Original article

Beneficial modification of functional renal parameters in 5/6 nephrectomized rats by nutraceutical. In view of a kidney-protective intervention

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Abstract. The objective of this study is to ascertain the potential beneficial effects of a novel phytoterapeutic formula (DTS, Kyotsu Jigyo, Japan) on renal function and morphological structure in 5/6 nephrectomized rats. Male Spraque-Dawley rats, 240-280 g, were divided into sham control (Group A) and nephrectomized (Group B and Group C) groups. The 5/6 nephrectomy was performed by removal of the right kidney and 2/3 ligation of left renal artery. After surgery, the animals were kept in individual cage for 6 weeks. Rats in Group A and Group B were fed with a normal protein diet only while those in Group C were fed normal protein diet added with DTS (10 mg/rat/day). The DTS supplementation was started a day after surgery. After 5 weeks, all rats were subjected to renal function study and then their left kidneys were isolated for morphological study. There were no significant differences in body weight, blood pressure, and heart rate among groups. DTS supplementation significantly increased ($p < 0.05$) plasma creatinine concentration, glomerular filtration rate, effective renal plasma flow, and urine flow rate in nephrectomized rats when compared to sham control (Group A) and untreated nephrectomized (Group B) controls. In contrast, plasma urea concentration and morphological structure were not significantly modified by DTS supplementation in nephrectomized animals. These data suggest that feeding with a normal protein diet and DTS supplementation improves renal function without any morphological effect in 5/6 nephrectomized rats if not a slight preservation.(www.actabiomedica.it)

Key words: nephrectomized rats, DTS supplementation, renal function, renal morphological

The most widely studied model for the pathophysiology of chronic renal failure is the 5/6 nephrectomized or the remnant kidney model. It has been shown that 5/6 experimental nephrectomy brings about systemic hypertension, proteinuria, a decrease in renal function and a progressive glomerulosclerosis (1). Different experimental approaches aimed to reduce the progression of renal insufficiency in the remnant kidney and dietary modifications are the ones most commonly employed. These include protein restriction (2-4), phosphorus restriction (5) and modulation of calorie intake (6) all showing some degree of hemodynamic and morphological alterations taking place in the nephrectomized model. The nephrectomized rats maintained on moderate protein restriction survived longer and developed less glomerular sclerosis when compared to those maintained on normal protein diet (2, 7). However, consuming low pro-

tein diet bears the unavoidable drawback to cause an inadequate calorie intake. Afshinnia and coworkers (6) showed that protein and calorie restricted diet effectively reduces serum urea nitrogen concentration in 5/6 nephrectomized rats. Renal morphology in 5/6 nephrectomized animals fed an high protein diet showed an higher degree of glomerular and tubular damage than those fed on low protein diet. Gao and coworkers (3) reported that the calorie, rather than the protein per se, was significantly related to the extent of non-glomerular lesion score.

As the beneficial effects of dietary modification especially those on moderate protein restriction, amino acid supplementation and adequate calorie intake had been reported on the 5/6 nephrectomized model representing the status of chronic renal failure (5). Thus, several foods have been developed for such purpose. We have recently shown that DTS, a novel functional food supplement used in clinics safely for general health purposes, might exert a beneficial regulation of GSH/GSSG redox status (8, 9) while conferring a protection against drug-induced DNA damage in the liver and kidney of either young and aged animals (10). The present study is designed to investigate the potential protective effect of DTS supplementation on renal function and morphology in 5/6 nephrectomized rats.

Materials and methods

Preparation of phytotherapeutic compound

DTS (panax pseudoginseng, eucommia ulmoides, 50/25 (%w/w), Kyotsu Jigyo, Tokyo, Japan) is produced under quality-controlled procedure from non GMO-modified crops and ISO 9001 and 140001 regulation and it was kindly donated by the Institute of Health Care with Oriental Herbs and Medicine, Tokyo, Japan. This compound is composed of palatable tiny grains of medium consistency which can be easily mixed with food.

