CHAPTER II

MATERIALS AND METHODS

MATERIALS

Beakers 100,250,500, and 1000 ml

Measuring volumetric flask 100, 250, 500, and 1000 ml

Cylinder flask 100, 250, 500,and 1000 ml

Pasture pipettes

Glass funnel

Cuvettes 1 ml

Spectrophotometer (Spectronic Henesys 5.)

Titertek Multiskan M 340 multiple reader

Microcentrifuge (Denver Instrument company, U.S.A)

HPLC (Consta Meric LDL Analyzer) U.S.A

Autopipetter 100-1000 µl, 10-100 µl, 20-200 µl

Glass pipettes 5 and 10 ml

Immuno Plate Maxisorp 96 F, (No. 442404) (NUNC, Denmark)

Waters Spherisorp 5 µm ODS2 4.6X250 mm Analytic cartidge

Spherisorp Guard Cartridge ODS2 (WSPSS83005)

Extended end fitting (WSPSS61410)

Pre-cut membrane Nylon, 0.45 um, 47 mm. (LMNY50470)

Micro Syringe HPLC-Rheodyne 250 µl (EXMSR250) (Japan)

Mucus Extractor # 6 (TGF-G-5006)

Filter membrane PTFE, 0.45 µm, 13mm (LMPT501300)

Microcentrifuge tube 1.5 ml

REAGENTS

The chemicals and reagents are analytical grade or purest as available.

5,5'-Dithio-bis (2-Nitrobensoic Acid) (DTNB) (D8130,Sigma)

Glutathione free acid std (G251, Sigma)

Thiobarbituric acid (TBA) (T5500, Sigma)

2-Amino-2-hydroxy methyl-1,3-propanediol (T6791)

Alpha-tocopherol acetate (T3001,Sigma)

n-Hexane HPLC grade (C2516, Lab-s)) 95%

Ethanol absolute AR 2.5 L (100983-250, Merck)

Dichloromethane HPLC grade (C2510L, Lab-s)

Methanol HPLC grade (C2517, Lab-s)

Mouse Anti Biotin-HRP Conjugate (No. 03-3720, Zymed, U.S.A)

HA umbilical cord (Sigma, Aldrich)

Bovine serum albumin (BSA)

EAH sepharose TM 4B (50 ml) (17-0569-01)

Hyaluronidase (Sigma, Aldrich)

HA standard (Healon) (Pharmacia & Upjohn (Uppsala, Sweden)

Peroxidase-mouse monoclonal anti-biotin (Zymed Laboratory, Inc. CA, USA)

Guanidine HCL (Sigma, Aldrich)

Trypsin inhibitor (Sigma, Aldrich)

$NaHCO_3$ (Merk, Darmstadt, F.R. Germany)

REAGENTS PREPARATION

Reagent for anticoagulant (Acid citrate dextrose: ACD)

Reagents for malondialdehyde (MDA) determination in TBARs-assay

Trichloroacetic acid (TCA) reagent. (100%)

Thiobarbituric acid (TBA) reagent

Normal saline solution (0.85)

Stock MDA standard (100 μM)

Reagents for glutathione determination (DTNB-method)

Precipitation solution

Phosphate solution (0.3 M, pH 7.4)

5,5-dithiobis-(2- nitrobenzoic acid) ,DTNB reagent

GSH standard (100 μg/ml)

Reagents for alpha-tocopherol (vitamin E) determination

Stock n- hexane solution.

Methanol (Analyzed grade for HPLC)

Absolute ethanol solution

7% (v/v) of dichoromethane

Standard alpha-tocopherol (100 mg/L)

Reagents for Hyarulonic acid (HA) determination

Phosphate buffer saline (PBS 10X) (pH 7.4)

0.05 M Tris buffer phsophate-Tweeen 0.1% solution (pH 8.6)

Citrate phosphate buffer (pH 5.0)

6 % Bovine serum albumin (BSA)

OPD substrate solution

Stock coating HA (1 mg)

Hyaluronan stock

Biotinylated HABP

Peroxidase-mouse monoclonal anti-biotin solution (1:2000)

Stop reaction solution (4M H_2SO_4)

METHODS

Subjects (see appendix, Picture 1)

The patients were examined, diagosis and suggested by clinicians. The parents of subjects had written informed consent before treatment and participation, they were explained about the normal treatment, advantages and the processes of study. All patients who were exclusion criteria were checked for a risk of severe anemia, low of platelets count, or clinical worse with superivisor by clinicians. Physical therapist cooperative performed with nurses and doctors in the ward to collecting of the samples and follow up all cases. All subjects were divided by randomize to two groups, for chest physical therapy in treatments A and B.

