CHAPTER I

INTRODUCTION

A. INTRODUCTION

I. Bilirubin Metabolism

Bilirubin is a linear tetrapyrrole, the four pyrrole rings being connected by methane bridges (Wang & Chowdhury, 2006) that is formed during the process of heme degradation by reticuloendothelial system (RE system). Most of the circulating bilirubin derives from senescent erythrocytes, the heme dissociates from hemoglobin and is oxidized by the membrane-bound enzyme heme oxygenase (EC1.14.99.3). Heme is cleaved specifically at the α-methene bridge by a reaction catalysed by microsomal heme oxygenases, resulting in the formation of biliverdin and 1 mole of carbon monoxide (CO), and the release of an iron molecule. The reaction consumes three molecules of oxygen and requires a reducing agent, such as NADPH. The α-methane-bridge carbon is eliminated as CO, and the iron molecule is released. Biliverdin is subsequently transformed to bilrubin by cytosolic biliverdin reductases (EC 1.3.1.24) at birth, coupled with a transient inability of the newborn to form bilirubin glucuronides in the liver and excrete them in the bile (Figure 1). Once the UGT*01 enzyme and the biliary excretory system reach maturity, at about 1 month of age, plasma UCB levels decrease and reach the adult levels of 20 μM (1.7 mg/dL)

Bilirubin exists in the serum in four major forms: as unconjugated bilirubin, as the monoglucuronide, as the diglucuronide, and as albumin-bound bilirubin (McGeary *et al.*, 2003). Most of the bilirubin produced in the body is mono- or di-glucuronidated in the liver by UDP-glucuronosyltransferase1 enzyme (UGT*01), and these water-soluble products are excreted in the bile. Bilirubin is obtained industrially by

extraction of either cattle or pig bile, and it can be isolated as light-sensitive orange-red crystals. Plasma unconjugated bilirubin (UCB) levels are usually elevated in normal infants during the first two weeks of postnatal life (< 200 μM or 11.7 mg/dl) because of the marked and sudden breakdown of fetal erythrocytes.

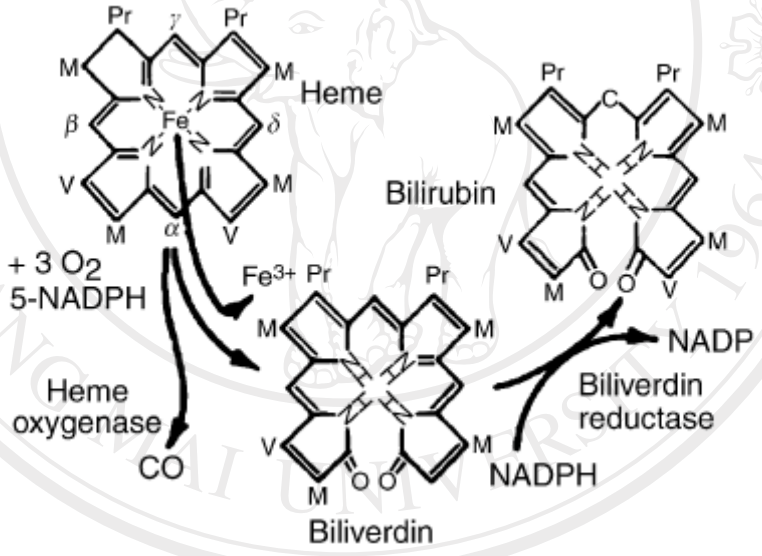

Figure 1. Enzymatic mechanism of bilirubin formation.

II. Function of Bilirubin in Serum

Bilirubin is created by the activity of biliverdin reductase (BVR) on biliverdin. Bilirubin, when oxidized, reverts to become biliverdin once again. This cycle, in addition to the demonstration of the potent antioxidant activity of bilirubin, has led to the hypothesis that bilirubin's main physiologic role is as a cellular antioxidant (Baranano & Snyder, 2001). Oxidation-reduction cycles for bilirubin and reduced glutathion (GSH) is shown in Figure 2. Lipophilic reactive oxygen species act directly on bilirubin, leading to its oxidation to biliverdin. BVR catalyzes the reconversion of biliverdin to bilirubin, permitting bilirubin to detoxify a 10,000-fold excess of oxidants. Soluble oxidants are detoxified by GSH, a cycle that requires 2 enzymes, GSH peroxidase and GSH reductase (http://www.benbest.com/nutrceut/BVR_GSH.jpg).

