CHAPTER 1

INTRODUCTION

1.1 Statement and Significance of the Problem

Heavy metals are a mixed group of elements with metallic properties. Some of these elements are actually necessary for humans while others are carcinogenic or toxic (1). Heavy metals are probably the oldest toxin known to human population. Poisoning due to heavy metals has traditionally been thought to occur only in the setting of occupational exposure; however, this is now uncommon in modern industrialized economies where environmental exposure is increasingly recognized.

Cadmium (Cd) is one of the hazard heavy metals that impact greatly on human health even at concentrations below the provisional safe levels set by the World Health Organization (2-4). It is an earth's crust natural element and is usually found as a mineral in combination with other elements such as oxygen, chlorine, or sulfur (5). Over the past two centuries, anthropogenic and industrial activities, such as mining, paints, electroplating and particularly nickel-cadmium battery, have led to high emissions of cadmium into the environment at concentrations significantly exceeding those originating from natural sources. It has been reported that approximately 30,000 tons of cadmium is released into the atmosphere each year, with an estimated 4,000-13,000 tons coming from industrial activities (6). Since cadmium is not degraded in the environment and can enter the food chain, the risk of human exposure, particularly non-occupational inhabitants, is constantly increasing

(5, 6). It is generally acknowledged that cadmium is not essential for the human body, since it is not involved in known enzymatic processes or other biological activities (7). By contrast, exposure to this heavy metal causes damage to various organ systems such as the kidneys, liver, lungs, bone, and reproductive system (8).

The kidney has been recognized as a critical target organ for cadmium toxicity (1, 8, 9). It has been reported that cadmium that is taken up into the body is rapidly cleared from the circulation and approximately 50% of the amount contaminated is deposited mainly in the kidney (1, 9). Although the storage form of cadmium in the body may change, the cadmium ion itself is biologically indestructible. Thus, unlike other organic toxicants, which can often be degraded metabolically to less toxic derivatives, cadmium remains intact in biological systems (5, 6). Cadmium has an extremely long biological half-life (15-30 years) that essentially causes it a cumulative toxin in the kidney, which makes up the bulk of total body burden (2).

Cadmium can produce a variety of renal toxic effects involving both the glomerulus and renal tubules (9, 10). These abnormalities are believed to be irreversible at advanced stages. Increased mortality was found among individuals showing signs of cadmium renal toxicity compared with those without signs, suggesting that renal toxicity may be an early warning of complications, sub-clinical or clinical morbidity (11). A better understanding of how cadmium mediates its serious nephrotoxic effect, in particular at the molecular levels, as well as identification of the agents that ameliorate the functional consequences of cadmium could therefore have significant clinical and financial impact.

Although several chelating agents and antagonists are established to reduce the cadmium toxicity, some of them are burned with undesirable side effects (1, 12). In addition, chelation therapy has been shown to be ineffective in the case of chronic cadmium exposure (1, 12). Due to the intrinsic limitations and variability of efficacy of heavy metal chelating agents, cadmium intoxication therapy is looking for the development of new therapeutic agents with different mode of actions, especially from the natural products.

1.2 Literature Review

1.2.1 Cadmium exposure

Cadmium is a silvery white, malleable, ductile, divalent metal that belongs to the group II of the periodic table. This non-essential heavy metal is an environmental and industrial pollutant that causes a serious health problem in both humans and animals. Cadmium has become increasingly prevalent in the environment because of its wide usage in the manufacture of products such as paints, chemical stabilizers, plating, alloys, and, in particular, nickel-cadmium batteries (1, 7). The risk of human exposure to cadmium is constantly increasing because cadmium is not degraded in the environment and it enters the food chain (5, 6).

Occupational cadmium exposures are to a large extent by inhalation of contaminated dust, while exposures in the general environment are almost entirely from ingestion of contaminated food and water (8). Cigarette smoking is also another significant source of cadmium exposure found in human (1, 2, 6). It is estimated that one cigarette may contain 1-2 μg cadmium (varies depending on the type and brand) and roughly 10% of cadmium oxide produced during cigarette smoking is inhaled

with an approximate 50% absorption in the lung (1, 3). Studies have shown that the concentration of cadmium in smokers is 4-5 times higher in blood, and 2-3 times higher in the kidneys, when compared with non-smokers (1). Although there is some evidence of cadmium exposure from environmental tobacco smoke in children, it does not seem to be a source of cadmium exposure in adults (3) .

