CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

I. Instruments:

Instruments used in this study were listed as following:

- Analytical balance AR 2130 (Ohaus Corp. N.J., USA)
- Autoclave SX-500 (Tomy Koqyo Co., Tokyo, Japan)
- BioRad Gel Doc 2000 System
- Centrifuge (Kubota 5200, Japan)
- DNA Electrophoretic Set Apparatus (MJ-105 Mini Horizontal Electrophoresis System, Taiwan)
- Electrical balance (Shimadzu, Japan)
- Hot plate (E.G.O.,Germany)
- Microcentrifuge (Eppendrof, Germany)
- Microwave R-216 (Sharp, Japan)
- pH meter, Cyberscan 510 (Eutech, Singapore)
- Power supply MP-300N (Major science, Taiwan)
- Roller mixer SRT1 (Stuart scientific, UK)
- Trip balance (Florham park, N.J., USA)
- UV-2450 UV Visible Spectrophotometer (Shimadzu, Japan)
- Vortex mixture VM-300 , (Gemmy Industrial, Taiwan)
- Water bath WB 22 (Memmert, Germany)

II. Chemicals and Reagents

Essential Chemicals were list as follows:

- Acetic acid glacial (Merck, Damstadt, Germany)
- Acetaldehyde (BDH Chemicals Ltd. England)
- Agarose powder (Promega, Spain)
- Bovine serum albumin (Sigma Chemical Co., USA)
- Bilirubin (SRM 916A, NIST, USA)
- Calf thymus DNA (lyophilized, MW = 1 x 10^6) (Sigma Chemical Co., USA)
- Cupric chloride (Sigma Chemical Co., USA)
- 2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline (Sigma-Aldrich Co., Germany)
- Diphenylamine (Sigma Chemical Co., USA)
- EDTA (BDH Chemicals Ltd. England)
- Ethanol (Merck, Darmstadt, Germany)
- Ethidium bromide (Promega, USA)
- Ferrous chloride (BDH Chemicals Ltd., England)
- Hydrochloric acid (Merck, Damstadt, Germany)
- Mannitol (Sigma Chemical Co., USA)
- Perchloric acid (Merck, Damstadt, Germany)
- S_1 nuclease (Promega Co., USA)
- Sodium acetate (Merck, Damstadt, Germany)
- Sodium azide (Merck, Damstadt, Germany)
- Sodium hydroxide (Merck, Damstadt, Germany)
- Sodium carbonate (Merck, Damstadt, Germany)

- Sulfuric acid (Merck, Damstadt, Germany)
- Tetramethoxypropane (Sigma Chemical Co., USA)
- Thiourea (Sigma Chemical Co., USA)
- Thiobarbituric acid (BDH Chemicals Ltd., England)
- Tris (USB Co., USA)
- Zinc sulphate (BDH Chemicals Ltd., England)
- Zinc chloride (Sigma Chemical Co., USA)

B. Methods

I. Spectrophotometric Determination of Bilirubin Absorption Spectra

A 0.342 mM stock unconjugated bilirubin standard dissolved in 10 mM of Tris-HCl buffer, pH 7.5 was diluted to 50 μM (2.9 mg%) by the same buffer and scanned for the absorption spectra in a Shimadzu, UV-Visible 2450 Spectrophotometer at the absorbance between 300-500 nm using 10 mM of Tris-HCl buffer, pH 7.5 as a blank. The maximum absorption and the absorbance of unconjugated bilirubin was recorded and used as reference absorption spectra of bilirubin in the absence of any effectors.

