CHAPTER III

MATERIAL AND METHODS

SUBJECTS AND METHODS

The subjects were 20 outpatients associated with nephrotic syndrome aged ranging from 12 to 39 years old (mean 21 yrs); 10 patients with mesangial proliferative nephrotic syndrome (MesP-NS), 10 patients with focal segmental glomerulosclerosis (FSGS) and 10 controls aged ranging from 10 to 40 years old (mean 20 yrs).

Inclusion criteria

All patients are idiopathic nephrotic syndrome with consent form and approval by the Ethical Committee who have complete renal functional studies, kidney biopsy and have minimal follow-up of one year duration.

Exclusion criteria

All secondary forms of nephrotic syndrome and who does not meet the above criteria.

Endothelial cell cytotoxicity and antioxidative defense were assessed as described in detail as follow.

Patients with enhanced endothelial cell cytotoxicity and antioxidative defect received treatments in accordance with the clinical severity. The initiation of treatment is dependent upon the initial assessment of magnitude of endothelial cell cytotoxicity, the incidence of oxidant and antioxidant imbalance, and the degree of renal functional impairment. For instances, the MesP-NS patients with mild degree of endothelial cell cytotoxicity, magnitude of oxidant and antioxidant imbalance and mild impairment in renal function were treated with enalapril 5-10 mg/day, vitamin C 1,000-3,000 mg/day, vitamin E 400-800 IU. The FSGS patients generally associated with greater magnitude of endothelial cell cytotoxicity, and moderately impaired renal function were treated with enalapril 10-20 mg/day, vitamin C 1,000-3,000 mg/day and vitamin E 400-800 unit/day. The doses of therapeutic agents were titrated in accordance with the clinical indices namely the level of endothelial cytotoxicity, the level of oxidant and antioxidant status and the status of renal function namely creatinine clearance and FE magnesium and with the clinical response to treatment i.e. the vasodilator dose was increased to maintain the blood pressure within the normal range, and / or to improve the renal function as much as possible.

Treatment received for a period of at least 12 months.

Six weekly follow-up was performed to assess the following parameters : blood pressure, blood biochemistry, 10-hour urinary examination for urinary protein excretion, creatinine clearance and urinary magnesium excretion.

Reassessment of endothelial cell cytotoxicity and antioxidative defense were performed at 12 weeks.

1-year follow-up of renal plasma flow (RPF) and glomerular filtration rate (GER) were determined in severe nephrosis (FSGS).

I. Endothelial Cell Cytotoxicity

We performed an endothelial cell cytotoxicity test using sera from nephrotic patients as previously described[48]. In brief, the human endothelial cell line ECV 304 (American Tissue Culture Collection) in medium 199 with 10% fetal bovine serum, approximately 2 x 10^4 cells / well of 96-well tissue culture plates was incubated overnight at 37°C in a 5% CO_2 atmosphere. Sera from nephrotic patients were added in duplicate wells. The culture medium and 10% Triton X were used as controls that showed no cell lysis and 100% cell lysis, respectively. The testing cultures were incubated as above for an additional 48 h. After incubation, each well was washed with phosphate-buffered saline and then stained

with crystal violet. The stained cells were lysed with acid alcohol solution, and the optical density (OD) was determined by using a microtiter plate reader (model 3550; Biorad) at 550 nm. The percentage of cytotoxicity was calculated by :

$$\text{Percent cytotoxicity} = 1 - \frac{OD_{testing} - OD_{Triton\ X}}{OD_{control} - OD_{Triton\ X}} \times 100$$

II. Oxidant and Antioxidant Study

Twelve millititers of blood was drawn from the vein. Blood was placed into heparinized tube and centrifuzed at 1,000 g for 10 minutes. The supernatant layer was removed and assayed for malondialdehyde (MDA), glutathione (GSH), vitamin C and vitamin E.

