CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Twelve male New Zealand White (NZW) rabbits of body weight between 1.5 – 2.0 kg were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. The animals were housed one per cage at the Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand and acclimatized for two week before being entered into an experiment. All animals were kept in a controlled humidify room at a constant temperature of 25 ± 2 °C and maintained on a 12-hour alternate light-dark cycle. They were allowed to freely access to food (Appendix E) and drinking water.

2. Instrument

The following instruments were used in the experimentation.

1. Analytical balance (Precisa, Switzerland)
2. Autopipettes 20, 100, 200 and 1000 µL (Gilson, France)
3. Freezer -80 °C (Thermoelectron, USA)
4. Glass homogenizing vessels (Heidolph, Germany)
5. Mikro 22R centrifuge (Hettich, Germany)
6. pH meter (Therma, Canada)
7. Surgical equipments
8. Timer
9. UV-spectrophotometer (Shimadzu, Japan)
10. Vortex mixer (Clay Adams, USA; CT Laboratory)

3. Chemicals

The following chemicals were used in the experimentation.

Chemicals used in determination of total peroxide (TP)

- Ammonium ferrous sulphate (Sigma, USA)
- Butylated hydroxytoluene (BHT) (Sigma, USA)

- Hydrogen peroxide (H_2O_2) (Carloerba, Italy)
- Methanol (Merck, Germany)
- Sulfuric acid (H_2SO_4) (Merck, Germany)
- Xylenol orange (Sigma, USA)

Chemicals used in determination of total antioxidant status (TAS)

- 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) (Sigma, USA)
- Di-potassium hydrogen phosphate (K_2HPO_4) (Merck, Germany)
- Glacial acetic acid (Merck, Germany)
- Hydrogen peroxide (H_2O_2) (Carloerba, Italy)
- 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox, $C_{14}H_{18}O_4$) (Sigma, USA)
- Potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany)
- Sodium acetate (CH_3COONa) (Merck, Germany)

Chemicals used in determination of paraoxonase1 (PON1) activity

- Calcium chloride ($CaCl_2$) (Merck, Germany)
- Diethyl *p*-nitrophenyl phosphate (paraoxon) (Sigma, USA)
- Hydrochloric acid (HCl) (Merck, Germany)
- Phenyl acetate (Merck, Germany)
- Sodium hydroxide (NaOH) (Merck, Germany)
- Tris [hydroxymethyl] aminomethane hydrochloride (Sigma, USA)

Chemicals used in preparation of cell lysate

- Di-potassium hydrogen phosphate (K_2HPO_4) (Fluka, Switzerland)
- Di-sodium phosphate (Na_2HPO_4) (Merck, Germany)
- Potassium chloride (KCl) (Merck, Germany)
- Potassium dihydrogen phosphate (KH_2PO_4) (Fluka, Switzerland)
- Sodium chloride (NaCl) (Merck, Germany)

Chemicals used in determination of protein assay

- Bovine serum albumin (BSA) (Sigma, USA)
- Brilliant Blue G-250 (Sigma, USA)

- Methanol (Merck, Germany)
- Phosphoric acid (Merck, Germany)

Chemicals used in determination of paraoxonase2 (PON2) activity

- Calcium chloride ($CaCl_2$) (Merck, Germany)
- Dihydrocoumarin (DHC) (Sigma, USA)
- Hydrochloric acid (HCl) (Merck, Germany)
- Methanol (Merck, Germany)
- Sodium hydroxide (NaOH) (Merck, Germany)
- Tris [hydroxymethyl] aminomethane hydrochloride (Sigma, USA)

Chemicals used in determination of paraoxonase3 (PON3) activity

- Calcium chloride ($CaCl_2$) (Merck, Germany)
- Hydrochloric acid (HCl) (Merck, Germany)
- *p*-nitrophenyl butyrate (Sigma, USA)
- Sodium hydroxide (NaOH) (Merck, Germany)
- Tris [hydroxymethyl] aminomethane hydrochloride (Sigma, USA)

4. Drug

Simvastatin was purchased from an accredited drug store. (Bangkok, Thailand).

