CHAPTER III

RESEARCH/METHODOLOGY

Research and Methodology

Subjects

Research design: Cross sectional study research

We received serum specimens from patients in the outpatient department of the Uttaradit Hospital for routine laboratory tests. This study was performed using residual specimens after performing and reporting all test ordered by the physicians. While, the total 292 volunteers in this study are the patients and general subjects who 203 with diabetes and participate in attending the our institute were in this study. These patients are subdivided into three groups by the blood creatinine concentrations estimated in these patients between 2.0 mg/dl and exceeded 6.0 mg/dl. While collecting 10 milliliter of blood were collected by veinipuncture after an overnight fasting and collected random urine 5 ml. Serum was separated from the whole blood tube (that without anticoagulant) by centrifugation and divided into 2 micro-tubes for the following purposes, blood chemistry parameters, oxidative stress, and total antioxidant capacity (TAC), respectively from 203 participants, stratified as three groups. Group (I) comprise with 79 renal disease patients without hemodialysis who had serum creatinine levels about 2-5.9 mg/dl. Group (II) comprise with 51 renal disease patients without hemodialysis who had serum creatinine $\geq$ 6.0 mg/dl. Group (III) comprise with 73 renal disease patients with hemodialysis who had serum creatinine level $\geq$ 6.0 mg/dl. And group (IV) with 89 healthy control were nondiabetes subjects sex, age-matched normal subjects were included in the study as control group. This group consists of their ages were between 30-60 years old. There had normal blood glucose levels and Serum creatinine levels of these patients varied 0.9 -1.3 mg/dl. The inclusion criteria of renal disease patients in the present study included without acute illness and clinical signs of ischemia, myocardial infarction, unstable angina or stroke, free of active infections and autoimmune disease. None of the patients received immunosuppressive treatment, non- steroidal anti-inflammatory

drugs, or antioxidants such as, vitamin E, C, or allopurinol and any changes in treatment over 30 days prior to inclusion in this study, Controls were healthy volunteers from a general population. Inclusion criteria for healthy controls included: absence of any history of CHD, absence of hypertension, any condition limiting mobility, life-treatening diseases, or any other disease or condition that would impair compliance. Exclusion criteria for those groups were the smoking and intake of antioxidant supplementation in the 2 months previous to their inclusion in the study. All biochemical parameters in serum (glucose, BUN, creatinine, cholesterol, triglyceride, HDL-C, LDL-C, uric acid), EDTA blood (HbA1C, and CBC) and Urine (Microalbumin, and urine creatinine) were analyzed in laboratory at the same day of collection. All samples were stored at – 20 °C until lipid hydroperoxide, malondialdehyde (MDA), total antioxidant capacity (TAC), and activity of urinary NAG activity analysis were carried out. This study was approved by the human experimental ethical committee of Naresuan University.

Analytical methods

Materials and Instruments

1. Polypropylene test tubes (13 ml) with screw caps
2. Polypropylene microtube (2ml) with screw caps that contain O-ring seal were used for containing blood samples.
3. Spectro 22 spectrophotometer, being available for visible, near ultraviolet, and near infrared spectral wavelength, was employed for qualitative and quantitative analysis.
4. The Sunrise Touchscreen (Sunrise RC/TS-TC/TW/BC/6 Filter, Tecam Austria), the fully automatic 96-well micro plate reader, is utilized for the measurement of absorbance (optical density) of samples from the biological or non-biological by reading twelve well simultaneously.
5. Becman DXC 800 biochemistry (Japan) automatic analyzer is the instrument for performing chemical clinical analysis. It was used to analyze glucose, BUN, creatinine, cholesterol, triglyceride, HDL-C, LDL-C, uric acid and were evaluated in serum and random urine was taken for microalbumin and urine creatinine by using commercial analytical kits

6. Beckman automatic analyzer is the instrument for performing chemical clinical analysis. It was used to analyze complete blood cell count

7. HA 8611 automatic analyzer is the instrument for performing chemical clinical analysis. It was used to analyze HbA1C

8. Continuously heat hotplate with heat control room temperature to 100°C or over.

Chemical reagents

1. Ammonium ferrous sulfate (Sigma-Aldrich, USA)
2. Xylenolorange (Sigma-Aldrich, USA)
3. Triphenylphosphine (Sigma-Aldrich, USA)
4. D-sorbitol (Sigma-Aldrich, USA)
5. Cumene hydroperoxide (Sigma-Aldrich, USA)
6. Phosphoric acid (Sigma-Aldrich, USA)
7. Thiobarbituric acid (Sigma-Aldrich, USA)
8. Tricholoacetic acid (MERCK, USA)
9. Phosphoric acid (Sigma-Aldrich, USA)
10. ABTS (Sigma-Aldrich, USA)
11. Trolox (Sigma-Aldrich, USA)
12. Myoglobin (Sigma-Aldrich, USA)
13. Citric acid solution (Fluka)
14. Sodium citrate solution (Fluka)
15. p-Nitrophenyl-N-acetyl-β-D- glucosaminide
16. p-Nitrophenol working standard solution (Sigma-Aldrich, USA)

1. Routine blood cell count

Whole EDTA blood was initially taken for complete blood cell count analysis by Beckman and for HbA1C by HA 8611

2. Routine biochemical test

All biochemical parameters (glucose, BUN, creatinine, uric acid, cholesterol, triglyceride, high density lipoprotein cholesterol; HDL-C), low density lipoprotein cholesterol; LDL-C) were evaluated in serum and random urine was taken for microalbumin and urine creatinine by using commercial analytical kits from Beckman with DXC800 automatic analyzer.