A. Lipid Peroxidation (MDA)

Malondialdehyde (MDA) was asssessed by thiobarbituric acid (TBA) colorimetric assay of hydroperoxides. The TBA assay was performed using a modification of the technique described by Askawa and Matsushita[49]

B. Glutathione (GSH)

Determination of glutathione in the erythrocytes was made by colorimetric methods of Beutler E et al[50] using the glutathione disulfide reductase-DTNB [5-5'-dithiobis (2-nitrobenzoic acid)] to react with sulhydril compound and yield a stable yellow color. GSH concentration in the erythrocytes was expressed as μ mol/g Hb[51]. The hemoglobin concentration was assayed by using a cyanmethemoglobin technique.

C. Vitamin C

Ascorbic acid in serum and plasma was determined by specific enzymatic spectrophotometric method. Samples were analysed indirectly by measuring the absorbance at 593 nm[52].

D. Vitamin E

Vitamin E was assayed by the modified Emmeric and Engle's method[53]. The oxidation of xylene-extracted tocopherols from the blood sample by ferric chloride and the red colored complex of ferrous ions with bathophenanthroline was measured colorimetrically at 536 n.

III. Renal Function Studies

A. Glomerular Function

A glomerular filtration rate (GFR) was performed by the radioisotope technique using ^{99m}Tc-labeled diethylene triamine pentaacetic acid (DTPA) or 10-hour endogenous creatinine clearance (CCr) and the value was converted to the body surface area of 1.73m^2 by the method of calculation.

$$\text{Body surface area} = \frac{\text{Body weight (kg) x 4 + 7}}{\text{90 + body weight (kg)}}$$

B. Tubular Function

Tubular transport was assessed by a 10-hour urinary collection after early dinner. Blood drawn at the end of the test and urine were analysed for creatinine, magnesium and protein. A reflection of tubular transport was derived from the determination of fractional excretion (FE) of filtered magnesium (Mg). The FE Mg was calculated through the formula

$$\text{FE Mg} = \frac{^{U}/_{P}\ \text{Mg}}{^{U}/_{P}\ \text{creatinine}} \times 100\%$$

The normal value for FE Mg is $\leq$ 2.2%

C. Vascular Function

Simultaneous assessments of effective renal plasma flow (RPF) using ^{131}I-labeled para-aminohippurate and of glomerular filtration rate (GFR) using ^{99m}Tc-labeled DTPA had been accomplished in all of these 19 patients during pre-treatment period as well as during subsequent post-therapy in all of these 8 FSGS. Intrarenal hemodynamics were calculated and based on modified Gomez's equation. Clinical data such as mean arterial pressure (MAP) was strictly recorded as previously described[54]. For calculation purpose, the effective filtration pressure across the glomerular capillary (PF) is assumed to be 35 mm Hg when the blood pressure is normal (BP 120/80 mm Hg or less) and 40 mm Hg when the blood pressure is high (BP > 120/80 mm Hg). The hydrostatic pressure in Bowman's space (Ht) is assumed to be 10 mm Hg, the RPF and GFR are in ml/s/1.73m^2. From the above assumptions, the following equations are derived

$$GFR = KFG \times PF \quad \text{.....(1)}$$

where KFG, PF are the gross filtration coefficient of glomerular capillaries (ml/s/mm Hg) and effective pressure across the glomerular capillaries respectively.

$$PG = \frac{GFR}{KFG} + Ht + 5\left(\frac{TP}{FF} \times \log \frac{1}{1 - FF} - 2\right) \quad \text{.....(2)}$$

where PG, Ht, TP, FF are glomerular hydrostatic pressure, hydrostatic pressure in Bowman's space, plasma total protein (gram) and filtration fraction respectively

$$RA = \frac{MAP - PG}{RBF} \times 1328 \quad \text{.....(3)}$$

where RA, MAP, RBF are afferent arteriolar resistance in $dyne.s.cm^{-5}$, mean arterial pressure = diastolic pressure + $^1/_3$ pulse pressure and renal blood flow $\frac{RPF \times 100}{100\text{-hematocrit}}$ respectively

$$RE = \frac{GFR}{KFG \times (RBF - GFR)} \times 1328 \quad \text{.....(4)}$$

where RE is efferent arteriolar resistance in $dyne.s.cm^{-5}$

STATISTICAL ANALYSIS

All results are expressed as mean $\pm$ SD. The datae were analysed using analysis of variance (ANOVA) and Student's paired t-test. The difference was statistically significant when the P value was less than 0.05.