Cadmium contamination via gastrointestinal tract is absorbed by a divalent metal ion transporter 1 (DMT1) in the duodenum and uptaken into the systemic circulation (1). Cadmium in the circulation, either from pulmonary or gastrointestinal absorption, is initially bound to albumin in plasma (8). This binding form of cadmium is predominantly taken up by the liver (Figure 1-1) where it forms complexes via sulfhydryl groups with small peptides such as the tripeptide glutathione (GSH) or with proteins such as the ~6-kDa high affinity metal binding protein metallothionein (MT) (1, 6, 8). Cadmium is then either secreted into the bile as CdGSH or, predominantly, released back into the circulation as CdGSH or CdMT complexes (1, 6, 8). The complexed cadmium or the free cadmium ion is taken up by target cells and tissues where toxicity ensues.

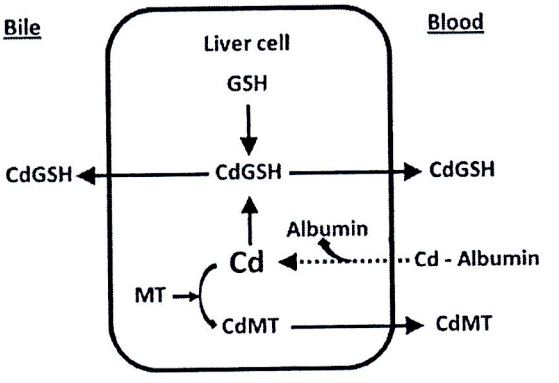

Figure 1-1 Schema of cadmium in blood and liver

(Modified from Nordberg, 2009 (8))

1.2.2 Cadmium toxicity and the kidney

The kidneys are the tissues most affected by cadmium. The S1 segment of the proximal tubule is the main site of cadmium accumulation as well as a target of cadmium toxicity (9). This nephron segment is supersensitive to damage by cadmium because it has a large luminal area for cadmium exposing, numerous mitochondria and varieties of transporters and receptors (13). Circulating cadmium, both as complexed cadmium and as free cadmium, is freely filtered at the glomerulus because of their small molecular mass and mainly reabsorbed by the epithelial cells lining the proximal tubule (1, 6). Between the two cadmium complexes, it is suggested that CdMT complex represents the major form in which cadmium is delivered to the kidneys *in vivo* (9).

Reabsorption of the CdMT complex occurs at the apical membrane of the proximal tubule by receptor-mediated endocytosis, possibly using the ZIP8 transporter (Figure 1-2) (1, 8). After uptake, CdMT complex enters the lysosomes and endosomes in the tubular cells. The metallothionein moiety is then degraded by acidic protease and free cadmium is released back into the cytosol by a carrier-mediated process, possibly using the DMT1 (1, 9). The free cadmium ion, which is filtered through the glomerulus, is also transported into the cytosol of the proximal tubule via Zn^{2+} and/or Cu^{2+} transporters (9).

Studies have shown that less than 10% of the total cadmium taken up at the luminal membrane of the proximal tubule is subsequently transported across the basolateral membrane (9). This indicates that most of the transported cadmium is resided within the proximal tubular cell. Cadmium has a very long half-life and excretion into urine or feces is extremely limited (2, 9). As a result of this

characteristic metabolic profile, free cadmium accumulates in the kidney overtime and renal damage occurs whenever the kidney defense and detoxification system are overwhelmed.

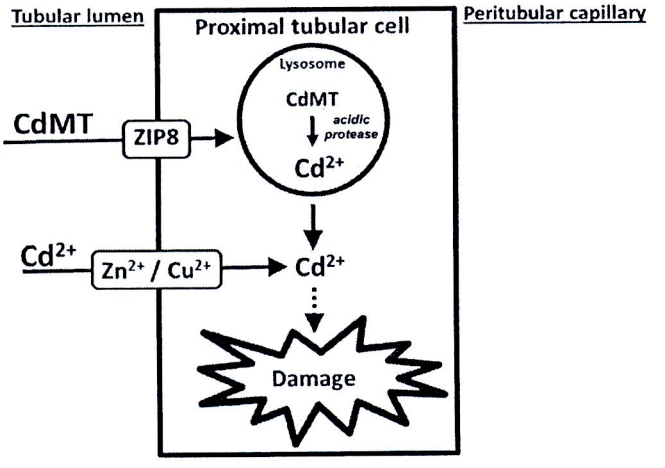

Figure 1-2 Putative cadmium uptake pathways in proximal tubule

(Modified from Travenod, 2003 (9))