The potent biological properties of bilirubin particularly as an antioxidant has prompted a number of investigations into this molecule concerning its *in vitro* and *in vivo* properties. Wu *et al.* (1994) have studied the protective effects of bilirubin against oxidation of human low-density lipoprotein (LDL). Oxidation of LDL is implicated in plaque formation in blood vessels leading to atherogenesis, and there is evidence that prevention of this oxidation reduces the incidence of coronary heart disease.

III. Hyperbilirubinemia and Clinical Significance of Bilirubin in Human Serum

In humans, accumulation of bilirubin in the bloodstream causes yellow pigmentation of the plasma, in turn causing the skin and sclerae to become yellow, appearing clinically as jaundice. Normal human serum levels of bilirubin are in the range of 5-17 μM (0.3-1.0 mg/dL), with levels above around 43 μM (2.5 mg/dL) manifesting as jaundice.

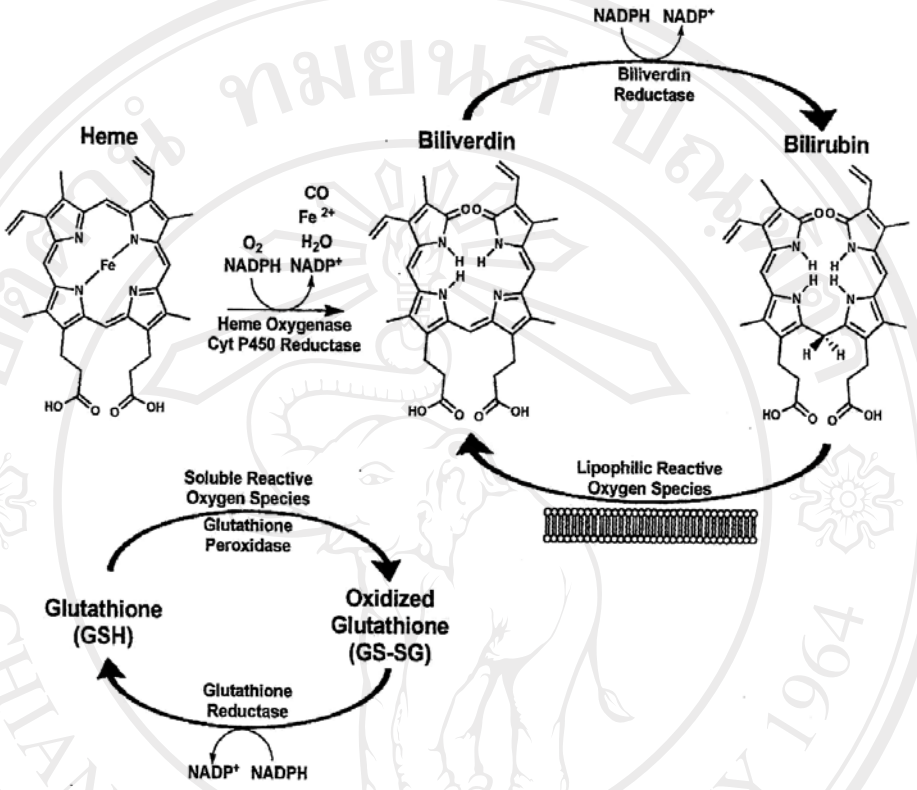

Figure 2. Oxidation-reduction cycles for bilirubin and reduced glutathion (GSH); a proposed physiologic role of bilirubin (See Text).

For clinical purposes, the predominant type of bile pigments in the plasma can be used to classify hyperbilirubinemia into two major categories

1.Plasma elevation of predominantly unconjugated bilirubin due to the overproduction of bilirubin, impaired bilirubin uptake by the liver, or abnormalities of bilirubin conjugation.

2.Plasma elevation of both unconjugated and conjugated bilirubin due to hepatocellular diseases, impaired canalicular excretion, and biliary obstruction.

In some situations, both overproduction and reduced disposition contribute to the accumulation of bilirubin in plasma. The frequency with which the different causes of jaundice occurs will vary markedly depending upon multiple factors such as age, geography, and socioeconomic class (Reisman *et al.*, 1996).

IV. Neonatal Hyperbilirubinemia

Neonatal hyperbilirubinemia, defined as a total serum bilirubin level above 86 μM (5 mg/dL), is a frequently encountered problem. It can be found up to 60% of term newborns and 80% of preterm newborns. In term newborns, they have clinical jaundice in the first week of life, few have significant underlying disease. Causes of neonatal hyperbilirubinemia are summerized as follows.