II. Investigation of the Absorption Spectra of Bilirubin in the Presence of Effectors by Spectrophotometric Method.

1. The effect of transition metal ions on bilirubin absorption spectra

1.1 types of transition metal ions

Each type of cationic metal ions, 500 μM of Copper ($CuCl_2$) or Iron ($FeCl_2$) or Zinc ($ZnCl_2$) prepared in 10 mM Tris-HCl buffer, pH 7.5 was mixed with increasing concentration of bilirubin solution, 50 to 500 μM in each reaction tubes

(0.5 mL), respectively. The absorption spectra of bilirubin and bilirubin in the presence of various type and different concentration of metal ions were recorded by spectrophotometric method as described in I. The absorption spectra of bilirubin in the presence of transition metal ions, either by Cu(II), Zn(II) or Fe(II), was compared with bilirubin reference solution in which the volume of metal ion added was compensated by 10 mM Tris-HCl, pH 7.5. All the scans were made against 10 mM Tris-HCl, pH 7.5(Blank).

1.2 The effect of transition metal ion concentrations on the absorption spectra of bilirubin

The transition metal ions dissolved in 10 mM Tris-HCl, pH 7.5 were diluted to various concentrations and mixed with 500 μM bilirubin solution prepared in 10 mM Tris-HCl, pH 7.5 buffer. The final concentrations of each kind of metal ion in the reaction mixture of 0.5 mL were between 50-500 mM, respectively. The 0.5 mL reaction mixtures were incubated at 37 ºC in a water bath for an hour before scanning for the absorption spectra from 350-500 nm in the spectrophotometer. The change in the peak of absorption spectra of bilirubin in the presence of various types of metal ions as compared with the control bilirubin without the transition metal ions demonstrated that there may be some interactions of bilirubin molecules with the indicated metal ions.

2. The effect of albumin on the interaction of transition metal ion with bilirubin observed by Spectrophotometric method

Bilirubin in plasma is loosely bound to albumin for transportation in circulation. Bilirubin and albumin concentrations in plasma can be varied according to pathological conditions. The role of albumin is as a reservoir for bilirubin which while binding, albumin keeps it out of the tissues (Ahlfors & Wennberg, 2004). Bilirubin

and albumin ratio in plasma can affect the appearing amount of unbound bilirubin concentration. The aim of this experiment is to observe the effect of bilirubin/albumin ratio which may result in increase the unconjugated bilirubin concentration which then can interact with various kinds of divalent cationic metal ions described in the experiments [method II.(1.1) and II.(1.2)].

In this study, 0.5 mL of reaction mixtures containing 500 μM bilirubin in 10 mM Tris-HCl, pH 7.5 buffer, selected type and concentration of transition metal ion, and albumin as a molar ratio of bilirubin or albumin/bilirubin ratios of 0.5:1.0, 1.0:1.0 or 1.5:1.0 were scanned in a spectrophotometer at the wavelength between 300-500 nm using 10 mM Tris-HCl, pH 7.5 buffer as a blank. The absorption spectra obtained were compared with the spectrum of bilirubin control reaction mixture without albumin. The changes in absorption spectra of bilirubin reveal that the excess amount of free bilirubin from binding with albumin in the reaction mixture can interact with metal ions and then cause the changes of the absorbance peaks.

3. Confirmation of bilirubin binding with interaction metal ion

The aim of this experiment is to prove that bilirubin can bind with the interaction metal ion such as the copper ion in the reaction mixture. The observed method was performed by using the polyphenolic flavonoid quercetin which has been reported that it can bind preferently with the copper molecule and giving the absorption spectra at 407 nm. The absorption spectra of bilirubin binding with copper molecule were compared with those spectra of quercetin – copper complex and bilirubin-copper complex interacted with quercetin to show the characteristic of bilirubin and copper binding. The experiment was performed as the following.

Two milliliters of five reaction mixtures were bilirubin alone, bilirubin mixed with Cu(II), quercetin alone, quercetin mixed with Cu(II), bilirubin mixed with Cu(II) and quercetin were incubated at room temperature for 10 minutes before scanning from 325-600 nm in a UV-2450 Spectrophotometer. The absorbance peaks of bilirubin in the presence of Cu(II) or quercetin or both were compared with reference bilirubin control reaction.

III. Method for Investigation of the Effect of Bilirubin interacted with transition metal ions on DNA Degradation *in vitro*.