The nephrotoxic effect of cadmium depends on the dosage, the chemical form of the metal, and the duration of exposure (7). Blood cadmium is considered to reflect not only recent exposure to this heavy metal but also lifetime body burden (2). The urinary cadmium is also influenced by the body burden of cadmium, and it is proportional to the accumulation of cadmium within the kidney (1-3, 7). Studies have shown that blood cadmium correlates well with urinary cadmium. This high correlation facilitates the use of urinary cadmium as the dose estimation of total body cadmium load in exposed populations (3). The provisional guidelines safe levels set for the toxicant by the World Health Organization is 2 μg/g creatinine for environmental exposure, 5 μg/g creatinine for occupational exposure, and > 10 μg/g

creatinine for possible renal damage caused by cadmium (2). However, experimental and epidemiological studies have shown a propensity towards development of kidney disease even at the lower exposure (3, 4, 8).

Cadmium-induced renal damage is initially characterized by proximal tubular reabsorptive dysfunction (1, 3, 10, 14). The kidney cadmium concentration at which renal tubular dysfunction develops is estimated to be about 200 μg/g wet weight (9) or urinary cadmium levels of 2-3 μg/g creatinine (1). Although cadmium-induced tubular impairment is asymptomatic at this stage, the earliest signs that can be detected are increased urinary excretion of low-molecular weight proteins (LMWP), such as β2-microglobulin and retinol binding protein. The urinary excretion of markers of cytolysis such as the lysosomal enzyme *N*-acetyl-*β*-D-glucosaminidase (NAG) also increases (1-3).

As tubular injury progresses, more generalized tubular dysfunction occurs with urinary losses of glucose, amino acids, bicarbonate and phosphate, all features of a renal Fanconi syndrome (1, 14). Phosphate wasting and impaired vitamin D metabolism, through loss of vitamin D binding protein as well as reduced conversion of 25-OH to 1,25-OH vitamin D, could contribute to renal calcium losses and bone disease (commonly known as the Itai-itai disease) as well as to renal stone formation (1, 10). Continued cadmium exposure causes glomerular damage, leading to albuminuria and a progressive decline in glomerular filtration rate, eventually causing renal failure. Renal damage may further develop to end stage renal disease and death, if exposure is high and prolonged, or if it occurs along with predisposing factors such as diabetics or hypertension (1, 3, 8, 14).

1.2.3 Cadmium toxicity and oxidative stress

Although cadmium-induced toxicity has intensely been investigated both *in vitro* and *in vivo* using a variety of experimental setup in different cell types and organs, the mechanism underlying its toxic effect remains unsettled. However, several studies are able to show that cadmium toxicity is linked to oxidative stress and subsequent renal cell death (15-17). Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) and the capacity of antioxidant defense systems, leading to the oxidative damage of DNA, RNA, lipids, and proteins (5). Oxidative damage has been implicated in the cause of many diseases including a broad spectrum of renal injuries (18-21).

Cadmium itself is unable to generate ROS directly because it is a non redox-reactive metal (5, 22). However, enhanced production of ROS such as superoxide anion, hydrogen peroxide, hydroxyl radicals and reactive nitrogen species have been reported after exposure of various cell culture systems or intact animals to cadmium (15, 16, 22). Evidence exists that cadmium-mediated ROS production is a consequence of its displacement of endogenous metal cofactors from active sites and these redox active metals subsequently enhance ROS formation through the Fenton and Haber Weiss reactions (5, 23).

A disturbance in antioxidant defense mechanisms has also been documented after cadmium exposure. Thiols are the major cellular antioxidant defenses and redox signaling, which is thought to be the first line of defense against cadmium toxicity (1). Cadmium shows a high affinity for thiol binding and it is suggested that the depletion of intracellular thiols by cadmium is the prerequisite for ROS generation (24). Glutathione, the most abundant low molecular weight thiol in living organisms, has

been reported to be decreased concomitantly along with the non-enzymatic antioxidants such as vitamin C and vitamin E in animals with cadmium nephrotoxicity (22, 25). The decreased activities of various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) have also been observed in the renal tissues of cadmium-intoxicated animals (16, 22, 25). Cadmium-induced oxidative challenge has further supported by the observations of increased lipid peroxidation, protein oxidation, modulation of DNA, and induction of several stress response genes following cadmium exposure, which may eventually lead to cellular dysfunction and death (5, 6, 15, 25).