1) Increased bilirubin production resulting from increased hemolysis (e.g.,in Rh or ABO incompatibility or in G6PD deficiency). Erythroblastosis fetalis, which usually results from Rh blood group incompatibility between an Rh - negative mother and an Rh-positive neonate**.** In this disorder, bilirubin accumulates as a result of antibody-induced hemolysis, which releases hemoglobin that is sequentially converted to heme, biliverdin and bilirubin.

2) Delayed maturation of the hepatic conjugation system (e.g., in prematurely born neonates)

Delayed maturation of hepatic transport processes results in significant retention of UCB even in healthy, term neonates (Gourley, 1997). In addition, the neonate lacks anaerobic ileo-colonic flora that convert UCB to urobilinogens, leaving more unmetabolized UCB available for absorption into the portal blood, thus increasing the entero-hepatic circulation of UCB (Ostrow *et al*., 2003b).

3) Genetic abnormalities (e.g., mutations in the UGT*01 gene, such as in patients with the Crigler-Najjar syndrome) (Wang & Chowdhury, 2006)

Crigler-Najjar syndrome is an inherited disorder of bilirubin metabolism. It is an autosomal recessive disorder, meaning that an individual needs to receive two copies of the defective gene, one from each parent, in order to develop the syndrome

Conversion to glucuronides is essential for the efficient biliary excretion of bilirubin. Bilirubin glucuronidation is catalysed by a specific isoform of uridinediphosphoglucuronate glucuronosyltransferase, termed UGT1A1. Delayed development of UGT1A1 is the most important cause of neonatal unconjugated hyperbilirubinaemia. This delayed development can be exaggerated because of some ill-defined factors in the maternal serum, which may cause a prolongation of severe hyperbilirubinaemia for several weeks and may even cause kernicterus. A mild form of unconjugated hyperbilirubinaemia (bilirubin levels ranging from normal to 85 μM or 5 mg/dL), termed Gilbert syndrome, is found in up to 5% of Caucasian, Black and South Asian populations. This condition is associated with a promoter variation (insertion of a TA residue in the TATA element) of UGT1A1 More severe unconjugated hyperbilirubinaemia is found with mutations or short deletions within

the five exons that constitute the UGT1A1 mRNA. A complete loss of UGT1A1 activity resulting from these rare genetic lesions causes Crigler-Najjar syndrome type 1 (serum bilirubin levels of 250-650 μM or 14.6-38 mg/dL) (Chuwdhury *et al*., 2001). Crigler-Najjar syndrome type 1 is associated with kernicterus unless vigorously treated with phototherapy, and eventually requires liver transplantation.

When the plasma levels of UCB in neonatal hyperbilirubinemia are excessively elevated and surpass the capacity of albumin for high-affinity binding of UCB, the unbound (free) fraction of the pigment increases. This fraction may also be elevated in the plasma of newborns with "physiology jaundice" in association with the following conditions: 1) low blood pH (acidosis); 2) reduced capacity of plasma albumin for high-affinity binding of UCB; and 3) use of drugs that compete with UCB for binding to plasma albumin (e.g., sulfonamides). Free UCB can easily enter the cells by passive diffusion and cause toxicity. The most vulnerable site is the central nervous system. UCB binds to discrete brain areas, such as the basal ganglia (kernicterus), and produces a wide array of neurological deficits collectively known as bilirubin encephalopathy. These include irreversible abnormalities in motor, sensory (auditory and ocular), and cognitive functions (Kapitulnik, 2004)

Newborn infants display an increased susceptibility for brain damage because of the lower UCB-binding capacity of their plasma albumin and the temporal immaturity of their bloodbrain.

Classification of neonatal hyperbilirubinemia

1. Physiologic Jaundice: Physiologic jaundice follows a regular pattern in healthy term neonates, usually peaking between the 2nd and 4th day of life to 5-6 mg/dL and then declining over the first week. The increased synthesis and enterohepatic

circulation of bilirubin and the transient limitation of bilirubin conjugation in the liver of newborn infants cause it.