Study design

All experiments were performed in male Sprague-Dawley rats, weighing 240-280 g. which were fed *ad libitum* with a standard rat chow containing 24% protein and allowed free access to tap water. All studies were performed in accordance with NIH and American Physiology Society standards for the care and use of research animals. Rats were divided into 3 groups. Group A(n=15) was sham operated rats. Group B (n=15) was served as control nephrectomized rats. Group C (n=15) was 5/6 nephrectomized rats fed with DTS supplementation (Kyotsu Jigyo, Tokyo, Japan) at dose of 10 mg/rat daily. DTS was mixed with the regular chow food. Before operation, rats were placed in a metabolic cage to collect urine for 24 hours. The 24-hour urine samples were used for measurement of urinary protein/creatinine ratio. Blood samples were collected by cutting tip of rat's tail for measurements of glucose, urea nitrogen, and packed cell volume (PCV). After blood collection, rats in Group A underwent sham operation while Group B and Group C underwent 5/6 nephrectomy. After the operation, each rat was kept in the individual cage fed *ad libitum* with a standard rat chow containing 22% protein and allowed to tap water for 6 weeks. The DTS supplementation for nephrectomized rats was administered once a day, starting 1 day after surgery until 6 weeks. One day before renal function study, rats were placed in metabolic cages for 24 hours. At the end of 6 weeks, all rats underwent renal function study. Finally, the left kidney of each rat was collected for morphological study.

Operative procedure of 5/6 nephrectomy

After first 24-hour urine collection, each rat was anesthetized by intraperitoneal injection with sodium pentobarbital (60 mg/kg body weight (BW). The 50 μl of blood was collected by cutting the tail's tip and allowed to drop into a heparinized tube for determination of blood urea nitrogen concentration and stored at −20°C for other biochemical measurements. Sham operation was performed in Group A by opening the abdomen, exposing the kidney, and then moving the kidney back and forth. In Group B and Group C, 5/6 nephrectomy was performed by a small mid-abdominal incision, removal of right kidneys, and then ligating two of the three branches of left renal artery

Operative procedure of renal clearance study

After 6 weeks of treatment, rats were anesthetized by intraperitoneal injection with a combination of ketamine hydrochloride (70 mg/kg). Tracheotomy was carried out and a PE240 catheter was inserted into the trachea for aspirating secretion and used as an artificial airway. The right femoral artery and vein were cannulated with PE50 catheters. The right femoral artery was used to monitor arterial blood pressure by connecting to a pressure transducer with a polygraph recorder. A polyethylene catheter was inserted into the right femoral vein for infusion of inulin and PAH solution. The left femoral artery was used for blood sampling. The abdominal midline incision was performed, both ureters were carefully located and the PE10 catheter was then inserted for urine collection. In Group B and Group B, only the remaining left ureter was cannulated for collecting urine. Urine was collected into a pre-weighed eppendorf.

Renal clearance study

Clearance study was carried out by infusing a mixture of 0.1 g PAH, 1.0 g inulin and 6 g mannitol dissolved in 0.9% saline solution at the rate of 0.01 ml per kg BW per hour continuously for 45 minutes to stabilize plasma inulin and PAH concentrations. After equilibration period, three consecutive 20-minute urine collection and arterial blood sampling at midpoint of each urine collection were carried out. Urine volume was measured from the weight changes of preweighed eppendorf. Blood samples were used to determine PCV. Plasma and urine were kept at −20°c for further analysis of PAH and inulin concentration.

Morphological study

Animals from each group were studied for histological changes in the kidney. Following renal clearance study, each kidney was fixed by perfusing with normal saline followed by 18% glutataraldehyde in 0.02 M cacodylate buffer (pH=7.2) for 20 minutes and then 3% glutaraldehyde in 0.1 M cacodylate buffer (pH=7.2) for 20 minutes. All kidneys were collected and peritoneal fat was freed. The kidney cortical area was sliced into a small piece (1 x 1 mm.) and was post-fixed in 3% cacodylate buffer. Tissue blocks were processed also for electron microscopic study. The remaining kidney was fixed in 10% buffered formalin solution and processed for histological evaluation. Paraffin sections were stained with Hematoxylin and Eosin (H&E) and treated with Periodic Acid Shift reagent (PAS). A minimum of 100 glomeruli were evaluated per animal by an observer blinded to the origin of the tissue.

Blood and urinary samples assay

Plasma and urine from clearance tests were used for determination of inulin, PAH, electrolytes, creatinine and osmolarity by a NOVA16 autoanalyzer (NOVA Biomedical, Waltham, MA).

