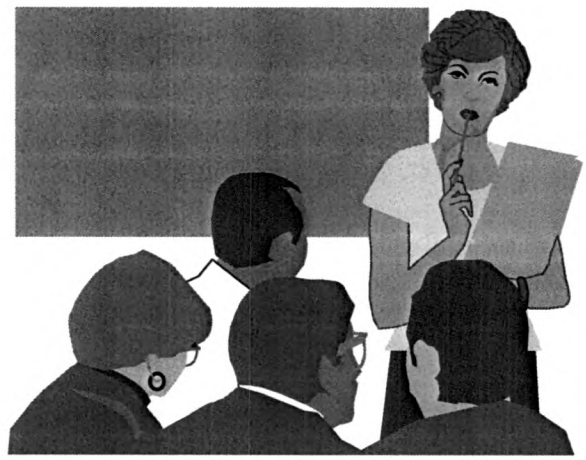
CHAPTER 5



DISCUSSION

5.1 *IN VITRO* STUDY

The *in vitro* free radical scavenging activity of green tea extract, melatonin, lovastatin and resveratrol were tested by their ability to bleach the stable radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). It is one of the free radicals generally used for testing preliminary radical scavenging activity of a compound or a plant extract. This assay provides information on the reactivity of test compounds with a stable free radical, independently of any enzymatic activity. Because of its odd electron, DPPH gives a strong absorption band at 516nm in visible spectroscopy (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up (Russo et al., 2002). The study showed that green tea extract, melatonin and resveratrol possessed moderate DPPH quenching capacity, while lovastatin fails to scavenge the DPPH radicals.

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (Halliwell and Gutteridge, 1985). The NBT (Nitro blue tetrazolium) reduction method showed that green tea extract and resveratrol possessed significant superoxide radical scavenging activity while melatonin and lovastatin fails to scavenge superoxide radical generated in riboflavin-NBT-light system *in vitro* as compared to the standard.

Nitric oxide (NO), because of its unpaired electron, is classified as a free radical and displays important reactivities with certain types of proteins and other free radicals. Recently, Lui et al. (2001) found that ischemia-reperfusion injury increased the production of NO and induced the expression of iNOS mRNA in the kidney. During ischemia, xanthine dehydrogenase is converted very rapidly to xanthine oxidase, which is the O^{-}_{2} producing oxidase (McCord, 1985), and hypoxanthine, which is an oxidizable substrate for this enzyme, accumulates in ischemic tissues as a result of ATP catabolism. Molecular oxygen provided by the blood that flows during reperfusion after ischemia is converted to the O^{-}_{2} radical. Under these conditions, NO will react rapidly with O^{-}_{2} to form $ONOO^{-}$. The reactivities of the free radicals NO and O^{-}_{2} were found to be relatively low, but their metabolite $ONOO^{-}$ was extremely reactive and directly induced toxic

reactions, including SH-group oxidation, protein tyrosine nitration, lipid peroxidation and DNA modifications (Moncada et al., 1991). Furthermore, $ONOO^-$ decomposed to give the hydroxyl radical (OH), which also had strong oxidant activity and induced renal injury (Beckman et al., 1990). It is thought that NO, O^-_2 and their metabolic products $ONOO^-$ and OH^- are closely associated with the causative mechanisms of renal injury and the damage caused by NO and O^-_2 becomes extremely serious due to the toxicities of their metabolites. The study showed that green tea extract, melatonin, and lovastatin possessed significant NO quenching capacity while resveratrol had a mild NO scavenging activity. The NO inhibition may be responsible for the upregulation of eNOS by statins (Pelat et al.2003).

Lipid peroxidation is destructive free radical mediated process for biological membranes, which has been implicated in a variety of disease state. It involves the formation and propagation of lipid radicals. Increased lipid peroxidation can result in change in cellular metabolism of various tissues (cheeseman and slater, 1993). The study showed that green tea extract, melatonin, lovastatin and resveratrol possessed significant inhibition of lipid peroxidation.

5.2 *IN VIVO* STUDY

5.2.1 Doxorubicin induced cardiotoxicity

Doxorubicin has been used as a single agent as well as in combination with other chemotherapeutic agents to control and regress a variety of neoplastic conditions. However, the beneficial effects of this drug are complicated by the acute as well as serious chronic side effects. Most of the acute side effects such as myelosuppression, nausea, vomiting and arrhythmias are not life threatening and are reversible as well as clinically manageable (Lefrak et al., 1973). It is the chronic side effects of the development of cardiomyopathy and ultimately congestive heart failure, which are critical and life threatening and thus limit the clinical usefulness of doxorubicin [Buja et al., 1973). These cardiotoxic effects in patients have been found to correlate with the cumulative dose of the drug administered (Cortes et al., 1975; Von Hoff et al., 1979).