5. Water

Autoclaved 18 Ω deionized water was used for preparation of reagents in cell lysate preparation. The reagents for all other tests were prepared by using 18 Ω deionized water without autoclaved.

Methods

1. Preparation of *Curcuma comosa* (*C. comosa*) rhizome

C. comosa rhizome was kindly provided by Professor Dr. Apichart Suksamrarn, Faculty of Sciences, Ramkamhaeng University, Thailand. In brief, dried rhizome of *C. comosa* was collected from Nakornpathom province. A voucher specimen (BKF No. 97298) was deposited at the Forest Herbarium, Royal Forest

Department, Ministry of Agriculture and Cooperatives, Bangkok. *C. comosa* rhizomes were sliced and dried at 50-60 ^{0}C. The dried rhizomes were pulverized before giving to the animals.

Chemical identification

C. comosa was characterized by Suksamrarn et al. (unpublished data). The major constituent in *C. comosa* powder were two diarylheptanoids: 1,7-diphenyl-(6*E*)-6-hepten-3-ol and 1,7-diphenyl-(4*E*,6*E*)-4,6-heptadien-3-ol.

2. Animal treatment

Twelve NZW rabbits were randomly divided into three treatment groups of 4 rabbits each as followings:

1. Group 1: NZW rabbits were given orally with 1.0% cholesterol diet for 1 month. After 1 month, the animals were continually given 0.5% cholesterol diet for 3 months.
2. Group 2: NZW rabbits were given orally with 1.0% cholesterol diet for 1 month. After 1 month, the animals were continually given 0.5% cholesterol diet and simvastatin at the dosage of 5 mg/day for 3 months.
3. Group 3: NZW rabbits were given orally with 1.0% cholesterol diet for 1 month. After 1 month, the animals were continually given 0.5% cholesterol diet and *C. comosa* at the dosage of 400 mg/kg/day for 3 months.

C. comosa suspension for animal administration were prepared daily by dissolving 400 mg of *C. comosa* powder with 1 mL of deionized water to make a concentration of 400 mg/mL of *C. comosa* suspension and mixed before feeding to experimental animals.

3. Samples collection

At the end of the treatment, animals were fasted for 12 hours before anesthesized with sodium pentobarbital by intravenous at the dosage of 100 mg/kg before collecting blood. Blood samples were collected into EDTA containing tubes and in plain tubes. Plasma samples were separated by centrifugation at 3,500 r.p.m.

for 15 min at 4 °C and aliquoted for lipid parameters and oxidative stress index (OSI) analysis. Serum samples were separated by centrifugation at 3,500 r.p.m. for 15 min at 4 °C and aliquoted for PON1 and PON3 activities. Both plasma and serum samples were stored at −80 °C until analysis. After collecting blood sample, the abdominal aorta was immediately removed from the body of rabbits, rinsed with PBS buffer pH 7.4 (KH_2PO_4 Na_2HPO_4, KCl and NaCl) for 3 times, transferred into microcentrifuge tube and then stored at −80 °C until analysis of PON2 activity.

4. Clinical blood chemistry

The following clinical blood chemistry parameters in plasma samples were analyzed by using auto-analyzer (Hitachi 917) at Professional Laboratories Management. Co. Ltd., Bangkok, Thailand. Total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) were determined.

5. Determination of total peroxide (TP)

Reagents

Solution A:

A volume of 180 mL methanol was used to dissolve 158.4 mg of BHT and thereafter 15.2 mg xylenol orange was added.

FOX2 Reagent:

A quantity of 9.8 mg ammonium ferrous sulphate was dissolved in 10 mL of 250 mM H_2SO_4. This solution was then added to 90 mL of solution A.

Blank Reagent:

The blank reagent was prepared by mixing 90 mL of solution A with 10 mL of 250 mM H_2SO_4.

