CHAPTER II
MATERIALS AND METHODS

2.1 Instruments, equipments, chemicals and reagents

All chemicals are of analytical quality and the highest purity. Lists of chemicals and reagents are shown in appendix A, B and C.

2.2 Green tea sample

Fresh tea shoots were harvested from Royal Project Foundation tea fields and immediately dried in a microwave cabinet (3 minutes, 800 watts, 100°C) (Ounjaijean, 2004; Srichairatanakool et al., 2006). Dry tea leaves were extracted with hot deionized water (80 °C) for 15 minutes and filtered through 0.45-μm membrane (cellulose acetate type, Millipore, Maidstone, England). Green tea (GT) crude extract was dried under vacuum, and the powder was stored at -20°C till further use.

Catechin derivatives in GT extract (GTC) was analyzed using a reverse-phase HPLC technique (Chen et al., 2001). Conditions of the HPLC separation include analytical column (Waters SpheroSorb-ODS2, 250x4.7 mm, 5-μm), mobile-phase solvent (0.05% H_2SO_4 : acetonitrile : ethylacetate = 86:12:2, *v*/*v*/*v*), a flow rate of 1.0 ml/min, and a detection of eluents of 280 nm. Eluted catechins were quantified by comparing with authentic standard catechins: C, EC, EGC, EGCG and ECG.

The EGCG was fractionated from the GT crude extract using semi-preparative HPLC method under following condition: a column (Phenomenex ODS-2, 250x10 mm, 5 μm), mobile-phase solvent (methanol : deionized water = 29:71, *v*/*v*) and a flow rate of 1.0 ml/min. The column was first calibrated with the known standard catechin species to position the EGCG. Eluents were collected 1 ml/fraction and measured absorbance at 280 nm, only the eluted EGCG was pooled. Purity of the EGCG was checked with the analytical HPLC as described above (Zhang et al., 1997). The EGCG fraction was evaporated at 60°C to remove existing methanol, dried under vacuum, and stored at -20°C until use.

Amounts of EGC, C, EC, EGCG, ECG and total catechins were shown in **Table 2.1**, and the single big batch of the GT crude extract was used in entire study.

Table 2-1 Amount of individual catechins and total catechins of the GT crude extract.

Amount	Catechins species (mean±SD, n = 9)					
	EGC	**C**	**EC**	**EGCG**	**ECG**	**Total**
mM	6.7±9.2	0.3±0.5	3.7±3.0	3.9±1.6	0.2±0.3	14.8±8.4
mg/g wet weight	25.8±31.2	1.5±2.4	14.8±11.7	24.9±12.2	1.2±2.1	68.3±2.9
mg/g dry weight	102±140	5.1±7.9	53.9±42.9	88.7±368	4.3±7.3	255±136

2.3 Chitosan sample

Chitosan used in entire experiments was purchased from the Bannawach Bioline Company, Amphur Muang, Samutsakorn. Preparation and specification of the chitosan product were mentioned briefly below.

Chitin preparation

β-Chitin was prepared from squid pen (*Dosidicus gigas*) according to Kurita et al. method. Briefly, desiccated squid-pen particles (100 g.) was ground, scaled off through 80 mesh sieve and reacted with 2.0 liters of 2 N. sodium hydroxide solution overnight to deproteinize the particles. The solution was cooled and filtered through a cellulose-acetate membrane. Particulate was treated with 2.0 liters of 2 N. sodium hydroxide again to remove more residual protein. After filtration 1 N. hydrochloric acid was added to achieve 10% (*w*/*v*) solid acidic solution and let stand at room temperature overnight.

Chitosan preparation

The β-chitin was then deacetylated with 25% sodium hydroxide solution at 112°C for 150 minutes, and 40% sodium hydroxide solution at 125°C for 180 minutes to obtain the chitosan. The chitosan matrix was comminuted to aluminum wire gauze (nominal 80-mesh size), air-convection dried at 65°C and stored under refrigeration (4 °C) in a sealed container.

To obtain a variety of chitosan matrice, depolymerization of the chitosan can be performed by chemical means (e.g. hydrochloric acid, phosphoric acid, nitrous acid, hydrofluoric acid, hydrogen peroxide, ferric chloride, copper ion, ascorbic acid

and UV-H_2O_2), physical means (UV- and γ-radiation, sonication and shearing) and enzymatic means (papain, lysozyme, chitosanase, cellulase, lipase, amylase and pectinase). In the assay, β-chitin and chitosan (1 g.) are dissolved in 100 ml of 2% acetic acid solution (1%, *w*/*v*, pH ~4.0). In this case, acid depolymerization technique of the chitosan was performed.

