synthase. Driven by a proton gradient across the inner membrane, ATP synthase that is a large protein complex, about 560,000 Da, embedded in the membrane, can synthesize ATP from ADP and Pi (Yang, 2008, chap. 1; Berg, 2002) The ATP synthase (complex V) that transports H+ is a complex molecular machine and is composed a head-portion, F1-ATPase (catalytic unit), and a transmembrane proton carrier, Fo (1) (Shen, 2007). The F1 contains the catalytic activity of the ATP synthase and the isolated F1 also has ATP hydrolysis activity. F1 consists of five unique subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ as shown in Figure 1.22. The three α-subunits and three β-subunits arrange alternatively to form a hexameric ring. The γ-subunit protrudes into the center of the $\alpha_3\beta_3$ hexamer. The N- and C-terminal regions of γ-subunit form a coiled-coil structure, which has interaction with α,β-subunits. The δ- and ε-subunits wrap around the bottom portion of γ-subunit and γ, δ and ε together form the central stalk (Abrahams, Leslie, Lutter & Walker, 1994; Kabaleeswaran, Puri, Walker, Leslie & Mueller, 2006). The Fo part mainly consists of three subunits with the

stoichiometry: ab_2c_{10-14}. It acts as a proton pore that converts the energy of the proton gradient to mechanical energy. Subunit a contains a proton channel, through which protons flow from one side of the membrane to the other side and makes rotation of subunits c (Elston, Wang & Oster, 1998). The subunit b acts as a stator holding the F1 part and preventing it from rotating (Walker & Dickson, 2006).

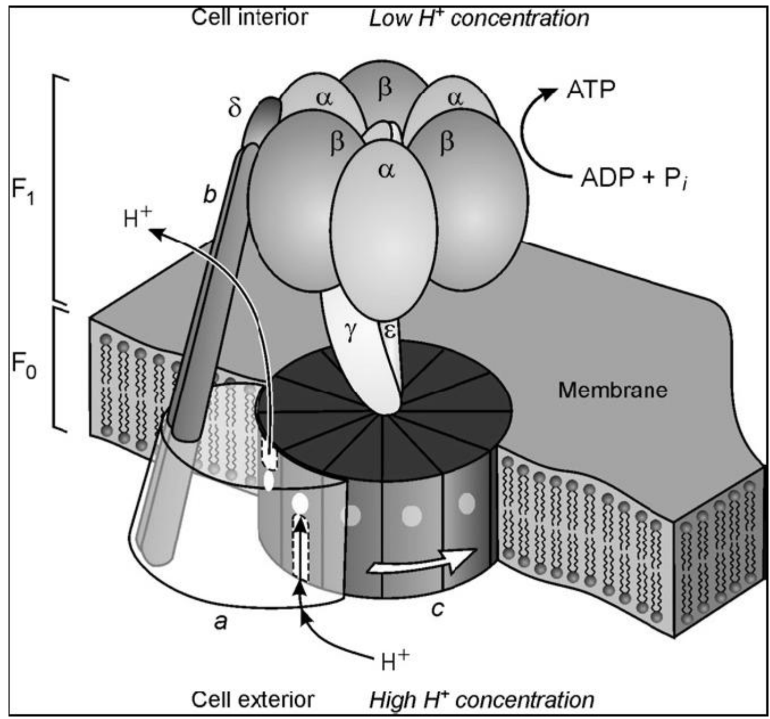


Figure 1.22 The reaction mechanism of ATP synthase.

1.3.2 Electron Leak

Mitochondrial proton and electron leak have a major impact on mitochondrial coupling efficiency and production of reactive oxygen species (Jastroch, Divakaruni, Mookerjee, Treberg & Brand 2010). One of the consistent sources of oxygen radicals among tissues is mitocnondrial respiratory chain (Chance, Sies & Boveris, 1979; Boveris & Cadens, 1982). When electron transfer from substrates to oxygen proceeds along respiratory chain, not all the oxygen is tetravalently reduced to form water via cytochrome oxidase. Instead, a small portion of oxygen molecules can accept single electron transfer to form superoxide radicals between NADH and the site of antimycin block by so called "electron univalent leak" or "electron leak" pathway.

Under normal physiological condition, the superoxide in the mitochondria can be metabolized by Mn^{++} superoxide dismutase (Mn-SOD) and other scavenging enzymes, and the steady-state concentrations of superoxide and hydrogen peroxide in vivo are maintained at about 10^{-11}M and 10^{-9}M respectively (Boveris & Cadens, 1982; Lippman, 1981; Rochter, 1994; Forman, 1982).

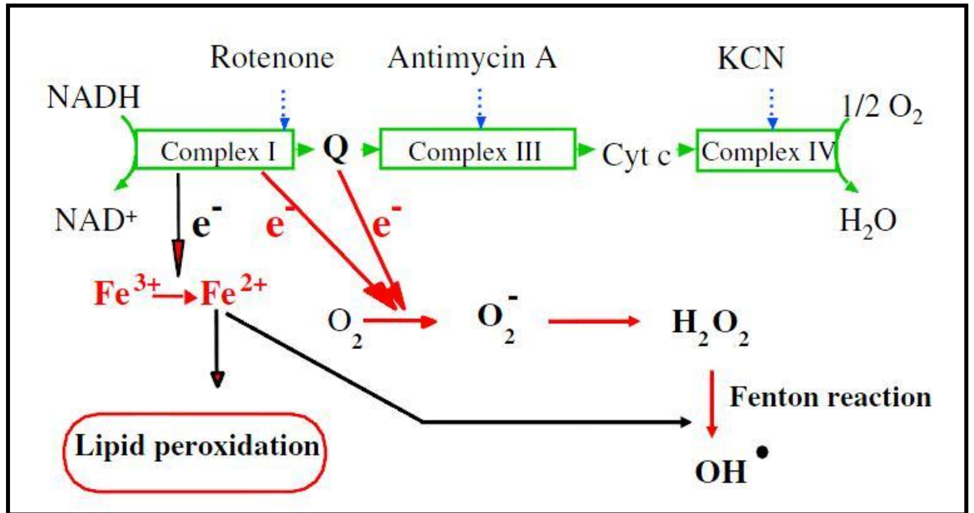


Figure1.23 Electron leaks schema from respiratory chain (complex II is omitted in this scheme).

There are two kinds of electron leak in the respiratory chain as shown in Figure 1.23, which lead to production of superoxide anions and reduction of free iron ions. The reduced free iron ion (Fe^{2+}) initiates lipid peroxidation (Kang, Kim & Hamasaki 2007).

1.3.3 Antioxidant

Antioxidants (oxidation inhibitors) represent a class of substances that very widely in chemical structrure that reduce oxidative damage. Antioxidants have been found to act as defensive and protective agents against oxidative species in the human body, food and plants, inhibiting the decompositions of oxidation products which result in decreased nutritional values and sensory quality. The damaging effects of O_2 could be attributed to the formation of oxygen radicals (Al-Turki, 2008).

Exposure to oxygen produces toxic reactive oxygen species (ROS) in the human body. ROS form naturally and are normally quenched quickly by antioxidants, so that damage to cellular molecules is minimal. There are two kinds of ROS: radicals and nonradicals. Radical ROS include superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radical ($RO_2^{\cdot}$)', alkoxyl radical ($RO^{\cdot}$) and hydroperoxyl radical ($HO_2^{\cdot}$). Non-radical ROS include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3) and singlet oxygen ($^1O_2$). Oxidative stress from ROS causes damage to many types of cellular molecules, including lipids, proteins and DNA. If such damages remain unrepaired, oxidative stress can lead to cell death or even induce cancer (Zhu, 2006, p. 1).

Enzymatic and non-enzymatic antioxidants are two types of biological antioxidants. Enzymatic antioxidant Superoxide dismutase (SOD), phospholipid hydroperoxide glutathione peroxidase, selenium glutathione peroxidase, and catalase are important antioxidant enzymes in the human body (Siu & Draper, 1982; Krinsky, 1992; Halliwell & Gutteridge, 1986; Ames, Shigenaga & Hagen, 1993). SOD, present in mitochondria and cytosol, can convert the superoxide anion into oxygen and the less toxic hydrogen peroxide H_2O_2 (Halliwell, Gutteridge & Cross, 1992; Siu et al., 1982; Krinsky, 1992; Halliwell & Gutteridge, 1986; Ames et al., 1993; Cheeseman et al., 1993). Glutathione peroxidase can eliminate H_2O_2 and organic peroxide through the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). Reduced GSH can be regenerated by glutathione reductase (Halliwell, Gutteridge & Cross, 1992) Catalase in peroxisomes helps to eliminate hydrogen peroxide by converting H_2O_2 to water. However, no enzymatic antioxidant is known to detoxify singlet oxygen. The human body contains a variety of non-enzymatic antioxidants, including GSH, uric acid, a-tocopherol (vitamin E), and ascorbic acid (vitamin C). Several other nonnutritive antioxidants are supplied in the diet such as carotenoids and flavonoids. There are three main non-enzymatic mechanisms for controlling lipid peroxidation: 1) the free radical chain-breaking (CB) mechanism; 2) metal chelation; and 3) single oxygen quenching (Figure 1.24).

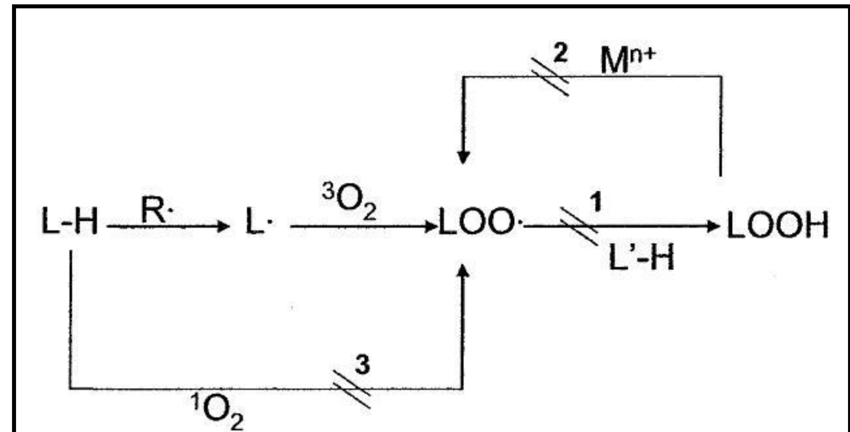


Figure 1.24 Non-enzymatic mechanisms to control lipid peroxidation:
1) Chain-breaking donor (CB-D); 2) Metal-chelation; 3) Singlet oxygen quenching.

1.3.3.1 Capsaicin

Capsaicin take part in the non-enzymatic antioxidant mechanism group. *Capsicum* species (Solanaceae), or hot peppers, are important plants and have been used worldwide as foods, spices, and medicines. The pungent principal component of red peppers is a group of acid amides of vanillylamine and C8 to C13 fatty acids, which are known generally as capsaicin (Figure 1.25). More than 16 other capsaicinoids have been found as minor components (Suzuki & Iwai, 1984).

Figure 1.25 Structre of capsaicin.

Capsaicin has many useful properties. However, its use as a spice or drug is limited by its strong pungency and nociceptive activity (Kobata, Todo, Yazawa, Iwai & Watanabe, 1998). Capsaicin inhibited the lipid peroxidation significantly. Capsaicin was found to scavenge radicals both at/near the membrane surface and in the interior of the membrane. The phenolic OH group of capsaicin is not associated

with the radical scavenging reaction (Kogure et al., 2002). Capsaicin has included an amide group that was associated with antioxidant activity and this site suggested that is the most probable site for free-radical attack in molecules (Henderson, D. E., & Henderson, S. K. (1992)). Capsaicin inhibits generation of reactive oxygen species in rat peritoneal macrophages. Phorbolester induced activation of nuclear factor-kappa B (NF-nB) and activator protein-1 (AP-1) induction of apoptosis and electron transfer in bovine heart mitochondrial complex I (Joe & Lokesh, 1994; Han et al., 2001; Macho et al., 1999; Lee, Nam & Kim, 2000; Jung, Kang & Moon, 2001; Miyoshi, 1998).

1.4 Lipid Peroxidation

Lipid peroxidation is either a causative or an associative factor in various pathological conditions, such as atherosclerosis, macular degeneration, ischemia-reperfusion injury, tumorigenesis, and a variety of nervous disorders that results from the oxidative deterioration of polyunsaturated fatty acids (Hanlon & Seybert, 1997; Xu & Sayre, 1998). Lipid peroxidation begins with a free radical mechanism that yields lipid hydroperoxides as the major reaction products. The lipid hydroperoxides then decompose to products that have a wide range of damaging effects, including various α,β-unsaturated aldehydes as end products (Uchida et al., 1998). The α,β-unsaturated aldehydes are relatively stable and therefore can migrate to other regions in the system to react with molecules not directly adjacent to the location where they were generated (Rahman et al., 2002).Even though these α,β-unsaturated aldehydes are end products of lipid peroxidation, they are free to react with other biomolecules present in the system. One of the consequences of lipid peroxidation is the age-related accumulation of lipofuscin and ceroid in biological samples (Xu et al., 1998; Uchida et al., 1998; Pryor & Porter, 1990; Rahman et al., 2002; Friguet, Stadtman & Szweda, 1994).

1.4.1 The Lipid Peroxidation Chain Reaction

Oxidative stress can cause oxidative damage to biomolecules such as lipids, proteins, and DNA. These effects may result in increased risk for cancer (Ziedman, 2012). Lipid peroxidation mechanism includes a free radical chain mechanism consisting of three major phases; initiation, propagation and termination. Oxidation of lipid is initiated by ROS that abstract one hydrogen radical ($H^{\cdot}$) from polyunsaturated fatty acids (PUFA) (equation 1). Rapid propagation follows through reaction with ground state oxygen to give the peroxyl radical $LOO^{\cdot}$ (equation 2) and abstraction of another $H^{\cdot}$ from neighbor PUFA by $LOO^{\cdot}$, resulting in the formation of lipid peroxides (LOOH) and a new radical (equation 3). Lipid peroxides decompose through cleavage of double bonds resulting in the formation of carbonyl products,such as malondialdehyde (MDA) and 4- 3 hydroxynonenal (4-HNE). The chain reaction is terminated when two radicals combine and form a new non-radical compound (equations 4-6) (Zhu, 2006).

Initiation

$L\text{-}H + \text{oxidant} \rightarrow L^{\cdot} + \text{oxidant-H}$ (1)

Propagation

$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$ (2)

$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH$ (3)

Termination

$L^{\cdot} + L^{\cdot} \rightarrow$ non-radical products (L-L) (4)

$L^{\cdot\cdot} + LOO^{\cdot} \rightarrow$ non-radical products (LOO-L) (5)

$LOO^{\cdot} + LOO^{\cdot} \rightarrow LOOL + O_2$ (6)

In addition, the initiation phase can be triggered by an oxygen molecule (Rawls & Van Santen, 1970). In this situation, singlet oxygen is 1500 times more reactive than triplet oxygen. As a result, the peroxyl radical LOO- can be formed in the presence of singlet oxygen (equations 7-8) (Zhu, 2006).

$L\text{-}H + {}^3O_2 \rightarrow L^{\cdot} + HOO^{\cdot}$ (7)

$L\text{-}H + {}^1O_2 \rightarrow H^{\cdot} + LOO^{\cdot}$ (8)

1.5 Eukaryotic models

For many years, numerous data was accumulated referring to such organisms, model organisms have been used to recieved some information involved to other species including humans which are more difficult to investigation directly. Primary eukaryotic models are fungi, yeast, cell culture, rat, mouse, hamster, cobay. The rat as the eukaryotic model shows similar genomes with human. We have studied with Male Sprague Dawley adult rats as eukaryotic model for cisplatin toxicity on different tissue; barin, kidney, heart, liver and lung.