3. Total antioxidant capacity (TAC)

Total antioxidant capacity assay is measure by trolox equivalence antioxidant assay developed by Evans and Halliwell [7, 54].

3.1 Reagent preparation of ABTS Assay

3.1.1 Phosphate buffered saline (PBS) 1 litre of Phosphate buffered saline (PBS) pH 7.4 by Sambrook, Fritsch and Maniatis prepare as follows: (http://protocolsonline.com/featured-articles/phosphate-buffered-saline-pbs/) start with 800 ml of distilled water. Add 8 g. of NaCl. Add 0.2 g. of KCl. Add 1.44 g. of Na_2HPO_4. Add 0.24 g. of KH_2PO_4. Adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1 liter.

3.1.2 Myoglobin stock solution 25 µM : Dissolve 0.01 g myoglobin in 2,850 µl Phosphate buffered saline (PBS) pH 7.4 (Myoglobin stock solution : Reconstitute the myoglobin catalog number M1882 by adding 285 ml of ultrapure water to the vial and vortexing well. Store in working aliquots at -20 °C. The myoglobin stock solution remains active for 6 months at -20 °C.)

3.1.3 Myoglobin working solution – before use, dilute the required amount of myoglobin stock solution 100- fold with Phosphate buffered saline (PBS) pH 7.4 and mix well.

3.1.4 Preparation of trolox standard according to Table 3

Table 1 Trolox standards

Tube	1.5 mM torlox (µl)	Assay Buffer (µl)	Trolox concentration (mM)
1	0	500	0
2	5	495	0.015
3	15	485	0.045
4	35	465	0.015
5	70	430	0.21
6	140	360	0.42

3.1.5 One litre of stock ABTS g (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) 300 µM : Dissolve 0.16 g ABTS in distrilled water 1000 ml.

3.1.6 Prepare ABTS substrate working solution by adding 25 ml. 5% hydrogen peroxide solution to 10 ml of ABTS substrate solution. Use within 20-30 minutes.

3.2 Method

In well for the trolox standard curve, add 10 ml of a trolox standard from tube 1-6 in ELISA plate. In well for sample, add 10 ml of sample in ELISA plate. Add 50 µl of myoglobin working solution. Add 150 µl of ABTS working solution. Incubate for 5 minutes at room temperature. Read absorbance at 405 nm by using ELISA reader.

4. Thiobarbituric acid reactive substant Assay

Thiobarbituric acid reactive substance was measured using methods applied from original method by Tangvarasittichai S., et al. [45].

4.1 Reagent preparation of TBARS Assay

4.1.1 Phosphoric acid solution, 0.44 mol/L. Dilute 10 ml of "ultra-pure" H_3PO_4 reagent (relative density 1.69 g/ml, 0.44 mol/L, 850 g/L; J.T. Baker Co., Phillipsburg, NJ) to 1 L with water (distilled water is used throughout).

4.1.2 Thiobarbituric acid solution (42 mmol/L, TBA solution) 0.6 g of 4, 6-dihydroxy-2-thiopyrimidine (no. T-5500, molecular mass 144.2 Da; Sigma Chemical Co., St. Louis, MO) was dissolved in approximately 80 ml of hot water (50-55°C), then cool the solution to 25 °C and dilute to 100 ml with water. Stored at room temperature, this reagent is stable for two weeks.

4.1.3 TEP standard solution (10, 20, 30, 40 and 50 µmol/L) 60 µl of 1, 1, 3, 3-tetraethoxypropane reagent (anhydrous, relative density 0.918, molecular mass 220.31 Da, purity 97%, no, T-9889; Sigma Chemical Co.) was diluted in a 25 ml volumetric flask with distilled water. This standard solution prepared freshly each month and store at 4 °C. For an intermediate standard, pipette 0.5 ml of this TEP stock standard solution into a 50-ml volumetric flask and dilute to the mark with distilled water. Prepare freshly each fortnight and store at 4 °C. To prepare TEP working standard solution (10, 20, 30, 40 and 50 µmol/l), pipette TEP intermediate standard into 12X75 ml test tube 0.1, 0.2, 0.3, 0.4 and 0.5 ml, respectively, and dilute the contents with distilled water 0.9, 0.8, 0.7, 0.6 and 0.5 ml. These solution were prepared weekly and stored at 4 °C.