Physicochemical examinations

The chitosan product is fine, ground, odorless, white-color powder packed in a 10-kg plastic bag. It is water insoluble, but can be solubilized in acid solutions such as 1% hydrochloric acid, 1% acetic acid and 1% citric acid. It has bounce density = 0.51 g/ml, tap density = 0.74 g/ml, ash = 0.26%, moisture content <10%, and swelling index = 2.12±0.17% in deionized water, 2.87±0.15% in bicarbonate buffer, pH 8.3.

Molecular weight (MW) of the chitosan determined using gel-permeation chromatography (GPC) technique was 125 - 500 kD. Conditions of the GPC were TSK G4000PWXL (Tosoh) and OHPak SB-803 HQ (Showa Denko) column in series after a Shodex precolumn (OHPak SB-G), flow rate of 0.5 ml/min, 25°C, a mobile-phase solvent of 0.2 M. acetic acid/0.1 M. sodium acetate and refractive index (RI) detection.

Degree of deacetylation (DD) value of the chitosan was determined by using Fourier-Transform Infra-Red (FTIR) spectroscopic technique. Theoretically, the FTIR spectroscopy is a priority technique used to assay the %DD value of β-chitin and chitosan. In practice, FTIR spectra of the chitosan solution are scanned with a FTIR machine over the wave number range of 4600 to 400 cm^{-1}. The characteristic band at 3449 cm^{-1} is attributed to $–NH_2$ and –OH groups stretching vibration, and the band for amide I is observed at 1655 cm^{-1} is seen in the infrared spectrum of chitosan. DD value of the tested chitosan was 80-90%.

2.4 Animal models

Thalassemic mice

Adult male C57/BL6 mice (9-12 months old), wild type ($mu\beta^{+/+}$)(WT) and heterozygous β^{KO} type ($mu\beta^{th\text{-}3/+}$) (BKO), were provided by Professor Suthat Fucharoen at Thalassemia Research Center, Institute of Science and Technology for

Research and Development, Mahidol University, Salaya Campus. For details, β^{E}-transgenic (TG) mice, carrying the entire human β-globin locus with codon $26^{Glu \rightarrow Lys}$ mutation were generated on a C57BL/6J background by using GET recombination system. $\beta^{IVS\ II\text{-}654}$ was generated by the replacement of a 21 kb region encompassing the mouse β^{major} and β^{minor} genes with a 5.7-kb DNA fragment of the human β-globin gene with the IVS II-654 (C→T) splicing mutation (severe β^{+}-thalassemia) using a plug and socket method. Homozygous $\beta^{IVS\ II\text{-}654}$ with β^{E} transgene, called 'rescued' or β-thal/HbE mice (R) were crossbred between two $\beta^{IVS\ II\text{-}654}$ double heterozygotes (Jamsai, 2005, 2006). Pathophysiologically, the WT mice have normal levels of hemoglobin, SI and ROS production. The BKO mice show decreased hemoglobin level, increased SI and ROS productions, but depressed HRV value indicating cardiac complications (Incharoen, 2007). Their mild anemia and depressed HRV complications are similar to those observed in human thalassemia intermedia.

All animal studies were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) of Faculty of medicine, Chiang Mai University (Reference Number -3/2548). Mice were housed in stainless steel cages in the conventional clean room where the temperature and humidity were maintained at 25±2 °C and 60±5%, respectively at 12-hours light/dark cycle. The mice were access freely to normal chow diet (control diet) and drinking water *ad libitum*.

Rats

Wistar rats (male albino 6-8 week old, 300-350 grams weigh) were purchased and shipped from the National Animal House, Mahidol University Salaya campus.

All animal studies were conducted with approval of the Institutional Animal Care and Use Committee (IACUC) of Faculty of medicine, Chiang Mai University (Reference Number 14/2551). Rats were housed in stainless steel cages in the conventional clean room where the temperature and humidity were maintained at 25±2 °C and 60±5%, respectively at 12-hours light/dark cycle. They were access freely to normal chow diet (control diet) and drinking water *ad libitum*.