CHAPTER TWO
MATERIAL AND METHOD

2.1 Animals and Cisplatin Injection

Male Sprague Dawley adult rats (12weeks, 450-500 grams) were housed for 4 week before the experiments. Rats have been housed in a temperature-controlled room and in an air conditioned room on a 12-h light, 12-h darkness Schedule, fed commercial rat chow and water ad libitum (Minami, Okazaki, Kawabata, Kuroda & Okazaki, 1998; Kumagai, Sugiyama, Nishida, Ushijima & Yakushiji 1996).

After single injection of cisplatin was given to Male Sprague Dawley adult rats (5 mg/kg, intramuscularly), rats were anesthetized by sodium pentobarbital with intravenous at 1st, 4th, 7th, 14th days and then brain, liver, lung, heart and kidney were collected immediately after sacrificing the rats. The control group rats received single dose serum physiologic at 1st day.

Block type diet was used to feed for rats, these blocks includes barley, corn, wheat, cotton seed pulp, sunflower seed pulp, nut pulp, scurf, sorghum, tapioca. Rat blocks contents is dry matter 88%, crude protein 14%, crude cellulose %11, crude ash %10, calcium %1.3-2.0, phosphor %1, sodium %0,5-1.0, NaCl %1, vitamine A 10000 IU/kg, vitamine D_3 IU/kg, vitamine E 30 IU/kg.

2.2 Capsaicin Preparation & Injection

Male Sprague Dawley adult rats (450-500 grams) have been used in experiment and capsaicin powder has been dissolved in ethanol to reach a final concentration of ethanol 0.625% in serum. Cisplatin (5 mg/kg) were injected in a single dose followed by 7 days capsaicin (10mg/kg) were injected intramuscularly. Capsaicin dose has been chosen based on the previous literature involved in protective effect of capsaicin to drug toxicity (Yuka et al., 2005). The initial capsaicin injection were carried out just after cisplatin injection. Single dose serum physiologic were injected to rats at 1st day for control groups.

2.3 Crude extract preparation

2.3.1 Mitochondrial preparation

3–15 times isolation buffer volumes; 10, 20, 30, 40, 50, 60 seconds homogenization periods were studied. The best isolation was determined such as; the thawed samples were resuspended in isolation buffer (1:15 weight/volume) containing 5mM HEPES, pH 7.4, containing 1 mM EDTA, 300 mM sucrose. Sample tissues were homogenized at 8000 and 9500 rpm for different seconds based on tissue types in the ice. Heart tissue has been homogenized 9500 rpm for 50 seconds, lung, liver, kidney and brain tissues were homogenized 8000 rpm for 50, 40, 20 and 20 seconds, respectively. Tissue suspensions were ground in 1.5 ml plastic vials and centrifuged at 2000 rpm for 15 minutes and cell debrises were removed. The supernatant was centrifuged at 12000 rpm for 15 minutes. Final pellet contains mitochondria. Before assaying, the mitochondrial pelletes were resuspended in isolation buffer and used for succinate dehydrogenase and cytochrome c oxidase activity assay.

2.3.2 Cytosolic preparation

Tissues homogenized in isolation buffer, (1:15 w/v), all tissues except heart were homogenized, at 8000 rpm for 90 seconds in the ice, only heart tissue has been homojenized at 9500 rpm for 90 second and homogenization periods were 3x30 seconds. Samples suspension was ground in 1.5 ml plastic vials and centrifuged at 2000 rpm for 15 minutes and cell debris was removed. The supernatant was used for CAT assay.

2.3.3 The preparation of sample for cisplatin level determination

The brain, heart, lung, liver, kidney and lung tissues were placed at 105°C for approximately 24 hours until the weight remained constant. 9 mL HCl and 3 mL HNO_3 were directly added onto the dry tissues, then these remained microwave oven

for UV decomposition about an hour. Their volumes completed to 20 mL with distillated water. The samples filtrated with black band type of filter papers. Terminal half life of cisplatin is 58-73 hours. Therefore 1st and 4th days of all studied tissues were analysed for cisplatin levels with ICP/MS.

2.3.4 Sample preparation for nucleotide level determination

The samples were prepared using a modified procedures of Cardoso et al. and Masubuchi et al. (Cardoso, Pereira & Oliveira, 1999; Masubuchi, Suda & Horie, 2005). Chilled tissues were homogenized and then 1 M $HClO_4$ (w/v) transferred into the homogenates in a volume equal to 5 times their weights. They were centrifuged for 15 mins at 5000 rpm. The supernatants were neutralized with 1M K_2CO_3 and then again centrifuged. The clear supernatants were injected into HPLC for determining the levels of cytosolic adenine nucleotides.

2.4 Enzyme Activity Assay

2.4.1 Succinate Dehydrogenase Activity Assay (Complex II)

Succinate dehydrogenase in mitochondrial pellet was assayed by measuring the initial rate of decrease in dichloroindophenol (DCIP) absorbance at 600 nm. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 1.0 mM EDTA, 20 mM sodium succinate, 3 mM sodium azid, 5 μl enzyme solution and 32 μM DCIP (Hatefi, & Galante, 1981).

2.4.2 Cytochrome c Oxidase Activity Assay (Complex IV)

Cytochrome c oxidase in mitochondrial pellet was assayed by measuring the initial rate of decrease in cytochrome c absorbance which was reduced by ascorbic acid at 550 nm. The reaction mixture contained 87.5 mM potassium phosphate buffer, pH 7.0, 30 μM reduced cytochrome c and 50 μl enzyme solution. Cytochrome c in 10 mM potassium phosphate buffer, pH 7.0 was reduced by adding ascorbic acid and monitoring the absorbance at 550 nm and 565 nm. Blank solution include 90 mM potassium

phosphate buffer, pH 7.0, 30 μM reduced cytochrome c and potassium ferricyanide ($K_3[Fe(CN)_6]$) 2.5 mM. $K_3[Fe(CN)_6]$ is included in the blank only in order to completely oxidize the reduced cytochrome c (Wharton, & Tzagoloff, 1967).

2.4.3 Catalase Assay

Catalase (CAT) activity in cytosol was determined in crude extract by the method of Aebi (Aebi, 1974).

2.5 Cisplatin Determination

2.5.1 ICP/MS Condition

The cisplatin concentration was measured by ICP/MS. This method conditions are RF power:1550 W; RF matching:1,78 V; sample depth:7,8 mm; carrier gas:0,87 L/min; make-up gas:0,1L/min; integration time:0,1 sec; acqusition time:22,76 sec; Nickel sampling and skimmer cone were used.

2.6 Adenine Nücleotids Assay

2.6.1 HPLC Conditions for Adenine Nücleotids

The HP 1100 HPLC system used was equipped with a photodiode detector. 50 mM aqueous triethylamin (TEA) buffer (adjusted with phosphoric acid to pH 6.5; A) and acetonitrile (B). Gradient elution was performed from 99 A/1B in 10 min to 95A/5B and changed in another 10 min to 92.5A/ 7.5B. Each run was followed by a 5-min wash with 70B/30 parts 0.1% phosphoric acid Detection wavelength, flow rate, column temperature were set to 254 nm, 1 ml/min, 20 ^{o}C (Ganzera, Vrabl, Wörle, Burgstaller & Stuppner, 2006).

2.7 Lipid Peroxidation

Tissues homogenized in isolation buffer, (1:3 w/v), pH 7.5. 500µL homogenate was transferred into 2.5 ml 10% TCA, incubated 90ºC for 15 minute, cooled and then centrifuged at 3900 rpm for 10 minute. 2 ml supernatant was added into 1 ml % 0.675 TBA solution. The mixture was incubated 90 ºC for 15 minute. After cooling, the absorbance was measured 532 nm. Malondialdehyde (MDA), an end product of fatty acid peroxidation, reacts with TBA and forms a coloured complex. This complex has maximum absorbance at 532 nm. MDA values in nanomoles were calculated from the absorbance coefficient of MDA-TBA complex at 532 nm, 1.56×10^{5} mol^{-1} x cm^{-1} (Buege & Aust, 1978).

2.8 Protein Determination

The protein content was determined by the method of Bradford et al. (1976) (Bradford, 1976). Bovine serum albumin as standard was used.

CHAPTER THREE
RESULTS AND DISCUSSION

Cisplatin, cis-diamminedichloroplatinum, is an effective anticancer drug, particularly for testicular ovarian, lung, bladder, gastrointestinal cancers. The mechanism of cisplatin uptake is not completely understood, however, both passive diffusion and carrier-mediated transport have been implicated. After its transport to the cytoplasm, cisplatin undergoes aquation, due to lower chloride concentration in the cytoplasm. The aquated form is thought to be the more reactive form of cisplatin. Cisplatin toxicity can lead to severe effects on the kidneys, nervous system and auditory system.

In different cancer cells, the primer target of cisplatin is mitochondria which plays an important role in generation of ATP through oxidative phosphorylation (OXPHOS). Some drug bring about alteration in mitochondrial membrane and the altered mitochondrial membrane potential can cause decreases in ATP production because of the lack of available protons in the inter membrane spaces for ATP synthase utilization, as well as failure of protein translocation, and interference with other mitochondrial functions (Mans, 2010).

The electron transport chain (ETC) via oxidative phosphorylation, is known to be the major intracellular site for the generation of reactive oxygen species (ROS) by continuously converting 1 to 2% of consumed oxygen as well as for ATP production. Aerobic cells have been normally protected from oxidative damage in various ways, involved in the activities of SOD, CAT and glutathione peroxidase. However under oxidative stress, the exposure of ROS to biological membranes induces progressive degeneration of membrane structure that is rich in polyunsaturated fatty acids (PUFA) and termed lipid peroxidation (LPO). Initiation of LPO is caused by abstract a hydrogen atom from a methylene group and multiple peroxidation reactions. Lipid hydroperoxides and malondialdehyde (MDA) form result in multiple peroxidation reactions of PUFA.

According to the some reports, the generation of ROS is a critical event that initiates damage to the various cells, leading to cell loss after cisplatin treatment (Kaur, 2012). However, the mechanisms underlying the transition from ROS generation to the manifestation of toxicity is not clearly defined. Therefore, we have studied whether cisplatin, used at 5mg/kg dose on five different tissues, three of which are composed of postmitotic cells (brain, heart) and two of slowly dividing cells (liver and kidney) as well as lung tissues in Sprague Dawley rats, changes mitochondrial electron transport chain by activates the mitochondrial complex II and IV and generation of adenine nucleotides (Kwong & Sohal, 2000). Since ETC also leads to form ROS, as an antioxidant enzyme catalase and indicative marker of membrane damage LPO were also studied under exposure of cisplatin. To understand the effect of capsaicin which is important antioxidant, changes of all studied parameters were also determined depending on capsaicin and cisplatin conditions.

3.1 Platin Levels in Different Tissues of Male Sprague Dawley Adult Rats

Effective anticancer agent cisplatin can uptake by both passive diffusion and carrier-mediated transport. According to the our results, cisplatin in Sprague Dawley rats was also transported to brain, liver, kidney, lung and heart tissues. In addition cisplatin levels for 1st day reached to maximum in liver as 0.38 ppm/gr tissue and the levels were ordered as kidney, brain, lung and heart as 0.36, 0.24, 0.24, 0.15 ppm/gr tissue, respectively. The results shows that cisplatin transported all studied tissues. Determinated cisplatin levels at 4th day were low in brain compaired to others tissues. Maybe explained by blood-brain barrier to protects by certain molecules. As can be seen in Table 3.1, cisplatin levels after 4th day of injection of cisplatin decreased for brain, heart, kidney, liver and lung as 100, 73.2, 43.7, 4.5 and 2.3%, respectively. The results showed coherence with the other article involved in rat showed that cisplatin levels in brain decreased markedly within a few days after injection of cisplatin probably to protect certain molecules from reaching central nervous system (Cerri et al., 2011). The decreases of cipslatin levels in kidney from 0.36 to 0.19

ppm/gr tissue may supports that Pt was excreted from kidney by urinary (Valentovic, Scott, Madah, & Yokel, 1991).

Table 3.1 Platin levels in 1gr of brain, liver, kidney, heart and lung tissues.

	Brain	**Liver**	**Kidney**	**Heart**	**Lung**
1st Day (ppm/gr tissue)	0.24	0.38	0.36	0.15	0.24
4th Day (ppm/gr tissue)	Non Detectable	0.36	0.19	0.039	0.23

3.2 Variations in SDH Activities of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

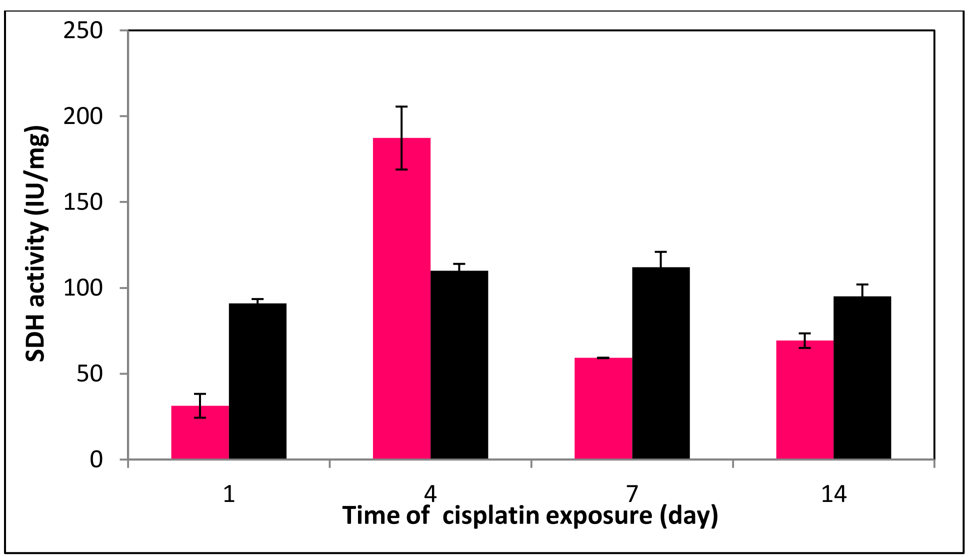


Figure 3.1 Variations in SDH activity in liver tissues depending on the days : liver tissues (—■—), control of liver tissues (—■—).

Succinate dehydrogenase, which actually belongs to the tricarboxylic acid cycle, is also assigned to the respiratory chain as complex II (Koolman et al., 2005). As shown in Figure 3.1, SDH activities in liver of cisplatin exposed to Male Sprague Dawley rats were lower compared to unexposed controls except for 4th day of injection. The results may showed evidence that cisplatin induced mitochondrial dysfunction is caused by inhibiton of complex II of ETC (Kruidering, De Water, De Heer, Mulder, & Nagelkerke, 1996). This inhibition is probably caused by

accumulation of cisplatin in the negatively charged inner space of the mitochondria because of the positive charge of aquated complexes of cisplatin (Kruidering et. al, 1996). Nevertheless, the increases in SDH activities in liver at 4th day of cisplatin exposure may supports adaptation of liver associated deleterious alterations in the liver of Male Sprague Dawley rats. It was obtained that SDH activity levels were highest in liver in comparasion with other studied tissues.

