CHAPTER IV

DISCUSSION AND CONCLUSION

I. DISCUSSION

The model of bilirubin binding with cationic metal ions utilized unconjugated bilirubin (UCB) to perform its physiological function. This form of bilirubin was toxic to brain cells if present in excessive amounts in the circulation. UCB, normally absorbs the visible light with a broad range between 450–460 nm. The absorbance peaks of UCB depend upon pH, the type of solubilizer and the aqueous diluent buffer. Bilirubin in slightly alkaline produces a broad peak at 420-430 nm. (Brodersen, 1979). In caffeine the average absorbance peaks of UCB are at 432 and 457 nm (Stanley *et al*., 2004). A maximum in absorption for the 50 μM (2.92 mg%) bilirubin in 10 mM Tris-HCl, pH 7.5 without albumin that was used as a reference peak in this study was broad and was obtained 420-430 nm. The results of spectrophotometric examinations on the effect of interactions between UCB and transition metal ions may be stated as follows: The formation of a bilirubin-interaction ion complex caused 1) A change in color and a shift in the peak of maximum absorption to blue-light or red-light wavelengths; 2) decreased and disappeared of absorption peak after the occurrence of the interaction.

Although bilirubin at low levels in serum is recognized as a physiological antioxidant, at high levels it also exhibits prooxidant activity both *in vitro* and *in vivo*, particularly in the presence of transition metal ions such as copper (Asad *et al*., 1999). In this study the levels of bilirubin used in all experiments were varied from borderline to abnormally high levels. Bilirubin at increasing concentrations from 50-500 μM (2.92- 29.2 mg%), including the level of recognition of jaundice(2.92 mg%) to the highly abnormal levels (500 μM or 29.2 mg%), which represents neonatal jaundice

(Tietz,1982), were used to react with increasing concentrations of metal ions. The various concentrations of metal ions used in this study, 50-500 μM in all cases, were very low as compared with those found in normal serum, which are between 11-24, 11.5-18.5 and 11-29 mmol/L for copper, zinc and iron, respectively (http://www.flash-med.com/Lab Normal.asp). However, these metal ion concentrations present in human brain cells are expressed as mass concentrations. The concentrations of iron and copper in neuromelanin (NM) isolated from the substantia nigra (SN) and locus coeruleus (LC) were 10,891 ± 1,416 ng/mg NM (mean ± SEM; *n* = 3) and 185 ± 24 ng/mg of NM (mean ± SEM; *n* = 3), respectively (Zecca *et al*., 2004). A Bilirubin concentration of greater than 250 μM (approximate borderline of normal level in neonatal hyperbilirubinemia) was used throughout the study of DNA degradation.

The effect of 3 types of fixed concentration of transition metal ions interacting with increasing bilirubin concentrations resulted in formation of a Cu(II)-bilirubin complex, causing a color change from yellow to greenish, and the bilirubin peak was shifted from 424 nm to 342 nm, along with two products which absorbed greatest at 594 and 644 nm, respectively. The change in the bilirubin absorption spectra caused by Cu(II) binding depended on the bilirubin concentration rather than the cationic metal ion concentration. Fe(II) and Zn(II) were less effective at binding with bilirubin than t Cu(II); however, it seems that Zn(II) is better than Fe(II) for interacting with bilirubin. The interaction of Zn(II) with bilirubin has been reported by the others (Adhikari *et al.*, 1998). The interaction of Zn(II) with bilirubin in previous studies has produced a UV region absorption spectrum of this complex similar to that of the Cu(II)–UCB complex. However, there is a difference in the visible region spectrum (peak at ~ 615 nm). The rate of formation of Zn(II)-UBC complex was slower than that of the

Cu(II)–UCB complex. Therefore the finding that concentrations of $ZnCl_2$ (or $FeCl_2$) greater than 100 µM interacted with 500 µM bilirubin and exhibited the same absorption spectra as that of a 100 µM concentration, as observed in this study, can be explained by the rate dependent behavior (Adhikari, *et al*., 1998).