4.2 Method

After thawing the samples, measurements of MDA in term of TBARS were performed for each of 200 samples by using method of Tangvarasittichai S., et al. [45]. Briefly, a 50 µL of TEP standard, plasma and control specimen was pipette into the 13-ml polypropylene test tube. 0.75 ml of phosphoric acid (0.44 mol/l) solution was added into the tubes and vortex-mix. Then 0.25 ml of TBA (42 mmol/l) was added into each tube. Distilled water (0.50 ml for reagent blank, 0.45 ml for TEP standard, plasma and control samples) was added to adjust the final volume to 1.5 ml. The test tubes were tightly capped and heated at 100 °C for 60 minutes after the sample was cooled in an ice water bath (0 °C). The optical density of the pink chromogen was read at 532 nm in a double-beam spectrophotometer (Spectro 22, a spectrophotometer work in the Visible, Near-Ultraviolet, and Near Infrared Spectral Range, Biomed, USA) [45].

5. Lipid hydroperoxide by FOX assay

Total plasma hydroperoxide concentrations were measured using method as described by Nourooz-Zadeh and Lester [6, 35] with minor modifications.

5.1 Reagent preparation of Fox assay

5.1.1 Fox reagent 1000 ml : Dissolve 0.07 g of xylenol orange. 0.01 g of ammonium ferrous sulfate, and 18.22 g in sulfuric acid 1000 ml

5.1.2 Butylated hydroxytoluene (BHT) and triphenyl phosphine (TPP) reagent 250 ml. Dissolve 22.04 g of BHT, and 0.66 g of TPP in methanol 250 ml

5.1.3 Preparation standard cumene hydroperoxide : Stock standard solution 1000 µM (Dissolve 55 µl cumene hydroperoxide in methnol 100 ml), working standard solution 100 µM, and dilute 2- fold from working standard solution 100 µM.

5.2 Method

90 µl of sample/standard was taken in test tubes. Add 10 µl TPP and 400 mmol/l BHT and mix well. Incubate for 15 minutes and add 900 µl of Fox reagent and vortex. Incubate 30 minutes at room temperature and measurement absorbance at 560 nm by spectrophotometer.

6. Spectrophometric assay for urine N-acetyl-β-D-glucosaminidase activity

6.1 Reagent preparation of Spectrophometric assay for urine N-acetyl-β-D-glucosaminidase activity

6.1.1 Citric acid solution 0.2 mol/L: dissolve 8.4 g of citric acid monohydrate in distilled water in a 200 mL volumetric flask, and store at 4 °C.

6.1.2 Sodium citrate solution 0.2 mol/L: dissolve 10.3 g of anhydrous trisodium citrate in distilled water in a 200 mL volumetric flask, and store at 4 °C.

6.1.3 Citrate buffer, pH 4.4, 0.1 mol/L: put 56 mL of citric acid solution and 44 mL of sodium citrate solution and allow the solution at 37 °C in water bath. Check the pH with pH meter and adjust to pH 4.40 (±0.05) by add citric acid solution and sodium citrate solution. Transfer the solution to 200 mL volumetric flask, dilute with distilled water and store at 4 °C.

6.1.4 Nag- substrate solution, 10 mmol/L: dissolve 342 mg of p-nitrophenyl-N-acetyl-β-D-glucosaminide in citrate buffer anddilute to the mark. This solution in 10 mL aliquots into screw capped and store the tube at -20 °C. Before use, thaw the NAG substrate solution and equilibrate it in a water bath at 37 °C.

6.1.5 AMP buffer, pH 10.25, 0.75 mol/L: dissolve 18.84 g of 2-amino-2-methyl-1-propanol hydrochloride in 50 mL of distilled water in 200 mL volumetric flask. Adjust the pH to 10.25 at 25 °C with a pH meter and adjust by adding NaOH, 6 mol/L solution and store at 4 °C.

6.1.6 p-Nitrophenol working standard solution, 100 µmol/l, transfer 1 mL of 10 mmol/L p-Nitrophenol stock standard solution into 100 mL volumetric flask and dilute to the calibration mark with NaCl solution. Prepare this solution immediately before use.

6.2 Method

Reagent blank, 0.15 mol/L NaCl solution, standard, p-Nitrophenol working ans add 100 µl of sample/standard/reagent blank was taken in ELISA plate. Add 100 µl NAG substrate in reagent blank, standard, and sample. and mix well. Incubate 37 °C for 15 minutes and add 100 µl of AMP buffer to all of the cuvette, and add 100 µl. NAG substrate to sample blank. And measurement absorbance at 405 nm using ELISA reader.

Statistics

The statistical analysis was performed by SPSS version 13 software. Volunteer's data are parametric statistic with normal distributions according to post hoc test differences between variable were using one way ANOVA. Simple correlation test were used to determine relationships between covariates. All data were expressed as mean ± SD. For variables used to evaluate the primary aim, results are also presented with 95% confidence intervals (95% CIs).