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Sterological analysis of podocyte mitochondria in adriamycin nephropathy rats

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Abstract: Objective: To disclose the relationship between mitochondrial morphology, density and pro-teinuria in adriamycin nephropathy rats. **Method:** Thirty Sprague Dawley rats of clean grade were divided into adriamycin group and control group. In adriamycin nephropathy group, rats were given adriamycin at dosage of 0.7 mg /100 g body weight by tail vein injection. The control rats received equal volume of sa-line. At 2 weeks (control group = 3, adriamycin group = 3) , 4 weeks (control = 3, adriamycin group =6) and 6 weeks (control = 8, adriamycin group = 7) after adriamycin injection, the rats were sacrificed and kidneys were harvested for preparation of ultra-thin sections. Electron microscopy was performed, and podocyte mitochondrial morphology was observed. Sterological analysis was performed on morphology and density of mitochondria in podocytes. **Results:** 4 weeks after adriamycin injection, the rats developed proteinuria until 6 weeks. Mitochondria in the podocytes from control rats showed ellipsoid shape. Differ-ent shaped and sized mitochondria were observed in podocytes of the adriamycin nephropathy rats. No sig-nificant statistical difference was revealed in the mitochondrial area, circumference, form factor and aspect ratio between adriamycin and control groups. Before development of proteinuria, the mitochondrial density increased significantly at 2 weeks after adriamycin injection compared with that in control rats (0.17±0.00 vs. 0.14±0.01, $t = 6.173$, $P < 0.01$). Meanwhile, the surface density of mitochondria showed an increasing trend (0.78±0.03 vs. 0.71±0.04, $t = -2.526$, $P = 0.065$). 6 weeks after adriamycin injection, the surface density of mitochondria decreased significantly compared with that in the control rats (0.71±0.11 vs. 0.87±0.12, $P = 0.02$) , the density of mitochondria did not change significantly. Conclusions Dysmorphic mitochondria are involved in the development of proteinuria in adriamycin ne-phropathy. The increase of mitochondrial density is an early event in the development of proteinuria. De-crease of mitochondria surface density is involved in podocyte injury and development of adriamycin ne-phropathy rats.

Keywords: Nephrotic syndrome; Adriamycin; Mitochondria; Morphology; Quantitative analysis
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0 Introduction

Nephrotic syndrome is a common disease in children, which seriously affects the quality of life of children and their families [1]. Large amount of proteinuria is its outstanding manifestation, and it is also an independent risk factor affecting the prognosis of chronic kidney disease. Podocyte injury has been proved to be the main pathophysiological basis of massive proteinuria in nephrotic syndrome [2-3]. The mechanism of podocyte injury has not been fully elucidated. Recent studies have found that mitochondrial gene mutations can lead to nephrotic syndrome or massive proteinuria [4-5], mitochondrial dysfunction participates in the mechanism of podocyte injury [6-8], and mitochondrial shape changes have been observed in podocytes of patients with nephrosis or proteinuria related to mitochondrial gene mutations. In 2001, Hotta o et al. [4] found that severely damaged podocytes contained extremely deformed mitochondria in four FSGS patients with mitochondrial A3243G mutation. In 2005, gucer et al. [9] found a large number of polymorphic mitochondria in podocytes of 2 patients with mitochondrial DNA heterozygous deletion and clinical manifestation of nephrotic proteinuria. However, there is still a lack of stereological research on the morphological and quantitative changes of mitochondria during podocyte injury, and it is not clear whether the morphological and quantitative changes of mitochondria are involved in the occurrence of other non mitochondrial related proteinuria. In this study, the classic adriamycin induced nephropathy model in rats was used as the research object. The morphological and quantitative changes of mitochondria in the dynamic process of the occurrence and development of proteinuria were analyzed by stereological methods to explore the relationship between the morphological and quantitative changes of mitochondria and the occurrence and development of proteinuria.

