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Original Article

Effect of *Salvia miltiorrhiza* Combined with Aromatic Amino Acids on the Impaired Renal Function in Rats with Chronic Renal Failure

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ABSTRACT

Background and Aim: At present, the main therapy for chronic renal failure (CRF) is dialysis and renal transplantation, but neither obtains satisfactory results. *Salvia miltiorrhiza* (SM) is a very popular medicinal plant that has been extensively applied to treat various diseases. It also has been reported that lowering the aromatic aminoacids (AAA) in the protein diet can improve renal function in rodents with CRF. This study seeks to employ the use of SM combined with low aromatic amino acid diet (LA-AAD) for treatment of CRF in rats. **Methods:** Animals were assigned into 3 groups: Control, CRF and Experiment. CRF models were induced by 2.5% adenine administered by gavage for 8 weeks. Control and CRF group received normal protein diet (18.8% proteins, AAA 0.024%), and Experiment group was divided into 3 subgroups, being treated with SM (1.0g/kg/d) and LA-AAD (18% proteins, AAA 0.009%) differently. Proteinuria, blood urine nitrogen (BUN), serum and urine creatinine (Scr, Ucr) were measured and creatinine clearance rate (CCR) was calculated accordingly. **Results:** Proteinuria, BUN, Scr analyses showed amelioration of functional parameters and increased the CCR significantly in LA-AAD + SM group. **Conclusion:** These results showed that the combination of SM and LA-AAD could be beneficial to the improvement of renal function in CRF rats and this method can be a novel strategy for CRF treatment.

Key words: Chronic renal failure, Kidney function, Low-aromatic amino acid diet, *Salvia miltiorrhiza*.

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INTRODUCTION

Chronic renal failure (CRF) is a condition of functional and structural dysfunction of the kidney with impairment longer than 3 months, caused by various factors. It is characterized by normal or abnormal glomerular filtration rate (GFR) from pathological glomerular injury, abnormal renal function indices in urine or blood, abnormal imaging findings, or decreased GFR for more than 3 months of unknown etiology (<60 mL/min·1.73m^2).[1] Nutritional status often becomes unbalanced during the course of CRF, and nutritional intervention is a mandatory component of the management of patients with CRF.[2] It has long been recognized that high protein intake has a deleterious impact on renal function in CRF patients, by increasing glomerular filtration rate (GFR), leading to progressive glomerular sclerosis.[3] Hence, a low protein diet (LPD) (i.e. 0.6 to 0.8 g/kg/day) is now commonly recommended for non-hemodialysis CRF patients, decreasing kidney workload and slowing renal function decline.[4]

Especially, low-aromatic amino acid diet (LA-AAD) seems to play a crucial role in the protection of chronic kidney disease and there have been some reports showing that LA-AAD could be as effective as LPD.

Also, CRF is treated mainly with hormone therapy and immune suppressors in addition to controlling urine protein, blood pressure, and blood glucose levels and maintaining homeostasis. Although these therapies can reduce kidney impairment to a certain degree, they are unable to radically delay the evolution of this disease. Furthermore, hormone and immune suppressors are only effective in part of the pathogenesis of kidney injury and lead to high rates of relapse. Their long-term application can also lead to severe infection and osteonecrosis of the femoral head, among other side effects.[5]

Therefore, a novel strategy of safe and efficacious drugs combined with proper nutrition management are needed for CRF treatment. *Salvia miltiorrhiza* (SM), often referred to as Danshen in DPR Korea and China or Tanshen in Japan, is widely distributed in Asian countries. *Salvia miltiorrhiza* root is frequently utilized in traditional medical systems for preventing and treating various forms of kidney disease. It is now becoming more widely known in other countries, as well. It contains diterpenoids

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including tanshinones and caffeic acid derivates such as danshensu and rosmarinic acid that impart ACE inhibiting, antioxidant, vasodilating, and blood thinning properties.[6] Danshen injection made from the aqueous extracts of SM has been widely used to treat a variety of renal diseases, such as acute renal failure and acute nephritis, with positive effects in clinical practice.[7]

Furthermore, the antioxidative and anti-inflammatory properties of *Salvia miltiorrhiza* as well as its protective effects on various organs have been investigated extensively.[8] Clinically, a number of studies have shown that SM administration can restore a certain degree of kidney function in CRF patients, but this has yet to be confirmed. Although previous studies strongly suggest that SM therapy and low-aromatic amino acid diet would be useful in treatment of CRF respectively, their combined effects have not been reported. Therefore, the purpose of this study was to investigate the use of SM combined with LA-AAD for the treatment of chronic renal failure in rats.