Functional parameters

Pulse Pressure (PP)	SP-DP
Glomerular Filtration Rate (GFR)	$\frac{\text{Uin V}}{\text{Pin}}$
Effective Renal Plasma Flow (ERPF)	$\frac{\text{Upah V}}{\text{Ppah}}$
Effective Renal Blood Flow (ERBF)	$\frac{\text{ERPF}}{\text{(1-PCV) x 100}}$
Filtration Fraction (FF)	$\frac{\text{GFR}}{\text{ERPF x 100}}$
Renal Vascular Resistance (RVR)	$\frac{\text{MAP}}{\text{ERPF}}$
Urinary Electrolyte Excretion	UeFr
Fractional Excretion of Electrolyte (FFe)	$\frac{\text{UeFr / Pe}}{\text{GFR x 100}}$

SP = systolic blood pressure; DP = diastolic blood pressure; Uin = urinary inulin concentration; Pin = plasma inulin concentration; UPAH = urinary PAH concentration; PPAH = plasma PAH concentration; Ue = urinary electrolyte concentration; Pe = plasma electrolyte concentration; Fr = urine flow rate.

Statistical analysis

The data were presented as mean ±SD. Student paired t-test was used to compare data within the same group. One-way analysis of variance (ANOVA) was used to determine the differences among the 3 groups. Duncan's multiple range test was used for pair-wise comparison. Kruskal-Wallis one-way analysis of variance rank test was used to analyze non-parametric parameters while Dunnett's method was used for pairwise comparisons between two-treatment groups. The result was considered to be statistically significant difference when p-value was less than 0.05.

Results

Body weight was not significantly different among groups either before or after treatment (Table 1). After 6 weeks of treatment, the plasma urea concentration significantly ($p<0.05$) increased in 5/6 nephrectomized rats with cereal supplementation, but the value was not significantly different among groups (Table 2). Plasma creatinine concentration was significantly higher in Group C as compared with Group B while PCV significantly decreased in both Group B and Group C ($p<0.05$) (Table 2). The urinary protein-creatinine ratio was not different among groups either before or after treatment.

Renal hemodynamics

Urine flow rate was not significantly different among groups (Table 2). Glomerular filtration rate and effective renal plasma flow were significantly lower in Group B when compared to Group A and Group C, the latter showing comparable values. There was no significant difference in filtration fraction among all groups. Renal vascular resistance of Group B was 2 times higher than that of Group A while that of Group B was reduced to a similar value as Group 1 ($p<0$-05 vs Group B).

Plasma and urinary electrolytes

There was no significant difference in plasma sodium, potassium, and chloride concentrations among all groups of rats (Table 3). Urinary sodium

Table 2. Renal hemodynamics in three experimental groups.

Parameters	Group A	Group B	Group C
Fr (µl/min)	49±5	41±6	50±6
GFR (µl/g BW/min)	2.3±0.4	1.0±0.3 *	1.7±0.5 **
ERPF(µl/g BW/min)	12.1±1.4	7.8±1,1*	11.7±2.6
ERBF (µl/g BW/min)	18.7±1.5	14.8±2.8	17.2±3.6
FF (%)	21.3±1.8	20.8±4.1	19.8±3.3
RVR (mmHg/ml/min)	6.2±1.4	11.3±2.1	5.8±1.9

Data reported as mean ± SD; *, ** data in the same row with difference superscripts differ significantly ($P<0.05$); Fr = urine flow rate; GFR = glomerular filtration rate; ERPF = effective renal plasma flow; ERBF = effective renal blood flow; FF = filtration fraction; RVR = renal vascular resistance

Table 1. Body weight, plasma urea concentration, plasma creatinine concentration, packed cell volume, blood glucose concentration, urinary protein-creatinine ratio and blood pH before and after 6 weeks of DTS supplementation

Parameters	Group A		Group B		Group C	
	Before	After	Before	After	Before	After
Body wt (g)	261±12	345±18*	244±13	323±21*	229±8 9	355±14*
Plasma urea (mg/dl)	31.3±3.9	54.2±7.1	44.4±5.3	57.3±12.3	28.4±5.2	69.8±6.5
Plasma creatinine (mg/dl)		0.43±0.05		0.58±0.02#		0.68±0.08#
Packed cell volume (%)	48,3±3	44,5±3	49,5±3	42,3±3*	51,8±1	42,1±2*
Blood glucose (mg/dl)	71,3±5.3	74,4±6.8	72,3±6,5	76,3±8.8	68,4±5,2	70,4±7,2
Urinary protein/creatinine ratio	1,6	3,4*	1,4	3,6*	1,9	1,5
Blood pH		7.30±0.02		7.22±0.03		7.31±0.03