2. Pathological Jaundice: Pathological jaundice is the neurologic consequences of the deposition of unconjugated (called Kernicterus) bilirubin in the basal ganglia and various brainstem nuclei. This occurs when the serum unconjugated bilirubin level exceeds the binding capacity of albumin, allowing unconjugated lipid-soluble bilirubin to cross the blood-brain barrier. Low albumin, infections, acidosis and prematurity may enhance the deposition of bilirubin in the brain. In the acute phase, clinical signs in neonates include lethargy, hypotonia, and poor suck. Later there is hypertonia of the extensor muscles, opisthotonus, and torticollis. Bilirubin encephalopathy can lead to developmental and motor delays, movement disorders, sensorineural deafness, and mild mental retardation.

Risk fators of neonatal hyperbilirubinemia (Glaser, 2006)

1) Maternal illness : gestational diabetes

2) Prematurity

3) Race (Chinese, Japanese, Korean, Native American)

4) Drugs (vitamin K, novobiocin)

5) Others (dehydration or delayed stooling)

V. Toxic effects of bilirubin on cellular functions and fragmentation of DNA in brain tissue

Bilirubin concentrations greater than 300 μM (17.5 mg%) are associated with the risk of development of neurological dysfunctions due to deposition of bilirubin in brain and its enhanced toxic effects on cellular functions in this tissue (Meuwissen *et al*., 1982; Schenker *et al.*, 1986). Several other toxic effects of bilirubin on cellular

functions, particularly inhibition of several membrane bound enzymes, have also been reported (Karp, 1979). The precise mechanism of bilirubin toxicity is uncertain. However, several studies have shown that biological antioxidants, of both plant and animal origin, such as uric acid (Shamsi & Hadi, 1995), flavonoids (Fazal *et al*., 1990) and tannic acid (Bhat & Hadi, 1994; Fazal *et al*.,1994) are themselves capable of acting as prooxidants either alone or in the presence of transition metal ions. The prooxidant reactions were shown to cause fragmentation of DNA and proteins. Similary, bilirubin which function is proposed as antioxidant (Baranano & Snyder, 2001), may act as prooxidant which is capable to interact with cationic metal ions and cause DNA degradation in brain cells. In this study, the interaction reactions and possible mechanisms of bilirubin toxicity in brain and the factors involved will be investigated *in vitro*.

B. LITERATURE REVIEW

Bilirubin was long considered a useless metabolite of heme catabolism, responsible for the clinical manifestation of jaundice, and potentially toxic in high doses, particularly in neonates (McGeary et al., 2003). About 60% of healthy, term neonates will develop recognizable jaundice. In pathological jaundice, many factors may interact with the high UCB concentrations, either preventing or predisposing to kernicterus. The following review is concentrated on the causes and the factors involved in neonatal hyperbilirubinemia.

I. Neonatal Jaundice and Bilirubin Encephalopathy

Plasma unconjugated bilirubin (UCB) levels are usually elevated in normal infants during the first two weeks of postnatal life (< 200 μM or 11.7 mg/dL) because of the marked and sudden breakdown of fetal erythrocytes at birth, coupled with a transient inability of the newborn to form bilirubin glucuronides in the liver and excrete them in the bile. When the plasma levels of UCB are excessively elevated and surpass the capacity of albumin for high-affinity binding of UCB, the unbound (free) fraction of the pigment increases. Free UCB can easily enter the cells by passive diffusion and cause toxicity. The most vulnerable site is the central nervous system. UCB binds to discrete brain areas, such as the basal ganglia (kernicterus), and produces a wide array of neurological deficits collectively known as bilirubin encephalopathy. These include irreversible abnormalities in motor, sensory (auditory and ocular), and cognitive functions (Shapiro, 2003)

II. Unconjugated Bilirubin Toxicity in Neural Cells

Previous studies established that mitochondria might be a major target for UCB neurotoxicity, as demonstrated by impairment in mitochondrial function leading to the uncoupling of oxidative phosphorylation (Mustafa *et al.*, 1969). Additional effects of UCB in neural tissues and neuronal cell lines include inhibition of DNA and protein synthesis (Yamada *et al.*, 1977), changes in carbohydrate metabolism (Park *et al.*, 2002), and modulation of neurotransmitter synthesis and release (Hansen *et al.*, 1988).