MATERIALS AND METHODS

Preparation of *Salvia miltiorrhiza* Rhizomes Extract

Salvia miltiorrhiza were obtained from Mannyon Pharmaceutical Company, DPR Korea and identified by comparison with the voucher specimen deposited at National Drug Certification Institute of Pyongyang, DPR Korea. The rhizomes were cleaned, dried, ground, weighed and homogenized in 92% ethanol at a ratio of 1:10 of sample to ethanol and left to soak for 3 days at 25°C with occasional shaking and stirring. The mixture was then filtered and the resulting liquid was concentrated under reduced pressure at 45°C in rotary evaporator to yield a dark gummy-yellow extract (7%, w/w). The concentrated extract was then kept in the incubator at 45°C for 3 days to evaporate the ethanol residue yielding the crude rhizome extract. Extracts were then dissolved in 10% Tween-20 before being orally administrated to animals in concentrations of 1.0g/kg body weight.

Animals

Adult Wistar rats (outbred, 240~260g) were provided by the Laboratory Animal Centre of the Pyongyang University of Medical Sciences and adapted in a lab environment before experiments for a week. All rats were group-housed in standard laboratory conditions with a 12-h light/dark cycle. They had free access to rats' formula feeds: containing 18.8% protein (aromatic amino acids 0.024%), 57% carbohydrate, 5.7% crude oil, 1.01% calcium, 0.65% phosphorus, 51μg/kg vitamin D, and water. The Institutional Animal Care and Use Committee of the Pyongyang University of Medical Sciences approved the animal protocol used in this study. After a week of acclimation, the rats were divided into three groups (n=50) and were fed designed experimental diets for 8 weeks as following:

Control (10 rats fed the normal protein diet, for 8 weeks)

CRF (adenine-treated 10 rats fed the normal protein diet, for 8 weeks)

Experiment: adenine-treated 30 rats were administrated by a different regimen of SM and LA-ADD.

LA-AAD (10 rats fed the low-aromatic amino acid diet: 18% proteins, aromatic amino acids 0.009%, for 8 weeks).

SM (10 rats fed the normal protein diet with gavage of SM 1.0g/kg/d, for 8 weeks).

LA-AAD + SM (10 rats fed the *Salvia miltiorrhiza* and low-aromatic amino acid diet, for 8 weeks).

CRF Model

In order to make chronic renal failure models, the rats in the CRF and Experiment group were administered 2.5% adenine (200 mg/kg/d) by gavage for 8 weeks. Adenine was dissolved in sterile 0.9% saline solution.[9]

Blood and Urine Sample Preparation

24-hour urinary samples were collected with the aid of metabolic cages. The animals were placed in individual metabolic cages for 24 h to collect urine samples after receiving access only to tap water on the day before the experimental rats were killed. Blood was collected from the heart using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, and the plasma was stored at temperatures below -20°C for further analysis.

Blood and Urine Analysis

The level of blood urea nitrogen (BUN) was measured with the BUN kit (Jian Cheng Biological Engineering Institute, Nanjing, China) based on the Fearon reaction. Briefly, under the acidic condition with heating, urea nitrogen and diacetyldioxime reactions generate a red condensation product, the absorbance values of which can be obtained at the wavelength at 520 nm to determine the BUN level. [10] Serum creatinine estimation was followed by Jaffe's method. All the reagents and the samples were brought to a reaction temperature of 37°C. A colored creatinine compound (orange color) produced in an alkaline media was measured at 520nm. Average results were expressed in mg/dL. Creatinine in urine was also assessed by Jaffe's method, adding NaOH and picric acid (ratio 5:1) to the samples and measuring the absorbance at 492nm. Based on the value of serum and urine creatinine, we calculated the creatinine clearance rate by the following formula.

$$\text{Creatinine clearance rate} = \frac{\text{Urine creatinine (mg / dL)} \times 24\text{ h Urine volume (mL)}}{\text{Serum creatining (mg / dL)} \times 1440(\text{min})}$$

Estimation of Proteinuria

To estimate the proteinuria, 50 μl of the diluted urine sample (1:5) is mixed with 100 μl of color reagent containing pyrogallol. The pyrogallol red reacts with the sodium molybdate, forming a complex which when combined with the protein, an acid medium develops a blue-colored chromophore. Proteinuria quantitation was made using a spectrophotometric method based on the 600nm absorbance relationship between the sample and the standard solution (50mg protein/dl). Proteinuria concentration results were obtained in mg/dL and corrected by the dilution factor. 24-hour protein level was expressed in mg/24h, calculated from the product of its concentration and the total volume of 24 hours in dL.