The clinical utility of doxorubicin is limited by its cardiotoxicity, and the development of a digitalis-unresponsive congestive heart failure in

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cancer patients treated with this drug is a major dose-limiting factor. Previous attempts to elucidate the molecular mechanisms of cardiac damage have emphasised the role of the quinone moiety, which is present in the tetracycline ring of doxorubicin and participates in reduction–oxidation processes. *In vitro* studies have shown that mitochondrial, nuclear and microsomal NAD(P)H oxidoreductases catalyse a one-electron reduction of the quinone group of doxorubicin, yielding a semiquinone free radical that regenerates the parent compound by oxidising with molecular oxygen (Davies et al.,1986; Powis, 1989). It follows that doxorubicin administration exposes tissues to substantial fluxes of superoxide and H_2O_2. According to the prevailing hypothesis, cells with high levels of superoxide- and H_2O_2-detoxifying enzymes resist the perturbing effects of doxorubicin metabolism, whereas cells with low levels are expected to be damaged. The latter should be the case of cardiomyocytes, as they contain less catalase, glutathione peroxidase, and superoxide dismutase than other cells (Dorr, 1991; Doroshow, 1991).

The clinical usefulness of doxorubicin is severely limited because of its cardiotoxicity manifested biochemically by elevated serum CK and LDH activity. The results of the present study demonstrated that acute as well as chronic administration of doxorubicin induced cardiotoxicity manifested by a significant increase in serum CK, LDH and SGOT. These results are consistent with previous studies (Sayed-Ahmed et al., 2001; Mostafa et al., 1999) that doxorubicin induced cardiotoxicity in normal rats. This increase in cardiac enzymes could be due to an increase in their release following doxorubin induced damage of cardiac membranes.

Doxorubicin treatment induces a progressive and severe deterioration of the repolarization phase in rats ECG (Jensen et al., 1984). QT interval is considered one of the most sensitive markers of doxorubicin induced ECG alterations (Villani et al., 1986). The results clearly demonstrated that there was increase in myocardial injury as indicated by ECG changes like increase in ST and QT interval in both acute as well as chronic doxorubicin treated group.

The haemodynamic changes, such as heart rate and blood pressure, give some clues about myocardial contractility (Singal et al., 1987; Cassidy et al., 1998). Doxorubicin influences the haemodynamic parameters (Cassidy

et al., 1998; Falcone et al., 1998; Sacco et al., 2001). Chronic administration of doxorubicin caused an increase in Systolic, Diastolic and Mean blood pressure. These results were consistent with the earlier studies (Cassidy et al., 1998; Falcone et al., 1998; Sacco et al., 2001) where it was hypothesized that the increase in BP may be due to catecholamine release. Also, the high blood pressure depends on several factors, including high peripheral resistance. The high peripheral resistance may be due to the high collagen content around the vessels (Murat Yagmurca et al., 2003).

Administration of doxorubicin caused decrease in heart rate. These results were consistent with the earlier studies where it was hypothesized that doxorubicin induced reactive oxygen species formation causes disturbances in calcium homeostasis. This could lead to a reduction of heart rate because a decrease in intracellular calcium can induce reduced excitability of pacemaker cells in the sinoatrial node and other cells in the cardiac conduction system (Xuwan, 2002). Vascular reactivity to drugs like noradrenaline, adrenaline, phenylephrine and angiotensin were decreased which may be due to severe cellular damage.

Acute as well as chronic administration of doxorubicin significantly increased MDA content (an index of lipid peroxidation) in cardiac tissues and the highly significant reduction in tissue levels of protective biological antioxidants like GSH, SOD and catalase as compared to the control animals indicated oxidative stress. These results correlate well with previous studies which have demonstrated the involvement of oxidative stress and lipid peroxidation in doxorubicin induced cardiomyopathy (Mostafa et al., 1999; Kotsinas et al., 1999; Nagi et al., 2000). Many investigators described the role of reactive oxygen species including superoxide and hydroxyl radicals in doxorubicin induced cardiotoxicity (Mimnaugh et al., 1985; Singal et al., 1995]. Doxorubicin is capable of generating oxygen radicals and increasing MDA levels in many experimental models (Geetha et al., 1990). $Na^{+}K^{+}$ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase are membrane bound enzymes. The inactivation or decrease of ATPase on doxorubicin administration could be due to enhanced lipid peroxidation by free radicals (Gubdjarson et al., 1983).

Histopathological changes in doxorubicin treated rats were similar to those of the literature. Santos et al. [2002] showed that doxorubicin treatment caused extensive vacuolization in the cytoplasm of myocardial

cells. Saad et al. [2001] demonstrated that doxorubicin induced cardiac damage and there were marked edema, disorganized myocardial fibers, and necrosis.

Therapeutic strategies are aimed at limiting free-radical-mediated cardiac injury (Al-Shabanah et al., 1998). Therefore, the possibility to counteract doxorubicin cardiotoxicity by treatment with antioxidants has been investigated both in vivo and in vitro, using well-known antioxidant molecules such as Trolox and α-TC (Wahab et al., 2000; DeAtley et al., 1999).