III. Association of Albumin and Bilirubin in Serum Effect to Cytotoxicity

Most of the neural toxicity data were obtained in cell cultures using excessively high UCB concentrations, exceeding its very low aqueous solubility (Hahm *et al.*, 1992) and the high-affinity binding capacity of plasma albumin (Ostrow *et al*., 2003b). Moreover, the source of the albumin or plasma that is often used in binding experiments is adult blood, whereas that obtained from newborns has a diminished binding capacity for UCB (Kapitulnik, 2004). These facts suggest that many of the published *in vitro* toxicity findings may be irrelevant to the *in vivo* conditions prevalent in most cases of neonatal jaundic. Additional confounding factors in the early *in vitro* studies are: the type of cell used (astrocytes versus neurons), species differences in the cellular systems employed, dependence of the effect on the chosen endpoint of toxicity, and length of exposure to UCB.

From the studies employed UCB concentrations that are below saturation of albumin and may be comparable with the UCB levels found in the central nervous system of most jaundiced infants, as well as better defined cell culture systems and appropriate endpoints. Amit and Brenner (1993), using primary cultures of fetal rat glial cells (which consist mainly of astrocytes), showed that UCB affects cell

morphology, cell viability, and mitochondrial function. UCB toxicity was dependent on its concentration and on the UCB/albumin molar ratio. It is interesting that the toxic effects of UCB were directly related to the cells' age in culture. Both cell viability and mitochondrial function were considerably impaired upon exposure to UCB of cells cultured for 2 days, whereas extension of the culture time abolished their sensitivity to the toxic effects of UCB. These data correlate with the increased *in vivo* neurotoxicity of UCB in newborns compared with adults.

Grojean et al. (2000) reported that low levels of UCB (0.5 μM or 0.03 mg/dL) induce programmed cell death (apoptosis) in primary cultured neurons from the embryonic rat forebrain. The apoptotic process involves caspase activation and requires the participation of glutamatergic *N*-methyl-D-aspartate receptors. Moreover, UCB enhanced the effects of hypoxia in these immature neurons by facilitating glutamate-mediated apoptosis (Grojean *et al.*, 2001). On the other hand, UCB inhibited glutamate uptake in cultured rat cortical astrocytes, which play a major role in the transport of synaptically released glutamate (Silva, R. *et al.*, 1999). This inhibition was directly correlated with the UCB/albumin molar ratio and was observed at a molar ratio as low as 0.8. This effect of UCB was pH-dependent and occurred at pH 7.4 and 8.0.

In accordance with these findings in cell cultures, intrastriatal injections of *N*-methyl-D-aspartate, an excitatory glutamate analog, caused an increased atrophy of the striatum and hippocampus in jaundiced (jj) compared with nonjaundiced (Jj) Gunn rats (McDonald *et al.*, 1998). It is interesting that astrocytes are more susceptible than neurons to the UCB-mediated inhibition of glutamate uptake and MTT reduction (an indicator of mitochondrial function). In contrast, neurons are more sensitive than

astrocytes to the UCB-induced loss of cell viability (as measured by the release of LDH), disruption of the cytoskeleton, and apoptotic cell death. These findings stress the importance of the cell type and endpoint used for analysis of UCB mediated neurotoxicity (Silva *et al*., 2002).

IV. Mechanism of Unconjugated Bilirubin Toxicity in Neural Cells

The molecular events leading to apoptotic cell death were characterized in cultures of developing rat brain neurons exposed to purified UCB (Rodrigues *et al*., 2002c). UCB stimulated neuronal apoptosis even at nonsaturating UCB/albumin molar ratios of 1.0 or less. UCB induced mitochondrial depolarization by diminishing the mitochondrial transmembrane potential, and increased the translocation of the pro-apoptotic Bax protein to mitochondria, leading ultimately to activation of caspase 3. The apoptotic effect of UCB may be mediated by its physical interaction with the mitochondrial membrane. UCB increased lipid polarity and fluidity, as well as protein mobility, resulting in an increased permeability of this membrane and release of cytochrome *c* (Rodrigues *et al*., 2002b). Ursodeoxycholic acid, a mitochondrial membrane-stabilizing agent that prevents the changes in mitochondrial transmembrane potential, almost completely abolished the UCB-induced membrane perturbation in isolated mitochondria and inhibited the UCB-mediated apoptosis of neurons and astrocytes (Silva *et al*., 2001).

Rodrigues et al. (2002a) reported that the apoptotic effect of UCB in primary cultures of rat neurons and astrocytes decreases as a function of age-in-culture of these cells, thus confirming the earlier findings in primary cultures of fetal rat glial cells. In contrast, the UCB induced mitochondrial membrane permeabilization and cytochrome

c release were 2-fold greater for mitochondria derived from older rats compared with younger rats (Rodrigues *et al*., 2002b). These results can be concluded that the young rats are relatively resistant to UCB toxicity.