Information about previous history, medical evaluation, and treatment were recorded from medical chart and computer data. Medical treatment about ventilating setting and arterial blood gases as FIO_2, Tidal volume, and ventilator pressure as PEEP, PaO_2 and $PaCO_2$, were recorded for six days. Additionally, laboratory data were collected for clinical chemistry and hematology.

The method of physical therapy began with assessment for the pulmonary disorder and localized lesion in the lung which was comfirmed by chest X-ray films. Treatment was performed with different methods, A and B as following;

Group A: Treatment A (see appendix, Picture 3)

The sequence of treatment was following as:

- Positioning for postural drainage approximately 5 to 15 minute for each lesion, and total time of treatment equal or less than 20-30 minutes

- Percussion and vibration with various frequency that depends on the patients during postural drainage.

- Manual hyperinflation was done during suction for remove secretion from tracheal airway.

Group B: Treatment B (see appendix, Picture 4)

The sequence of treatment was following as:

- Aerosol therapy with Normal saline solution by MDI for 5 to 10 minutes before treatment with postural drainage, percussion, vibration and suction.

- Position for postural drainage approximately 5 to 15 minutes for each lesion, and total time of treatment equal or less than 20-30 minutes

- Percussion and vibration with various frequency that depends on the patients whatever using postural drainage.

- Manual hyperinflation was done during suction for remove secretion from tracheal airway.

Frequency of treatments per day was three times, it depends on the severity and demanded for treatment. All patients were treated within 10-30 minutes for each visiting.

PREPARATION METHOD

Samples Preparation

A 2.8 ml of the blood was separated from the blood puncture in routine investigation or new draw from venous vein into the micro-centrifuge tubes. A tube with 0.2 ml of anticoagulant (ACD) was prepared for 1.3 ml of blood, and the other tube was used for 1.5 ml of blood.

The 0.4 ml of blood with ACD was analyzed for GSH concentration and the residual blood was centrifuged at 3,000 g for 5 minutes, and separated the plasma for analysis the alpha-tocopherol later by keeping in refrigerator at -20 °C.

The 1.5 ml of blood without ACD or clot blood was centrifuged at 3,000 g for 5 minute and separated the serum to analyze the level of MDA and urea concentration by freezing at - 20 °C.

Standardized procedure of tracheal aspirates (TA) was performed by collection from routine suction with 100 mmH_2O of negative pressure. TA was aspirated into a sterile specimen trap, mucus extractor # 6 (Endomed company limited) with a few of 0.9% sodium chloride. TA samples were kept in freezer at -20 °C. The collection of the TA was between 6.00 am. to 9.00 pm every day. After collection, TA were mixed and kept in microtubes in the refrigerator.

The TA samples were determined for the levels of GSH and MDA within a week, and separated some amount to analyze the alpha-tocopherol and HA concentrations later by freezing them below -20 °C until testing.

ANALYTICAL METHODS

The Thiobarbituric acid reactive substances (TBARs) Assay (Santos, et al., 1980)

Principle

The Thiobarbituric acid (TBA) test is used for measuring the peroxidation of fatty acid. Aldehydes are produced as lipid peroxide products of metabolism in biological system. Malondialdehyde (MDA) is the intermediate substance of lipid peroxidation which is the most abundant aldehyde and interact with thiobarbituric acid (TBA) giving the pink colored product as the following;

$$\text{TBA} + \text{MDA} \xrightarrow{\text{heat}} \text{TBA-MDA adduct} + 2\,H_2O$$

The TBA-MDA adduct product can be detected by measuring with spectrophotometer at 532 nm.

