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Ganoderma lucidum suppresses endothelial cell cytotoxicity and proteinuria in persistent proteinuric focal segmental glomerulosclerosis (FSGS) nephrosis

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Abstract. A persistent proteinuria is commonly observed in nephrotic patient with focal segmental glomerulosclerosis (FSGS) under treatment with prednisolone ± cyclophosphamide or with vasodilators (ACEI + AII receptor antagonist, calcium channel blocker and antiplatelet agent). Fourteen such patients with persistent proteinuria were subject to be treated with *Ganoderma lucidum*. Initial study revealed an enhanced endothelial cell cytotoxicity induced by patient's serum, and an altered immunocirculatory balance with predominant proinflammatory cytokine TNF alpha activity in the presence of defective anti-inflammatory cytokine interleukin-10. Treatment with *Ganoderma lucidum* suppressed endothelial cell cytotoxicity, restored immunocirculatory balance and successfully suppressed proteinuria in all of these 14 patients.

Keywords: Endothelial cell cytotoxicity, focal segmental glomerulosclerosis (FSGS), proteinuria immunocirculatory balance, *Ganoderma lucidum*

1. Introduction

A persistent proteinuria and reduction in renal perfusion are generally encountered in nephrotic patients associated with focal segmental glomerulosclerosis (FSGS). Treatment with combined formula consisting of ACEI, AII receptor antagonist, calcium channel blocker and antiplatelet agent have been demonstrated to improve the renal perfusion and restore the renal function. However, such treatment has a minor effect on the clinical persistence of proteinuria in many patients [1,2]. Although the mechanisms for such persistent proteinuria remain to be further elucidated, multiple factors, such as injury

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to podocyte, oxidative stress and hemodynamic maladjustment, have been proposed [3–7]. Recently, a glomerular endothelial dysfunction with enhanced endothelial cell cytotoxicity is documented in FSGS nephrosis [8]. Also, an immunocirculatory disturbance, associated with enhanced proinflammatory cytokine TNF alpha in the presence of defective anti-inflammatory cytokine interleukin-10, is detected in both serum and T-lymphocyte of patients with FSGS nephrosis [9,10]. The defective immunocirculatory balance is likely to induce a prolonged inflammatory effect upon glomerular endothelial function, and this may be responsible for the persistent proteinuria commonly encountered in this refractory to-be-treated group of nephrosis. The associations between enhanced endothelial cytotoxicity, altered immunocirculatory balance and persistent proteinuria documented in these FSGS patients appear to be an interesting observation. A search for therapeutic agents to suppress the proteinuria in this refractory group of patient would be desirable. In this regard, *Ganoderma lucidum* is selected to serve for this purpose because it has many pharmaceutical effects and has long been used as a home remedy. In fact, *Ganoderma lucidum*, is known to inhibit angiotensin converting enzyme activity [11] with immunomodulatory property [12]. Moreover, it is shown to protect adriamycin induced cytotoxicity in rats [13]. Since adriamycin can induce nephrotic proteinuria in experimental model in animal [5], it would be interesting to see whether *Ganoderma lucidum* can suppress proteinuria in clinical setting of human nephrosis associated with FSGS. In fact, a pilot study has recently demonstrated that *Ganoderma lucidum* can suppress proteinuria in 5 FSGS patients who have been associated with altered immunocirculatory balance and enhanced endothelial cytotoxicity [1]. The aim of this study, therefore, is to extend the observation of the effect of *Ganoderma lucidum* in other FSGS patients and also to study the mechanism of such proteinuria suppression.

2. Material and method

Fourteen patients with biopsy confirmation of FSGS with moderate degree of tubulointerstitial fibrosis who presented with persistent proteinuria under the treatment of prednisolone $\pm$ cyclophosphamide and vasodilators (ACEI + AII receptor antagonist, calcium channel blocker, antiplatelet agent) were included. The study was approved by the ethical committee of the Institution and the patients gave their informed consent. All patients had moderately impaired renal function and the degree of tubulointerstitial fibrosis correlates with the fractional excretion of magnesium [14]. With respect to the persistent proteinuria, we prospectively treated all these 14 patients with *Ganoderma lucidum*. They were subjected to the following investigations and treatment.

2.1. Endothelial cell cytotoxicity test

An endothelial cell cytotoxicity test using sera from nephrotic patients was performed as previously described [15]. In brief, the human endothelial cell line ECV 304 (American Tissue Culture Collection) in medium 199 with 10 per cent fetal bovine serum approximately 2×10^4 cells/well of 96-well tissue culture plates was incubated overnight at 37°C in a 5 per cent CO_2 atmosphere. Sera from nephrotic patients were added in duplicate wells. The culture medium and 10 per cent Triton X were used as controls that showed no cell lysis and 100 per cent lysis, respectively. The testing cultures were incubated as above for an additional 48 hours. 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 using the formula as follows:

$$\% \text{ cytotoxicity} = 1 - \frac{\text{OD}_{\text{Testing}} - \text{OD}_{\text{Triton X}}}{\text{OD}_{\text{Control}} - \text{OD}_{\text{Triton X}}} \times 100.$$

2.2. Cytokines study

TNF alpha and interleukin-10 were measured in plasma in accordance with manufacturer's recommendations. TNF alpha and interleukin-10 were determined with commercially available ELISA kits (Predicta, provided by Genzyme Corporation, Cambridge, MA, USA). In brief, 50 μl of buffered protein base (sample deluent) was placed in each well; then 50 μl of standard or sample was added, incubated at 37°C for 90 minutes and washed with 400 μl of buffer. Thereafter, 100 μl TNF alpha biotinylated antibody was added into each test well and incubated at 37°C for 30 minutes. After washing, 100 μl of TNF alpha streptovidin reagent was added into each test well and incubated at 37°C for 15 minutes. After washing, 100 μl of working substrate solution was added into each test well and incubated for 10 minutes. The reaction was stopped with 100 μl of stop solution. The optical density was determined at 450 nm within 30 minutes after the reaction had been stopped results were expressed as pc/ml.