1.2.4 Mitochondria and oxidative stress

A mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells. It composes of compartments that carry out specialized functions. These compartments include the outer membrane, the intermembrane space, the inner membrane, the cristae and the matrix (Figure 1-3).

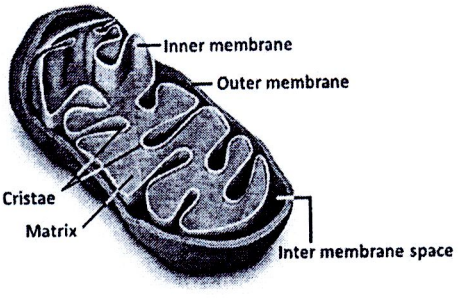

Figure 1-3 Structure of mitochondria

(Modified from www.curebd.com/2011/04/function-of-mitochondria-and.html (26))

The outer membrane is a semi-permeable membrane. It contains a lot of integral proteins called porins, which form channels that allow molecules less than 5000 Daltons to freely diffuse into the other side, and a large multisubunit protein called translocase of the outer membrane (TOM), which allows large protein molecules to enter the mitochondria (27). The intermembrane space, the space between the outer membrane and the inner membrane, is similar in composition to the cytosol. Cytochrome *c* is also localized in this space and disruption of the outer membrane causes cytochrome *c* to leak into the cytosol, leading to certain cell death. The inner membrane contains an unusual phospholipid, cardiolipin, which make the membrane impermeable to all molecules (28). It also contains the enzymes responsible for oxidative phosphorylation (2). The cristae are the compartment formed by folding of the inner membrane to increase surface area to accommodate more ability to produce ATP. The matrix is a semi-fluid enclosed by the inner membrane which contains the enzymes that are responsible for the citric acid cycle reactions (29).

Mitochondria have long been recognized as a powerhouse of cell. These organelles generate energy in the form of adenosine triphosphate (ATP) through the oxidative phosphorylation, in which electrons are passed along a series of protein complexes situated in the inner mitochondrial membrane known as the electron transport chain (ETC). This process is also the major source of intracellular ROS generation (2, 7, 29).

Electrons generated from reduced nicotinamide adenine dinucleotide (NADH) are collected by complex I (NADH dehydrogenase) and from succinate by complex II (succinate dehydrogenase). The electrons collected at these sites are

passed to complex III (cytochrome bc_1) via coenzyme Q (CoQ) and to complex IV (cytochrome c oxidase) using cytochrome c (Cyt c) as a carrier, and finally to O_2 to form water (Figure 1-4). The energy produced from the electron flow is used to drive out protons (H^+) at the level of complexes I, III, and IV. As the inner mitochondrial membrane is almost impermeable, an electrochemical gradient is formed and is used to reintroduce protons through the proton channel of complex V (ATP synthase). The proton flow drives the condensation of adenosine diphosphate (ADP) and inorganic phosphate (Pi) to form ATP. The ATP is exchanged with cytosolic ADP by adenine nucleotide translocator (ANT) (2, 7, 29, 30). During the transfer of electrons along these electron transport complexes, single electrons sometimes leak out and result in a single electron reduction of molecular oxygen to form a superoxide anion ($O_2^{\cdot-}$). This occurs mainly at complex I and complex III of the ETC and these superoxide radicals may serve as the precursor of other reactive species (2, 29).