Procedure

Sample preparation and incubation

1. Add 0.45 ml of NSS to 0.1 ml of plasma or Tracheal aspirates (TA)
2. Add 0.2 ml of TBA reagent and then add 1.0 ml of TCA reagent and mix
3. Incubate in 95-100 °C water for 30 minute in the water bath.
4. Cool the tubes on cold water and add 3 ml of distill water
5. Centrifuge at 3,000 rpm for 10 minutes
4. Transfer the supernatant to a cuvette and read OD at 532 nm.

Preparation of standard curve. (see appendix, Figure 34)

Standard MDA was prepared by diluting the stock 100 μM TMP that hydrolyzed with strong acid, with distilled water yielding 10, 15, 30, 35 and 40 μ.M and keeping the working standard MDA at -20 °C and dark.

Modified DTNB-method (Beutler, et al., 1963)

Principle

A simple photometric procedure was using 5,5'-dithio bis- (2-nitrobenzoic acid) (DTNB). Only three stable reagents, including precipitating solution, phosphate reagent and DTNB reagent, are required for the reaction postulated to be as follow:

GSH + DTNB ⟶ GSTNB + 2-nitro-, 5-thiobenzoic acid (TNB)

GSH + GSTNB ⟶ GSSG + 2-nitro-, 5-thiobenzoic acid (TNB)

The first reaction product in the equation is yellow color and can be measured at 412 nm.

Procedure

1. Pipette 0.4 ml of whole blood or TA into test tube and add 1.6 ml distilled water, mix to hemolyze.

2. Add 3.0 ml of precipitating solution and mix.

3. Allow to stand 5 minutes at room temperature or centrifuge at 2000 g for 10 minute and then filter through coarse-grade filter paper Whatman No.1.

4. Duplicate 1 ml of supernatant from filtration and add phosphate buffer 4 ml

5. Vortex after adding 0.5 ml of DTNB reagent and read absorbance at 412 nm within 5 minutes.

6. Calculation the GSH in blood and in TA.

$$\frac{\text{(Optical density of the test) X (standard GSH concentration) X 12.5 (Dilution factor)}}{\text{Optical density of the standard}} = \text{mg/ dl of whole blood}$$

or calculate in mg/dl of erythrocyte

$$= \frac{\text{mg/dl of whole blood}}{\text{Hct / 100}}$$

Preparation of standard curve. (see appendix, Figure 36)

Stock standard GSH at 100 µg/ml was prepared by mixing 0.001 mg of pure standard GSH (Sigma) in 100 ml of distilled water. Working standard GSH at 20, 30 and 40 µg/ml were prepared by diluting with distill water.

Competitive Inhibition ELISA technique (Pothacharoen, 2000)

Principle:

Hyaluronic acid (HA) concentrations in serum and TA were determined by ELISA based-assay. The HA in sample or standard (6% BSA-PBS) was incubated with biotinylated HA-binding proteins. The levels of B-HABP, which bound to the cells, was determined by the addition of peroxidase conjugated anti-biotin

antibody, followed by peroxidase substrate (OPD) and the absorbance at 492/690 nm was determined by a microplate reader.

Procedure:

1. Microtiter plates (Maxisorp, Nunc) were coated overnight at 4 °C with umbilical cord HA (100 µg/well) in coating buffer.

2. Blocked with 150 µl/well of 1 % (w/v) BSA in incubating buffer for 60 minutes at 25 °C.

3. Washing with 150 µl of PBS-Tween three times and drying.

4. Add 100 µl of the mixture, samples or standard competitor (HA healon; range 3.9-1000 ng/ml) in B-HABP (1:200) and incubating for 60 minute at 25 °C.

5. Plates were washed three times with 150 µl PBS-Tween buffer and drying.

6. Peroxidase-mouse monoclonal anti-biotin (100 µl/well:1:2000) was added and incubated for 60 min, 25 °C.

7. The plates were washed three times again with PBS-Tween buffer.

8. Peroxidase substrate (100 µl/well), OPD was added and incubated at 37 °C a few time for color development.

9. Stop reaction by adding of 50 µl/well of 4 M H_2SO_4. The absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader.

10. Calculation: Each TA samples was diluted with PBS 1000 time. The concentration of HA was compared with HA standard control (from 10000 ng/ml to 19.53 ng/ml) in a same plate. Therefore, the concentration of HA in TA had 1000 time.