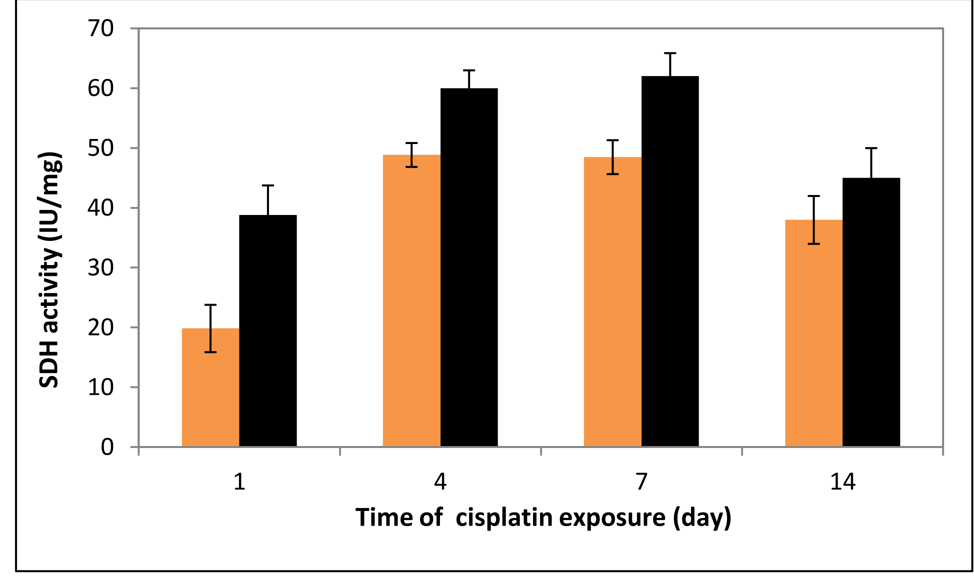


Figure 3.2 Variations in SDH activity in lung tissues depending on the days: lung tissues (—■—), control of lung tissues (—■—).

As shown in Figure 3.2, SDH activities levels in lung of cisplatin exposed to Male Sprague Dawley rats were significantly lower compared to control four all studied days of cisplatin exposed. Although SDH activity at 4th days of exposure to cisplatin increased significantly according to 1st day but it was not higher compared to control in contrast to liver. Consistent with previous literature the toxicity occurred in lung where SDH activities were significantly decreased to be 48.9, 18.6, 21.8, 15.6 % in comparison with control at 1st, 4th, 7th, 14th days, respectively. The observed decreases in the enzyme activities may also indicated suggested that cisplatin adversely effect oxidative phosporylation of the respiratory components. It was determinated that SDH activity levels were lowest in lung compared to other studied tissues.

As can be seen in Figure 3.3, decreases in SDH activities levels in heart of cisplatin exposed to Male Sprague Dawley rats were also determined to control for first four days of cisplatin exposed. However, SDH activities markedly increased on 7th and 14th days and they nearly reached the control levels after 7th day of cisplatin exposure. Since the increases in SDH activity of exposure cisplatin in heart continually begining from 1st day to 7th day, the activity levels reached control at 7th day. The results can be explained by rapid adaptation of heart because of the lowest levels of cisplatin in heart at 1th day compared to other tisues.

Decreases in SDH activity both in lung and heart tissues as shown in liver, it may explain cisplatin induced SDH inhibiton. SDH enzyme activity at 4th day increased compared to 1st day of exposeure cisplatin in lung and heart, it suggest that ETC produced ATP for detoxification mechanism or DNA repair mechanism against to DNA damage.

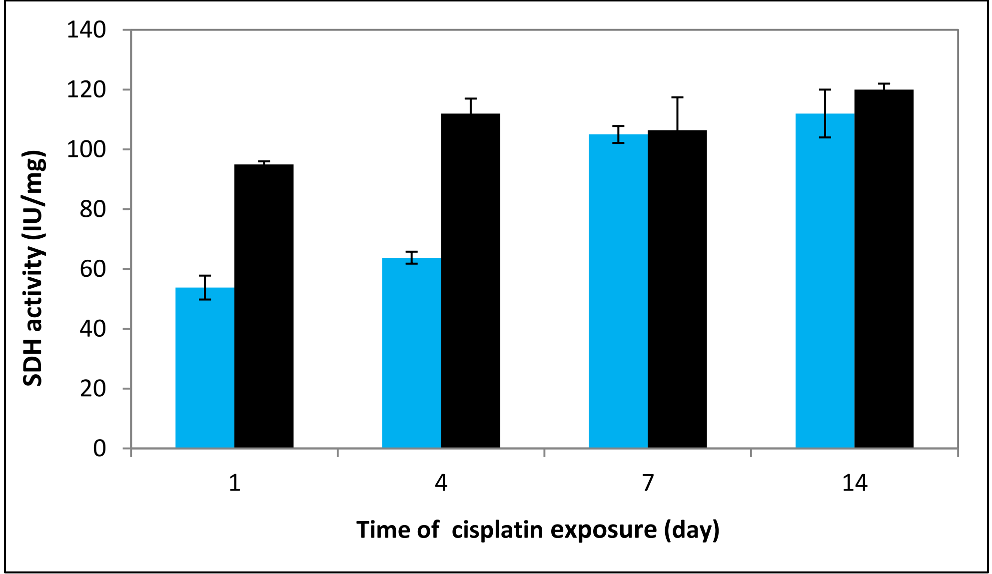


Figure 3.3 Variations in SDH activity in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

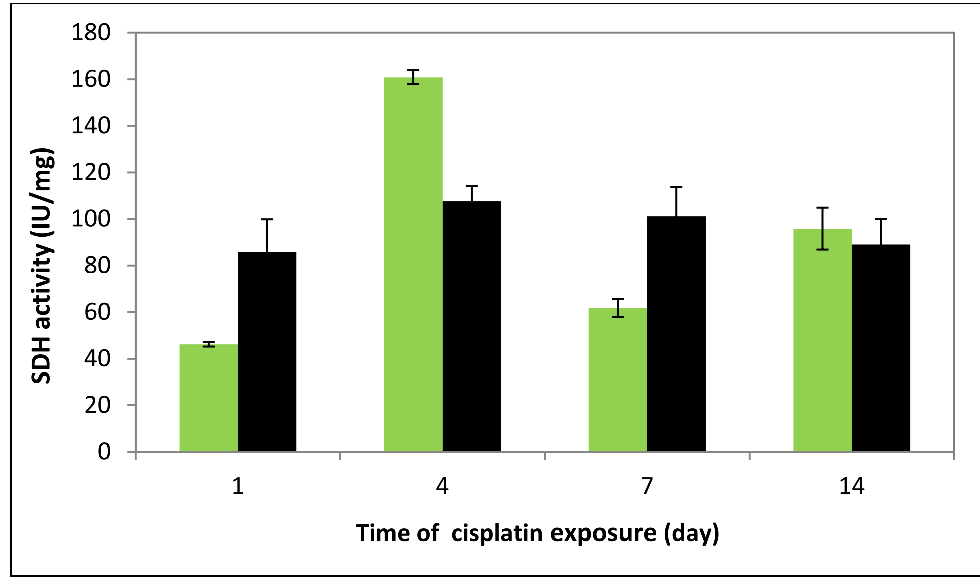


Figure 3.4 Variations in SDH activity in brain tissues depending on the days: brain tissues (—■—), control of brain tissues (—■—).

Figure 3.4 shows that SDH activity in cisplatin group of brain decreased 1.85 fold at 1th day compared at the control. However, SDH activity increased 3.5 fold at 4th day compared to 1th day. The SDH activity level of brain was also higher than the control group tissue for 4th day parallel to liver tissue. To reduce neurotoxicity and protect the brain, platin levels reached non-detectable level in cisplatin injection group of brain tissue at the end of the 4th day. And this situation may explain that brain need more ATP for cisplatin excretion from brain barrier.

Figure 3.5 indicate that SDH activity in cisplatin injection group of kidney reduced significantly for first day. The result showed coherence with the previous publish article which showed that cisplatin cause disruption of the mitochondrial respiratory chain as a result of inhibition of SDH by inducing nephrotoxicity (Nowak, 2012). The SDH activity belong to kidney for all investigated days showed similar trends with the corresponding changes in the liver. The determination of higher SDH acitiviy levels in cisplatin exposed tissues at 4th day also supported that the effect of rapid transportation rate of cisplatin from these tissues. Increases in SDH activity of kidney at 14th compared to 7th day may indicate that kidney

adaptation was determined with lower cisplatin level by excretion of cisplatin from kidney.

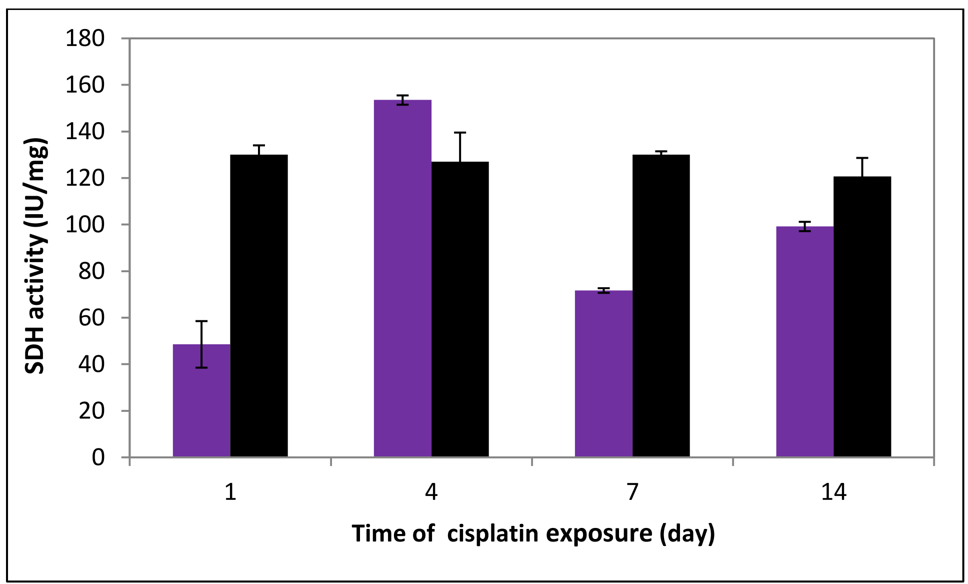


Figure 3.5 Variations in SDH activity in kidney tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

3.3 Variations in COX Activities of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

Cytochrome c oxidase is a membrane protein responsible for the oxidation of cytochrome c, reduction of oxygen to water, and proton pumping to generate an electrochemical gradient across the membrane necessary for the production of ATP. As shown in Figure 3.6, COX acitivities in liver of cisplatin exposed to Male Sprague Dawley rats were lower compared to control group for 1st day and it nearly reached the control groups for 7th day. COX activity significantly increased for 4th and 14th day according to unexposed control group. COX activity in liver of cisplatin injected rats group at 1st, 4th and 7th days by showing parallelism with SDH activity in Figure 3.1.

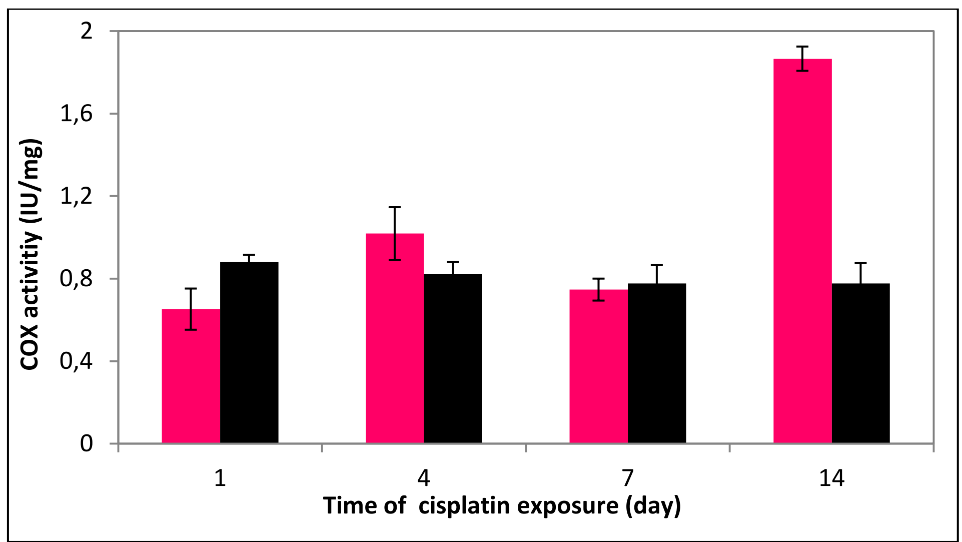


Figure 3.6 Variations in COX activity in liver tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

As can be seen in Figure 3.7, COX activities levels in lung of cisplatin exposed to Male Sprague Dawley rats show similar tends with SDH activity. COX activities of cisplatin groups were significantly reduced to be 43.1, 41.5, 14.9, 43.7 % in comparison with control at 1st, 4th, 7th, 14th days, respectively.

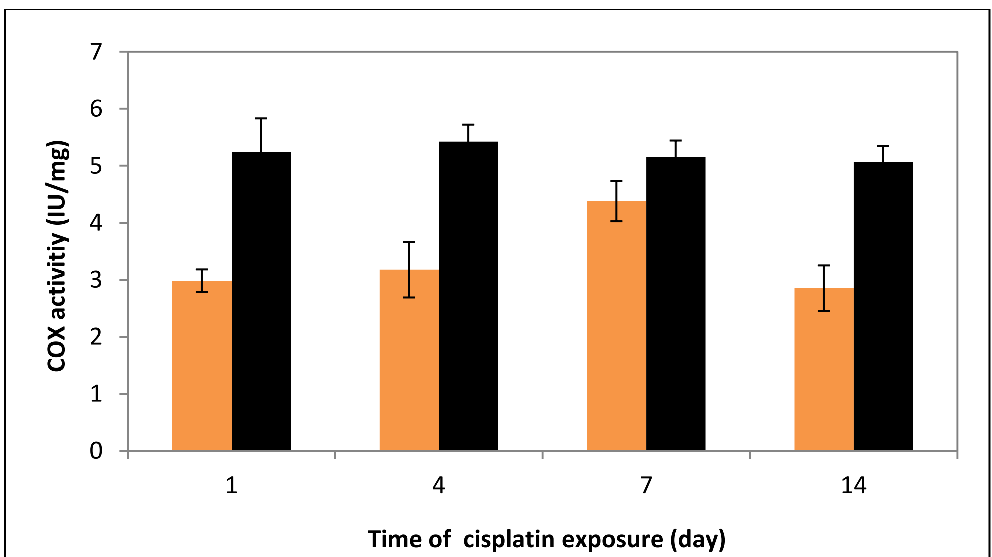


Figure 3.7 Variations in COX activity in lung tissues depending on the days: lung tissues (—■—), control of lung tissues (—■—).

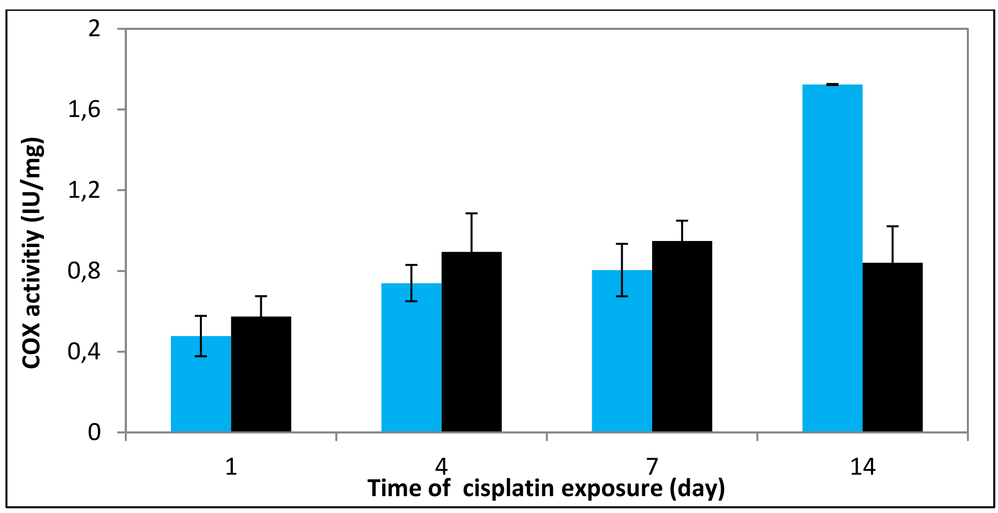


Figure 3.8 Variations in COX activity in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

As shown in Figure 3.8, COX activity levels group of heart decreased compared to control for first 7th days. COX activity in cisplatin group decreased 1.20, 1.21, 1.18 fold at 1st, 4th and 7th days, respectively.