2.5 Diets

Normal chow diet

Normal chow (control) diet contains moisture 12%, crude protein 24%, fat 4.5%, fiber 5%, metabolic energy 3040 kcal/kg, calcium 1%, phosphorus 0.9%, sodium 0.2%, potassium 1.17%, magnesium 0.23%, manganese 171 ppm, copper 22 ppm, zinc 100 ppm, iron 180 ppm, cobalt 1.82 ppm, selenium 0.1%, vitamin A 20000 IU/kg, vitamin D 4000 IU/kg, vitamin E 100 mg/kg, vitamin K 5 mg/kg, vitamin B1 20 mg/kg, vitamin B2 20 mg/kg, vitamin B6 20 mg/kg, vitamin B12 mg/kg, niacin 100 mg/kg, folic acid 6 mg/kg, biotin 0.4 mg/kg and choline chloride 1500 mg/kg.

Iron-supplemented diet

Ferrocene-supplemented (Fe) diet was prepared by remixing ferrocene (0.2%, *w*/*w*) with the normal chow diet. The Fe diet was pellet for use entire experiments.

High-fat diet

High-fat (HF) diet was modified from the normal chow diet by adding lard, corn oil and multiple essential elements and vitamins. Corn oil emulsion (corn oil 600 g, sodium taurocholate 5 g, Tween-20 18.2 g, deionized water 150 in a total volume of 750 ml).

2.6 Animal treatments

Iron loading

For iron loading, WT and BKO mice were fed with ferrocene-supplemented chow diet (0.2%, *w*/*w*) (Fe diet) and sterile drinking water *ad libitum* for 90 days. Iron status including blood hemoglobin and plasma NTBI levels were detected weekly until the NTBI was detectable.

Intervention of iron-loaded mice with GT products

When complete iron loading, the mice were randomly divided into subgroups (12 mice each) for indicated treatments. WT1 and BKO1 mice (placebo) were orally administered with PBS solution, WT2 and BKO2 mice with GT extract (300 mg/kg/day), WT3 and BKO3 mice with EGCG (50 mg/kg/day, and WT4 and BKO4 mice with DFP (50 mg/kg body weight) as a reference oral iron chelator. All the mice were still fed with the Fe diet as well as sterile drinking water *ad libitum* for further

60 days. At termination the mice were sacrificed by diethyl ether anesthesia, their heart blood was collected in heparinized tubes for analyses of the surrogate markers.

Liver, spleen and heart were dissected, fixed immediately in 10 % neutral buffered formalin, dehydrated with a graded series of ethyl alcohol and embedded in paraffin boxes. Slide tissue sections were stained with hematoxylin & eosin (H&E), Pearls' supravital and Manson trichrome dyes as mentioned below. Histochemical examination was performed by an expert pathologist and photographed with a digital camera. Hepatic non-heme iron content was determined by the bathophenathroline method (Grundy et al., 2004; Vacha et al., 1978) and expressed as mg iron/mg tissue wet weight.

Fat loading

Rats were divided into two main groups, pre-fatten group (HF1 – HF5, n = 12/group) and non-prefatten group (NF1 – NF3, n = 12/group). The NF rats were fed with a normal chow diet for 12 weeks. The HF rats were fed (gavaging) with emulsional corn oil preparation (corn oil 600 g, sodium taurocholate 5 g, Tween-20 18.2 g, deionized water 150 in a total volume of 750 ml), a dose of *measured body weight divided by 3* daily (4.00 – 5.00 pm) together with the HF diet for 12 weeks. For intervention, the HF rats were fed with different diets as following; HF1 rats with corn starch-supplemented diet, HF2 rats with 5% chitosan supplemented diet, HF3 rats with 10% chitosan-supplemented diet, HF4 rats with 0.25% orlistat-supplemented diet, and HF5 rats 0.25% cholestyramine-supplemented diet. Similarly, the NF rats were fed with different diets as following; NF1 rats with corn starch-supplemented diet, NF2 rats with 5% chitosan supplemented diet, and NF3 rats with 10% chitosan-supplemented diet. Dose and time schedule for the intervention with selected chitosans and hypolipidemic drugs were indicated in **Table 2.2 – 2.4**.

Their body weight and food intake were recorded and calculated daily, and correction for spilled food was made. Blood samples were collected from tail vein into anti-coagulant heparinized tubes before and after the intervention. Heparinized plasma was separated and stored at –20°C until further analysis. Feces were collected and accumulated for determination of unabsorbed fat. Following the chitosan treatment for 2 months, the rats were given normal chow concomitantly with

administrating corn oil emulsion (washing period). Plasma lipid profiles, liver function test and kidney function test were evaluated.