V. Mechanism of Unconjugated Bilirubin Toxicity in Neural Cells Neonates

Bilirubin-induced encephalopathy in the newborn has been described for over a Century. The term is mainly used to describe a picture of reversible lethargy and alterations in cerebral evoked potentials through to be benign (Hansen, 2000). But theses manifestation share a common graded mechanism or reflect different entities is not settled (Silva *et al*., 2002).

Previously, neuronal death has been resulted from either necrosis or apoptosis (Leist & Nicotera, 1998). However, it now seems that apoptosis and necrosis represent extreme ends of a spectrum of possible biochemical and morphologic deaths, so that a more dynamic boundary between apoptosis and necrosis has been suggested. Recently, several studies have reported that UCB primarily induces apoptosis both in primary cultures of fetal cortical and cerebellar rodent neurons (Grojean *et al*., 2000;Rodrigues *et al*., 2002a) in cultures of fetal rodent astrocytes (Silva *et al*., 20001) and in murine hepatoma cells (Seubert *et al*., 2002). Despite these studies, it remains unclear

1) whether the cells die by apoptosis, necrosis, or both

2) whether the UCB concentration is decisive for the relative proportion of apoptosis and necrosis

3) how neuronal death involves with time.

VI. Effect of Concentrations and Times of Unconjugated Bilirubin Expose to the Cells

Hanko et al. (2005) demonstrated that increasing UCB concentration from < 1-250 μM or < 0-11.7 mg/dL at an UCB to BSA ratio of 1.5 caused a dose dependent increase in NT2-N neuronal cell death *in vitro*. Moreover they observed that: *1*) low concentration (5 μM or 0.3 mg/dL) UCB exposures were associated with apoptosis as evidenced by fragmented nuclei, DNA laddering, and cleavage of the DNA repair enzyme and death substrate poly (ADP) ribose polymerase (PARP); whereas *2*) moderate (10 μM or 0.6 mg/dL) to high (≥ 25 μM or 1.5 mg/dL), UCB concentrations were associated with morphologic nuclear variants typical of necrosis and an absence of DNA laddering and PARP cleavage. Consistent with these findings are the observations of Silva and colleagues (2001) who noted that although short duration (4 hours) UCB exposures resulted in similar degrees of apoptosis and necrosis in rat astrocytes *in vitro*, necrosis predominated over apoptosis at higher UCB concentrations and longer exposure times. In this case UCB concentration, is critical to the death pathway activated and that stimuli that lead to apoptosis are typically milder than those that cause necrosis.

VII. Role of Bilirubin Induced Cells Apoptosis

Some studies have suggested that apoptosis plays a role in bilirubin induced cytotoxicity *in vitro* in murine neurons, astrocytes, and capillary endothelial cells, and that programmed cell death in this context appears to be caspase dependent (although caspase independent alternative cell death mechanisms cannot be entirely excluded at

this time). Such evidence includes activation of caspase 3, a key distal effector of apoptosis; PARP cleavage decreased mitochondrial transmembrane potential increased mitochondrial cytochrome *c* release (Rodrigues *et al*., 2002b), DNA fragmentation (Cowger *et al*., 1965) and transmission electron microscopic ultrastructural nuclear changes (Akin *et al*., 2002).

VIII. Evidence Involved in Initiation of DNA Degradation

The activation of small molecules (NO, O_2, CO, CO_2, N_2, etc.) has a significant impact in biology, medicine, industrial catalysis and environmental protection. Each activation process has its own requirements depending primarily on the desired effect such as change of the reaction pathway, selectivity, effectiveness, yields, new processes or reactions which are not allowed thermodynamically and/or kinetically from the substrate ground state, lower energy input, *etc*. These goals could be achieved in a thermal or photochemical manner in homogeneous or heterogeneous systems as direct or indirect processes. Metal ions and compounds play a crucial role in thermal and photochemical activation of small molecules. They not only can mediate the actual active form of the small molecule, but also control its spatial concentration dynamics.

Reactive Oxygen Species (ROS) in systems containing metal compounds can be achieved either through thermal or photochemical paths. As a result of photochemical activation electron or energy transfer occurs. In the case of the electron transfer, radicals are mainly generated while energy transfer is responsible rather for singlet oxygen generation (Macyk & Stochel, 2005).