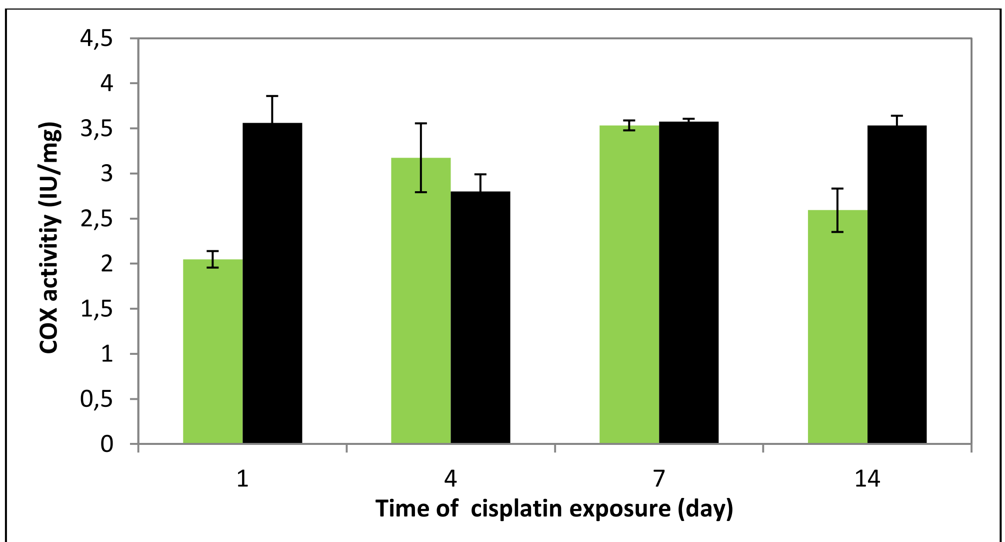


Figure 3.9 Variations in COX activity in brain tissues depending on the days: brain tissues (—■—), control of brain tissues (—■—).

As can be seen in Figure 3.9, COX activities in brain of cisplatin exposed to Male Sprague Dawley rats reduced for 1st day compared to control groups. The maximum enzyme activites observed at 4th day compared to control group.

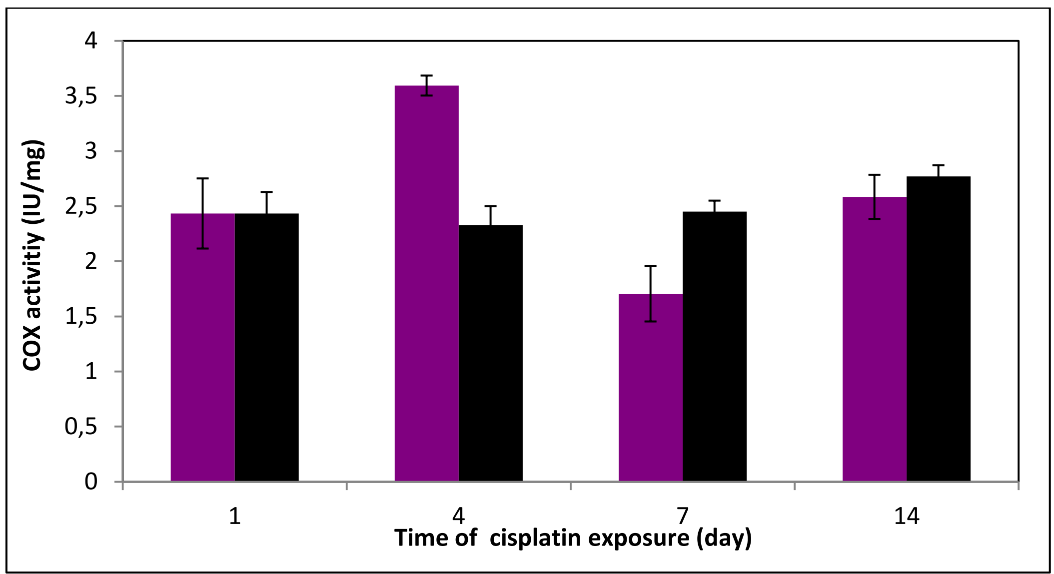


Figure 3.10 Variations in COX activity in kidney tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

As shown in Figure 3.10, COX activity levels group of kidney increased 36% compared to control for first 4th days. However, COX activities in cisplatin groups decreased 1.4, 1.1 fold at 7th, 14th days, respectively.

3.4 Variations in CAT Activities of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

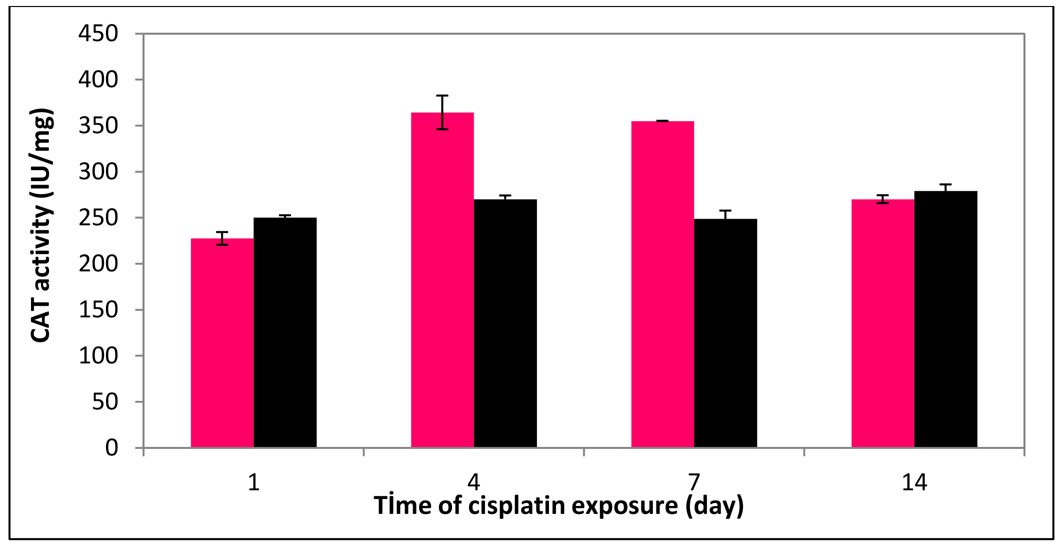


Figure 3.11 Variations in CAT activity in liver tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

As an important antioxidant enzymes catalase, plays important role on protection of mitochondrial dysfunction against to ROS which is produced by electron leakage in ETC system. As can be seen in Figure 3.11, while CAT activity in liver of cisplatin injected rats group at 1st day not change compared to control the level of cisplatin group increased from 227.3 to 364.2 IU/mg at 4th day by showing parallelism with SDH and COX. These data may suggest that cisplatin may lead to increases intracellular levels of ROS, in particular H_2O_2 and therefore CAT overexpressed to prevent cell. Despite descreses in SDH an COX, CAT activity increased in liver at 7th day, it may suggest that cisplatin may induced ROS production with other mechanism On the other hand, decreases in CAT activity of cisplatin group at 14th day by reaching similar level of control may indicate adaptation of cells to balance oxidative stress.

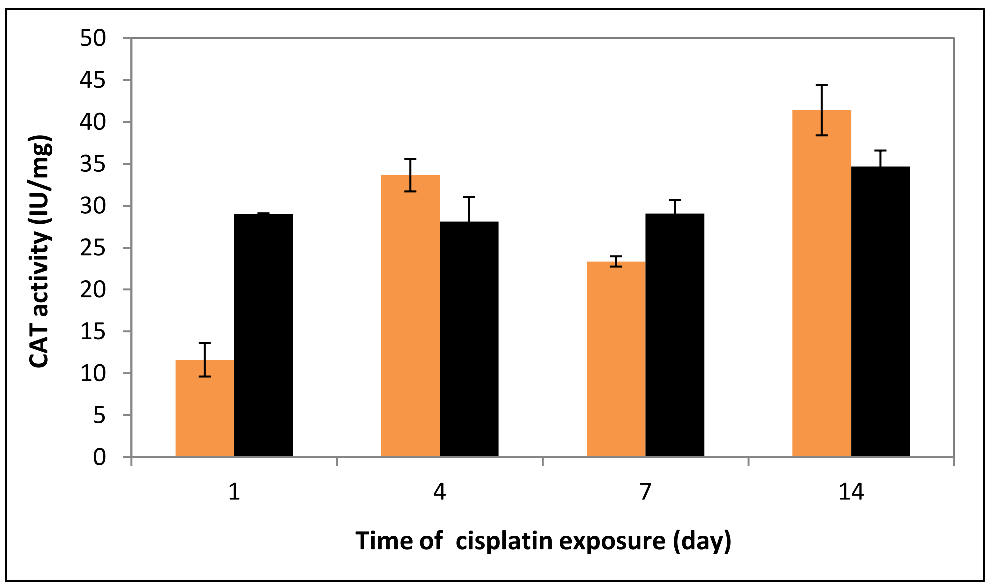


Figure 3.12 Variations in CAT activity in lung tissues depending on the days:lung tissues (—■—), control of lung tissues (—■—).

Figure 3.12 shows that, CAT activity in lung of cisplatin exposed to Male Sprague Dawley rats were lower compared to unexposed controls for 1st day of injection while the CAT activity were higher for cisplatin group at 4th day. The similar alterations trends in CAT activity besides cisplatin levels between liver and lung tissue may suggest that cisplatin has a critical role on both ETC and antioxidant system (Oral, George, & Haskard, 2000). CAT activity in lung tissues at 1st and 7th

days by showing parallelism with SDH and COX enzyme activity. Increases in CAT activity at 4th and 14th days may explain ROS production were induced by other mechanism in lung tissues.

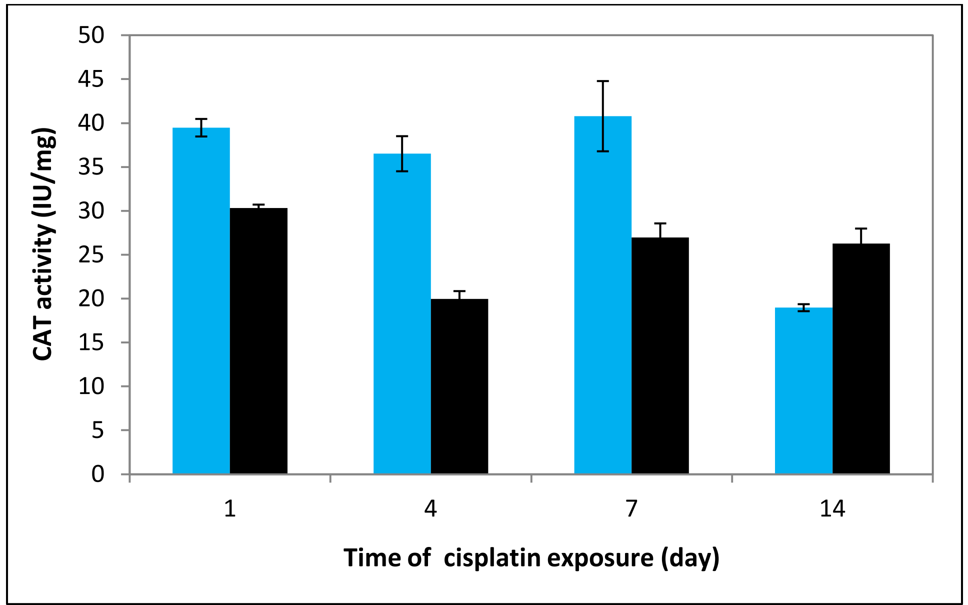


Figure 3.13 Variations in CAT activity in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

As shown in Figure 3.13, CAT activity were significantly higher in cisplatin injection group of heart for the during seven days compared with controls group. The higher levels were determined as 30, 82 and 51% for 1st, 4th and 7th days, respectively. In addition, at the end of the 14th days, CAT activity decreased according to control groups. CAT activity in heart tissue not showing parallelism with SDH and COX, it may explain H_2O_2 was produced by other mechanism aside from ETC enzymes.

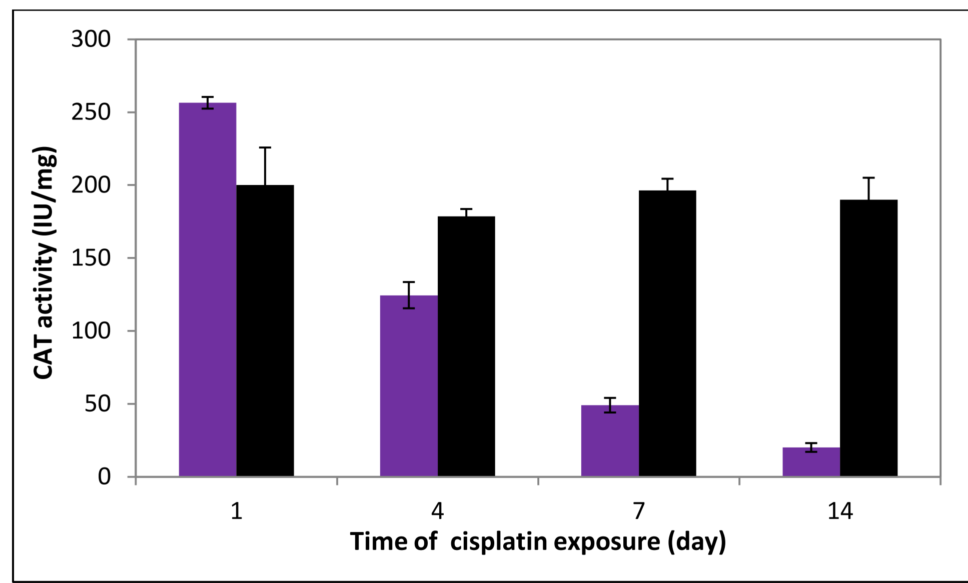


Figure 3.14 Variations in CAT activity in kidney tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

As can be seen in Figure 3.14, CAT activity increased 1.28 fold compared to control group by showing response to H_2O_2 which is produced by cisplatin exposure. Cisplatin levels in kidney at 1st day were also highest compared to other studied tissues. This situation can be explained by highest H_2O_2 level in kidney at 1st day since cisplatin uptake was also highest. Nevertheless CAT activity is in liver decreased continuosly during exposure time by showing correlation with decrease in cisplatin level. These results may suggest that CAT as an antioxidant enzyme was effected by cisplatin exposure during 14 day.

According to the our results, the highest levels of CAT were found in liver and kidney whereas the lowest CAT activity were determined in heart and lung cells. These results may indicate that CAT is produced efficiently in peroxisomes rich cells contained tissue which generates H_2O_2 (Oral, George, & Haskard, 2000). In addition, CAT activity in brain were not determinated because of low activiy CAT peroxisome (Uysal et al., 2005).