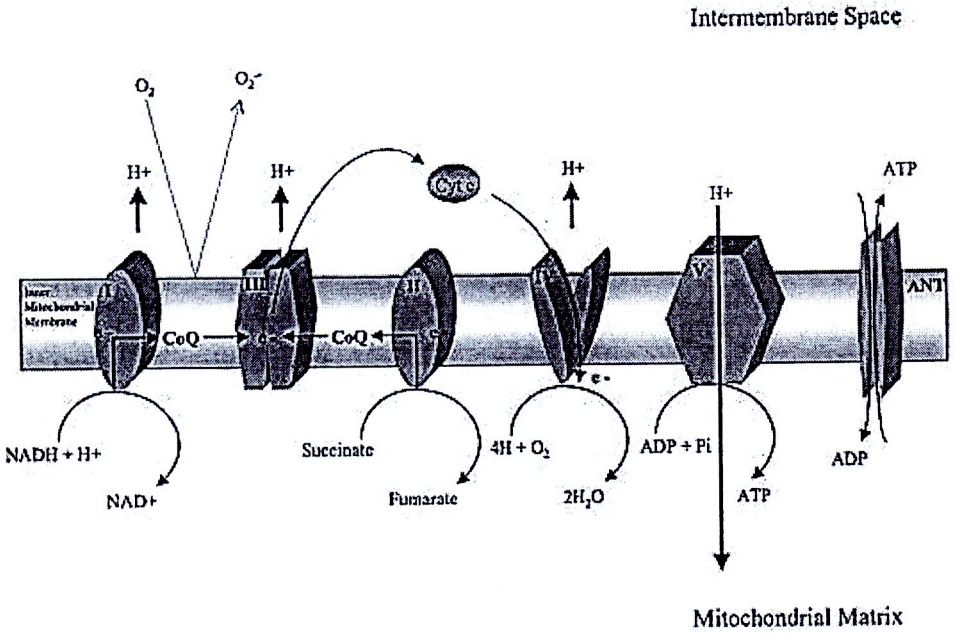

Figure 1-4 Mitochondrial oxidative phosphorylation

(Jassem et al, 2002 (30))

Superoxide anion produced in the mitochondria is normally neutralized by the mitochondrial antioxidant system, which comprises an enzymatic system that is based on superoxide dismutase and glutathione peroxidase (29, 30). Superoxide anion is converted to hydrogen peroxide (H_2O_2) by the activitiy of matrix manganese superoxide dismutase (MnSOD) as well as Cu, Zn-SOD in the intermembrane space (Figure 1-5) (29, 30). H_2O_2 is reduced to H_2O by glutathione peroxidase (GPx) with GSH as substrate to produce the oxidized glutathione (GSSG). This reaction is combined with reduction of GSSG by NADPH-dependent reductase to form GSH and oxidized nicotinamide adenine dinucleotide phosphate (NADP). However, when not metabolized, H_2O_2 may generate the hydroxyl radical ($OH^{\cdot}$) through the metal catalyzed Fenton reaction (23, 29, 30).

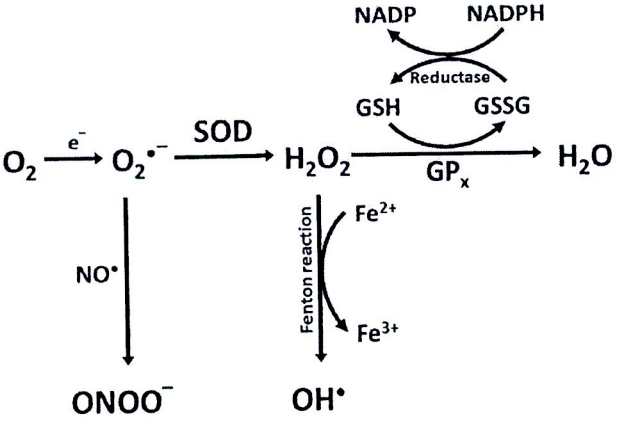

Figure 1-5 Generation of ROS and mitochondrial antioxidant system

(Modified from Jassem et al, 2002 (30))

Superoxide radical may also react with nitric oxide ($NO^{\cdot}$) to form the cytotoxic peroxynitrite ($ONOO^-$). Both hydroxyl radical and peroxynitrite are highly reactive and generally believed to act essentially as a damaging molecule, affecting

multiple networks that control redox balance within mitochondria (23, 29, 30). Under physiological conditions, it is estimated that about 1-4% of total mitochondrial oxygen consumed is completely reduced and leads to the production of ROS (30, 31). However, ROS production substantially increases once the mitochondrial antioxidant defense systems are overwhelmed, and excessive ROS contribute to mitochondrial oxidative stress and damage (2).

Although energy production is a primary role of mitochondria, a new paradigm of mitochondrial function is emerging in the past few years, which includes the control of cell signaling, cell proliferation and programmed cell death as well as the synthesis of amino acids, nucleotides, lipids and the maintenance of calcium and ion homeostasis (2, 7, 32). Based on present knowledge of mitochondrial function, the mitochondrial-related events such as a decline in ATP production, the onset of mitochondrial permeability transition, increased concentrations of free calcium and bioavailable of iron, and oxidative damage to key mitochondrial constituents appear to be important contributors to the demise of cell and, thus, malfunctioning mitochondria have been implicated in a range of pathologies (7, 33).