At the end of study, the rats were anesthetized with diethyl ether and sacrificed. Liver, spleen and heart were excised and weighed. One part of the tissues were immediately frozen in liquid N_2 at and stored at –20°C until analysis, the other tissues were fixed immediately in 10 % neutral buffered formalin, dehydrated with a graded series of ethyl alcohol and embedded in paraffin boxes. Slide tissue sections were stained with hematoxylin & eosin (H&E), Pearls' supravital and Manson trichrome dyes as mentioned below. Histochemical examination was performed by an expert pathologist and photographed with a digital camera. Hepatic non-heme iron content was determined by the bathophenathroline method (Grundy et al., 2004; Vacha et al., 1978) and expressed as mg iron/mg tissue wet weight.

Table 2-2 Details of mice, iron loading and GT extract treatment.

Studied rats			Iron loading	Intervention	
Code	**Group**	**n**	**Fe diet**	**Substance/Drug**	**Dose**
WT1	Wild type	4	-	-	-
WT2	Wild type	4	0.2% (*w*/*w*) ferrocene	PBS	-
WT3	Wild type	4	0.2% (*w*/*w*) ferrocene	GT extract	300 mg/kg body weight
WT4	Wild type	4	0.2% (*w*/*w*) ferrocene	DFP	50 mg/kg body weight
BKO1	Thalassemic mice	2	0.2% (*w*/w) ferrocene	PBS	-
BKO2	Thalassemic mice	3	0.2% (w/w) ferrocene	GT extract	300 mg/kg body weight
BKO3	Thalassemic mice	2	0.2% (w/w) ferrocene	DFP	50 mg/kg body weight

Table 2-3 Time table for intervention of iron-loaded mice with GT extract.

Activities	Iron loading period	Intervention period (week)									
		1	2	3	4	5	6	7	8	9	10
Feeding Fe diet	←	←									→
Intervention with GT product		←							→		
Record body weight (every day)	←	←							→		
Blood collection (every 2 weeks)	←	←									→

Table 2-4 Details of studied rats, fat administration and chitosan treatment.

STUDY RATS			INTERVENTION		ADMINISTRATION OF FAT	
Code	**Group**	**n**	**Substance/Drug**	**Dose**	**Corn oil gavaging**	**HF diet feeding**
Baseline	At beginning	5	-	-	-	-
NF1	Non-fatten	6	Corn starch	5% (*w*/*w*)	g. body weight/3	Recorded
NF2	Non-fatten	6	Chitosan	5% (*w*/*w*)	g. body weight/3	Recorded
HF1	Pre-fatten	5	Corn starch	5% (*w*/*w*)	g. body weight/3	Recorded
HF2	Pre-fatten	6	Chitosan	5% (*w*/*w*)	g. body weight/3	Recorded
HF3	Pre-fatten	6	Orlistat	25 mg/kg bodyweight	g. body weight/3	Recorded

Table 2-5 Time table for intervention of fatten rats with the chitosans.

Activities	Pre-fatten period	Intervention period (week)									
		1	2	3	4	5	6	7	8	9	10
Gavaging colloidal corn oil											
Feeding supplemented diet											
Intervention with chitosan											
Record body weight (every day)											
Blood collection (every 2 weeks)											
Fecal collection (every week)											

2.7 Biochemical analyses

2.7.1 Plasma lipid profiles

Plasma lipid profiles including triglyceride, total cholesterol, VLDL-cholesterol, HDL-cholesterol and LDL-cholesterl were determined in the treated rats using assay procedures previously described by a manufacturer.

Plasma triglyceride (Bucolo and David, 1973; Megraw et al., 1979)

Triglyceride in plasma is hydrolyzed by lipase to release glycerol and fatty acids. The glycerol is catalyzed by glycerol kinase and then glycerol phosphate oxidase to produce hydrogen peroxide. The hydrogen peroxide is oxidized by peroxidase and gives electron to 4-aminoantipyrin in the presence of phenol to form a red-colored quinineimine product. In the assay, plasma (10 µl) was added to Triglyceride Enzyme reagent containing lipase, glycerol kinase, glycerol phosphate oxidase/horse-radish peroxidase/*p*-aminoantipyrine (1.0 ml) and incubated at 37 ^{o}C for 10 minutes. Absorbance of the product was measured at 505 nm.