Procedures

Total peroxide concentrations of plasma samples were determined by FOX2 method as previously described (Kosecik et al., 2004). Oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) by various types of peroxides contained within the plasma samples, produced a colored ferric-xylenol orange complex whose absorbance was measured. A 100 μL of plasma was mixed with 900 μL of FOX2 reagent. After incubation at room temperature for 30 min, the mixture was centrifuged at 14,000 r.p.m. for 10 min.

Absorbance of the supernatant was then determined at 560 nm by spectrophotometer. Total peroxide content of plasma samples was determined as a function of the absorbance difference between test and blank tubes using a solution of H_2O_2 as standard. Each sample was performed in triplicate.

6. Determination of total antioxidant status (TAS)

Reagents

Reagent 1: acetate buffer 0.4 mol/L pH 5.8

Solution A:

A quantity of 32.8 g CH_3COONa was dissolved in 1,000 mL of deionized water.

Solution B:

A volume of 22.8 mL glacial acetic acid was diluted to 1,000 mL with deionized water.

(Reagent 1 was prepared by mixing 940 mL of solution A with 60 mL of solution B then adjusted pH 5.8, the buffer solution was stable for at least 6 months at 4 °C).

Reagent 2: $ABTS^{\bullet+}$ in acetate buffer 30 mmol/L pH 3.6

Solution C:

This solution was prepared by dissolving 2.46 g of CH_3COONa in 1,000 mL of deionized water.

Solution D:

A volume of 1.705 mL of glacial acetic acid was diluted to 1,000 mL with deionized water.

Solution E:

Solution E was prepared by mixing 75 mL of solution C with 925 mL of solution D then adjusted pH 3.6.

Solution F:

Solution F was prepared by diluting 278 µL H_2O_2 solution (35%) with 1,000 mL solution E.

(Reagent 2 was prepared by dissolving 0.549 g of ABTS in 100 mL of solution F. After 1 hour of incubation at room temperature, the characteristic color of $ABTS^{\bullet+}$ appeared, the reagent 2 was stable for at least 6 months at 4 °C).

Procedures

Total antioxidant status of plasma was determined as previously described by Erel (Erel, 2004) with minor modifications. The reduced ABTS molecule is oxidized to $ABTS^{\bullet+}$ using hydrogen peroxide in acidic medium (30 mM acetate buffer pH 3.6). Deep green $ABTS^{\bullet+}$ molecules remained more stable in the acetate buffer. While it was diluted with a more concentrated acetate buffer solution at high pH values (0.4 M acetate buffer pH 5.8), the color was spontaneously and slowly bleached. Antioxidants in the plasma sample accelerated the bleaching rate to a degree proportional to their concentrations. The bleaching rate was inversely proportional related with TAS of the plasma. The mixture of 25 μL of plasma and 1,000 μL of reagent 1 was used to set zero absorbance at 660 nm. Then, 100 μL of reagent 2 was added to determine plasma TAS by measuring absorbance at 660 nm after 5 min incubation. The TAS value of the plasma samples tested was expressed as an equivalent of the millimolar concentration of Trolox solution. Each sample was performed in triplicate.

7. Determination of oxidative stress index (OSI)

The percent ratio of total peroxide level to TAS level was accepted as oxidative stress index (OSI), an indicator of the degree of oxidative stress (Kosecik et al., 2004). To perform the calculation, the result unit of total peroxide was changed from μmol/L to mmol/L and the OSI value was calculated as below formula;

$$\text{OSI} = \frac{\text{Total Peroxide, (mmol/L)}}{\text{Total Antioxidant Status, (mmol Trolox equivalent/L)}} \times 100$$

8. Determination of serum PON1 activity

PON1 activities were determined by using paraoxon and phenyl acetate as substrates (Eckerson et al., 1983).

8.1 Paraoxonase (PON) activity assay

Reagents

1. 100 mM Tris-HCl buffer pH 8.0 containing 2 mM $CaCl_2$ and 1.1 mM paraoxon.