Cations of Cu(II), Fe(II) and Zn(II) have unpaired electrons that allow their participation in redox reactions involving mostly one electron loss (oxidation) or gain

(reduction). The unpaired electrons also allow the chemical classification of most metals as free radicals (Halliwell & Gutteridge, 1999). Several of the biological effects, mostly toxic, of these elements can be explained by their capacity to catalyze the initiation of free radical reactions or the decomposition of peroxides and other unstable molecules, allowing the propagation of deleterious free radical reactions. Following the recognition of the participation of free radicals (reactive oxygen species, oxygen radicals, oxidants) in a number of biological processes and pathological states, metals (free or bound to chelators or proteins) were identified as participants in most of the free radical reactions, acting as pro-oxidant or antioxidant entities (Fraga, 2005).

Within the brain, the trace metals: copper, iron and zinc play an important role as components of proteins essential for neural functioning. However, these metals have also been implicated in neurodegenerative diseases where it is thought that the normally tight regulation of the concentration of free ions is disrupted. The mechanisms by which any of these metals may elicit damaging effects in disease states is unknown although oxidative stress and energy failure (Lai & Blass, 1984; Sheline et al.,2000; Amstrong *et al*., 2001) may be primary routes of action.

Copper(Cu), iron(Fe) and Zinc(Zn) were found in five different regions (amygdala, hippocampus, inferior parietal lobule, superior and middle temporal gyri, and cerebellum) (Deibel *et al.*, 1996). The flameless atomic absorption method was applied to reveal regional differences in heavy metals between suckling and adult rat brains. Iron concentrations in the cerebrum of the suckling brains were approximately half those in the adult brains. Zinc and copper concentrations in medulla from the suckling rats were higher than those in the same region from the adult rats (Kishi *et al.*, 1982).

The relationship between copper (Cu), iron (Fe) and zinc (Zn) in normal growth and brain development and differentiation is well known (Walravens, 1980; Cousins, 1985). Iron is used by the enzymes of the mitochondria electron transport chain, which provides cellular ATP for the maintenance of axonal transport and synaptic transmission (Rojkind & Dunn, 1979). The role of excess brain iron and its involvement in neurodegeneration and progressive neurodegenerative diseases are well documented (Howell *et al.*, 1984; Youdim, 2000). Neurological and psychiatric symptomatology with zinc exposure and the essential role of zinc in several enzymes involved in nucleic acid synthesis were described (Conde-Martel *et al.*, 1992). Metabolic studies of copper in rat's brain development show changes in time consistent with the development of numerous enzymatic activities such as monoamine oxidases, dopamine-β-hydroxylase and superoxide dismutase (Howell *et al.*, 1984). Neurological and neuropsychological symptoms of copper toxicity occur during the Wilson's disease development (Strausak *et al.*, 2001). In conclusion, the brain is vulnerable to either a deficit or an excess of available trace elements (Ferri *et al.*, 2003).

It has been proposed that considerable DNA damage may be caused by endogenous metabolites produced during the body's normal metabolic processes. For example, it has been shown that Dopamine, formed by the decarboxylation of L-DOPA, condenses with acetaldehyde, a product of ethanol metabolism, to generate 1-methyl-6,7-dihydroxy-1,2,3,4 tetrahydroisoquinoline (Salsolinol) (Dostert *et al.*, 1988). Salsolinol is considered to be involved in the etiology of Parkinson's and Huntington's diseases and has been detected in the cerebrospinal fluid of parkinsonian patients. Thus, dopamine can be considered a precursor of an endogenous neurotoxin.

From previous studies shown that L-DOPA, in the presence of the transition metal ion Cu(II), causes DNA cleavage by generating hydroxyl radicals (Asad *et al.*, 2000). However, Copper(II) complexes with diverse drugs have been the subject of a large number of research studies (Purnell *et al.*, 1975), presumably due to the biological role of copper(II) and its synergetic activity with the drug (Sorenson, 1989). Mononuclear carboxylato copper complexes in the presence of nitrogen donor ligands have been isolated either in neutral or in cationic form (Gagne *et al.*, 1977). In the literature, it has also been reported that copper complexes with drugs are much more active in the presence of a nitrogen donor heterocyclic ligand, such as 2,2′-bipyridine (=bipy) and 1,10-phenanthroline (=phen) (Tangoulis *et al*., 1996; Bakalbassis *et al*., 1998; Psomas *et al*., 2000; Dendrinou-Samara *et al*., 2001).