Green tea extract contains gallocatechin, epigallocatechin, epicatechin, epigallocatechin gallate and epicatechin gallate. It has been reported that catechins are important constituents of green tea that are responsible antioxidant and protective effects. We verified the catechin content of extract using ethyl acetate as medium and found that it contains 35% catechins. In one of the study involving doxorubicin induced fatty acid composition modification in cardiomyocytes, it was revealed that only one of the GTE which was rich in catechin contents was able to counteract the detrimental changes induced by doxorubicin and elevation of conjugated dienes (Hrelia et al., 2002). It seems that antioxidant agents can protect the heart from doxorubicin induced assault as confirmed by several studies and reviewed (Quiles et al., 2000). Tea components possess antioxidant, antimutagenic and anticarcinogenic effects and could protect humans against the risk of cancer by environmental agents (Mukhtar et al., 1992). Sano et al (1995) studied the inhibitory effects of green tea leaves against tert-butyl hydroperoxide induced lipid peroxidation and a similar antioxidant effect on the kidney was observed after oral administration of a major tea polyphenols. Shim et al (1995) studied the chemopreventive effect of green tea among cigarette smokers and found it block the cigarette-induced increase in sister chromatid exchange frequency. Anti-hyperglycemic effect of black tea has been reported earlier by Gomes et al. (1995). Streptozotocin diabetic rats showed increased sensitivity to platelet aggregation and thrombosis and this abnormality could be improved by dietary catechin of green tea (Yang et al., 1999; Choi et al., 1998). Green tea polyphenols has the antioxidant properties that result from their ability to sequester metal ions and to scavenge reactive oxygen species. Further, it is also reported that

green tea extract exhibits more potent antioxidant activity than other conventional antioxidants like vitamin E and C at the same time green tea extract also showing anti cancer action (Ahmad and Mukhtar, 1999).

Pretreatment with green tea extract offered a significant protection from the doxorubicin induced acute cardiotoxicity. Green tea extract pretreatment significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in doxorubicin induced acute cardiotoxicity. The reduction in serum markers of cardiotoxicity like LDH, CK, and SGOT levels by green tea extract may be due its membrane stabilizing activity, which prevented the release of lysosomal enzymes. ECG alterations induced by acute doxorubicin treatment were prevented by administering green tea extract. Na^+K^+ATPase, Ca^{2+}ATPase, and Mg^{2+}ATPase are the membrane bound enzymes and the levels of Na^+K^+ ATPase, Ca^{++} ATPase; Mg^{++} ATPase was reduced in heart of doxorubicin treated rats. Since these membranes bound enzymes are 'SH' group containing enzymes, which are lipid dependant. The restored activities of ATPase enzymes suggest that the ability of green tea extract to protect the sulfhydryl group from oxidative damage through inhibition of lipid peroxidation.

Administration of green tea extract along with chronic treatment of doxorubicin causes significant decrease in LDH, CK, SGOT and lipid peroxidation near to that of control group. These data suggest that green tea extract may protect the myocardial tissue against doxorubicin induced chronic cardiotoxicity. Green tea extract treatment along with chronic doxorubicin administration restored blood pressure and heart rate towards normal value.

Further results also led to the belief that administration of green tea extract improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of lipid peroxidation. These protective effects were also supported by the restoration of serum marker enzymes, EGC changes and histopathology study. It seems that antioxidant agents can protect the heart from doxorubicin induced assault as confirmed by several studies and reviewed (Hrelia et al., 2002; Quiles et al., 2000).

Melatonin (*n*-acetylmethoxytryptamine) is produced mainly by the pineal gland and has been suggested to have antioxidant and prophylactic

properties (Reiter, 1993). In particular, melatonin was recently shown to reduce lipid peroxidation during cyclophosphamide therapy (Kaya et al., 1999), inhibit cerulein-induced acute pancreatitis, (Qi et al., 1999) protect human erythrocytes from oxidative haemolysis, (Tesoriere et al., 1999) show radioprotective effects during cell division (Badr et al., 1999), exhibit prophylactic effects on lead-induced inhibition of haem biosynthesis, and display oncostatic actions (El-Missiry et al., 2000; El-Missiry, 2000). Several investigators believe that melatonin's antioxidant properties are due to its ability to scavenge reactive oxygen species and increase cellular antioxidants (Reiter et al., 1997; Zhang et al., 1999). The role of melatonin in prevention of acute gastric lesions induced by stress, ethanol, ischaemia and aspirin has been studied previously. These studies suggest that the inhibition of ulceration by melatonin is probably via its antioxidative nature in addition to a mechanism involving the central nervous system (Kato et al., 1997) and was associated with a reduction in polymorphonuclear granulocyte activation (De la Lastra et al., 1999). Many free radical scavengers are compartmentalized within cells due to their specific solubility characteristics and, therefore, they cannot protect against •OH or $ONOO^-$ damage in other locations. Melatonin seems unique among cellular antioxidants as it is located in both lipid and aqueous compartments of the cell in sufficient concentrations to ameliorate the damage caused by the highly toxic •OH and ONOO-(Reiter et al., 1995). Melatonin is an important component of the antioxidant profile of many tissues and cells. Recently, Reiter et al. (1999) documented that melatonin is an efficient scavenger of •OH, $ONOO^-$, $•O_2^-$, •NO and peroxy radicals. Moreover, it enhances the ability of cells to resist oxidative damage by inhibiting the pro-oxidant nitric oxide synthase (Pozo et al., 1997).