Plasma total cholesterol (Richmond, 1973)

Cholesterol ester is hydrolyzed by cholesterol esterase to release unesterified cholesterol and fatty acid. The unesterified cholesterol is then oxidized by cholesterol oxidase to generate cholestynone and hydrogen peroxide. In the presence of peroxidase, *p*-aminoantipyrine and phenol the hydrogen peroxide is oxidized to produce red-colored quinoneimine. In the assay, plasma (10 µl) was added to Cholesterol Enzyme reagent containing cholesterol esterase/cholesterol oxidase/horse-radish peroxidase/*p*-aminoantipyrine) (1.0 ml) and incubated at 37 ^{o}C for 10 minutes. Absorbance of the colored product was measured at 505 nm.

2.7.2 Assay of plasma liver enzymes

Liver function was evaluated in the treated rats to see whether the chitosan is harmful to the liver or not. The biomarkers are described and measured in serum of the treated rats as described in a manufacturer protocol. These are:

Glutamate-oxaloacetate transaminase activity (Reitman and Frankel, 1957)

Serum aspartate aminotransferase (AST) also know as serum glutamic oxalacetic trasaminase (SGOT) is a tissue enzyme that catalyzes the exchange of amino and keto groups between alpha-amino acid and alpha-keto acid. AST is widely in tissue principally cardiac, hepatic, muscle and kidney. Injury to these tissue result

in the release of the AST enzyme to general circulation. This assay system was evaluated and optimize in 1960 by Henry et al. In the assay, test tubes: “blank”, “standard”, “patient or sample” and “control” were labeled. Working reagent 1.0 ml was added to test tube and warm to 37°C for 5 minutes. Serum, standard and water 0.1 ml were added in sample tube, standard tube and blank. After that, incubate at 37°C for 5 minutes and determine the average absorbance at 545 nm.

Glutamate-pyruvate transaminase activity (Reitman and Frankel, 1957)

Glutamate-pyruvate transaminase (GPT) or alanine aminotransferase (ALT) catalyzes transfer of amino group from alanine to α-ketoglutarate to generate pyruvate. The pyruvate reacts with 2,4-dinitophenylhydrazine (DNP) in alkaline solution and gives a golden-brown dinitrophenylhydrozone product. In the assay, plasma (0.1 ml) was incubated with the GPT substrate (0.25 ml) at 37^{o}C for 30 minutes. DNP coloring reagent (0.25 ml) was added to and further incubated at room temperature for 5 minutes. Sodium hydroxide solution (0.4 N.) was added to develop the colored product absorbtion at 545 nm against deionized water.

2.7.3 Quantification of hepatic glutathione

Reduced glutathione (GSH), a tripeptide (γ-glutamylcysteinylglycine) is the major free thiol in most living cells and is involved in many biological processes such as detoxification of xenobiotics, removal of hydroperoxides, and maintenance of the oxidation state of protein sulfhydryls. It is the key antioxidant in animal tissues. Glutathione is present inside cells mainly in the reduced form (90- 95% of the total glutathione). Oxidation of glutathione leads to the formation of glutathione disulfide (GSSG). Intracellular GSH status appears to be a sensitive indicator of the overall health of a cell, and of its ability to resist toxic challenge. High levels of GSH in the cell may indicate pathological changes.

Liver tissue was deproteinized with 5-sulfosalicylic acid solution (5%). Clear supernatant was measured glutathione concentration using a colorimetric method. Basically, reduced glutathione (GSH) converts 5,5’-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoic acid (TNB), and produced oxidized glutathione (GSSG) was recycled by glutathione reductase and NADPH to be the GSH. Absorbance of the yellow-colored product, TNB, was measured at 412 nm against reagent blank.

2.7.4 Quantification of liver lipid content (LLC)

Total lipid in the liver was extracted with chloroform/methanol (2:1, *v*/*v*) solvent in a proportion of 1:5 (*w*/*v*) in a screw-cap tube overnight. The mixture was centrifuged at 3,000 rpm, 4 °C for 20 minutes to sediment tissue pellet. Chloroform-extractable lipid (upper layer) was transferred to a new test tube and evaporated to dryness under nitrogen gas stream. Residue was reconstituted in methanol, and finally amounts of cholesterol and triglyceride in the liver extract were measured by the enzymatic techniques as described below.