In general, quinolones can act as antibacterial drugs that effectively inhibit DNA replication and are commonly used as treatment for many infections (Neu, 1987). The interaction of diverse metal ions with quinolones as ligands has been thoroughly studied. In the literature, the study and the crystal structures of Cu(II) complexes with ciprofloxacin, cinoxacin (Ruiz *et al*., 1995; Casanova *et al*., 1997) and ofloxacin (Macias *et al*., 2001; Drevensek, 2003) have already been reported. Additionally, mixed ligands neutral mononuclear copper(II) complexes of phenanthroline with nalidixic acid, cinoxacin and ciprofloxacin (Wallis *et al*., 1996; Drenvensek, 2003; Saha *et al*., 2004) have also been reported, as well as ionic copper(II) complexes of protonated norfloxacin (Iztok Turel, 1996). Enrofloxacin, as a typical quinolone, can act as bidentate ligand through the pyridone oxygen and one carboxylate oxygen atom when coordinated to metal atoms.

The interaction of Cu(II) with the quinolone enrofloxacin in the presence or not of a nitrogen donor heterocyclic ligand (bipy, phen) has been studied in an attempt to examine the mode of binding, possible synergetic effects and the biological behavior of the resulting complexes. In conclusion, the synthesis and characterization of three mononuclear copper complexes with the quinolone antibacterial drug enrofloxacin in the presence or not of a nitrogen donor heterocyclic ligand phen and bipy has been realized with physicochemical and spectroscopic methods. In all complexes, enrofloxacin is bound to Cu(II) via the pyridone and one carboxylate oxygen atoms. Interaction of Cu(II) and deprotonated enrofloxacin results to the formation of the neutral mononuclear complex $Cu(erx)_2(H_2O)$. For the complexes prepared in the presence of phen or bipy the crystal structures of the neutral mononuclear complex Cu(erx)(phen)Cl or the cationic $[Cu(erx)(bipy)(H_2O)]Cl$ have been determined with X-ray crystallography. These structures are the first reported crystal structures of complexes of deprotonated enrofloxacin. Electron paramagnetic resonance (EPR) spectroscopy indicates that the complexes in solution behave differently. The interaction of the complexes with calf-thymus DNA has revealed that all complexes are bound to calf-thymus DNA by the intercalative mode. The antimicrobial activity of the complexes has been tested on three different microorganisms and the best inhibition is provided by $Cu(erx)_2(H_2O)$ (MIC = 0.125 μg mL^{-1}) against *E. coli* and *P. aeruginosa* (Efthimiadou *et al.*, 2006).

IX. Alternative Study on DNA Fragmentation *in vitro*

There are two terms described neurotoxicity in the newborn. Bilirubin-induced encephalopathy is mainly used to describe a picture of reversible lethargy and

alterations in cerebral evoked potentials thought to be benign (Hansen, 2000). The term *kernicterus* has been used to describe more severe clinical manifestations like seizures, opisthotonos, and hyperthermia in the acutely ill newborn, followed by neurologic sequelae in survivors or death (Hansen, 2000). Previously, neuronal death has been thought of as the result of either necrosis or apoptosis (Leist & Nicotera, 1998), both terms caused by DNA fragmentation. Several studies described previously have reported that UCB primarily induced apoptosis in primary cultures of fetal cortical and cerebellar rodent neurons (Grojean *et al.*, 2000; Rodrigues *et al.*, 2002) in cultures of fetal rodent astrocytes (Silva *et al*., 2001) and in murine hepatoma cells (Seubert *et al*., 2002). All those studies remains unclear that how much the concentration of UBC and the time dependent of UBC exposed on neuronal cells can cause cells death. This thesis used the calf thymus DNA to study directly on how and the mechanism of UBC affected on DNA degradation in order to characterize the pathogenesis of bilirubin neurotoxicity. Furthermore, this study try to investigate the other factors such as cationic metal ions which involved with UBC in degradation of DNA to explain how the possible initiated mechanism of toxic effect can be occurred in neural cells which then induced neonatal kernicterus. This study simulated the phenomenon to substantiate that bilirubin induced neuronal cell death caused by DNA degradation observed *in vitro* is a phenomenon that can be occurred *in vivo*.

C. OBJECTIVES

1. To investigate the effect of bilirubin, cationic metal ions and the factors involved in the degradation of DNA *in vitro*.

2. To investigate the possible mechanism of DNA degradation by bilirubin in the presence of interacting metal ions.