Pretreatment with melatonin offered a significant protection from the doxorubicin induced acute cardiotoxicity at 6 mg/kg. Melatonin significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in doxorubicin induced acute cardiotoxicity. Melatonin directly scavenges hydrogen peroxide to form N^1-acetyl-N^2 formyl-5-methoxykynuramine, which, by the action of catalase forms N^1-acetyl-5-methoxykynuramine [Tan et al., 2000]. These biogenic amines could also scavenge hydroxyl radical and reduce lipid peroxidation. The reduction in

serum enzymes like LDH, CK, and SGOT levels by melatonin may be due its membrane stabilizing activity, which prevented the release of lysosomal enzymes. ECG alterations induced by acute doxorubicin treatment were prevented by administering melatonin. Na^+K^+ATPase, Ca^{2+}ATPase, and Mg^{2+}ATPase are the membrane bound enzymes. The restored activities of ATPase enzymes suggest that the ability of melatonin to protect the sulfhydryl group from oxidative damage through inhibition of lipid peroxidation. The indole moiety of the melatonin molecule is the reactive center of interaction with oxidants due to its high resonance stability and low activation energy barrier towards the free radical reactions. The methoxy and amide side chains also contribute significantly to melatonin's antioxidant capacity (Tan et al., 2002).

Treatment of melatonin along with chronic administration of doxorubicin causes significant decrease in LDH, CK, SGOT and lipid peroxidation near to that of control group. Melatonin treatment along with chronic doxorubicin administration restored blood pressure and heart rate towards normal value. Further results also led to belief that administration of melatonin improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of lipid peroxidation. These protective effects were also supported by the restoration of serum marker enzymes, EGC changes and histopathology study.

Inhibitors of 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase (or statins) represent a newly discovered family of chemically related molecules, selected for their lipid-lowering effect. Statins are extensively used in medical practice and large clinical trials have demonstrated that this class of lipid-lowering drugs greatly reduces cardiovascular related morbidity and mortality in patients with and without coronary disease (Illingworth et al., 1972). Lovastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase that has been used in the clinic to treat hyper-cholesterolemia (Downs et al., 1998). Lovastatin has also been shown to arrest tumor and normal cells in the G1 phase of the cell cycle (Rao et al., 1998) and has demonstrated antitumor effects in experimental murine models (Matar et al., 1999). Although clinical studies on the antitumor activity of lovastatin have been initiated (Thibault et al., 1996), its

effectiveness in clinical tumor therapy will probably depend on whether combination therapies are found in which lovastatin used with other drugs is shown to exert potentiated antitumor effects. As shown recently by Agarwal et al. (1999), pretreatment with lovastatin increased apoptosis induced by chemotherapeutic agents in tumor cells *in vitro*. Lovastatin has also been shown to strengthen the antitumor activity of cisplatin (Feleszko et al., 1998) and tumor necrosis factor ∝ (Feleszko et al., 1999) in murine tumor models.

The decrease in serum markers of myocardial injury, ECG and histopathological abnormalities indicated the protection offered by pretreatment of lovastatin in doxorubicin induced acute cardiotoxicity. The lower dose (3 mg/kg) may not be sufficient enough to impart the protection. The lovastatin treatment resulted in positive changes in markers of oxidative stress indicating the role of antioxidant activity in protective effect.

Treatment of lovastatin along with chronic administration of doxorubicin causes significant decrease in LDH, CK, SGOT and lipid peroxidation near to that of control group. Lovastatin treatment along with chronic doxorubicin administration restored blood pressure and heart rate towards normal value. In the present study, administration of lovastatin improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of lipid peroxidation. These protective effects were also supported by the restoration of serum marker enzymes, EGC changes and histopathology study. These results correlate well with several reports showing the antioxidant activities of lovastatin (Feleszko et al., 2000). However the antioxidant activity was earlier correlated to its lipid lowering effect and pleiotropic effects involving improvement in nitrogen oxide mediated protection. There were no changes found in serum lipid levels as lovastatin being administrated in normolipidemic animals. This study provides evidence for the first time that lovastatin inhibited lipid peroxidation and nitric oxide in *in-vitro* studies. This effect might also contribute crucial role in protective effects shown by lovastatin on doxorubicin induced myocardial injury apart from other pleiotropic effects reported earlier. Further, it may be hypothesized that nitric oxide scavenging activity of lovastatin may be playing some role in initiation of feedback in

response to decreased nitric oxide availability leading to increase eNOS expression as observed in previous studies (Pelat et al., 2003).

5.2.2 Cisplatin induced nephrotoxicity

Cisplatin (*cis*-diamminedichloroplatinum II) is one of the most potent antitumor agents. Activity has been demonstrated against a variety of tumors, particularly for head and neck, testicular, ovarian, bladder and small cell lung cancers (Rosenberg, 1985). However, cisplatin has serious side effects on non-tumor cells and causes nephrotoxicity. The major side effect, nephrotoxicity, is dose limiting and occur either acutely or after repeated treatments. In rats, cisplatin exerts its effects mainly in the S3 segment of the proximal tubule, which results in necrotic lesions (Doyban et al., 1980); in man, additional damage is found in the distal part of the tubules (Gonzalez-Vitale et al., 1977). A wealth of histopathological data, mainly in rats and mice, is available about cisplatin induced nephrotoxicity; in addition, several bio-chemical effects have been described *in vitro* as well as *in vivo*, among which alterations in capacity of several active transport systems (Miura et al., 1987), loss of mitochondrial function (Gordon and Gattone, 1986), Na^+K^+ATPase activ-ty (Brady *et al.*, 1993; Kim et al., 1995) and lipid peroxidation (Zhang et al., 1994; Hannemann and Baumann, 1988).