2.7.5 Quantification of liver iron content (LIC) (Imbert-Bismut et al., 1999)

Non-heme iron in the liver was measured using a colorimetric technique as described by the method established by Fischer and Price. All glass-wares were cleaned with 6 N. HCl overnight, and washed 4-5 times with deionized distilled water; otherwise, plastic-wares were used. Liver tissues were lyophilized for 24 hours, weighed (expressed as mg dry weight) and transferred to the Erlenmeyer flask. Dry liver tissue was digested with a mixture of concentrated sulfuric acid and concentrated nitric acid (1:1, *w*/*v*) at 65 °C for 20 hours. After cooling down to room temperature clear yellow solution was transferred to a clean test tube, and adjusted a final volume to 10 ml exactly with deionized water. The tissue iron was reduced with ascorbic acid and reacted with 2,4,6-tripyridyl-*s*-triazine (TPTZ) at room temperature (25 °C) for at least 10 minutes to form an intense violet-colored product. Optical density (OD) of the solutions was measured at 595 nm against reagent using a spectrophotometer. Standard iron solution at 2 μg/ml was used for calculation. Liver iron concentration (TIC) was calculated using the equation as shown below.

$$\text{TIC } (\mu g\ Fe/g\ tissue) = \frac{(OD_{tissue} - OD_{blank})}{(OD_{standard} - OD_{blank})} \times \frac{1}{g.\ tissue\ dry\ weight} \times 200$$

which OD_{tissue} is OD at 535 nm of tissue extract,

$OD_{standard\ iron}$ is OD at 535 nm of standard iron,

OD_{blank} OD at 535 nm of reagent blank

2.7.6 Thiobarbituric acid reactive substance (TBARS) assay

Liver and plasma TBARS was measured by HPLC method as mentioned below (Chirico, 1994). Liver tissue (100 mg) was homogenized in the solution containing 50 mM phosphate buffer pH 2.8 (0.8 ml), methanol (0.1 ml) and BHT (50 ppm) in ice bath. A 0.5-ml aliquot of the homogenate was transferred to 1.1 ml of 10% (*w*/*v*) trichloroacetic acid containing BHT (50 ppm), mixed well and heated at 90°C for 30 minutes. After cooling to room temperature the mixture was centrifuged at low speed to achieve clear supernatant. The sample (0.5 ml) was mixed with 0.44 M H_3PO_4 (1.5 ml) and TBA solution (0.6%, *w*/*v*) (1.0 ml), incubated in a water bath at 90°C for 30 minutes. Plasma sample (50 µl) was mixed with 2% BHT solution (2.5 µl), followed by 0.44 M H_3PO_4 (750 ml) and TBA solution (0.6% *w*/*v*) (250 µl). The mixture was incubated at 90°C for 30 minutes and then allowed to cool. The solution was filtered through 0.45-µm syringe filter and measured absorbance at 532 nm. A standard curve was constructed from the peak heights of standard 1,1,3,3-tetramethoxypropane. Plasma TBARS concentration was determined from the standard curve and reported as MDA equivalents.

2.8 Histochemical examination

2.8.1 Paraffin embedding

Formalin-preserved liver tissues were prepared in sequences as shown in **Table 2.6**. The paraffin embedment was done on a Tissue-Tek Embedding Console System TEC-5, and cut with a sliding microtome (Pteratome CRM440, Sakura Seiki) at 5 µm thickness. Thin sections were obtained and incubated on hot plate at 37 °C overnight. After paraffin sections were cooled down, they were stained with H & E, Masson trichrome and Pearls' Prussian blue supravital dyes for histological evaluation, collagen content and iron deposit respectively.

Table 2-6 Steps for processing tissues before embedding in paraffin blocks.

Process	No.	Reagent	Period	Temperature
Dehydration	1	70% Alcohol	3 h	Room temp
	2	80% Alcohol	3 h	Room temp
	3	90% Alcohol	3 h	Room temp
	4	95% Alcohol	3 h	Room temp
	5	Absolute alcohol (I)	24 h	Room temp
	6	Absolute alcohol (II)	24 h	Room temp
Clearing	7	Methyl benzoate + Benzene (I)	1-2 h	Room temp
	8	Methyl benzoate + Benzene (II)	3 h	Room temp
	9	Benzene (I)	1-2 h	Room temp
	10	Benzene (II)	3 h	Room temp
Pre-embedding	11	Paraffin+Benzene	20-30 min	58-60 °C
	12	Paraffin (I)	20-30 min	58-60 °C
	13	Paraffin (II)	20-30 min	58-60 °C
	14	Paraffin (III)	20-30 min	58-60 °C

The stained sections were then dehydrated with 95 – 100 % ethyl alcohol and preserved in xylene.