Data reported as means ± SD; Group A, sham control; Group B, nephrectomised without DTS supplementation; Group C, nephrectomized with DTS supplementation; # data in the same row with different superscripts differ significantly ($p<0.05$); *significant differences as compared to baseline value before treatment ($P< 0.001$); ** data reported as median

Systolic pressure, diastolic pressure, mean arterial pressure, and heart rate were not significantly different among the three groups (data not shown)

Table 3. Plasma electrolyte concentrations, urinary excretion and fractional excretion of electrolytes, and urinary and plasma osmolality ratio

Parameters	Group A	Group B	Group C
P_{Na} (mEq/ml)	132.7±3.5	129.7±4.2	138.5±3.2
P_K (mEq/ml)	4.2±0.3	3.7±0.5	3.9± 3.4
P_{Cl} (mEq/ml)	132.6±6.2	125.8± 6.7	131.4±4.4
$U_{Na}V$ (mEq/min)	4.4±0.6	3.8±0.7	5.2±0.9
U_KV (mEq/min)	1.1±0.2	0.8±0.1	1.3±0.2 *
$U_{Cl}V$ (mEq/min)	3.3±0.6	3.1±0.8	3.2±0.5
Fe_{Na} (%)	1.6±0.4 *	2.7±0.6	2.5±0.7
Fe_K (%)	12.2±2.7 *	29.6±6.8	25.8±4.3
Fe_{Cl} (%)	1.2±0.4	1.7±0.3	1.5±0.4
Uosm/Posm	1.8±0.5	1.7±0.5	1.7±0.6

Data reported as mean + SD; *p<0.05, indicating a significant difference of data in the same row; UNaV = urinary excretion of sodium; UKV = urinary excretion of potassium; UClV = urinary excretion of chloride; FENa = fractional excretion of sodium; FEK = fractional excretion of potassium; FECl = fractional excretion of chloride; PNa = plasma sodium concentration; PK = plasma potassium concentration; PCl = plasma chloride concentration; Uosm/Posm = urinary and plasma osmolality ratio

and chloride excretion was not significant different among groups while urinary potassium excretion was significantly higher in Group C when compared to Group A. Both nephrectomized groups (Group B and Group C) displayed about two times higher fractional potassium excretion than sham control rats but significant difference was found only between Group B and Group A. In contrast, fractional sodium and chloride excretion and urinary plasma osmolarity ratio were not significantly different among groups.

Morphological structure

Dilated glomeruli were observed in both nephrectomized groups (Fig. 1). Some showed thickening of capillary wall and hypercellularity while glomerular shrinkage was also shown. Group B had tubular dilatation with hyaline droplets in the proximal epithelial cells. Some of the proximal epithelia developed hydropic degeneration and dark-dense nuclei while others were sloughed off (Fig. 2). DTS supplementation in Group C caused slightly tubular dilatation with dark-dense nuclei in proximal epithelia and some of them were sloughed off. Mild degree of intertubular edema was also observed Overall, this represented a mild morphological preservation as compared to group B.

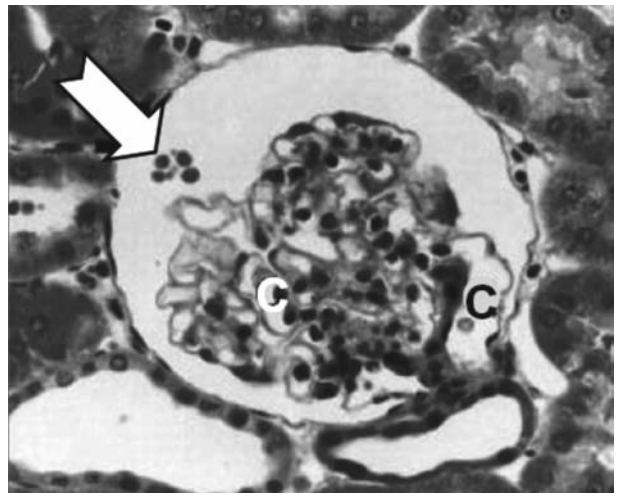

Figure 1. Group B: Glomerular capillary appeared dilated (C). Few droplets were found in Bowman's space (arrow) (H&E, 40x)

Under electron microscope, Group B and Group B displayed similar alterations. These included slightly swollen podocytes and endothelial cells, foot process fusion, insinuation, cytoplasmic debris in capillary lumen, and round droplets in the glomerular space (Fig. 3).