2.3. Glomerular function

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

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

2.4. Tubular function

Indirect tubular transport was assessed by a 10-hour urinary collection as previously described [14]. Diuretic was not administered during or within 24 hours before the test. Briefly, after a regular supper, no additional food except drinking water ad lib was allowed. The patients were instructed to void at 7 PM, and then urine was collected from 7 PM to 5 AM. Clotted blood from venipuncture was drawn at the end of the test for the analysis of creatinine and magnesium levels. Urine samples were analyzed the same as blood samples by the Renal Metabolic Laboratory Unit. Analyses of (i) creatinine was determined by the method described by Faulkner and King and (ii) magnesium was determined by atomic absorption spectophotometer (model 1100 G; Perkin Elmer, Norwalk, CT). A reflection of tubular transport was derived from the determination of FE Mg which was calculated through the formula:

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

2.5. Mode of therapy

In addition to the present medication consisting of (i) vasodilators namely angiotensin converting enzyme inhibitor (enalapril 10–40 mg/day), calcium channel blocker (isradipine 10–20 mg/day), dipyridamole 50–100 mg/day plus baby aspirin 1 gr/day, and with or without AII receptor antagonist (Losartan 50–100 mg/day). (ii) vitamin C 1000–3000 mg/day and vitamin E (400–800 IU/day), *Ganoderma lucidum* 900–1125 mg/day was additionally given to each of these 14 patients.

2.6. Statistical analysis

Values in text are expressed as mean $\pm$ SEM. The difference between pre- and post-treatment was performed by the Student's paired t-test. The difference was statistically significant when the p value was less than 0.05.

3. Results

A significant elevation of endothelial cell cytotoxicity and altered immunocirculatory balance with predominant proinflammatory cytokine TNF alpha were noted prior to the treatment (Table 1). Following treatment, endothelial cell cytotoxicity and immunocirculatory disturbance were converted back to normal. Table 2 showed altered renal function with decreased creatinine clearance, elevated FE Mg and total urinary protein. Following treatment, improvements in renal function were achieved with statistic significance.

Table 1
The effect of *Ganoderma lucidum* on the endothelial cell cytotoxicity and the immunocirculatory balance (cytokine studies)

	Initial	Normal	Follow-up after *Ganoderma lucidum*	P value
Endothelial cell cytotoxicity (%)	28.7 ± 11	1 ± 0.6	1.9 ± 3	<0.001
Immunocirculatory balance (TNF alpha/interleukin-10 ratio)	3.7 ± 1.2	2.5 ± 0.2	2.2 ± 0.4	<0.001

Table 2
The effect of *Ganoderma lucidum* on the renal function

	Initial	P value	Follow-up after *Ganoderma lucidum*	Normal
Creatinine clearance (ml/min/1.73m$^{2)}$	50 ± 24	<0.001	70 ± 23	120
Fractional excretion of magnesium (FE Mg) (%)	6 ± 3	<0.001	3.4 ± 2	1.6 ± 0.6
Total urinary protein (g/24)	2.2 ± 1.6	<0.001	0.2 ± 0.3	<0.3

4. Discussion

The result of this study indicates that *Ganoderma lucidum* can effectively suppress proteinuria in these nephrotic patients who had enhanced endothelial cell cytotoxicity and altered immunocirculatory balance. An enhanced endothelial cell cytotoxicity induced by the patient's serum in the presence of predominant activity of TNF alpha may reflect TNF alpha inducing glomerular endothelial dysfunction. A dysfunctioning glomerular endothelium favors proteinuria due to the loss of negatively charged surface (charge-selective proteinuria) [16]. In addition, a dysfunctioning glomerular endothelium also induces size-selective proteinuria secondary to the hemodynamic maladjustment which preferentially constricts the efferent arteriole. The hemodynamic maladjustment also induces podocyte detachment from the glomerular capillary basement membrane [2]. Such podocyte injury secondary to the hemodynamic mechanism as well as its direct toxic effect from the TNF alpha would exert additional mechanism inducing protienuria. The suppression of proteinuria in conjunction with the conversion of immunocirculatory balance back to normal as well as the suppression of endothelial cell cytotoxicity implies their cause – and – effect relationships even though the actual mechanism of such suppression of proteinuria remains to be determined. Nevertheless, *Ganoderma lucidum* possesses several known herbal components namely polysaccharides, triterpenoids, Ling Zhi-8 and nucleosides. The suppressive effect of proteinuria may work through their biologically active ingredients consisting of immunomodulating, anti-inflammatory, antioxidant and vasodilating effects [17–20]. Thus, the persistent proteinuria and the reduction in renal perfusion which are the common threats in nephrosis associated with FSGS can be corrected by *Ganoderma lucidum* and vasodilators respectively.

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