Preparation of standard HA concentration. (see appendix, Figure 39)

The standard HA concentration was determined parallelly with samples by making two fold diluting concentration from 1000 ng/ml with PBS.

Alpha-tocopherol determination by HPLC method (Shearer, 1986)

Principle:

Alpha-tocopherol is found in both plasma and cellular elements of blood, includes erythrocytes, and platelets. In plasma, vitamin E is transported with lipoproteins and measured with a reverse phase, high performance liquid chromatography (RP-HPLC). In this study, spherisorb ODS-2 instead of μ-Bondapack C 18 column was used for analyzing the concentration of Vit E.

Procedure:

1. Pipette 100 μl of plasma or TA into a micro-centrifuge tubes.

2. For testing in serum; Add 100 μl of a solution of alpha-tocopherol (Sigma) in ethanol (60 mg/L) into serum sample as the internal standard and vortex mix for 30 second.

3. For the TA samples; add 100 μl of absolute ethanol and vortex mix for 30 second.

4. Pipette 200 μl of n-hexane as the extraction solvent into the tube and vortex mix vigorously for 30 second and shaker and shake the content for 10 minutes.

5. Centrifuge for 5 minutes and separate 100 μl of an upper hexane layer from a lower aqueous ethanolic layer.

6. Transfer the upper hexane layer, containing the alpha-tocopherol and internal standard alpha-tocopherol, tapped tubes and evaporate to dryness with

speed vaculator at room temperature for about one and half hours amd kept at -20 °C until analysis.

7. Lyophilized sample was reconstituted in 200 µl of absolute ethanol, mixed vigorously and manually filtered through the 0.25 µm pre-cut Nylon membrane. A 50 µl of readily filtered sample was analyzed for Vit E concentration using the HPLC based-assay.

8. For the TA samples, before injection, add 100 µl of external standard alpha-tocopherol (100 mg/ml) was spiked into the sample before injection.

9. Calculation:

The concentration of serum and TA were converted from OD values that compared with the OD of standard alpha-tocopherol at 60 mg/L and 100 mg/L respectively. The dilution factor in this assay was 4. (see appendix, Figure 42-43)

Preparation of standard curve of alpha-tocopherol curve.

The purity of alpha-tocopherol at a finial concentration of 100 mg/L derived from stock standard alpha-tocopherol concentration (1000 mg/L) was analysed

$$E^{1\%}_{cm} = 71 \quad (294 \text{ nm})$$

$$\text{Purity} = \frac{\text{(Optical density of the test)}}{0.71}$$

On the other way, standard alpha-tocopherol at 100 mg/L was prepared by diluting 1 ml of stock solution (10 g/L) with 9 ml of absolute ethanol and kept in dark at - 20 °C (see appendix, Figure 40)

EVALUATION OF PRECISION AND ACCURACY OF THE ASSAY

Precision test

Twenty aliquots of pooled whole bloods, plasma, serum, and tracheal aspirates were used to assess the precision of each assay. Inter-assay precision was determined by using standard of MDA at 20 μM, whole blood and serum.

Recovery test

The analytical recovery of whole blood GSH, serum MDA, plasma alpha-tocopherol and serum HA was determined by using pooled whole blood, plasma and serum which has been added with known amount of standard GSH, MDA, alpha-tocopherol and HA concentrations.

STATISTIC ANALYSIS

Data are shown as mean±SE. Normal distribution of the data was previously checked using the Kolmogorov-Smirnov test.

Two way ANOVA was used to compared the concentrations of whole blood GSH, serum HA, plasma Vit E, serum HA, lung injury score and oxygenation index (PaO_2/FiO_2) on the first day of treatment with those on the sixth day of treatment with physical therapy.

The concentrations of whole blood GSH, serum MDA, plasma alpha-tocopherol and HA in blood and TA, lung injury score and oxygenation index on the 1st day were analyzed for correlation by Pearson correlation statistic analysis. The concentrations of GSH, MDA, alpha-tocopherol and HA on the first day and sixth day were compared using related paired t-test. P less than 0.05 (two-tail test) was considered significant.