Discussion

Irrespective of dietary treatment, nephrectomized rats showed a significantly reduced PCV at the end of experiment as compared to those of control. This is likely to be a result of renal mass reduction and subsequently reduced erythropoietin production. Increased plasma glucose concentrations after surgery were observed in all groups. The changes may be due to stress induced hyperglycemia (11). In the present study, the renal function of rats subjected to 5/6 nephrectomy alone were decreased. Glomerular filtration rate and renal plasma flow were reduced by half while filtration fraction did not change and higher RVR was observed. These results are in agreement with Bouby and coworkers (12) who showed that 4-6 weeks after operation, the 5/6 nephrectomized rats had lower level of GFR and ERPF. At this stage, the hyperfiltration takes

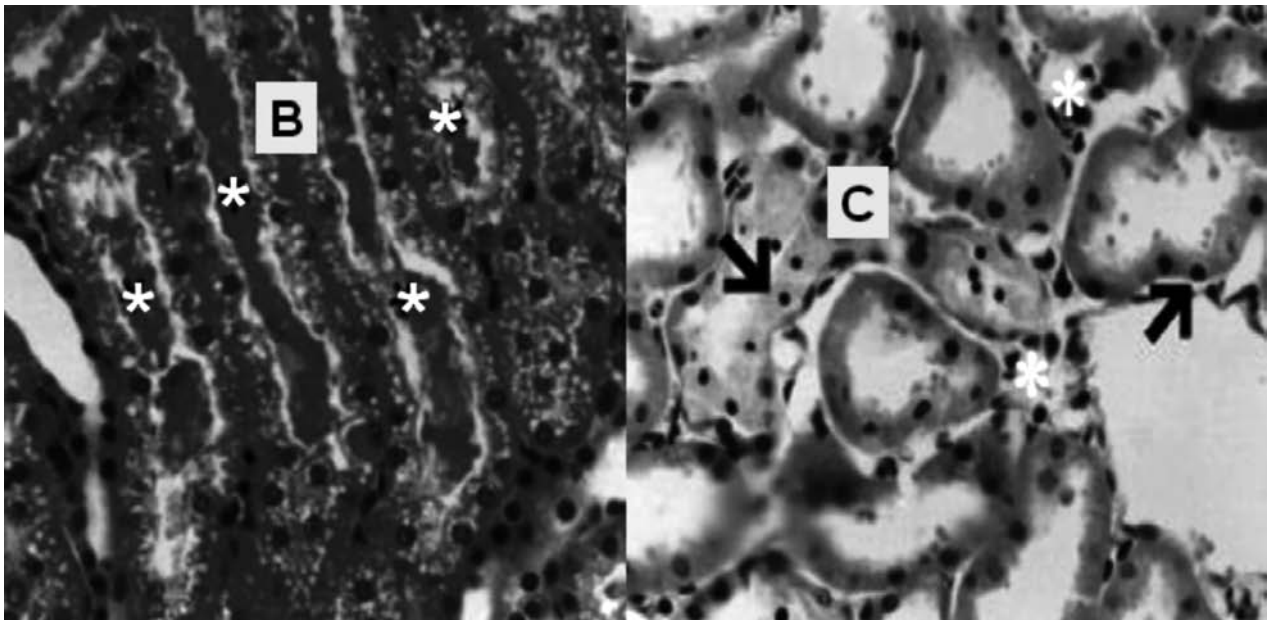

Figure 2. Group B: Proximal epithelial lining adjacent to the renal capsule displayed vacuolarization. Casts were found in proximal tubular lumen (star). (H&E, 40x). Group C: Tubular dilation was present. Proximal tubular cells were irregular in shape and size with dark and dense nuclei (arrow). There was slightly intertubular edema (star). (H&E, 40x)