1 Materials and methods

1.1 Establishment and selection of adriamycin induced nephropathy model in rats

This study was approved by the experimental animal ethics committee of the first hospital of Peking University. Thirty clean grade male spra gue Dawley rats weighing 120~160 g were randomly divided into two groups: adriamycin group (16 rats) and control group (14 rats). Adriamycin group was given 0.7 mg/100 g body weight of adriamycin (sigma) via caudal vein. The rats in the control group were injected with the same amount of normal saline through the caudal vein. All rats were kept in metabolic cages, fed with standard feed and water, and maintained a 12 h day night cycle. The experimental time was 6 W. The rats in the experimental group and the control group were killed at 2 w (3 rats in the control group and 3 rats in the adriamycin group), 4 w (3 rats in the control group and 6 rats in the adriamycin group) and 6 w (8 rats in the control group and 7 rats in the adriamycin group). 24 h urine samples were collected at 2 W, 4 W and 6 W after adriamycin injection for 24 h urine protein quantitative analysis. After the rats were killed, the kidneys were collected immediately. The renal cortex of the upper pole of the left kidney was taken, cut into 1 mm3 tissue blocks, and immediately fixed with precooled 2.5% glutaraldehyde for the preparation of transmission electron microscope samples [2].

1.2 Preparation of transmission electron microscope specimen of renal tissue and image capture

The kidney tissue samples fixed with glutaraldehyde were fixed with 1% osmic acid for 1 h, dehydrated with gradient alcohol, embedded with EPON 812 resin, sliced with ultra-thin microtome (Leica, Germany), stained with 2% uranium acetate and lead citrate, and positioned under transmission electron microscope (jeol-1230, Japan). The magnification was set to 25000 times, and moved in Z-shape from the edge of glomerulus. When podocytes were included in the visual field, photos were taken, About 50 mitochondria were taken from each case until the whole glomerulus was moved and photographed.

1.3 Stereological measurement of mitochondrial morphology in rat renal cortex

A group of grids are randomly superimposed to cover the whole picture. The distance between the vertical and horizontal lines of the grid is 1000 nm. The mitochondria that intersect with the grid line are taken as effective measurement targets, including the mitochondria that the test line passes through and the mitochondria that do not pass through but are closely connected with the grid line. For the latter, in order to avoid selection bias, The principle of judging whether the mitochondria are effective follows the principle introduced by Professor Yang Zhengwei in practical stereological methods [8], and the horizontal line follows the principle of priority above, that is, if the mitochondria are above the line segment, no gap between them is regarded as an effective target, and if the mitochondria are below the line segment, no gap between them is regarded as an invalid target. Similarly, the vertical line follows the principle of right first. In the analysis image processing software, the effective mitochondrial outline is outlined, and the area, perimeter, maximum aspect ratio and shape factor of mitochondria are measured.

1.4 Measurement of mitochondrial density in rat renal cortex

In order to understand the density of mitochondrial cross-section in podocyte and podocyte cytoplasm, this study analyzed the area number density of mitochondria, that is, the effective number of mitochondria in each photo divided by the area represented by the grid line used to measure podocyte or podocyte cytoplasm in this photo. The average value of relevant values in all photos of each sample represents the area number density of mitochondria in podocyte or podocyte cytoplasm in this case. Since only part of the images in the photo are podocytes, some of the grid lines must be located outside the podocytes, so it is difficult to calculate their area through line segments. Therefore, the area used for measurement is measured by the number of grid line intersections located in the podocytes, that is, the number of effective intersection points × one × one μm2. Criteria for judging whether the intersection is effective: the disputed one shall be subject to the upper right corner, that is, if the structure to be tested is in the opposite direction, the structure to be tested shall be effective in the upper right corner. If the structure to be tested is in the upper right corner, it shall be effective as long as there is no gap with the upper right corner [10]. At the same time, the surface density parameters of mitochondria were obtained by intersection counting method.

1.5 Statistical analysis method

SPSS statistical software was used for data analysis. The data were expressed in mean ±

standard deviation. T-test was used for comparison between the two groups. $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 Changes of urinary protein content in adriamycin rats

Two weeks after injection, there was no significant proteinuria in the adriamycin group compared with the control group. After 4 weeks, the 24h urinary protein of adriamycin group rats increased significantly, which was statistically significant compared with the control group at the same time point (150.5 ± 87.7 mg vs. 16.0 ± 9.2 mg, $P = 0.013$). After 6 weeks, the 24h urinary protein of adriamycin group rats further increased, and the difference was statistically significant compared with the control group at the same time point (226.9 ± 106.9 mg vs. 12.0 ± 4.4 mg, $P < 0.01$,
Table 1).