Studying of the effect of albumin on the interaction of UCB with metal ions simulated neonatal hyperbilirubinemia. The ratio of albumin : bilirubin lower than 1.0 in the circulation resulted in having excess UCB, which could enter the brain cells (Shapiro, 2003). At a 0.5: 1 albumin-bilirubin ratio, there was an excess of bilirubin molecules, which could form complexes with transition metal ions, especially at high concentrations of $CuCl_2$. As compared with that reported *in vivo,* bilirubin can enter the brain cells, if it does not bound to albumin or is unconjugated, or if there has been damage to the blood–brain barrier. Albumin can bind bilirubin at a molar ratio of up to 1 or a maximum of 8.2 mg of bilirubin per gram of albumin. Therefore, newborn infants with a serum albumin concentration of 3 g/dL may have a serum concentration of albumin-bound bilirubin of approximately 25 mg/dL. If the serum albumin concentration is low, the binding of bilirubin is compromised and the risk of kernicterus increases (Phyllis *et a*l., 2001).

Since Cu(II) is the most effective transition metal ion that can interacted with bilirubin, the reaction of bilirubin-Cu(II) complex with quercetin can be used to confirm the interaction between bilirubin and Cu(II). The present study showed that the absorbance peak of the quercetin-Cu(II) complex was more or less the same as that of bilirubin-Cu(II) complex. Adhikari and colleagues have proposed that the coordination of copper to bilirubin at pH 7.0 is through the 4 nitrogens of tetrapyrroles and not through the carboxylate oxygens, as in albumin (Adhikari *et al*., 1998). The

polyphenolic flavonoid quercetin also binds to copper, giving rise to a charge transfer complex. A novel peak absorbing at 422 nm was obtained when the complex was formed in the presence of quercetin. The possibly represents a ternary complex of bilirubin-Cu-quercetin which may be implied that copper is capable of forming a co-complex with both bilirubin and quercetin. In conclusion, it can be proposed that coordination of copper to bilirubin occurs at more than one site. This is further suggested by the observation made earlier (Asad *et al.*, 1999) where on addition of Cu (II) to bilirubin an intermediate species absorbing at 404 nm is initially formed before the appearance of the band at 343 nm. Adhikari and colleagues have proposed a model for copper binding of bilirubin involving the four pyrrole nitrogens (Adhikari *et al*., 1998). These studies would suggest an additional possibility where two molecules of bilirubin coordinate with two copper ions through the terminal pyrrole rings. This model is supported by the formation of a ternary complex between bilirubin–Cu(II)–quercetin. Here coordination of copper would occur involving the nitrogen and oxygen of the terminal pyrrole rings I and IV of a bilirubin molecule and the 3´and 4´ hydroxyls of the B rings of quercetin molecules.

The previous report suggested that upon formation of the complex, Cu(II) is reduced by bilirubin to Cu(I) and this is accompanied by the generation of reactive oxygen species. The evidence for the appearance of Cu(I) in the bilirubin-Cu(II) complex reaction employed bathocuproine as a selective Cu(I) sequestering agent. The Cu(I) chelates have absorption maxima at 480 nm (Rahmane *et al*.,1989). Under the experimental conditions, neither Cu(II) nor bilirubin interferes with this maximum absorption, whereas bilirubin and Cu(II) reaction to generates Cu(I) (Asad *et al*., 1999

and Appendix A). The implication of this finding is that Cu(II) is reduced by bilirubin in a complex to generate Cu(I).

The DNA degradation caused by the interaction of metal ions in the reaction mixtures was observed qualitatively by agarose gel eletrophoresis. In the present study, calf thymus DNA was used as a DNA model to observe the degradation caused by bilirubin-metal ion complex interactions. Generally, calf thymus DNA is a representative eukaryotic DNA used in several of *in vitro* research studies concerning DNA (Chen *et al*., 2006; Labieniec & Gabryelak, 2006;Prashanth *et al*., 2007). However, results of agarose gel electrophoresis in this study showed some degradation of DNA alone after incubation at 37 ^{O}C for 4 hrs; therefore controls for DNA degradation using DNA alone were included in all of DNA electrophoresis experiments. DNA degradation is caused by the instability of commercial calf thymus DNA prepared by freeze-drying process, which can introduce oxidative damage of the DNA product (Wood *et al*., 2000).