place since the remnant kidney can compensatorily increase the amount of plasma. In Group supplemented with DTS (group C), GFR and RPF were significantly higher than those of Group B. The extent of increase in RPF is much higher than GFR causing a reduction in filtration fraction. Renal vascular resistances were reduced to values comparable to control thus indicating that a marked vasodilation occurred in the remnant kidney. Indeed, it has been shown that efferent arteriole dilation is an early phenomenon causing a reduction in filtration fraction (13). Although FF was reduced, a higher GFR in Group C suggested that an even more efficient hyperfiltration occurred in DTS-supplemented rats in parallel with increased urine flow rate. On the other hand, we showed that such hyperfiltration was not due to increased systemic blood pressure at least in our current experimental set up.

The lower renal vascular resistance in Group C compared to Group B may be due to some nutrients in the DTS. In particular, we have recently shown that DTS significantly reduced glomerular expression of MCP-1 (14) through a likely anti-oxidant/anti-inflammatory property exerted by the main components of the phytocompound (8, 9) resulting possibly in nitric oxide-induced vasodilation as recently suggested for eucomnia ulmoides (15) although this hypothesis needs experimental confirmation. Vos and coworkers (16) have reported that renal allografted rats when given an high dosage L-arginine in drinking water (1 g L-arginine in 100 ml) had lower renal vascular resistance as compared to those without supplementation. Urinary potassium excretions in Group C were slightly higher than Group B which was consistent to a slight increase in urine flow rate. The slightly higher urine flow rate in Group C is likely to be due to an increase in GFR after DTS supplementation. At 6 weeks after 5/6 nephrectomy, glomerula in the remnant kidney of both nephrectomised groups displayed several changes. Glomerular capillary dilation was observed under the light microscopic examination. Fusion of foot processes under electron microscopic examination suggested podocyte damages. However, glomerular basement membrane detachment was not observed and this was paralleled with an unaltered urinary protein-creatinine ratio. Whereas short-term histopathology observation didn't show any abnormality of glomerular basement membrane, a long-term progression of glomerulosclerosis cannot be