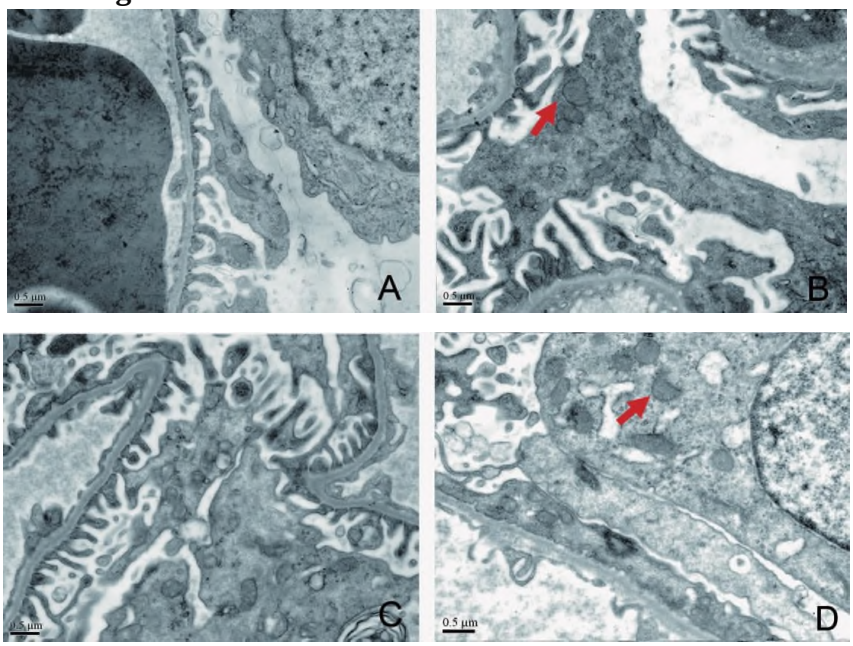
2.2 Ultrastructural changes of renal cortex in adriamycin rats

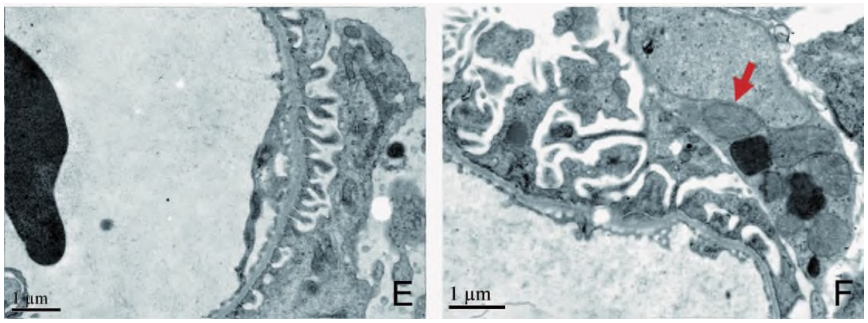
The results of transmission electron microscope observation on ultra-thin sections of rat kidney tissue showed that foot process fusion could be observed in the kidney tissue of adriamycin rats after 2 weeks of injection, and diffuse foot process fusion occurred after 4 and 6 weeks. The foot process structure of rats in the control group was intact (figure 1).

Table 1 Changes of 24-h urinary protein in adriamycin group at different time points

	2 w		4w		6w	
Control group	Mean±SD (mg)	n	Mean±SD (mg)	n	Mean±SD (mg)	n
Adriamycin group	6.0±4.4	3	16.0±9.23	3	12.0±4.4	8
	13.7±12.5	3	150. 5 ±87.7a	6	226. 8 ±106.9b	6

A. Compared with the control group at the same time point, $t = 3.717$, $P = 0.013$; b. Compared with the control group at the same time point, $t = -5.31$, $P < 0.01$





A. C and E are the transmission electron microscope pictures of the kidney tissue of the control group at 2 W, 4 W and 6 W, respectively. The morphology of the foot process is normal, and the mitochondria are oval; B. D and F are the transmission electron microscope pictures of the renal tissues of rats 2, 4 And 6 Weeks after adriamycin injection. The foot processes of the renal tissues of rats in adriamycin group were fused to varying degrees, with different sizes and shapes of mitochondria, swelling and irregular shapes of some mitochondria, and disordered arrangement of mitochondrial cristae.