3.5 Variations in LPO Activities of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

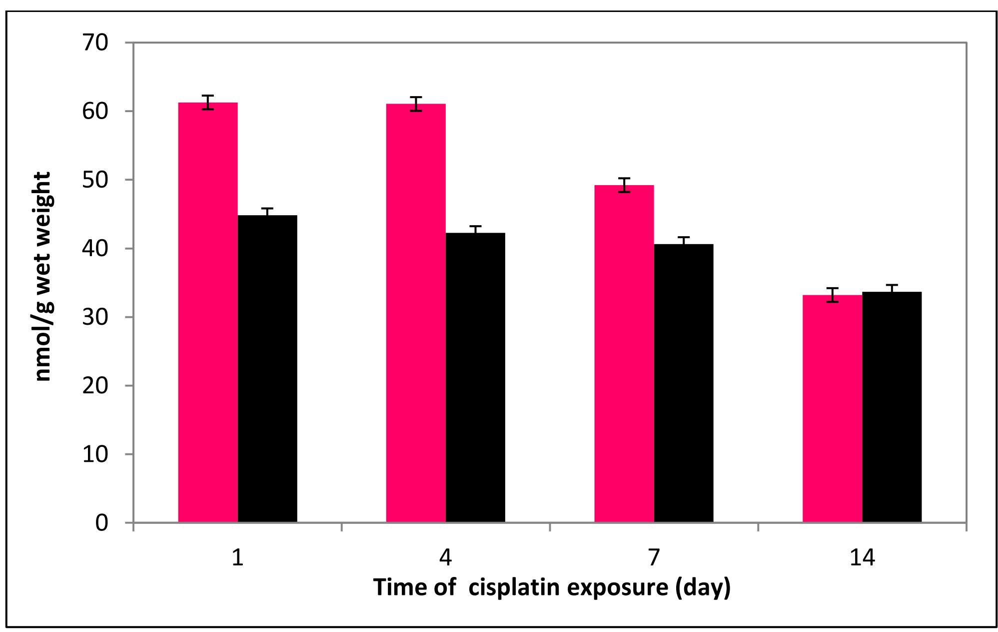


Figure 3.15 Variations in LPO level in liver tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

The malondialdehyde (MDA) concentrations in the liver, lung, heart, brain and kidney tissues of Sprague Dawley rats have been used a determination of lipid peroxidation. In this study, we have compared control groups of different tissues LPO levels with groups administered with cisplatin. As shown in Figure 3.15, the increases of LPO level in liver of cisplatin exposed rats, exhibited correlative effect with CAT activity for 1st day. It is proposed that decreased CAT activities of liver in the cisplatin injected rats increased lipid peroxidation which is established cell membrane damage. In additon, liver have high LPO level for 4th day, this situation may explain with liver had much higher SDH and COX activities because of quite a few occurred electron leak especially at 4th day according to reduces in CAT activity. And the last seven day LPO levels reached slowly to control group.

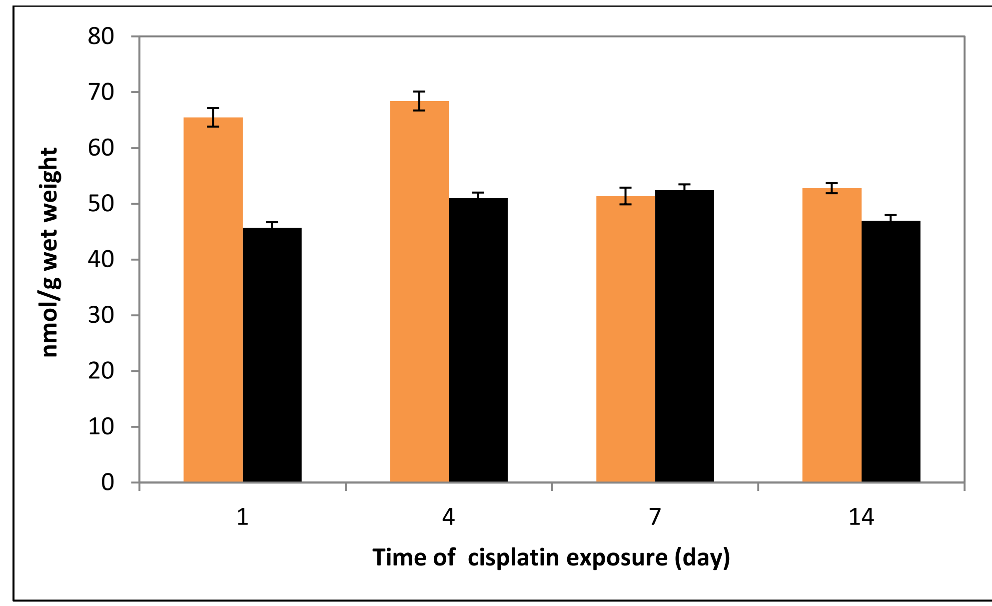


Figure 3.16 Variations in LPO level in lung tissues depending on the days: lung tissues (—■—), control of lung tissues (—■—).

As can be seen in Figure 3.16, LPO levels in lung after cisplatin injection group at 1st day increased compared to controls. This data are consistent with those of some previous studies (Srivastava et al., 2010), just after cisplatin treatment membrane lipid peroxidation levels increased while reduced some antioxidants levels. LPO levels increased for 4th day as similarly at 1st day, this data correlation with ROS forming because of SDH and CAT activity have higher value at 4th day in comparison with 1st day. On the other hand this results may explained the lowest cisplatin transport %2 carried out in lung tissue compaired to other studing tissues at 4th days, and thus accumulating cisplatin may induced membrane damage in lung at 1st and 4th days. Highly electron leakage involved in SDH and ROS can cause extensive tissue damage such as membrane lipid destruction.

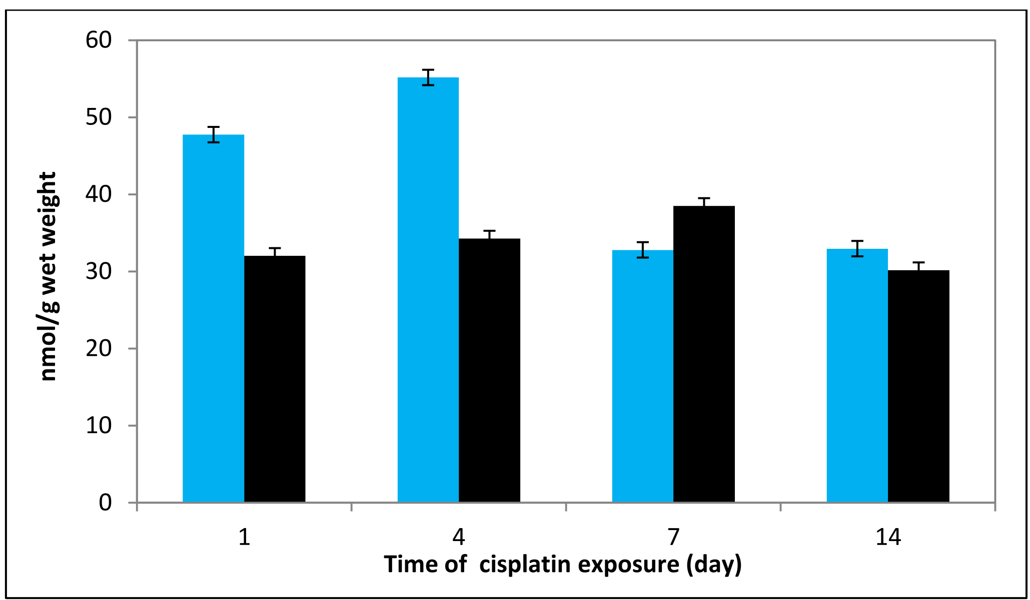


Figure 3.17 Variations in LPO level in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

According to Figure 3.17, LPO levels in kidney of cisplatin exposed rats have high value first four days in compared with last seven days.

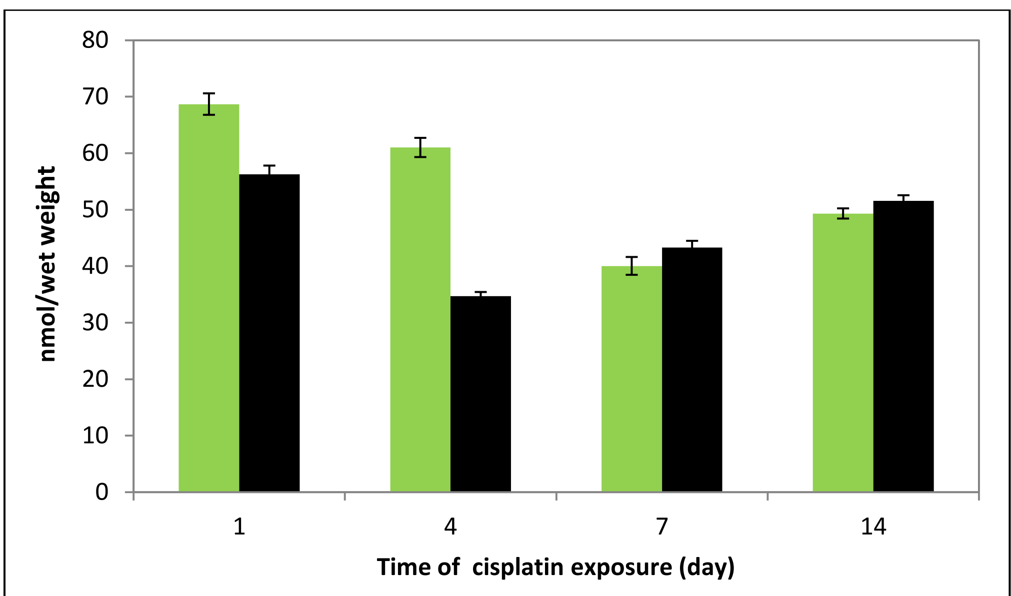


Figure 3.18 Variations in LPO level in brain tissues depending on the days: brain tissues (—■—), control of brain tissues (—■—).

This data demonstrate that there is an increase in LPO levels of cisplatin treated brain at 1st and 4th day (Fig. 3.18). LPO levels belong to 4th, 7th and 14th days show a

similar tends with SDH and COX activities. The results also showed the effect of electron leakage on membrane damage.

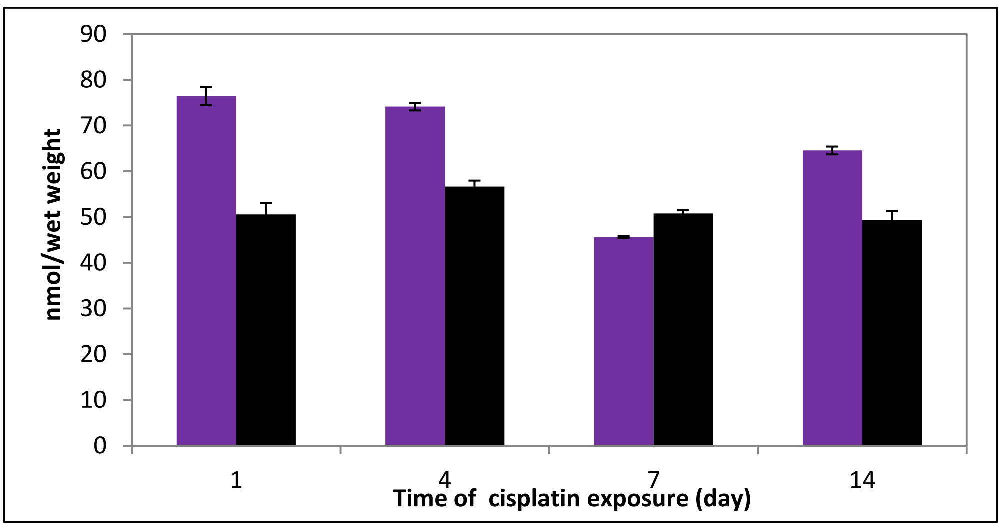


Figure 3.19 Variations in LPO level in kidney tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

As can be seen in Figure 3.19, LPO levels on kidney of cisplatin injection were much higher than control groups for 1st and 4th days. These values indicate similar tends with SDH, COX and CAT activities.

Despite decresas in SDH and COX enzyme activities and increases in CAT at 1st day in cisplatin exposed heart, brain and kidney compared to control, LPO levels increased it may explained CAT enzyme has not prevent cisplatin induced membrane damage.

3.6 Variations in Adenine Nucleotide Levels of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

3.6.1 Variations in ATP Levels of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

All living organisms need energy to continue their lives. For most energy consuming biological processes, adenosine triphosphate (ATP) is the direct fuel. In

eukaryotic cells, more than 95% of ATP is produced in a process called oxidative phosphorylation.

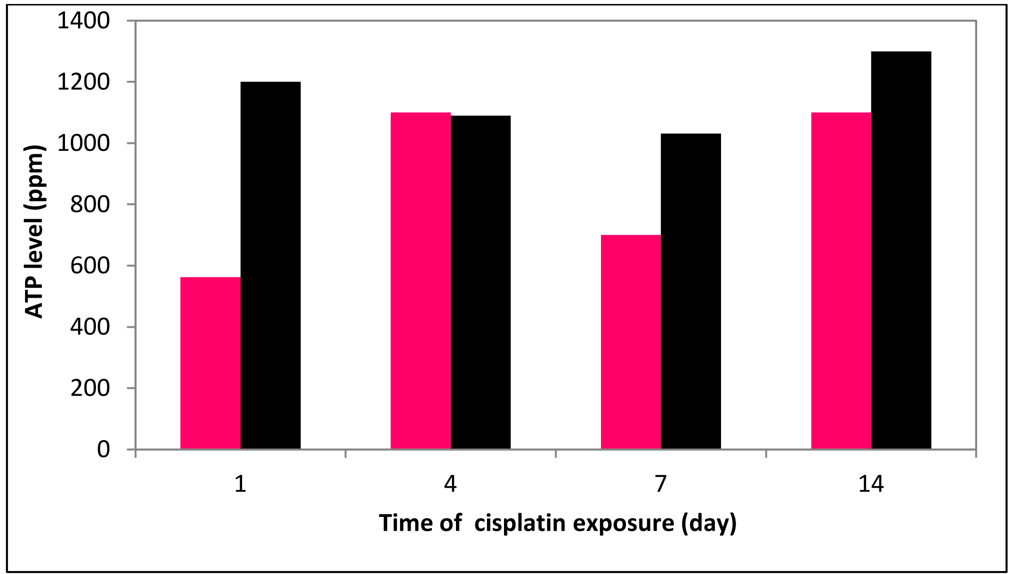


Figure 3.20 Variations in ATP level in liver tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

As can be seen in Figure 3.20-22, all ATP levels in cisplatin treated liver and heart tissue lower than control groups, except for 4th day which were accompanied by changes in SDH activities. ATP levels in lung tissue were lower than first four days according to control groups (Figure 3.21). ATP levels in lung were lower compaired to liver and this results indicated parallelism with SDH activity.

The results indicated that oxidative phosporylation and electron transport chain were the mitochondrial target of cisplatin and suggested that decreases in ATP levels can be caused by inhibiton of mitochondrial ATPase activity. Some research showed that cisplatin also inhibits mitochondrial phosphate transport, possibly by a direct interaction with the mitochondrial phosphate carrier (Nowak, 2012)..

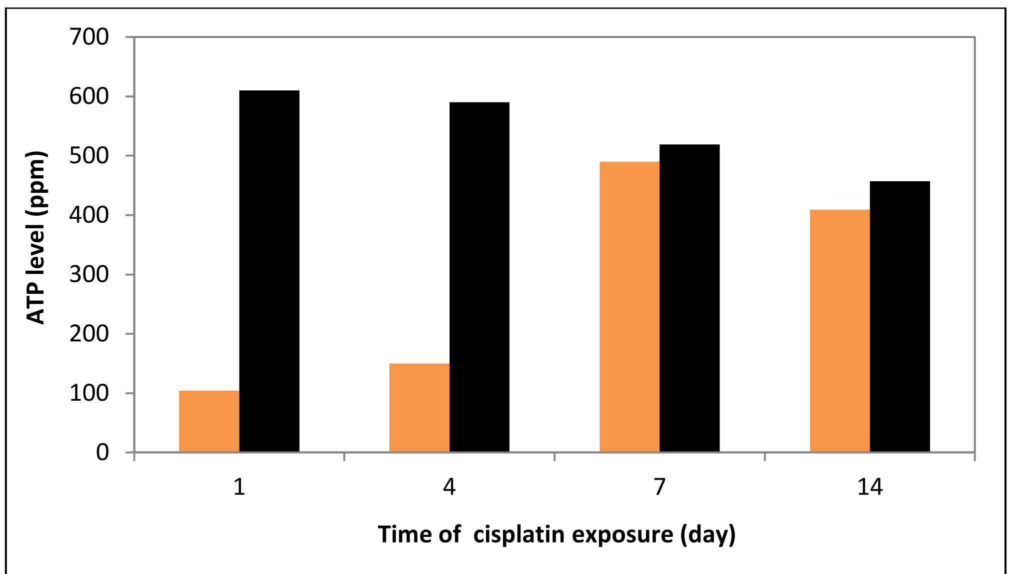


Figure 3.21 Variations in ATP level in lung tissues depending on the days: lung tissues (—■—), control of lung tissues (—■—).