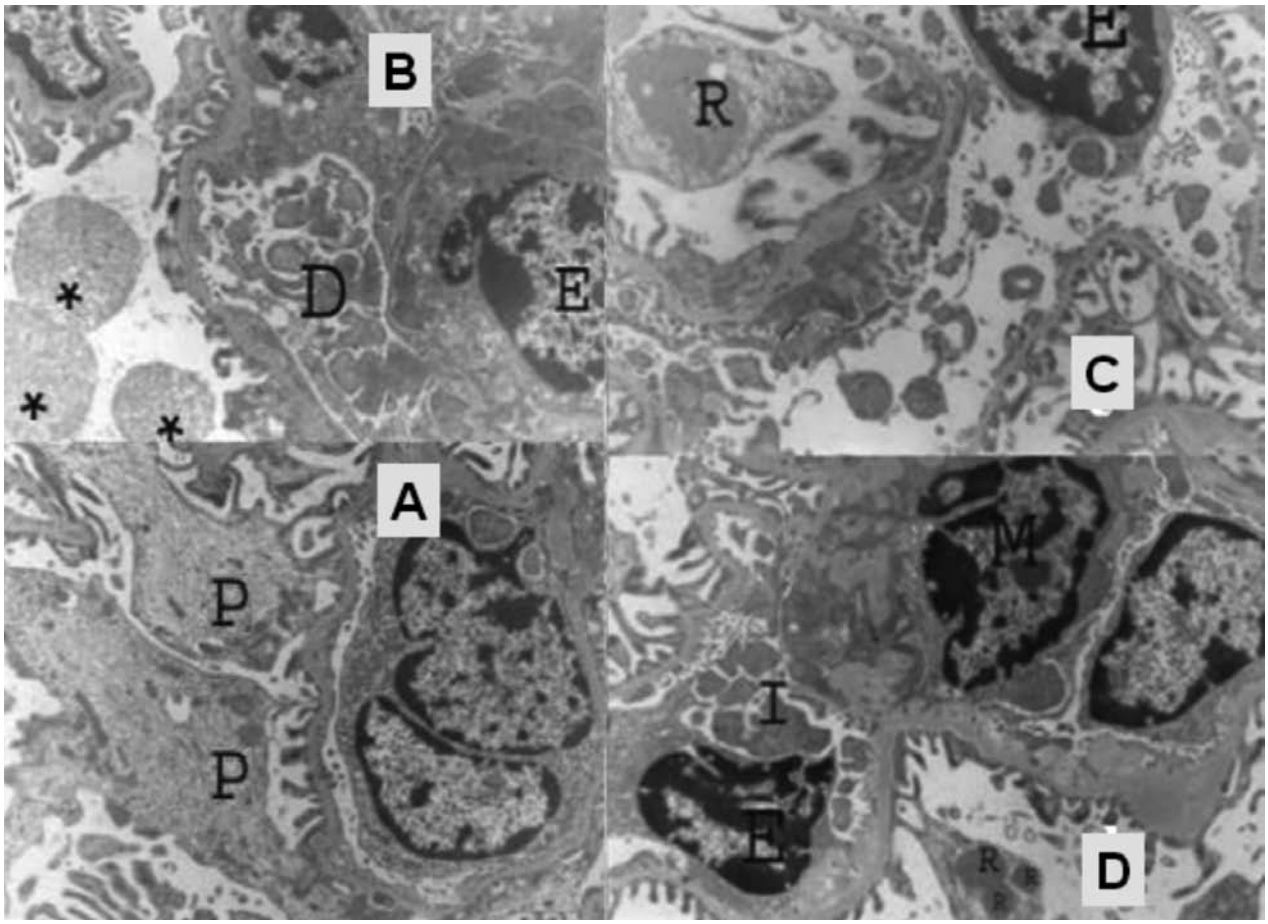

Figure 3. Electron microscopy findings. Group B (a - b) and Group C (c- d) show similar abnormalities which included endothelial cell insinuations (I), slightly Podocyte (P) swelling, Cytoplasic debris (D) in capillary lumen, electron dense droplets (R) in the podocyte cytoplasm, and round droplet in the glomerular space (*); M = Mesangial cell, E = Endothelial cell (9000x)

ruled out. Indeed, previous studies showed that rats with the remnant kidney had focal segmental glomerulosclerosis affected only 8% of glomeruli at day 30, and 24% at day 120 after nephrectomy (17). In young rats, uninephrectomy causes more pronounced compensatory hypertrophy of the remnant kidney than in adult, followed by a higher incidence of focal and segmental glomerulosclerosis (18). Nagata and Kriz (19) studied processes of glomerular hypertrophy in young Spraque-Dawley rats at 12 and 24 weeks after uninephrectomy, suggesting that glomerular hypertrophy contributed to local mesangial expansion, capillary dilation and structural changes in podocyte architecture. Although changes in podocyte structure were detected, no glomerular hypertrophy was observed in the present study.

Structural alterations in Group C included proximal and distal tubular dilation and degeneration of some tubular epithelia, which was consistent with the high urinary electrolyte excretion. Although glomerular capillary dilation was observed in some glomeruli, shrinkage of some glomeruli was also present. It is suggested that various degrees of glomerular alteration had occurred in this model. Round, pink, and PAS negative droplets that were found in the glomerular space in all groups of rats could be inulin that filtered from plasma. Hyaline droplets, which found in the proximal epithelium of rats from all groups, could be protein reabsorbed from the tubular lumen, which was previously reported by Confer and Panciera (20 1995). Electron microscopic examination of all nephrectomized rats showed enlargement of endothelial cells

that caused narrowing of capillary lumen. Fragmentation of endothelial cytoplasm was also observed. These changes suggest that altered endothelial cell ultrafiltration function may occur. Round electron-dense droplets that were found in podocyte in Group C could be reabsorbed droplets. The mesangial cells in Group C appeared normal. These results are consistent with undetectable proteinuria. Overall, these data suggest that DTS supplementation increases GFR and ERPF in nephrectomized rats leading to an efficient hyperfiltration process. DTS supplementation had no effect on renal structural changes in the remnant kidney of 5/6 nephrectomized rats. Further study should be done in a longer term to evaluate the effect of DTS supplementation on renal structural changes related to hyperfiltration. The components of DTS that are responsible for renal vasodilation should also be further determined, nitric oxide-induced vasodilation being a candidate mechanism.

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