1.Investigation of the effect of bilirubin with or without albumin and transition metal ions concentrations on DNA degradation *in vitro*

1.1 The effect of bilirubin concentrations on the degradation of DNA with fixed transition metal ion concentration.

The reaction mixtures of 0.5 millitres contained 10 mM Tris–HCl (pH 7.5), 500 μg of DNA, increasing concentrations of bilirubin (50–500 μM) and 500 μM (fixed concentration) of interaction metal ion;Cu(II) or Fe(II) or Zn(II). Incubation was performed at 37°C for 4 h. The effect of bilirubin concentration in the presence and absence (control reaction) of metal ions on the DNA degradation were investigated by agarose gel electrophoresis. S_1 nuclease digestion and the amount of DNA degradation by means of acid soluble deoxyribonucleotides were colorimetrically determined by the method described later.

1.2 The effect of transition metal ion concentrations on DNA degradation with fixed bilirubin concentrations

The 0.5 ml of the same reaction mixtures as described in II.1 (1.1) were prepared but at 500 μM fixed concentration of bilirubin and the increasing amount of

transition metal ions, Cu(II) or Fe(II) or Zn(II), respectively. Each reaction mixture was incubated at 37 ^{O}C for 4 hours and the effect of metal ions interacted with the fixed bilirubin concentration on the degradation of DNA and the amounts of DNA degradation were determined.

2.Time dependent kinetic of DNA degradation

The reaction mixtures were performed as previously described [see experiment II.1 (1.1 &1.2)] excepted for bilirubin and metal ion concentrations were at optimal concentrations (from previous experiments). Time dependent kinetic degradations of DNA were observed by incubating five identical reaction mixtures at 37 ^{O}C for 4, 8, 12, 24 and 48 hours, respectively. At the end of the incubation time, the effect of bilirubin interacted with metal ions on the DNA degradation were determined by agarose gel electrophoresis and the amounts of DNA hydrolysis were quantitated by diphenylamine coloremetric reaction.

3. Protocol of agarose gel electrophoresis (Burkitt et al., 1996; Asad et al., 1999), modified.

The technique of electrophoresis is based on the fact that DNA is negatively charged at neutral pH due to its phosphate backbone. For this reason, when an electrical potential is placed on the DNA it will move toward the positive pole. The DNA is visualized in the gel by addition of ethidium bromide. This binds strongly to DNA by intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light. The details of procedure and the modification used in this experiment were as followed.

In this study, 1% (W/V) of agarose gel was performed at the constant voltage of 80 Volts for an hour using 1X Tris-Acetate-EDTA (TAE) buffer as electrophoresis buffer. Electrophoresis was carried out as tracking dye is approximately 3/4 the way

across the gel. After electrophoresis, The gel was stained with 0.5 μg/ml ethidium bromide in TAE buffer for 30 minutes. After the gel was removed and placed in a tray of water to destain for 1- 5 minutes, the patterns of DNA degradation on the gel were visualized by using Biorad Gel Doc 2000 system.

4. Quantitation of DNA degradation

DNA degradation in all reaction mixtures were measured quantitatively after digesting the DNA by S1 Nuclease and the amount of DNA in reaction mixtures were determined by Diphenylamine reaction. The details of methods are as followed:

4.1 S1 Nuclease digestion of DNA (Rahman *et al*., 1989),modified

The Nuclease S_1 digestion is used for the selective removal of single stranded DNA or RNA. It degrades single-stranded nucleic acids to yield 5' phosphoryl mono- or oligonucleotides. It cleaves single-stranded DNA about 5 times faster than single-stranded RNA. Nuclease S_1 will also hydrolyze single-stranded regions in duplex DNA such as loops and gaps. The procedure was described as followed.