As can be seen in Figure 3.22, ATP levels in heart tissues at 1st and 4th days showed similar trends with the SDH and COX enzyme activity. Nucleotide levels in heart showed parallelism with complex II, IV and V enzyme complexes.

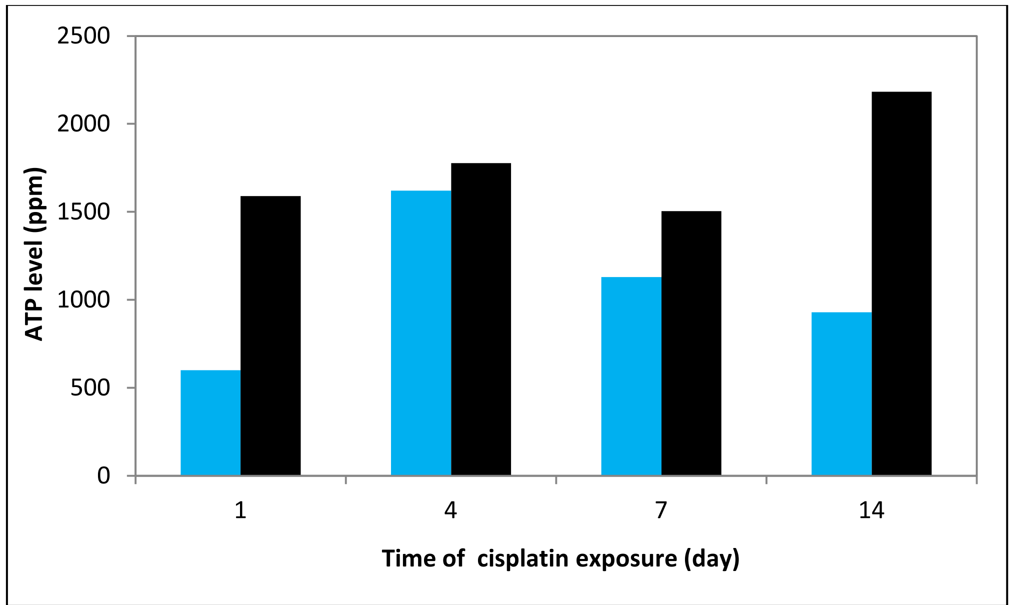


Figure 3.22 Variations in ATP level in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

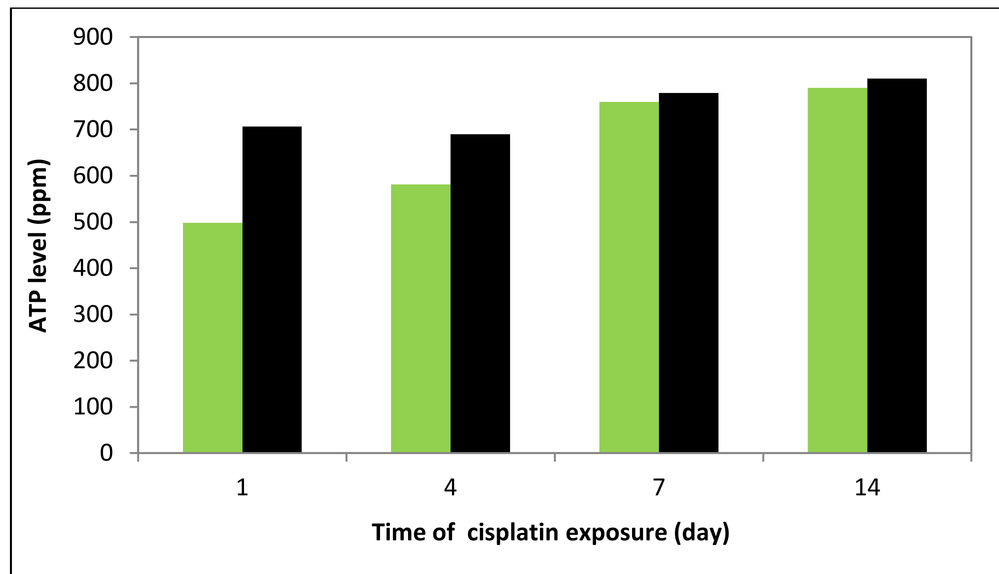


Figure 3.23 Variations in ATP level in brain tissues depending on the days: brain tissues (—■—), control of brain tissues (—■—).

As can be seen in Figure 3.23, ATP levels in brain at 1st day showed similar trends with SDH activity. ATP levels reduced at 4th day in cisplatin exposed brain although increases in SDH and COX at 4th days, it may explained that ATP is necessary for whole of cisplatin transport from brain at 4th days.

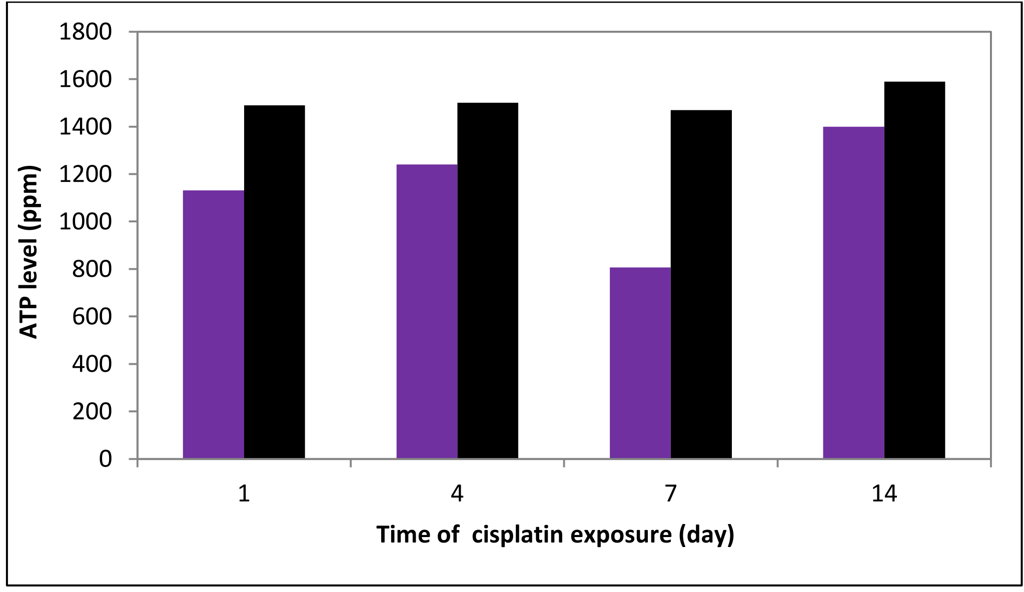


Figure 3.24 Variations in ATP level in tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

According to Figure 3.24, increases in ATP levels at 1st day showed similarity with SDH and COX enzyme activities. Although increases in SDH and COX activities in cisplatin exposed kidney at 4th days compared to control, ATP levels reduced at 4th days according to control groups, it may explained that ATP was used through cisplatin excretion because kidneys carry out cisplatin excretion %90 in body.

As shown in Figure 3.23-24, Nonetheless although markedly increases in SDH activity at 4th compared to 1st day determination of insignificant changes in ATP levels may evaluated that ATP was used for cisplatin excretion by kidney and brain to prevent toxicity in the cells.

ATP levels were significantly low compaired to control groups in all tissue at 1st, it may suggest that cisplatin inhibited the ATPase enzyme as shown in other literature (Daley-Yates & McBrien, 1982).

3.6.2 Variations in ADP Levels of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

As shown in Figure 3.25, ADP levels of cisplatin treated in liver tissue decreased significantly compared to control the decreases were 1.2, 3, 3.6, 4.7 fold for 1st, 4th, 7th and 14th days, respectively. This results may be explained that cisplatin induced inhibition of DNA, mRNA, protein synthesis (Nowak, 2002) which are necessary adenine nucleotide synthesis. Decreases in ADP levels in liver by showing with ATP production.

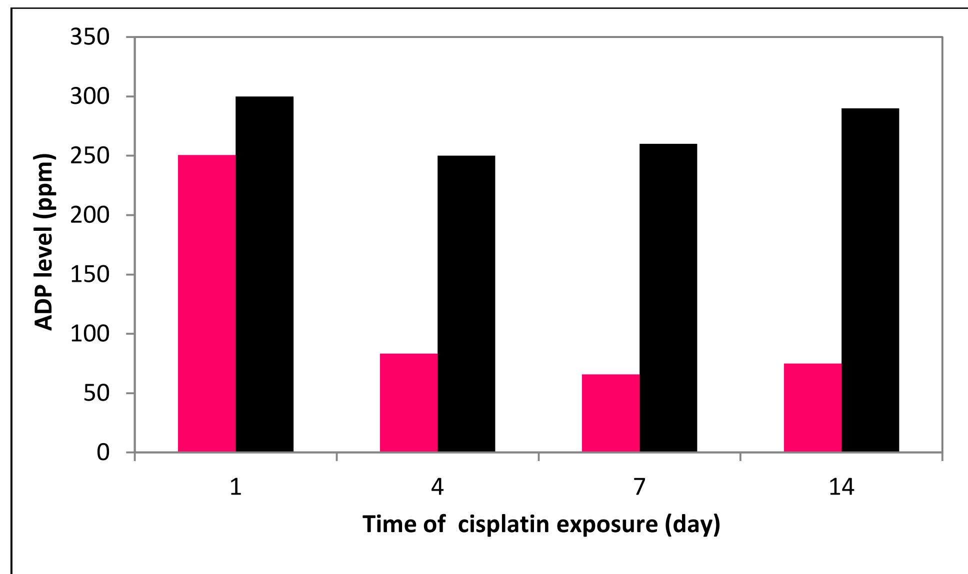


Figure 3.25 Variations in ADP level in liver tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

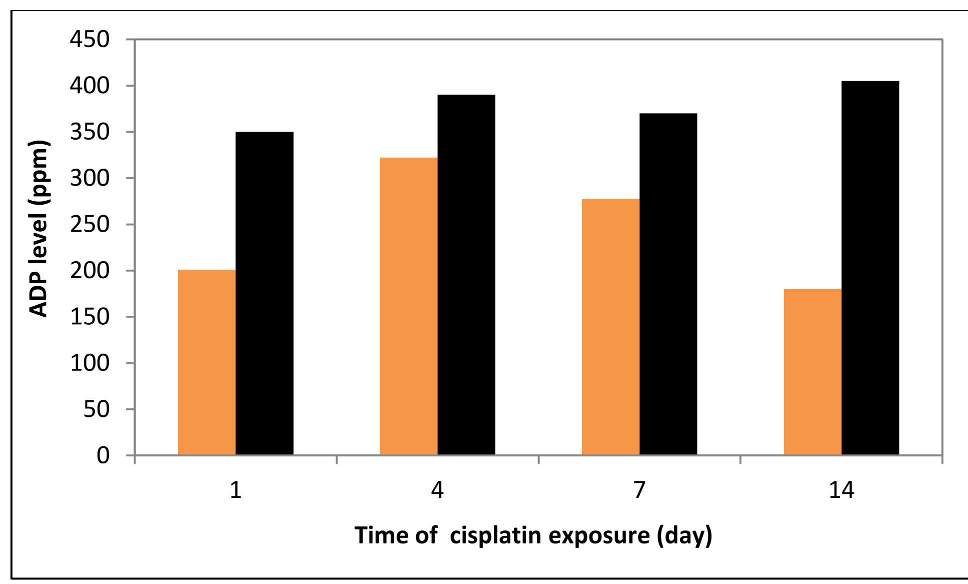


Figure 3.26 Variations in ADP level in lung tissues depending on the days: lung tissues (—■—), control of lung tissues (—■—).

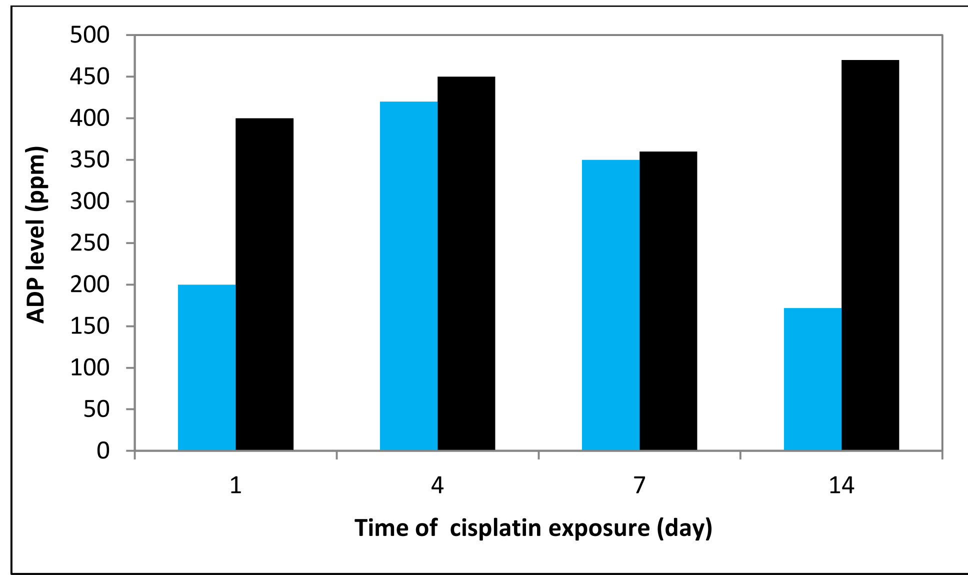


Figure 3.27 Variations in ADP level in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

As can be seen Figure 3.26-27, ADP levels of lung decreased 42.5, 17.3, 25, 55.5 %, while they were 50, 6.6, 2.7 and 63.4 % for heart. This situation showed that nucleotide synthesis in lung and heart were less affected compared to liver since cisplatin levels were also lower.

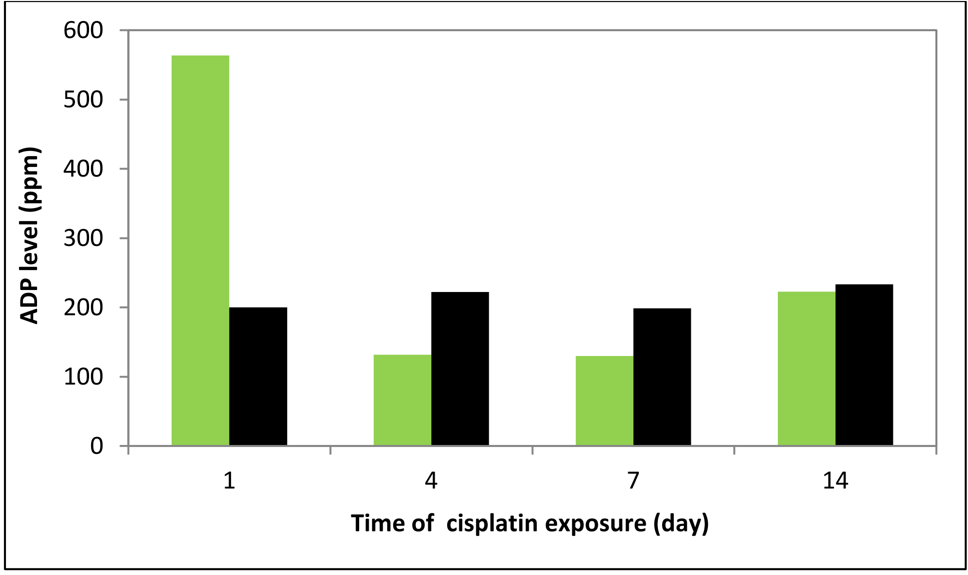


Figure 3.28 Variations in ADP level in brain tissues depending on the days: brain tissues (—■—), control of brain tissues (—■—).