The alterations in kidney function induced by cisplatin are characterized by signs of injury, such as changes in urine volume and in glutathione status, and cisplatin induced nephrotoxicity is closely associated with an increase in lipid peroxidation (Hannemann and Baumann, 1988). Cisplatin is highly mutagenic, inducing chromosome aberrations in peripheral blood lymphocytes in patients and in rat bone marrow cells (Antunes et al., 2000). This antitumoral was able to generate active oxygen species, such as superoxide anions and hydroxyl radicals [Matsushima et al., 1998), and to inhibit the activity of antioxidant enzymes in renal tissue. Cisplatin chemotherapy induces a fall in plasma antioxidant levels, which may reflect a failure of the antioxidant defence mechanism against oxidative damage induced by commonly used antitumor drugs [Weijl et al., 1998).

Previous reports suggest that cisplatin induced nephrotoxicity is by increase in lipid peroxidation (Hanneman and Baumann, 1991) and depletion of cellular thiols (Levi et al., 1980) in kidney tissues following

cisplatin treatment. It has been reported that administration of cisplatin (Devi Priya and Shyamala Devi, 1999) alone resulted in decrease in the activities of membrane bound ATPases (Na^+K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase).

Acute tubular necrosis is a prominent feature of cisplatin nephrotoxicity and is clinically manifested by elevations in blood urea nitrogen (BUN), serum creatinine, urea and uric acid due to decreased clearance by kidney (Goldstein and Mayor, 1983).

In the present study, Cisplatin induced acute as well as chronic nephrotoxicity, which was observed by biochemical parameters as a significant increase in serum creatinine, uric acid, urea and BUN, indicators of kidney damage. Kersten et al. (1998) and Husain et al. (1998) also observed that cisplatin administration resulted in renal failure following cisplatin injection. Our results are supported by a significant increase in lipid peroxidation and a significant decrease in antioxidant enzymes, SOD, catalase and reduced glutathione levels in renal tissues after treatment with cisplatin. These data are corroborated by previous studies reported by other investigators on cisplatin induced nephrotoxicity in normal rats. [Appenroth et al., 1997]. The levels of Na^+K^+ ATPase, Ca^{2+} ATPase; Mg^{2+} ATPase was reduced in kidney of cisplatin treated rats.

Treatment with green tea extract offered a significant protection from the cisplatin induced acute nephrotoxicity in acute as well as chronic nephrotoxicity. Green tea extract treatment significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in cisplatin induced nephrotoxicity. The reduction in serum levels of creatinine, uric acid, urea and BUN, the indices of renal functional impairment, and increase in the levels of membrane bound enzymes, may be due to the protective effect of the green tea extract on the kidneys. Histopathological studies also confirm the above findings.

Melatonin treatment offered significant protection in chronic nephrotoxicity as compared to acute nephrotoxicity. Pretreatment with the melatonin did not significantly alter the serum levels of creatinine, uric acid, indicators of kidney damage and the various antioxidant parameters in cisplatin induced acute nephrotoxicity. However, the melatonin significantly reduced the serum levels of creatinine, uric acid, urea and BUN in the

chronic nephrotoxicity. It also significantly reduced the lipid peroxidation and increased the levels of glutathione, catalase and SOD of kidneys, which suggests efficacy of melatonin in preventing free-radical induced damage. Treatment of melatonin along with cisplatin in chronic nephrotoxicity also significantly increased the activity of ATPases. These protective effects were also supported histopathology study.

Lovastatin treatment was fails to produce significant protection in acute as well as in chronic nephrotoxicity. Treatment with the lovastatin did not alter the serum levels of creatinine, uric acid, urea and BUN, indicators of kidney damage and the various antioxidant parameters in cisplatin induced nephrotoxicity.

5.2.3 Doxorubicin induced testicular toxicity

Doxorubicin is a widely used anticancer agent. In spite of its high antitumor efficacy, the use of doxorubicin in clinical chemotherapy is limited due to diverse toxicities, including testicular toxicity (Herman and Ferrans, 1998; Au and Hsu, 1980; Lui et al., 1986; Sawada et al., 1994; Imahie et al., 1995). Although a number of potential toxic mechanisms have been identified following exposure to doxorubicin, the major pathogenic mechanism appears to involve the generation of toxic reactive oxygen species (Goodman and Hochstein, 1977). Testicular membrane contains a high amount of polyunsaturated fatty acids, which are more vulnerable to attack by free radicals. Mitochondrial respiration is the main biological source of superoxide anion radicals under normal physiological conditions. Free radicals are generated in testis and are efficiently scavenged by the antioxidant defence system, which constitutes antioxidant enzymes such as superoxide dismutase, catalase, and glutathione reductase. Under normal physiological conditions, free radicals are generated in testis, which are subsequently scavenged by the antioxidant defence system. Further, the testicular membranes and sperm are more susceptible to lipid peroxidations; they are rich in unsaturated phospholipids and have been shown to contain a low amount of antioxidants. These membranes have been reported to undergo permeability changes following enhanced lipid peroxidation and glutathione depletion (Chance et al., 1979). It has been reported that the activities of antioxidant enzymes decreased and the levels of hydrogen peroxide and lipid peroxidation increased in the testis as well as in the

epididymis and epididymal sperm of rats after exposure to anticancer drugs (Jong-Koo Kang et al., 2002).