Procedures

PON1 activity toward paraoxon was measured after the reaction of paraoxon hydrolysis into *p*-nitrophenol and diethylphosphate catalyzed by the enzyme. PON1 activity was determined from measuring the liberation of *p*-nitrophenol at 37 °C and recorded at 405 nm for 50 seconds by spectrophotometer. The activity was measured by adding 20 µL of serum to 1 mL 100 mM Tris-HCl buffer pH 8.0 containing 2 mM $CaCl_2$ and 1.1 mM paraoxon. PON1 activity of 1 U/L was defined as 1 µmol of *p*-nitrophenol formed per minute per liter of serum. The molar extinction coefficient (ε) of *p*-nitrophenol is 18,700 $M^{-1}cm^{-1}$ at 405 nm, pH 8.0. Each sample was performed in triplicate.

Calculations

PON1 activity toward paraoxon was determined from the concentration (U/L) of the product formed (*p*-nitrophenol) per minute per liter of serum. PON activity was calculated as following;

$$\text{PON activity} = \text{(OD/min) x conversion factor}$$

$$= \text{(OD/min)} \times \frac{V}{\varepsilon \times v \times l}$$

Where V = Total volume (mL)

v = Serum sample volume (mL)

ε = Molar extinction coefficient (18,700 $M^{-1}cm^{-1}$)

l = Path length (cm)

8.2 Arylesterase (ARE) activity assay

Reagents

1. 10 mM Tris-HCl buffer pH 7.4
2. 10 mM Tris-HCl buffer pH 8.0 containing 0.9 mM $CaCl_2$ and 1.0 mM phenyl acetate

Procedures

PON1 activity was also determined by using phenyl acetate as substrate, as previously described by Eckerson (Eckerson et al., 1983). ARE activity was

determined from an increase in absorbance of phenol at 270 nm by using spectrophotometer. The reaction mixture contained 1.0 mM phenyl acetate and 0.9 mM $CaCl_2$ in 10 mM Tris-HCl buffer pH 8.0. The reaction was initiated by adding 20 µL of serum which was prediluted to 1:20 ratio with 10 mM Tris-HCl buffer pH 7.4. The enzyme activity was calculated from the molar extinction coefficient of 1,310 M^{-1} cm^{-1}. Each sample was performed in triplicate.

Calculations

PON1 activity toward phenyl acetate was determined from the concentration (U/L) of the product formed (phenol) per minute per liter of serum. ARE activity was calculated as following;

$$\text{ARE activity} = (\text{OD/min}) \times \text{conversion factor}$$

$$= (\text{OD/min}) \times \frac{V}{\varepsilon \times v \times l}$$

Where V = Total volume (mL)

v = Serum sample volume (mL)

ε = Molar extinction coefficient ($1{,}310\ M^{-1}cm^{-1}$)

l = Path length (cm)

9. Preparation of cell lysate

Reagents

1. 0.1 M Phosphate buffer, pH 7.4

One litre of 0.1 M Potassium phosphate buffer, pH 7.4 consisted of 4.27 g of KH_2PO_4 and 15.43 g of K_2HPO_4. The solution was adjusted to pH 7.4 with NaOH or HCl.

2. Phosphate Buffer Saline (PBS), pH 7.4

One litre of PBS buffer, pH 7.4 consisted of 0.12 g of KH_2PO_4, 0.72 g of Na_2HPO_4, 0.1 g of KCl and 4.0 g of NaCl. The solution was adjusted to pH 7.4 with NaOH or HCl.

Procedures

Abdominal aorta was weighed, cut into pieces and then rinsed with ice cold PBS buffer pH 7.4 for 1 time and wrapped with aluminium foil. The abdominal aorta was submerged in liquid nitrogen for 3 minutes, then crushed with mortar and pestle, and transferred to glass homogenizing vessel. Ice cold 0.1 M potassium phosphate buffer, pH 7.4 was added and homogenized for 30 minutes. The lysate was viewed under the microscope. The lysate suspension was separated by centrifugation at 5,000 $\times g$ for 20 minutes at 4 °C, and the supernatant was transferred into microcentrifuge tube and then stored at -80 °C until assays.

10. Determination of protein assay

Total protein concentration in supernatant of cell lysate was determined by using Bradford method (Bradford, 1976).