2.8.2 Liver fat deposit

Liver tissue was fixed in neutral buffered formalin solution, cut in cryostat and stained with Oil Red O dye for detection of small intracellular lipid droplets (neutral fat) and counterstained with H & E supravital dye for demonstration of cell morphology. Fatty change was graded the percentage of hepatocytes cotaining macrovesicular fat according to Kirsch and colleages' criteria (Kirsch et al., 2003). Degree of inflammation and necrosis was expressed and graded by an expertise pathologist from Department of Pathology, Faculty of Medicine, Chiang Mai University.

2.8.3 Liver iron content (Pearl's Prussian blue staining)

Liver iron deposit was evaluated chemically using Pearl's staining technique. In the assay, the liver tissue section was stained with hydrochloric acid-potassium ferrocyanide solution and visualized with a simple microscope (Olympus). Pink cytoplasm, red nucleus and Prussian blue granules were appeared. Degree of siderosis in hepatocytes, Kupffer cells and endothelial cells was graded from 0-4, as shown by Pearls' stain. Grade 1 represents minimal amounts, and grade 4 represents the degree seen in hepatocytes in fully developed untreated hemochromatosis. Hepatocyte siderosis correlates slightly better with chemical liver iron content than does the degree of endothelial and Kupffer cell siderosis (Aldouri et al., 1987).

2.8.4 Liver collagen content (Manson trichrome staining)

This method is used for the detection of collagen fibers in tissues such as skin, heart, liver, etc. on formalin-fixed, paraffin-embedded sections and frozen sections as well. The collagen fibers will be stained blue, the nuclei will be stained black and the background is stained red. In the assay procedure, liver tissue section was deparaffinized and rehydrated sequentially through 100% alcohol, 95% alcohol 70% alcohol. The section was washed in distilled water, stained with Weigert's iron hematoxylin working solution for 10 minutes and rinsed in running warm tap water for 10 minutes. The tissue section was stain in Biebrich scarlet-acid fuchsin solution for 15 minutes and washed in distilled water. The section was stained in phosphomolybdic-phosphotungstic acid solution for 15 minutes, then transferred directly (without rinse) to aniline blue solution and stained for 5-10 minutes. The tissue section was rinsed briefly in distilled water and dehydrated very quickly through a respective 95% ethyl alcohol and absolute ethyl alcohol. The tissue slide was clear in xylene and finally mounted with resinous mounting medium

2.8.5 Hematoxylin and Eosin (H&E) staining

The oxidation product of hematoxylin is hematin, which exhibits indicator-like properties, being blue and less soluble in aqueous alkaline conditions, and red and more soluble in alcoholic acidic conditions. In acidic conditions, hematin binds to lysine residues of nuclear histones by linkage via a metallic ion mordant, in this case aluminium. Undesirable colouration is selectively removed by controlled leaching in an alcoholic acidic solution, (acid alcohol). Differentiation is arrested by returning to an

alkaline environment, whereupon the hematin takes on a blue hue, the process of "blueing-up". The hematin demonstrates cell nuclei.

Full cellular detail is obtained by counterstaining with the eosin mixture. Colour enhancement is achieved by fortifying the stain with phloxine, a chemical member of the same family as eosin (halogenated fluorosceins). Visualisations are obtained by applying the dyes in acidic conditions, whereby more intense specific colourations are obtained, the more acidic tissue components taking up the dye to a greater intensity, hence the addition of acetic acid. Microscopic visualization of collagen (pale pink), muscle (deep pink), acidophilic cytoplasm (red), basophilic cytoplasm (purple), nuclei (blue), erythrocytes (cherry red) were evaluated by an expert pathologist and photographed.

2.9 Data analysis

The results were expressed as means$\pm$SD. Statistical significance of the data was determined by a Student's *t*-test or one-way analysis of variance (ANOVA), which $p<0.05$ is considered significant. When the SD is significantly different, a non-parametric test will be used.