Doxorubicin is known to disturb spermatogenesis in a dose-dependent manner in animal studies (Lui et al., 1986). With a low dose of doxorubicin, a significant but temporary reduction in spermatogenesis occurred (Imahie et al., 1995). Ward et al. (1988) also reported that doxorubicin induced reductions in testicular sperm count. In the present study, acute as well as chronic administration of doxorubicin significantly induces pathological changes in serum and biochemical markers indicative of toxicity and increases the free radical production. Acute as well as chronic treatment of doxorubicin significantly decreased the testosterone and sperm count. These results were consistent with earlier studies (Lui et al., 1986; Sawada et al., 1994; Imahie et al., 1995). The results clearly demonstrated that the testicular toxicity due to acute and chronic doxorubicin treatment as indicated by a significant increase in lipid peroxidation and a significant decrease in antioxidant enzymes, SOD, catalase and reduced glutathione levels. The levels of Na^+K^+ATPase, Ca^{2+} ATPase; Mg^{2+} ATPase was reduced in testes of doxorubicin treated rats.

Treatment with green tea extract offered a significant protection from the doxorubicin induced acute well as chronic testicular toxicity. Green tea extract treatment significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in the testes of doxorubicin treated rats. These protective effects were also supported by the restoration of sperm count, testosterone, and histopathological study.

Melatonin treatment offered significant protection in chronic testicular toxicity as compared to acute testicular toxicity. Pretreatment with the melatonin did not significantly affect the serum levels of testosterone, body and testes weight and the various antioxidant parameters in doxorubicin induced acute testicular toxicity. However, the melatonin significantly increased sperm count in the doxorubicin induced chronic testicular toxicity. It also significantly reduced the lipid peroxidation and increased the levels of glutathione, catalase and SOD of testes, which suggests efficacy of melatonin in preventing free-radical induced damage. Treatment of melatonin along with doxorubicin in chronic testicular toxicity significantly

increased the activity of ATPase. These protective effects were also supported histopathology study.

Lovastatin treatment failed to produce significant protection in acute as well as in chronic testicular toxicity. Treatment with the lovastatin did not alter the serum levels of testosterone, sperm count, indicators of testicular toxicity and the various antioxidant parameters in doxorubicin induced acute as well as chronic toxicity.

5.2.4 Cisplatin induced testicular toxicity

Cisplatin has proven useful against a wide spectrum of human cancers, including bladder, head and neck, ovarian and testicular cancers. However, the use of cisplatin is limited by the development of nephrotoxicity and ototoxicity (Ward and Fauvie, 1976; Loehrer and Einhorn, 1984), toxicities that involve alterations in the antioxidant defence systems (Somani et al., 1995; Husain et al., 1996). Cisplatin has also been shown to alter the levels of luteinizing hormone (LH and FSH), to reduce intratesticular testosterone, and to decrease sperm motility and count (Seethalakshmi et al., 1992; Aydiner et al., 1997). Histological examination of testes further indicates significant damage to Sertoli, Leydig and germ cell populations induced by cisplatin (Aydiner et al., 1997).

In the present study, acute as well as chronic administration of cisplatin significantly induces pathological changes in serum and biochemical markers indicative of toxicity and increases the free radical production. Acute as well as chronic administration of cisplatin significantly decreased the testosterone and sperm count. Our results are supported by a significant increase in lipid peroxidation and a significant decrease in antioxidant enzymes, SOD, catalase and reduced glutathione levels in testes after treatment with cisplatin. The levels of Na^+K^+ATPase, Ca^{2+}ATPase; Mg^{2+}ATPase was reduced in testes of cisplatin treated rats. These data are corroborated by previous studies reported by other investigators on cisplatin induced testicular toxicity in normal rats (Seethalakshmi et al., 1992).

Treatment with green tea extract offered a significant protection from the cisplatin induced acute well as chronic testicular toxicity. Green tea extract treatment significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in the testes of cisplatin treated rats.

These protective effects were also supported by the restoration of sperm count, testosterone, body weight and histopathology study.

Melatonin treatment offered significant protection in chronic testicular toxicity as compared to acute testicular toxicity. Pretreatment with the melatonin did not significantly alter the sperm count and the various antioxidant parameters in cisplatin induced acute testicular toxicity. However, the melatonin significantly increased serum testosterone, sperm count and body weight in the cisplatin induced chronic testicular toxicity. It also significantly reduced the lipid peroxidation and increased the levels of glutathione, catalase and SOD of testes, which suggests efficacy of melatonin in preventing free-radical induced damage. Treatment of melatonin along with cisplatin in chronic testicular toxicity significantly increased the activity of ATPase. These protective effects were also supported histopathology study.