Statistical Analysis of Data

Data were expressed as Mean ± SD. Statistical analysis was performed by ANOVA for multiple comparisons using SPSS 16.0. Statistical significance was considered at $P < 0.05$.

RESULTS

As shown in Table 1, urine protein quantity in the adenine-treated CRF group was higher than in the control rats from 4 weeks. In LA-AAD administration group, there was a certain degree of reduction but not a significant difference compared to CRF group. However, in the SM administration group there was a significant improvement in the urine protein quantity compared to CRF group at 8 weeks, especially much better in the LA-AAD + SM group (P<0.05).

Table 2 showed that the levels of Scr and BUN in the CRF group were higher than in the Control group respectively. In LA-AAD

Table 1: Effect of *Salvia miltiorrhiza* and low aromatic amino acid diet on the urine protein quantity in CRF rats (mg/24h).

	Control	CRF	Experiment		
			LA-AAD	SM	LA-AAD + SM
Baseline	11.34±3.56	10.45±2.46	11.21±9.34	12.94±4.65	10.58±5.43
4 weeks	21.34 ±11.54	38.34±7.45*	29.32±9.56*	28.72±10.2*	24.23±6.19*
8 weeks	20.85±13.28	128.11±19.33*	119.49±15.21*	94.27±11.28*#	65.65±3.86*#

Table 2: Effect of *Salvia miltiorrhiza* and low aromatic amino acid diet on the serum and urine creatinine in CRF rats at 8 weeks.

	Control	CRF	Experiment		
			LA-AAD	SM	LA-AAD + SM
Scr(mg/dL)	0.64±0.06	2.79±0.18*	2.53±0.23*	2.46±0.45*	1.58±0.05*#
BUN(mg/dL)	15.88±0.54	63.18±2.16*	58.43±3.11*	48.33±1.37*#	21.72±1.18*#

Data are presented as mean ± SD. *P< 0.05 comparison with control group and #P< 0.05 comparison with CRF group. CRF: Chronic renal failure; LA-AAD: Low aromatic amino acid diet; SM: *Salvia miltiorrhiza*.

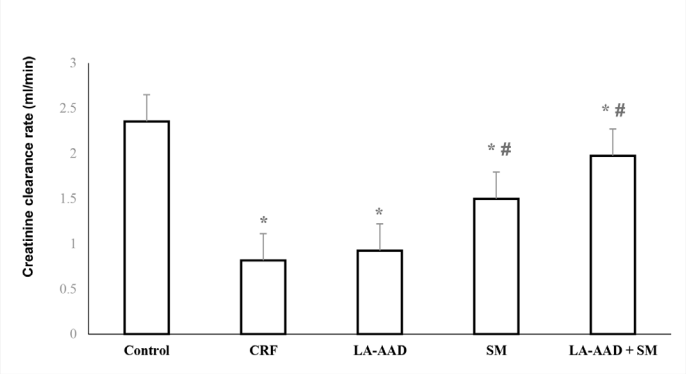

Figure 1: Effect of *Salvia miltiorrhiza* and low aromatic amino acid diet on the creatinine clearance rate in CRF rats (ml/min).

Data are presented as mean ± SD. * P< 0.05 comparison with control group and #P< 0.05 comparison with CRF group. CRF: Chronic renal failure; LA-AAD: Low aromatic amino acid diet; SM: *Salvia miltiorrhiza*.

administration group, reduction of Scr and BUN was not significant differences compared to CRF group. In SM group, the level of BUN was significantly decreased compared to CRF group but not the level of Scr. On the other hand, in LA-AAD + SM group, indices were significantly decreased compared to CRF group and achieved much better than SM group.

As shown in Figure 1, creatinine clearance rate in the CRF group were lower than in the control group respectively. LA-AAD administration seemed to increase the creatinine clearance rate in certain amount but not a significant increase compared to CRF group. However, there were significant increases in the SM administration group compared to CRF group, especially better improvement in LA-AAD + SM group (P<0.05).