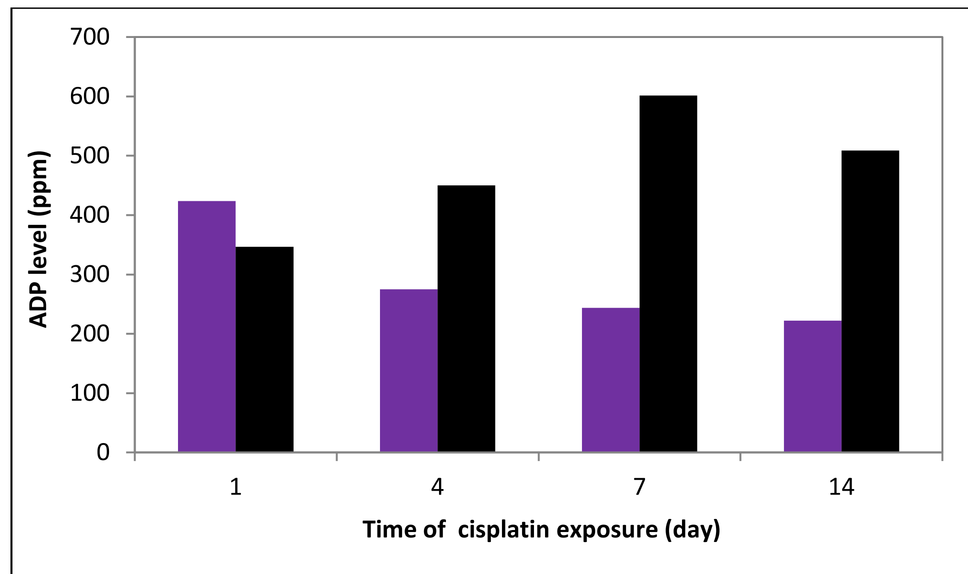


Figure 3.29 Variations in ADP level in kidney tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

According to Figure 3.28-29, despite increases in ADP levels in cisplatin exposed brain and kidney at 1st day, ADP levels at 4th and 7th days were reduce.

3.6.3 Variations in AMP Levels of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity

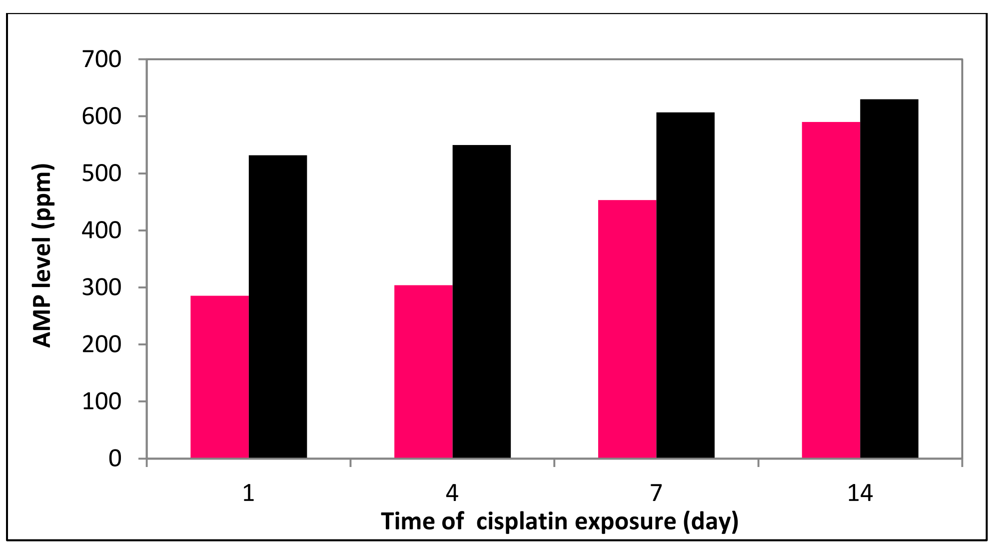


Figure 3.30 Variations in AMP level in liver tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

According to Figure 3.30-31, AMP levels in cisplatin injected group of liver and lung tissues show the similar tends for all days. AMP levels increased 1.8, 1.8, 1.3 and 1.1 fold in liver and 1.9, 1.8, 1.4 and 1.4 fold in lung at 1st, 4th, 7th and 14th days, respectively.

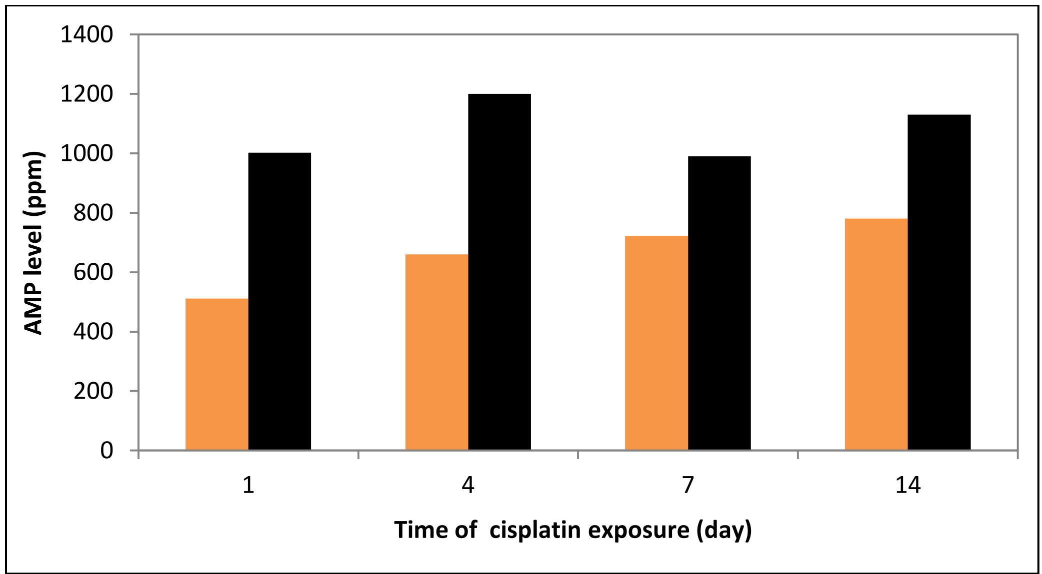


Figure 3.31 Variations in AMP level in lung tissues depending on the days: liver tissues (—■—), control of liver tissues (—■—).

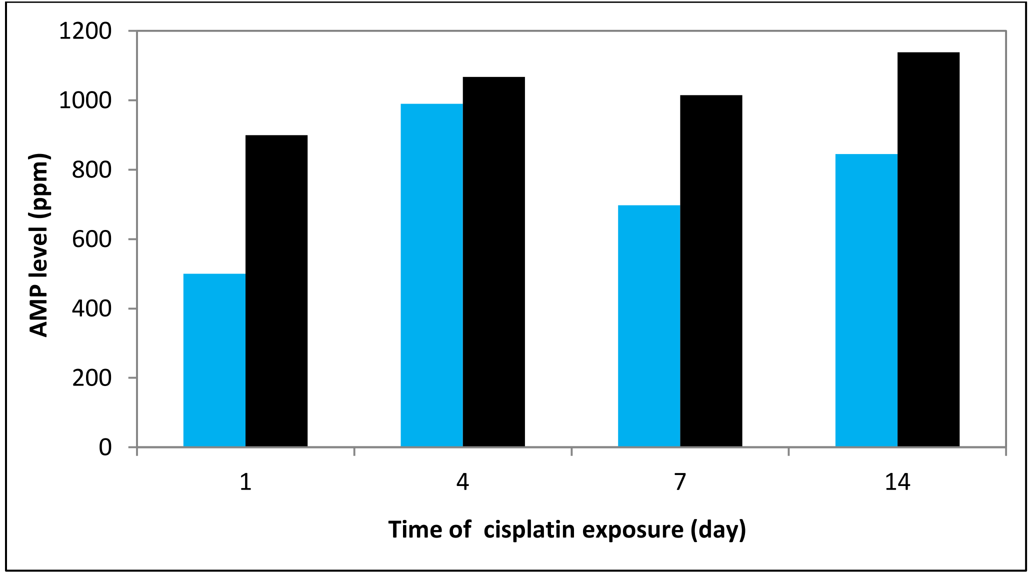


Figure 3.32 Variations in AMP level in heart tissues depending on the days: heart tissues (—■—), control of heart tissues (—■—).

As can be seen in Figrue 3.32, AMP levels in heart tissue of cisplatin treated groups decreased in 44.4, 7.2, 31.3 and 25.8% for 1st, 4th, 7th and 14th days, respectively. This results may be explained cisplatin induced inhibiton of AMP synthesis in liver, lung, heart and brain tissues.

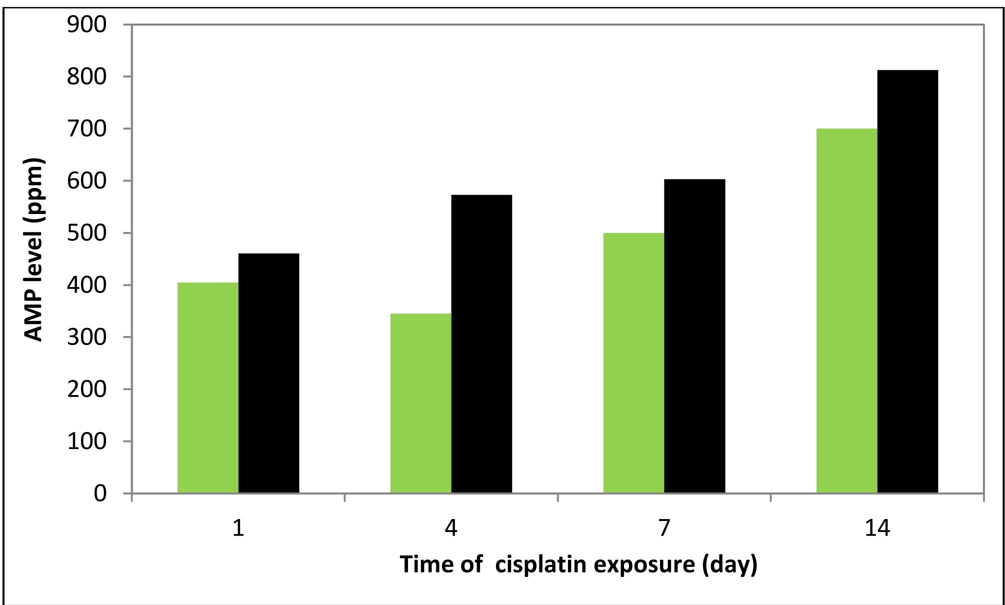


Figure 3.33 Variations in AMP level in brain tissues depending on the days: brain tissues (—■—), control of brain tissues (—■—).

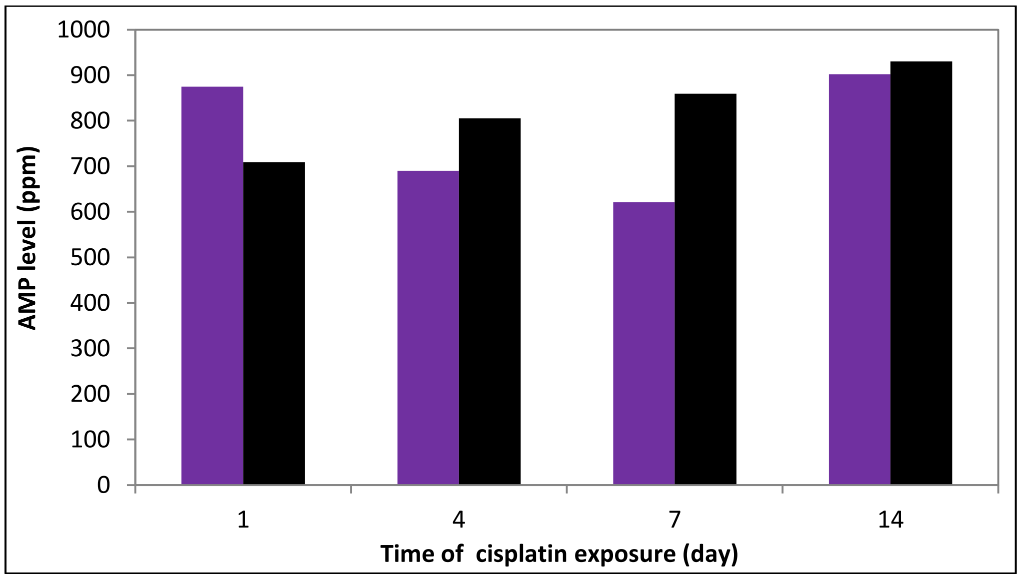


Figure 3. 34 Variations in AMP level in kidney tissues depending on the days: kidney tissues (—■—), control of kidney tissues (—■—).

According to Figure 3. 33-34, cisplatin toxicity shows similar reducing effect on AMP levels in brain and kidney tissue at 4th. On the other hand, AMP levels have contrast effect for 6r1st and 4th day, AMP levels in brain decreased 1.3 fold for 1st day and increased 0.8 fold for 14th day, while this level increased 0.8 fold at 1st and reduced 1.1 fold at 14th day.

3.7 Variations in SDH activity, COX activity, CAT activity, LPO levels and ATP, ADP, AMP Levels of Different Tissues of Male Sprague Dawley Adult Rats Depending on Cisplatin Toxicity and antioxidant effect of Capsaicin

Capsaicin (hot papers) is the antioxidant which is non-enzymatic antioxidant mechanism. Capsaicin is the most important antioxidant because of numerous people could use this plant so easily and for many years this plants have been used as foods, spices and medicines. In this present study, antioxidant effect of capsaicin against the cisplatin toxicity were evaluated in Sprague Dawley rats as an eukaryotic model. Capsaicin were injected each days during 7 days after single dose cisplatin (5 mg/kg) treated rats were injected with capsaicin (10mg/kg) by intramuscularly. The control groups treated with only single dose serum physiologic. Rats in the control, the cisplatin and the cisplatin+capsaicn groups were sacrificed at 7th day.

As can be seen from Figure 3.35, SDH activities in liver, heart, brain and kidney of cisplatin + capsaicin treated rats increased 54, 13 and 24 % compared to only cisplatin exposed groups although it decreased 41 % for lung, respectively.