Reagents

A quantity of 50 mg Coomassie Brilliant Blue G-250 was dissolved in 25 mL of 95% methanol and 50 mL of phosphoric acid then made up final volume of 500 mL with water.

Procedures

Bradford protein assay is a dye binding assay in which a differential color change of dye occurs in response to various concentrations of proteins. A volume of 20 µL sample was mixed with 1 mL of Bradford reagent and incubated at room temperature for 5 min. Total protein was determined at 595 nm by using spectrophotometer. Bovine serum albumin (BSA) was used as standard.

11. Determination of PON2 activity

Reagents

1. 50 mM Tris-HCl buffer pH 8.0 containing 1 mM $CaCl_2$ and 1 mM DHC (from a 100 mM stock, dissolved in methanol)

Procedures

PON2 activity was determined by using DHC as a substrate, with minor modification from the previous protocol described by Draganov (Draganov et al., 2000). PON2 activity was measured by adding 20 µL of supernatant of cell lysate to 1

mL of 50 mM Tris-HCl buffer pH 8.0 containing 1 mM $CaCl_2$ and 1 mM DHC. The hydrolysis rate of DHC was followed by measuring the librations of 3-(2-hydroxyphenyl)propionic acid at 270 nm at 37 °C using spectrophotometer. The enzyme activity was calculated from the molar extinction coefficient of 876 $M^{-1}cm^{-1}$. Each sample was performed in triplicate.

Calculations

PON2 activity was determined from the concentration (U/L) of the product formed (3-(2-hydroxyphenyl) propionic acid) per minute per liter of protein concentration in supernatant of cell lysate. PON2 activity was calculated as following;

PON2 activity = (OD/min) x conversion factor

$$= (OD/min) \times \frac{V}{\varepsilon \times v \times l}$$

Where V = Total volume (mL)

v = Supernatant of cell lysate (mL)

ε = Molar extinction coefficient (876 $M^{-1}cm^{-1}$)

l = Path length (cm)

12. Determination of serum PON3 activity

Reagents

1. 50 mM Tris-HCl buffer pH 8.0 containing 1 mM $CaCl_2$ and 1 mM *p*-nitrophenyl butyrate

Procedures

PON3 activity was measured following the hydrolysis of *p*-nitrophenyl butyrate as a substrate as previously described by Nagila (Nagila and Porntadavity, 2008). PON3 activity was measured by adding 10 µL serum to 1 mL 50 mM Tris-HCl buffer pH 8.0 containing 1 mM $CaCl_2$ and 1 mM *p*-nitrophenyl butyrate. The reaction was followed at 37 °C for 50 seconds by using spectrophotometer. The enzyme activity was monitored at 405 nm with the molar extinction coefficient of 18,700 M^{-1} cm^{-1}.

Calculations

PON3 activity was determined from the concentration (U/L) of the product formed (*p*-nitrophenol) per minute per liter of serum. PON3 activity was calculated as following;

$$\text{PON3 activity} = (\text{OD/min}) \times \text{conversion factor}$$

$$= (\text{OD/min}) \times \frac{V}{\varepsilon \times v \times l}$$

Where V = Total volume (mL)

v = Serum sample volume (mL)

ε = Molar extinction coefficient ($18{,}700\ M^{-1}cm^{-1}$)

l = Path length (cm)

13. Verification of the methods

Before using the methods to determine TP, TAS, PON1 activity (using paraoxon and phenyl acetate as substrates), PON2 activity and PON3 activity, the methods were verified by performing the precision assays. Twenty samples of control serum or 4 samples of normal rat vascular samples were used to perform the particular assay. The precision of the assay was shown as Optimal Condition Variance (OCV) and Routine Condition Variance (RCV) which were calculated as following:

$$\text{OCV} = \frac{\text{SD}}{\text{Mean}} \times 100$$

$$\text{RCV} = \frac{\text{SD}}{\text{Mean}} \times 100$$

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls and Kruskal-Wallis tests for normally and non-normally distributed parameters, respectively. Changes from baseline outcomes were analyzed using Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.