1.2.5 Cadmium toxicity and mitochondria

Mitochondria appear to be a primary intracellular target organelle for cadmium toxicity (1, 2, 7). Direct penetration of cadmium into mitochondria has been documented (2, 7). Accumulating evidence suggests the generation of oxidative stress as the first step in cadmium-mediated cytotoxicity, preceding mitochondrial damage, since an increase ROS level was detected within minutes after incubation of several cell lines to cadmium (5, 6, 34). Investigation into the effects of cadmium on

the individual complexes of the mitochondrial electron transfer chain from guinea pig liver, brain and heart demonstrated that cadmium inhibits the electron transfer and stimulates ROS production in all three tissues studied (31). Based on the electron spin resonance measurement, the impairment of electron transfer through complex III is considered to be the major site of ROS production induced by cadmium (31). Kinetic studies and electron turnover experiments suggested that cadmium may bind between semiubiquinone and cytochrome *b*566 of the Q_0 site of cytochrome *b* of complex III, thereby preventing electron delivery from semiubiquinone to *b566* and promoting the accumulation of semiubiquinones at the Q_0 site. The accumulated semiubiquinones are unstable and may be responsible for the transfer of one electron to molecular oxygen, thus forming superoxide radical (31). These findings may provide a possible mechanism for cadmium-induced generation of ROS in mitochondria.

Cadmium has also been shown to interact with specific protein thiols in mitochondria isolated from rat liver. This is followed by ROS generation together with Fe^{2+} mobilization leading to mitochondrial membrane lipid peroxidation (35). A more recent study using mitochondria isolated from human embryonic kidney cell lines demonstrates that cadmium can directly cause an increased mitochondrial membrane permeability, mitochondrial swelling, and membrane potential collapse, which is accompanied by enhancing levels of ROS, lipid peroxidation, and inhibition of several antioxidant enzyme activities (11). Considering the involvement of oxidative stress and mitochondrial dysfunction in cadmium-induced injury, it is possible that cadmium nephrotoxicity may be associated with mitochondrial dysfunction through mechanisms involving increased ROS production and the use of

antioxidant targeting on mitochondria may be an essential therapeutic approach in the alleviation of cadmium-induced nephrotoxicity. This possibility remained to be established.

1.2.6 Caffeic acid phenethyl ester

Propolis, sometimes also referred to "bee glue", is a strongly adhesive, resinous substance collected, transformed and used by bees to seal holes in their honeycombs, smooth out the internal walls and protect the entrance against intruders. Honeybees collect the resin from the cracks in the bark of trees and leaf buds. This resin is masticated, salivary enzymes added and the partial digestion material is mixed with beeswax and used in the hive (36). Caffeic acid phenethyl ester (CAPE) is a biologically active component of propolis (Figure 1-6). It has been used in traditional and alternative medicine in many countries for many years (19). CAPE has several biological and pharmacological properties, such as antioxidant, anti-inflammatory, anti-mitogenic, anticarcinogenic, antiviral, antifungal, and immunomodulatory activities (18-21). Studies have shown that CAPE can penetrate cell membrane and exhibits its antioxidant properties even in the micromolar concentration range. At a concentration of 10 μM, it completely blocks the production of free oxygen radicals in human neutrophils and suppresses the xanthine/xanthine oxidase system (37).

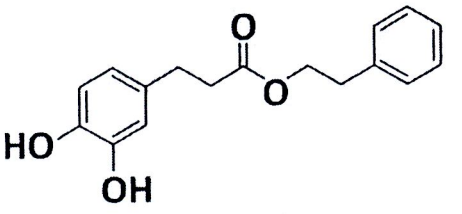

Figure 1-6 Chemical structure of CAPE

(Modified from Gocer&Gulcin, 2011 (38))

Numerous reports have demonstrated that CAPE is able to scavenge superoxide anion, hydroxyl radicals, singlet oxygen, and peroxyl radicals (39-41) . CAPE exerts its antioxidant activity by chelating the transition metal, suppressing lipid peroxidation, scavenging the reactive species, inhibiting xanthine oxidase, NADPH oxidase, and nitric oxide synthase (11, 19, 20, 42). There are also reports of CAPE to enhance the cellular antioxidant such as reduced glutathione, and antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (40, 43-45).