DISCUSSION

CRF is a slowly progressive renal damage and originating from diverse kidney diseases. CRF develops predominantly due to the injury and necrosis of renal proximal tubule cells as a result of ischemic or toxic insult. In recent years, researchers have found a variety of ways to cause CRF in rats such as renal resection, renal artery ligation, adenine and adriamycin, etc. The advantage of the adenine method is simple and controllable as well as has a high success rate. The disadvantage is high animal mortality. The mechanisms of the adenine-induced CRF rat include azotemia, accumulation of uremic toxins, metabolic imbalances of amino acids and electrolytes, as well as complex inflammatory response. [11]

In the present study, we used the SM (*Salvia miltiorrhiza*) to improve the impaired renal function in CRF rats. SM has been used as traditional herbal medicine against a variety of diseases. Rosmarinic acid, phenolic acids, carnosic compounds and flavonoids or their derivates exist in sage extract. It possesses antioxidative (lipid peroxidation inhibitory and radical scavenger impacts), anti-bacterial, anti-inflammatory, fungistatic and virustatic capabilities and as well as insulin sensitizer and hypoglycemic impacts on streptozotocin-induced hyperglycemic rats. SM has been widely used in the treatment of chronic kidney disease, and its antifibrosis effect has been recognized by the majority of physicians. Zhang *et al*. have reported that, SM can attenuate kidney injury in 5/6Nx rats, which is attributed to its anti-inflammatory activities through inhibition of the activation of the NF-κB and p38 MAPK signaling pathways. [12] In Li *et al*., SM may protect the renal function and improve the tubular function and renal pathology in rats with unilateral ureteral obstruction, which may be related to a reduction in inflammatory cytokines CCL5 and CXCL10 secretin.[13] In this study, we also combined a low aromatic amino acid diet with SM and demonstrated that it has a reno-protective effect.

Recently some researchers have demonstrated that a specific restriction in the intake of aromatic amino acids lowered some key uremic toxin (such as p-Cresyl sulfate and IS) concentrations and mitigated inflammation that play major roles in the progression of renal damage. p-Cresyl sulfate (PCS) and indoxyl sulfate (IS) are protein-bound uremic toxins produced by the gut microbiota and aromatic amino acids, tryptophan and tyrosine (and also to a limited extent phenylalanine), respectively. A strong association has been demonstrated between accumulation of PCS and cardiovascular damages. PCS concentrations are associated with the rise of inflammatory markers (e.g.: IL-6) and the increase in oxidative stress, through the production of reactive oxygen species. It has also been shown that IS levels are negatively correlated with the GFR and contribute to the progression of CRF.[14] Consequently, lowering uremic toxin levels in CRF patients appears to be a relevant strategy to prevent the deterioration of kidney function.

It is very likely that lower uremic toxin concentrations could have been reached using either very low protein diet (VLPD), or aromatic amino acid corresponding concentrations. Indeed, a recent study showed that VLPD was effective to beneficially modulate gut microbiota, improving intestinal permeability and reducing serum levels of total and free IS

and PCS in CRF patients.[15] However, some studies showed that low aromatic amino acid diet can exhibit a reno-protective effect without needing of reducing total protein content.[14] Therefore, we investigated the effect of *Salvia miltiorrhiza* combined with aromatic amino acids on the impaired renal function in rats with chronic renal failure. The result showed that, after CRF modeling, compared with control group, in CRF groups Scr, BUN and proteinuria levels were increased significantly. This suggested that, there was obvious renal damage in CRF rats. In the LA-AAD group, there was a certain degree of improvement in these indices of renal function but not a significant difference between the CRF group. However, in SM and LA-AAD + SM groups, there was a significant improvement in the indices of renal function, especially much better in the LA-AAD + SM group at 8 weeks.

CONCLUSION

These results showed that the combination of *Salvia miltiorrhiza* and low aromatic amino acid diet could be beneficial to improvement of renal function in CRF rats and this method can be a novel strategy for CRF treatment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CRF: Chronic Renal Failure; **SM:** *Salvia miltiorrhiza*; **AAA:** Aromatic Amino Acids; **Cr:** Creatinine; **GFR:** Glomerular Filtration Rate; **LPD:** Low Protein Diet; **EDTA:** Ethylene Diamine Tetra Acetic Acid; **BUN:** Blood Urea Nitrogen.

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