After incubation of bilirubin with interaction metal ions, the DPA reaction was also performed in order to measure the quantity of DNA degradation *in vitro*. The degraded DNA products were digested with S_1 nuclease. S1 nuclease recognizes distortions in the secondary structure of DNA caused by single strand breaks and damage by chemicals and UV light (Weigand e*t al*., 1975). The action of S_1 nuclease at sites with strand breaks results in generation of acid soluble deoxynucleotides which can be estimated colorimetrically (Schneider, 1957).

The Bilirubin-Cu(II) complex generated a dose dependent increase in DNA degradation. A similar increase in acid soluble DNA hydrolysis detected by diphenylamine reaction was found to be correlate with the DNA degradation patterns.

Bilirubin-Cu(II) complex generated a time dependent increase in DNA degradation, which reached the maximum degradation at 8 hours, slightly increasing up to 48 hours after incubation at 37 ^{O}C. This result suggested that the toxic effect of bilirubin can occur rapidly, if *in vivo*, as this free form of bilirubin (UBC) can traverse the cell membranes owing to its hydrophobic nature or, the association of UCB with copper ions may render bilirubin lipophilic enough to cross the cell membrane (Asad *et al.*, 2002).

The degradation of DNA *in vitro* depended on bilirubin and transition metal ion concentrations. These experiments simulated condition inside the cells, and investigated DNA degradation by the interaction of bilirubin with transition metal ions. The metals, Cu (II), Fe(II) and Zn(II) are widely distributed in body tissues and their natural role in human physiology, toxicity, interactions with various components and involvement in different diseases is well studied (Beckman *et al.*,1993; Berg & Shi, 1996). Copper, the most interesting interaction metal ion, is present as an integral part of the active site of many enzymes and plays an important role in physiology. Excess amounts of copper are toxic and have deleterious effect in living tissues (DiDonato & Sarkar, 1997). Copper is the one of the transition metal ions that has been reported to be a normal component of chromatin, and such endogenous copper can be mobilized by chemical agents such as 1,10-phenanthroline to cause internucleosomal DNA fragmentation (Burkitt *et al.*, 1996). Copper has also been reported to be neurotoxin as evidences by the brain pathology produced in patients with copper overload as a result of Wilson's disease (Hartard *et al.*, 1993). Iron is an essential element for normal cellular functions. Many enzymes in the electron transport chain, including cytochromes a, b, and c, and cytochrome oxidase, use iron as a cofactor for adenosine

triphosphate (ATP) synthesis (Gordon, 2003). Iron also plays specific roles in the central nervous system(CNS). It involves in myelin formation (Connor *et al.*, 1995), as well as in the production of several neurotransmitters such as dopamine (Ponting, 2001), norepinephrine and serotonin (Connor *et al*., 1995) and generation of GABAergic activity (Li, 1998). In addition, iron overload is implicated as a cause of neuronal death. Abnormally high levels of iron in the brain have been demonstrated in a number of neurodegenerative disorders (Wu *et al.*, 2004). Zinc supports a healthy immune system and is needed for DNA synthesis. Zinc has structural and catalytic roles for many proteins and plays a fundamental role in expressing genetic potential, *i.e.*, in the synthesis and repair of the structural integrity of nucleic acids (Ferri *et al*., 2003).

In this study, the maximum DNA degradation caused by the interaction of bilirubin with Cu (II) depended on bilirubin concentration and the molar ratio of bilirubin to $CuCl_2$. A bilirubin to Cu(II) ratio of 1: 1 (such as 500 μM : 500 μM) was optimal for strong denaturation of calf thymus DNA. In contrast to Cu(II) and as previously shown in the spectrophotometric analysis, the interactions of Zn(II) and Fe(II) transition metal ions with bilirubin were lower compared with Cu(II), and therefore resulted in less amount of DNA degradation. The results of diphenylamine reaction also agreed with results from agarose gel electrophoresis.