Lovastatin treatment failed to produce significant protection in acute as well as in chronic testicular toxicity. Treatment with the lovastatin did not alter the serum levels of testosterone, sperm count, indicators of testicular toxicity and the various antioxidant parameters in cisplatin induced acute as well as chronic toxicity.

5.2.5 DMBA induced mammary gland cancer

The present study shows for the first time to our knowledge that resveratrol offers a considerable protection against doxorubicin induced cardtiotoxicity in DMBA induced mammary carcinogenesis in female rats. Resveratrol (3,4_-trihydroxystilbene), a natural phytoalexin present in grapes and many other natural sources, has been suggested to play a role in reducing the risk of coronary heart disease and cancer (Kopp, 1998; Jang and Pezzuto, 1999). In addition, resveratrol intake has been reported to have antiinflammatory and antiatherosclerosis functions and to modulate hepatic alipoprotein and lipid synthesis, platelet aggregation, and production of antiatherogenic eicosanoids by human platelets and neutrophils (Fauconneau et al., 1997; Pace-Asciak et al., 1996). Resveratrol has also been reported to inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary organ cultures and the promotional stage of mouse skin carcinogenesis. Additionally, it mediates reduced aberrant colonic crypt foci formation and inhibits of the growth of a wide variety of human-derived tumor cells, including leukemic, prostate, breast,

and endothelial cells (Tessitore et al., 2000; Carbo et al., 1999; Hsieh et al., 1999; Surh et al., 1999). Other targeted cellular effects belonging to resveratrol involve inhibition of the enzymes protein kinase C, ribonucleotide reductase, cyclooxygenase, and nitric oxide synthase and inhibition of aryl hydrocarbon-induced cytochrome P-450 IAI (Subbaramaiah et al., 1999; Ciolino et al., 1999; Tsai et al., 1999). A close structural similarity exists between synthetic estrogen (4,4 dihydroxy-*trans*-diethylstilbene) and resveratrol. It is unclear, however, whether resveratrol is an estrogen receptor agonist or antagonist. Estrogen agonists have been reported to exert protective action against estrogen-dependent cancers, such as cancer of the breast and endometrium; presumably, resveratrol interacts with estrogen receptor to inhibit its activation (Lu and Serrano, 1999). Lu and Serrano (1999) reported that resveratrol acts as an estrogen receptor antagonist in the presence of estrogen, leading to inhibition of growth of human breast cancer cells.

The results of the present study demonstrated that administration of doxorubicin in DMBA induced mammary cancer produced a significant increase in serum markers of cardiotoxicity (CK, LDH and SGOT) and MDA content (an index of lipid peroxidation) in cardiac tissues and the highly significant reduction in tissue levels of protective biological antioxidants like GSH, SOD, catalase and membrane bound enzymes.

Treatment of resveratrol along with doxorubicin in DMBA induced mammary cancer causes significant decrease in LDH, CK, SGOT and lipid peroxidation near to that of control group. These data suggest that resveratrol may protect the myocardial tissue against doxorubicin induced cardiotoxicity in DMBA induced mammary cancer.

Further results also led to belief that administration of resveratrol improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of lipid peroxidation. These protective effects were also supported by histopathological study. It seems that resveratrol can protect the heart from doxorubicin induced assault DMBA induced mammary cancer as confirmed by several studies and reviewed (Hrelia et al., 2002; Quiles et al., 2000).

CHAPTER 6

SUMMARY AND CONCLUSIONS

Antineoplastic agents have been shown to produce oxidative stress in patients who receive these drugs during cancer chemotherapy (Faber et al., 1995; Weijl et al., 1998; Sangeetha et al., 1990). This is evident by the elevation of LPO products; the reduction in plasma levels of antioxidants such as vitamin E, vitamin C, and β-carotene; and the marked reduction of tissue glutathione levels that occurs during chemotherapy. Since many drugs used for cancer chemotherapy cause oxidative stress, which can interfere with antineoplastic activity, reducing this oxidative stress by administering antioxidants may enhance the effectiveness of the treatment. However, enhancing the cytotoxicity of antineoplastic agents would affect normal cells as well as cancer cells. Certain antioxidants appear to prevent the development of some chemotherapy induced side effects for e.g. coenzyme Q10 for anthracycline cardiotoxicity and glutathione for cisplatin nephrotoxicity (Conklin, 2000).

The present study on the *in vitro* testing of drugs green tea extract, melatonin, lovastatin and resveratrol showed that these drugs possessed scavenging activity of free radicals generated in *in vitro* systems. *In vivo* study such as doxorubicin induced cardiotoxicity, cisplatin induced nephrotoxicity in addition to testicular toxicity and doxorubicin induced cardiotoxicity in mammary cancer also proved that these drugs can ameliorate the tissue injuries inflicted by the release of free radicals as a result of the oxidative stress induced by anticancer drugs.

The following studies were conducted to substantiate these findings.

- Preliminary radical scavenging activity of green tea extract, melatonin, lovastatin and resveratrol was tested by their ability to bleach the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH). The study showed that the methanolic extracts of the drugs green tea extract, melatonin and resveratrol possessed DPPH quenching capacity while lovastatin did not show DPPH scavenging capacity.