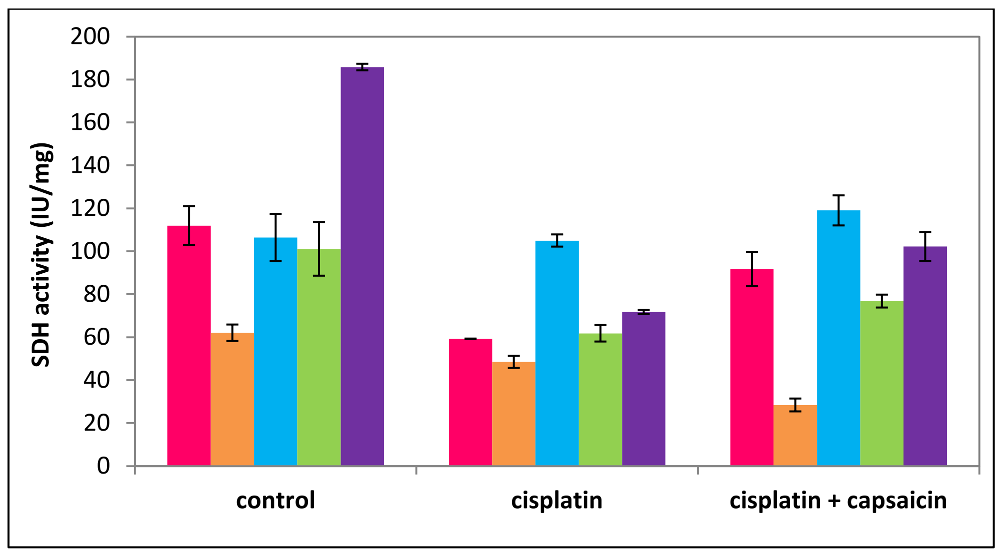


Figure 3.35 Variations in SDH activity in different tissues depending on the control, cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), brain tissues (—■—), kidney tissues (—■—).

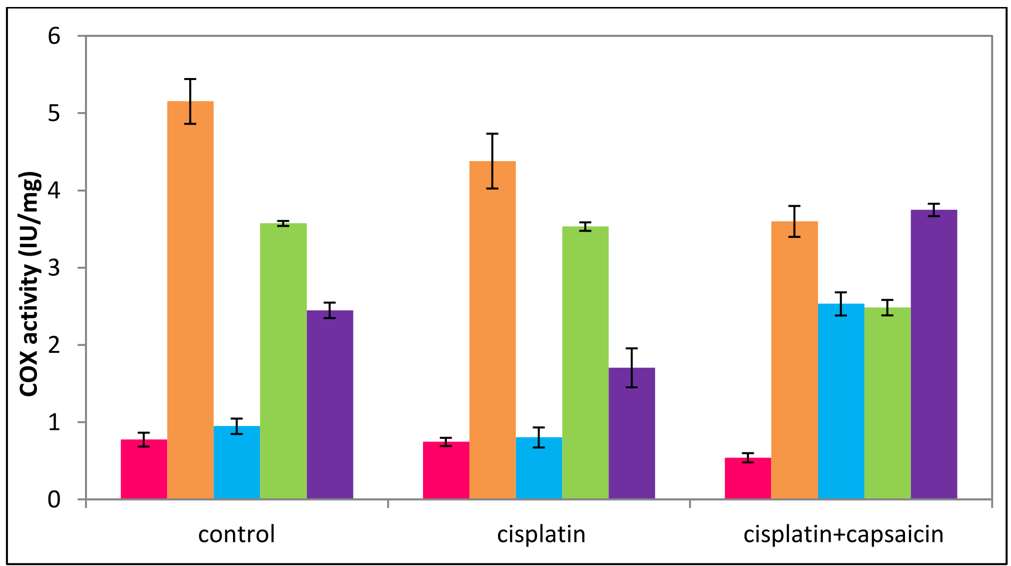


Figure 3.36 Variations in COX activity in different tissues depending on the control, cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), brain tissues (—■—), kidney tissues (—■—).

As shown in Figure 3.36, COX activity of cisplatin+capsaicin in heart and kidney tissues were higher compared to the control. It may suggest that capsaicin induced COX activity in heart and kidney. However, COX activity of cisplatin+capsaicin

exposure decreased compaired to the control 29.9 %, 29.5%, 30.3% in liver, lung and brain tissues. Figure 3.37 showed that CAT activities of liver, heart and kidney of cisplatin + capsaicin treated groups compaired to their cisplatin groups decreased as 32, 34 and 18 % while it increased 71 % for lung, respectively.

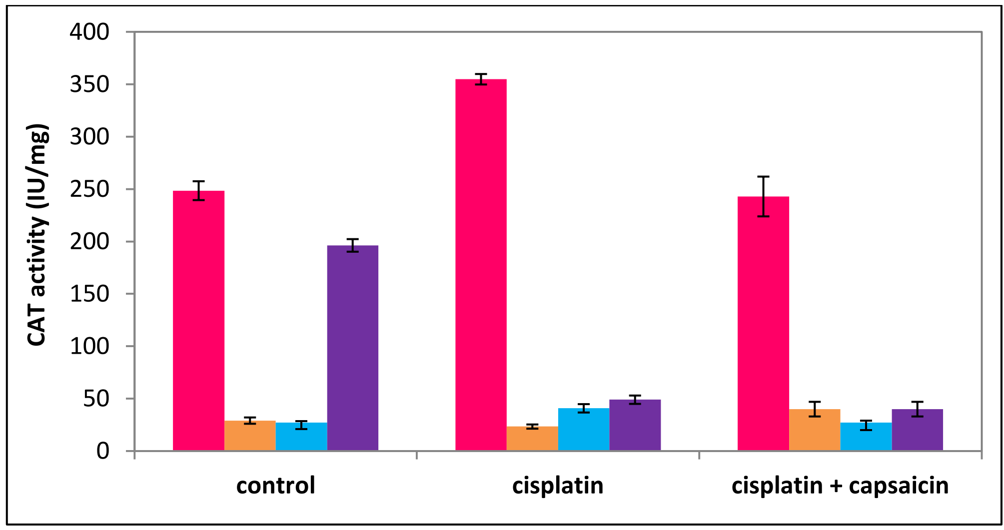


Figure 3.37 Variations in CAT activity in different tissues depending on the control, cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), kidney tissues (—■—).

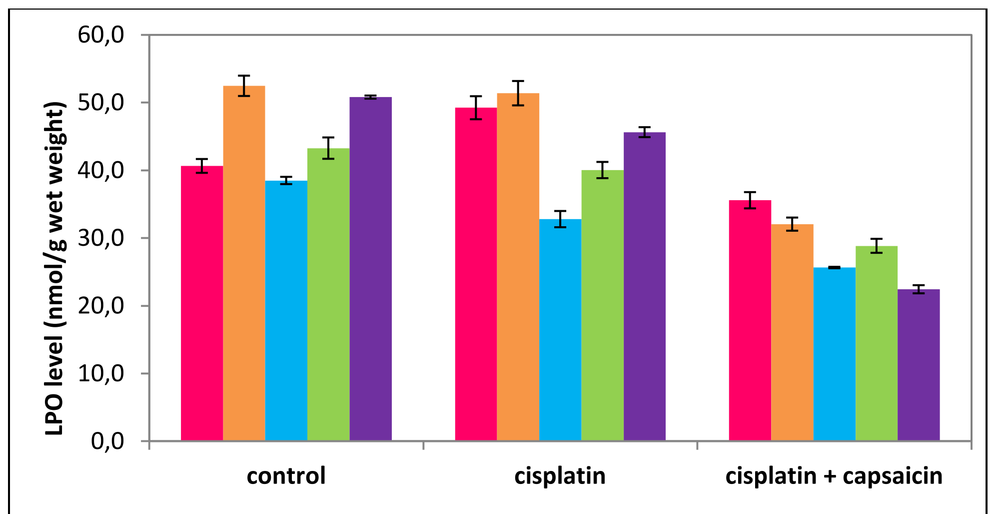


Figure 3.38 Variations in LPO level in different tissues depending on the control, cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), brain tissues (—■—), kidney tissues (—■—).

According to Figure 3.38, LPO levels of in liver, lung, heart, brain and kidney of cisplatin + capsaicin treated groups decreased 27.7, 37.6, 21.8, 27.9 and 50.8 %, respectively. The results showed that capsaicin inhibited the lipid peroxidation significantly. This results showed coherent with the previous article that capsaicin was found to scavenge radicals both at/near the membrane surface and in the interior of the membrane (Kogure et al., 2002). The phenolic OH group of capsaicin is not associated with the radical scavenging reaction (Kogure et al., 2002). Capsaicin has included an amide group that was associated with antioxidant activity and this site suggested that is the most probable site for free-radical attack in molecules (Henderson, 1992).

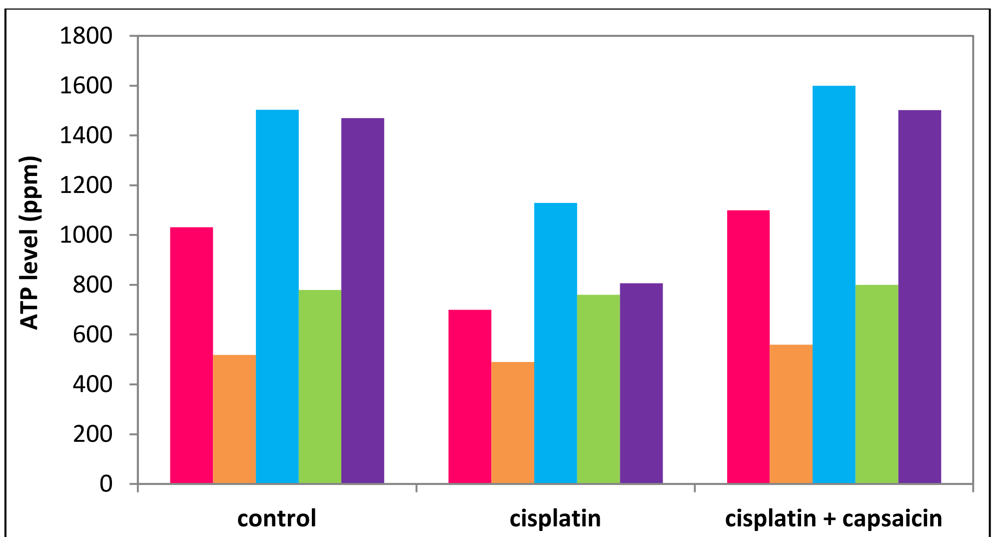


Figure 3.39 Variations in ATP level in different tissues depending on the control,cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), brain tissues (—■—), kidney tissues (—■—).

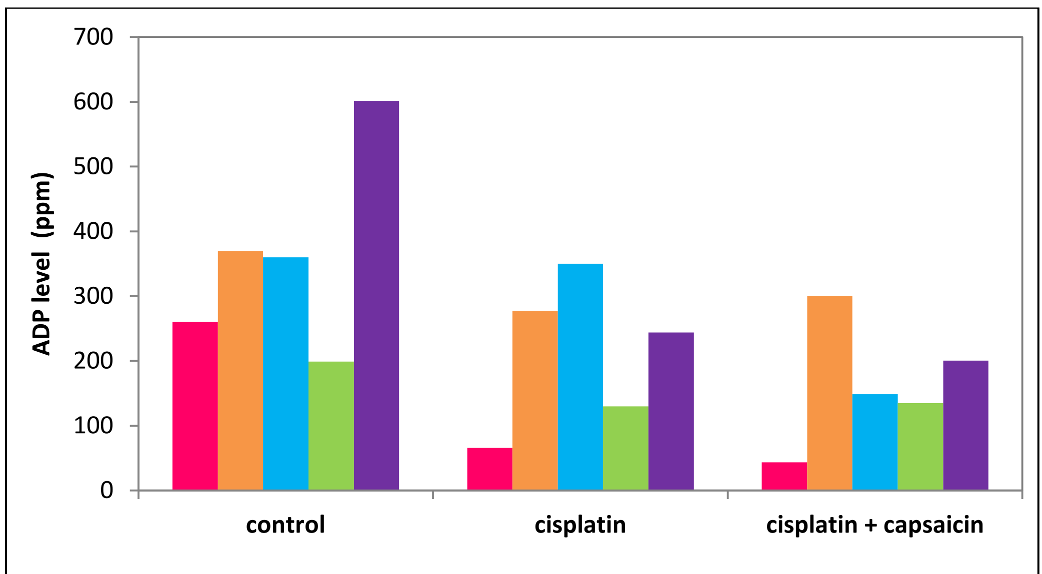


Figure 3.40 Variations in ADP level in different tissues depending on the control, cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), brain tissues (—■—), kidney tissues (—■—).

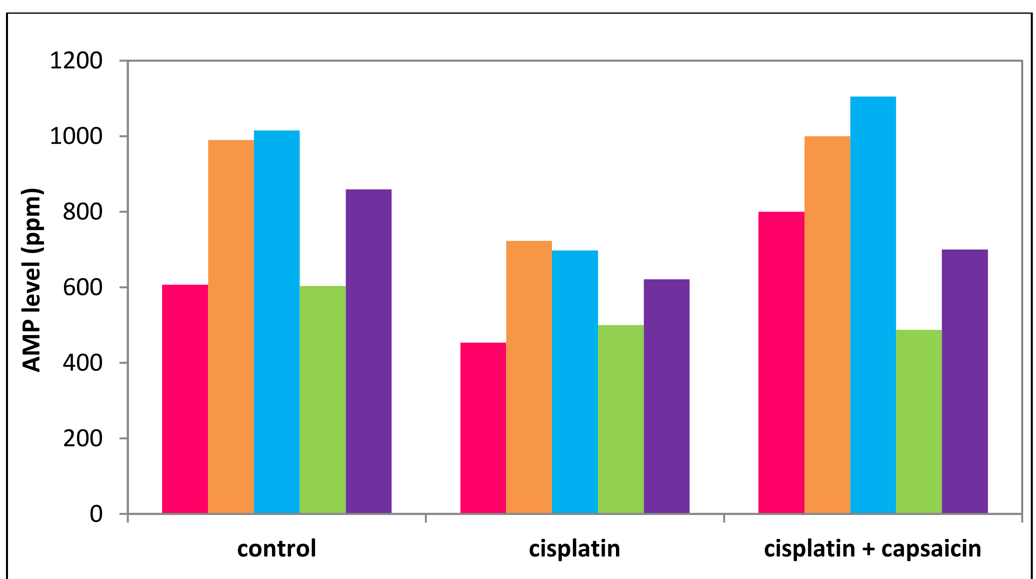


Figure 3.41 Variations in AMP level in different tissues depending on the control, cisplatin groups for 7th day and, capsaicin groups exposure followed by 7 days of cisplatin injection. Liver tissues (—■—), lung tissues (—■—), heart tissues (—■—), brain tissues (—■—), kidney tissues (—■—).

In conclusion, cisplatin is a effective anticancer drug that is widely used in the treatment of a variety number of solid tumors and is currently one of the most

important cytostatic drug (Kopelman et al., 1988; Gandara et al., 1989; Chirino et al., 2004). This study provides evidence that cisplatin induced mitochondrial dysfunction is caused by inhibiton of SDH and COX enzyme activities of electron transport system at 1st day which results in decrease intracellular level of ATP this selectivity for mitochondria is probably cause by accumulation by cisplatin in the negatively charged inner space of mitochondria because of the positive charge of aquated complex of cisplatin. In generally, SDH and COX levels in tissues of exposure to cisplatin were significantly higher compared to control at 4th days, it may explain cisplatin induce DNA damage as shown in other article therefore ETC system may induced to produce ATP for DNA repair mechanism (Gong et. al, 1999, Li et al, 2000). However, AMP levels in liver, lung, heart and brain except kidney tissues significantly low compaired to control it is suggested that cisplatin may effect AMP syntheses.

Furthermore cisplatin exposure in all studied tissues caused the increases in CAT activity except for kidney. It has been shown in very studies that cisplatin are overexpressed by showing association with increase formation of free radicals to prevent damage effect of ROS. Although CAT activity for kidney in cisplatin groups for 1st day were higher compaired to control they increased significantly during 7th days by excessing the control for 7th day.

According to the results, cisplatin induced toxicity in each tissue especially for 1st day were determined with higher LPO levels of exposure to cisplatin compared to the controls. The results can be explained by insufficiency in enzymatic and non-enzymatic antioxidant systems against to cisplatin toxicity therefore we added cisplatin + capsaicin which is non-enzymatic antioxidant to determine capsaicin effect on these system. Our results showed that LPO levels decreased although CAT activity decreased in the presents of capsaicin. The results suggest that capsaicin have antioxidant capacity to scavenge ROS to prevent membrane damage.

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