The mechanism of DNA degradation in cells has been thought to be caused by the reactive oxygen species produced by the lipid peroxidation process. In this study, the mechanism of DNA degradation *in vitro* caused by UCB interaction with the transition metal ions was also proposed to occur through the generation of reactive oxygen species. DNA damage by hydroxyl radicals involving bilirubin is well

established. There is considerable evidence that hydroxyl radicals play an important role in Alzheimer's diseases (AD). Celec and Prhodova (2003) hypothesized that ROS, Nitric oxide synthase (NOS) and Heme oxygenase (HO) are involved in the development of AD. The presence of Cu(II) in the human brain (Zecca *et al.*, 2004), the increase in HO activity (Celec & Prhodova, 2003) and evidence of bilirubin interacting with Cu(II) to generate ROS, together suggest the role of a bilirubin and copper complex as a cause of AD.

The mechanism of DNA strand cleavage by ROS mainly involves the formation of hydroxylated bases, such as 8-hydroxyguanine, and strand breakage and enhanced damage to deoxyribose of the DNA backbone (Asad *et al*., 2002). The free hydroxyl radical formed in the reaction mixtures was examined by the inhibition of free radical production by specific scavengers. The bilirubin-transition metal ion DNA breakage reaction is inhibited by 3 types of radical scavengers; sodium azide, thiourea and mannitol. Sodium azide is a singlet oxygen scavenger which removes superoxide anions (O_2^-) whereas mannitol and thiourea eliminate hydroxyl radicals (OH^-). Thiourea was the most potent inhibitor of DNA breakage (Asad *et al*., 2003).

The inhibition of DNA degradation determined by the MDA assay is based on the fact that degradation of deoxyribose of DNA by hydroxyl radicals results in the release of TBA reactive material which forms a colored adduct with TBA (Asad *et al*., 2003). The inhibition of hydroxyl radical formation decreased the % DNA hydrolysis, in good agreement with the DNA patterns that were previously elucidated by agarose electrophoretic patterns.

There are several methods of probing ROS production. However, such methods are unsuitable for detecting the generation of ROS in this study. The methods

include the short half-life of the hydroxyl radical (10.sup.-9s) (Lubec, 1996), and the interference by Fe(II) when $FeCl_2$ is used as an interactive metal ion (Jiang *et al.*, 1992). The carboxy-dichlorodihydrofluorescein dicetate (H_2DCF) method is suitable for use a a probe for cell culture experiments. Thus, MDA detection is always used to detect ROS production in *in vitro* experiments (Adhikari *et al*., 1998 ; Asad *et al*., 1999: Asad *et al.*, 2002).

The precise role of bilirubin in the development of kernicterus is not completely understood. If the serum UCB level exceeds the binding capacity of albumin, unbound lipid-soluble bilirubin crosses the blood-brain barrier (Porter & Dennis, 2002). Unconjugated bilirubin diacid (UCB) can passively diffuse across the plasma membrane of any cell (Zucker *et al.*, 1999). Some cells, such as hepatocytes and trophoblasts, can also take up UCB by saturable, carrier-mediated, facilitated diffusion (Pascolo *et al.*, 2001). Carrier-mediated uptake is predominant at low concentration of UCB, whereas passive diffusion is predominant at higher free fraction of unconjugated bilirubin (Mediavilla *et al.*, 1999; Zucker, Goessling *et al.*, 1999). With either mechanism, cellular uptake occurs from the free fraction of plasma UCB that does not bound to plasma proteins and lipoproteins (Wennberg, 2000). The results of this study suggest that the excess of Cu(II) that interacts with UBC and generates ROS in brain cells can leading DNA degradation may be at least in the part applied to explain the mechanism in vivo which has been observed by Zong and Thompson that the excess ROS production can lead to oxidative stress, damage of intracellular molecules and organelles, and ultimately necrosis (Zong& Thompson,2006).

II. CONCLUSION

Bilirubin can form a complex with the transition metal ions Cu(II), Fe(II) and Zn(II). Absorption studies indicate binding of bilirubin to form the complex with the interaction metal ions, and such a complex was shown to cause oxidative DNA damage. The DNA degradation reaction was suggested to be caused by the reactive oxygen species generated by the interaction of bilirubin with transition metal ions. Further study in the biologically active system will yield more information concerning the mechanism involved in the DNA degradation.