The protective effect of CAPE against oxidant-induced injury has previously been established in diverse organ systems under different pathogenic conditions (40, 46, 47). Focusing on the kidney, CAPE treatment has been reported to protect the kidneys from oxidant-induced injury caused by ischemia-reperfusion (18), shock wave (48), electromagnetic radiation (45), and various xenobiotics such as cyclosporine (21), doxorubicin (43), cisplatin (49) , methotrexate (50), gentamicin (51), amikacin (52), and vancomycin (53). It has also been shown to be effective in reducing nephrotoxicity induced by toxic substances such as carbon tetrachloride (19) and lithium (44).

Most recently, the beneficial role of CAPE at the mitochondrial level has begun to be explored. CAPE has been demonstrated to protect against glutamate-induced cerebellar granule neuron death by blocking Ca^{2+}-induced mitochondrial swelling and cytochrome *c* release (54). It has also been reported to limit the functional alterations of the isolated mouse brain and liver mitochondria submitted to *in vitro* anoxia-reoxygenation through its antioxidant activities (55). However, the

possible mitochondrial protective effect of CAPE in the setting of nephrotoxicity caused by cadmium has never been investigated.

1.3 Rationale, Model, and Hypothesis

Cadmium is a toxic heavy metal occurring in the environment naturally and as a pollutant emanating from industrial and agricultural sources. Based on available evidence, cadmium exposures, both environmental and occupational exposures, have no margin of safety and can cause adverse health effects even at very low concentrations. The kidneys are particularly susceptible to cadmium intoxication. Human studies demonstrate that 7-10% of the general population have renal dysfunction from cadmium exposure (9). Cadmium nephrotoxicity has provoked a significant public health concern as it can progress to chronic kidney disease and, eventually, end-stage renal disease.

Emerging evidence indicates that oxidative stress and ROS formed in the presence of cadmium may be responsible for its toxic effects (1, 5, 6). Molecular mechanisms accounting for cadmium-induced oxidative stress in the kidney are not well understood, but the involvement of mitochondria is highly plausible given that these organelles are ubiquitous sources of ROS production. Mitochondria are also involved in the control of cell signaling and cell death and defective mitochondria have been related to different pathological conditions (7, 32, 33).

Recent evidences indicate that mitochondria are key intracellular targets for cadmium, and mitochondrial dysfunction impacts greatly on the health of cells via mechanisms involving increased ROS production (1, 2, 5, 7). These findings imply that a strategy to preserve tissue mitochondria could preserve normal organ

functioning and further suggest that antioxidant targeting on mitochondria may find therapeutic benefit in diminishing the detrimental cellular and organs outcomes of cadmium.

In recent years, a great interest has emerged concerning the protective biochemical functions of naturally occurring antioxidants in biological system against oxidative damage caused by free radical species. Caffeic acid phenethyl ester (CAPE) is an active component of propolis obtained from honeybee hives. It has been shown to be a potent antioxidant even in the micromolar concentration range (37). CAPE has also been reported to protect against oxidative stress-related renal injury in different pathologic settings (18-21). Most importantly, the antioxidative effect of CAPE has recently become evident at the mitochondrial level (54, 55) .

In light of these observations, it is hypothesized that CAPE can offer protection against cadmium nephrotoxicity through mitochondrial mechanism. The present study, therefore, was established to address this issue using isolated rat kidney mitochondria exposed to cadmium as an experimental model. The outcome of using this model could provide essential information regarding the direct effect of cadmium on mitochondrial structure and function in the kidney of intact animals. The findings could also determine a promising role for CAPE as mitochondria-targeted antioxidant to combat the nephrotoxicity elicited by this toxic metal.

1.4 Objective of the Study

The present study was undertaken as an initial step with the main objective to determine the effects of caffeic acid phenethyl ester on kidney mitochondrial structure and function following cadmium exposure.

1.5 Scope of the Study

The current investigation consisted of 4 sets of experiments. The first set determined the effect of different concentrations of cadmium on the isolated rat kidney mitochondrial function. The second experiment explored the impact of CAPE at various concentrations on the isolated kidney mitochondrial function both in the presence and absence of cadmium exposure. The third experiment evaluated the contribution of oxidant mechanism to the protective potential of CAPE against cadmium-induced kidney mitochondrial dysfunction. Then, the last set of experiment examined the alteration of kidney mitochondrial structure following cadmium and CAPE administration.