- The methanolic extracts of drugs green tea extract and resveratrol were found to possess scavengers of superoxide radical generated in

riboflavin-NBT-light system *in vitro while* melatonin and lovastatin did not show superoxide radical scavenging capacity.

- The methanolic extracts of the drugs green tea extract, melatonin, and lovastatin possessed significant NO quenching capacity while resveratrol had a mild NO scavenging activity.

- The methanolic extracts of the drugs green tea extract, melatonin, lovastatin and resveratrol possessed significant inhibition of lipid peroxidation *in vitro*.

- In doxorubicin induced cardiotoxicity, The administration of green tea extract, melatonin and lovastatin resulted in significant decrease in the activities of serum markers of cardiotoxicity (creatine kinase, lactate dehydrogenase and GOT) and increase in the levels of endogenous antioxidants (SOD, catalase and reduced glutathione), reduction in lipid peroxidation and prevention of associated histopathological changes induced by doxorubicin myocardial injury in chronic as well as acute administration schedule. The administration green tea extract, melatonin and lovastatin offered significant protection as indicated by improvement in hemodynamic and ECG in chronic as well as acute doxorubicin cardiotoxicity. Thus, the results obtained from this study indicate that treatment with green tea extract, melatonin and lovastatin offered significant protection to heart (cardioprotective effect) and thus reduced the risk of doxorubicin induced cardiac damage by inhibiting lipid peroxidation and activating antioxidant defense enzymes in the organ.

- Cisplatin administration increased the serum levels of creatinine, urea, uric acid and BUN in acute as well as chronic administration schedule. It inhibited the activities of antioxidant enzymes (SOD and catalase), increased lipid peroxidation and depleted reduced glutathione in rat kidneys suggesting that cisplatin nephrotoxicity results from generation of reactive oxygen species. Treatment with the green tea extract significantly reduced lipid peroxidation and

increased the levels of glutathione, catalase and SOD in acute as well as chronic model, which suggests efficacy of green tea extract in preventing free radical induced damage. Melatonin treatment offered significant protection in chronic nephrotoxicity as compared to acute nephrotoxity. The reduction in serum levels of creatinine, urea, uric acid and BUN by the drugs, in the chronic model, may be due to protective effect on the kidneys. Thus, the results obtained indicate that the green tea extract and melatonin offer significant protection to kidney (nephroprotective effect) and reduce the risk of cisplatin-induced nephrotoxicity by their antioxidant mechanism of action.

- Acute as well as chronic doxorubicin administration decreased the serum testosterone level and sperm count. It inhibited the activities of antioxidant enzymes (SOD and catalase), increased lipid peroxidation and depleted reduced glutathione in rat testes suggesting that doxorubicin induced testicular toxicity results from generation of reactive oxygen species. Treatment with the green tea extract significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in acute as well as chronic model, which suggested that efficacy of green tea extract in preventing free radical induced damage. Melatonin treatment offered significant protection in chronic testicular toxicity as compared to acute testicular toxicity. Thus, the results obtained indicate that the green tea extract and melatonin offer significant protection to testes and reduce the risk of doxorubicin induced testicular toxicity by their antioxidant mechanism of action.

- Administration of cisplatin decreased the serum testosterone level and sperm count in acute as well as chronic study. It inhibited the activities of antioxidant enzymes (SOD and catalase), increased lipid peroxidation and depleted reduced glutathione in rat testes suggesting that cisplatin induced testicular toxicity results from generation of reactive oxygen species. Treatment with the green tea extract significantly reduced lipid peroxidation and increased the levels of glutathione, catalase and SOD in acute as well as chronic

model, which suggested that efficacy of green tea extract in preventing free radical induced damage. Melatonin treatment offered significant protection in chronic testicular toxicity as compared to acute testicular toxicity. Thus, the results obtained indicate that the green tea extract and melatonin offer significant protection to testes and reduce the risk of cisplatin induced testicular toxicity by their antioxidant mechanism of action.

- Administration of doxorubicin in DMBA induced mammary cancer produced a significant increase in serum markers of cardiotoxicity (CK, LDH and SGOT) and MDA content in cardiac tissues and the highly significant reduction in tissue levels of protective biological antioxidants like GSH, SOD and catalase. Administration of resveratrol improved the biochemical marker levels indicating decrease in oxidative stress as evident by increased levels of GSH, SOD and CAT with decreased production of lipid peroxidation and serum markers of cardiotoxicity (CK, LDH and SGOT). These protective effects were also supported histopathology study. It seems that resveratrol can protect the heart from doxorubicin induced assault DMBA induced mammary cancer. Thus, the results obtained from this study indicate that treatment with resveratrol offers significant protection to heart (cardioprotective effect) and thus reduces the risk of doxorubicin induced cardiac damage in DMBA induced mammary cancer by inhibiting lipid peroxidation and activating antioxidant defense enzymes in the organ.

With all these findings, it can be concluded that green tea extract, melatonin, lovastatin and resveratrol are effective in scavenging free radical *in vitro* as well as *in vivo* by protecting the various organs from the deleterious effect caused by anticancer drugs like doxorubicin and cisplatin.