One milliliter of reaction mixture contained 500 μg of calf thymus DNA in 10 mM Tris-HCl, pH 7.5, 1 mM of $ZnSO_4$ in 100 mM Sodium acetate, pH 4.5 and 100 units of S_1 nuclease. The reaction mixture was incubated at 37 ^{O}C for 4 hours. At the end of incubation period, the reaction was terminated by adding 0.2 ml of 10 mg/mL bovine serum albumin. After thoroughly mixed by shaking, 1 mL of ice-cold 14% perchloric acid was added and mixed. The reaction mixture was kept for 1 hour at 4 ^{O}C and then centrifuged to remove the precipitated protein and undigested DNA. The acid-soluble nucleotides were determined by diphenylamine reaction (Schneider, 1957).

4.2 The colorimetric diphenylamine reaction (Schneider, 1957)

This colorimetric assay is used to measure the concentration of DNA in cells or tissues (Schneider, 1957). The reagents used in the diphenylamine reaction include acetic acid and sulfuric acid. When these are heated with DNA they cleave the phospho-diester bonds and hydrolyze the glycosidic bonds between the sugar and purines. The free 2-deoxy ribose undergoes a dehydration reaction to form ω-hydroxylevulinyl aldehyde, which reacts with diphenylamine to produce a variety of blue-colored compounds showing a characteristic absorbance peak at 600 nm. The more DNA there is in the sample, the darker the blue color will be. Since the reaction is specific for 2-deoxy ribose, the sugar in DNA, there is no reaction with the ribose sugar of RNA. Thus, the presence of RNA in a sample will not interfere with the measurement of DNA.

4.2.1 DNA standard graph

Stock DNA solution was prepared by dissolving calf thymus DNA in 10 mM Tris – HCl, pH 7.5. The amount of DNA was checked by reading the absorbance at 260 and 280 nm in UV-2450 Spectrophotometer. The DNA solution was diluted with the same buffer to obtain 0.2 – 1.0 mg/mL before preparing the standard graph described as the following.

One mililiter of diluted DNA was mixed with 1 mL of 1N perchloric acid (V/V) and heated at 70 °C for 15 minutes. Then 1 ml of each concentration of DNA was mixed with 2 ml of diphenylamine reagent and boiled at 100 °C for 10 minutes. The reaction mixture was immediately cool on ice. When cool, the absorbance was read at 600 nm in a UV-2450 Spectrophotometer.

4.2.2 Percentage of DNA hydrolysis

In the determination of DNA degradation by acid soluble diphenylamine reaction, 1 mL of supernatant from S_1 nuclease digested sample was mixed with 2 mL of diphenylamine reagent. The reaction was heated (boiled) at 100 ^{O}C for 10 minutes. After cooling, the absorbance was read at 600 nm in a UV-2450 Spectrophotometer and the amount of DNA in the observed reaction tube was determined by comparing with the standard graph.

For determination of the percentage of DNA hydrolyzed, the amount of DNA in the observed reaction was compared with the 100% DNA hydrolyzed reaction described in 4.2.3

4.2.3 Determination of 100% DNA hydrolyzed

The same amount of DNA as in the previous experiments (500 μg of calf thymus DNA in 0.5 mL of Tris-HCL buffer, pH 7.5) was boiled at 100^{O}C for 10 minutes (100% DNA degradation). After cooling, 1 mL of the degraded DNA digested with 100 units of S_1 Nuclease was mixed with 2 mL of diphenylamine reagent. The reaction was boiled at 100 ^{O}C for 10 minutes. After cooling, the absorbance was read at 600 nm in a UV-2450 Spectrophotometer. The amount of DNA hydrolysis in the reaction tube determined from the DNA standard graph represented the 100 % DNA hydrolysis reaction.

IV. Method for Investigation of the Mechanism of Bilirubin Interacted with Metal Ion Influenced DNA Degradation *in vitro*

Studies in the previous experiments aim to show that bilirubin-copper complex can cause fragmentation of DNA. Therefore, bilirubin itself may act as prooxidant either alone or in the presence of transition metal ions such as Cu(II), Fe(II) or Zn(II).

A significant cause of DNA degradation may result from the redox recycling which gives rise to the formation of reactive oxygen species, particularly hydroxyl radical.

Therefore, the next experiment was performed to show the mechanism involved in the degradation of DNA especially to observe the formation of reactive hydroxyl radical ($OH^{\cdot}$) caused by the effect of bilirubin-copper complex on the DNA degradation *in vitro.* The $OH^{\cdot}$ formed can be determined by Thiobarbituric acid reaction (TBA) or the Malondialdehyde (MDA) Assay.

1. Standard graph of MDA

One hundred microliters of working Tetramethoxypropane was prepared by dissolving 25 μL of stock Tetramethoxypropane in 25 mL of distilled water (MDA standard). Then the MDA standard was diluted further with distilled water to get 0-35 μM standard solution before preparing the standard graph as follows.

Nine hundred microliters of each concentration of the standard was added with 0.5 mL of 1% (W/V) thiobatbituric acid (TBA) in 0.05 M NaOH and 0.5 mL of 2.8% (W/V) trichloroacetic acid. The reaction was boiled at 100 °C for 10 minutes to develop the colour. When cool, the absorbance was read at 532 nm against appropriate blanks.

2. Free radical generation resulted from the reaction of bilirubin-copper complex caused DNA degradation using the MDA assay

The principle of MDA assay is based on the fact that degradation of DNA by the effect of free hydroxyl radicals result in the release of a product which can form a colored adduct with TBA readable at 532 nm. (Quinlan & Gutteridge, 1987)

Reaction mixtures (0.5 mL) contained 10 mM of Tris - HCl (pH 7.5), 200 μg of DNA, 200 μM of bilirubin , 200 μM of either kind of divalents metal ions ($CuCl_2$,

$FeCl_2$ or $ZnCl_2$) and 50 mM of free radical scavengers (Thiourea, Sodium azide or Manitol) were included (Rahman *et al.*, 1989). Bilirubin reaction mixture was incubated at 37 ^{O}C for 4 hours. The DNA degradation and the amount of DNA hydrolysis were determined by agarose gel electrophoresis and DPA reaction as before. For the MDA assay, the volume of reaction mixture was adjusted to 0.9 mL with 10 mM Tris-HCl buffer, pH 7.5 and then 0.5 mL of 1% (W/V) thiobatbituric acid in 0.05M NaOH was added following by a 0.5 mL of 2.8% (W/V) trichloroacetic acid and mixed. The reaction mixture was heated for 10 munites at 100 °C to develop the colour. When cool, the absorbance was read at 532 nm against appropriate blanks.

3. Investigation of free radical generated by bilirubin degradation *in vitro* by the MDA assay.

This study aims to show that bilirubin alone treated with heat can produced hydroxyl radical or not. The positive reaction will give rise of a product that can form complex with thiobarbituric acid (TBA) and the product should have the maximum absorption at 532 nm. The method was performed by mixing in a 0.5 mL reaction tube with 200 μM bilirubin and 200 μM Cupric chloride. Anathor 0.5 mL reaction tube contained 200 μM bilirubin solution alone. The reactions were incubated at 37 ^{O}C for 2 hours. At the end of incubation time, 0.4 mL of 10 mM Tris-HCl buffer, pH 7.5 was added to each tube and mixed. After that 0.5 mL of 1% (W/V) thiobatbituric acid in 0.05M NaOH was added to both tubes following by 0.5 mL of 2.8% (W/V) trichloroacetic acid. The reaction mixtures were heated for 10 munites at 100 °C to develop the colour. When cool, the absorbance was read at 532 nm or scanned between 300-600 nm. against appropriate blanks. After the absorbance was read, an equal volume of chloroform was added in a tube and the tubes were shake vigorously,

then centrifuged for 15 minutes at 3500 rpm. The pigment in the reaction tubes, partition in two separated parts of two liquid in the tubes, were scanned between 300-600 nm in a UV 2450 Spectrophotometer.