Figure 1 Comparison of foot process morphology and mitochondrial morphology between adriamycin group and control group at different time points under electron microscope, transmission electron microscope, 25000 ×

Table 2 Comparison of morphological indexes and area number density of podocyte mitochondria between adriamycin group and control group at different time points

		N	Area (nm2)	Perimeter (nm)	Shape factor	Maximum aspect ratio	Area number density relative to cytoplasm (mean ± SD)	Area number density relative to cell body (mean ± SD)	Surface area density relative to cytoplasm (mean ± SD)	Surface area density relative to cell body (mean ± SD)
2w	Ctrl	3	84344.81 ±17668.41	1288.85±173.08	0.69±0.03	2.17±0.19	0.16±0.01	0.14±0.01	0.79±0.05	0.71±0.04
	Adr	3	74007.33 ±16573.03	1163.91±114.80	0.71±0.03	2.08±0.03	0.19±0.01a	0.17±0.00b	0.85±0.07	0.78±0.03c
4w	Ctrl	3	90796.00 ±16129.76	1297.02±97.79	0.70±0.02	2.06±0.19	0.16±0.05	0.16±0.05	0.74±0.21	0.69±0.20
	Adr	6	100938.95 ±19300.16	1327.34±137.37	0.74±0.03	1.92±0.21	0.14±0.04	0.13±0.03	0.60±0.18	0.56±0.15
6w	Ctrl	8	104815.43 ±31963.94	1345.85±174.48	0.72±0.05	2.01±0.24	0.19±0.03	0.17±0.03	0.87±0.12	0.77±0.11
	Adr	7	99290.14 ±19403.74	1310.77±128.88	0.73±0.03	1.94±0.16	0.17±0.02	0.16±0.02	0.71±0.11d	0.68±0.10

CTRL, control group; ADR, adriamycin group; a. Compared with the 2-W control group, t = 4.099, P = 0.015; b. Compared with the 2-W control group, t = 6.173, P = 0.003; c. Compared with 2 W control, t =-2.526, P = 0.065; d. Compared with 6 W control, t = 2.656, P = 0.019.

In the control group, the mitochondria of podocytes were mostly oval, with mitochondrial

cristae distributed in podocyte bodies and processes. Some mitochondria swelled and deformed after 2 weeks of adriamycin injection. The morphological changes of mitochondria in podocytes were more obvious after 4 and 6 weeks of adriamycin injection. The mitochondria were diverse in shape and size. Some mitochondria swelled and deformed, with irregular edges and fuzzy or disordered mitochondrial cristae (figure 1).

2.3 Stereological analysis of mitochondria in podocytes of renal cortex of adriamycin rats

Compared with the rats in the control group at the same time point, the morphological indexes of podocyte mitochondria, including mitochondrial area, perimeter, maximum length width ratio and shape factor, had no significant difference between the two groups at 2W, 4W and 6W after adriamycin injection (Table 2). Two weeks after adriamycin injection, the area number density of mitochondria in rat podocytes relative to podocytes and the area number density relative to podocyte cytoplasm increased significantly, with a statistically significant difference compared with the control group at the same time point (Table 2). Meanwhile, compared with the control group at the same time point, the mitochondrial surface area density per unit volume of podocytes in adriamycin group showed an increasing trend (P = 0.065, Table 2). After 6 weeks of adriamycin injection, although there was no significant difference in the area number density of mitochondria in podocytes compared with the control group at the same time point, the surface area density of mitochondria in podocytes relative to cytoplasm decreased significantly (P = 0.02, Table 2).

3 Discussion

Nephrotic syndrome is a common disease in children, and a large amount of proteinuria is its outstanding performance. A large number of studies have confirmed that podocyte damage in the outermost layer of glomerular filtration barrier is the key to the occurrence of proteinuria. The mechanism of podocyte injury has not been fully elucidated. A variety of podocyte hiatus membrane molecules, skeletal proteins and signal transduction molecules have been found to be involved [2-3]. Mitochondria are the energy factory of cells. Mitochondrial dysfunction is closely related to cell damage and apoptosis in a variety of diseases. Recent studies have found that abnormal mitochondrial function is also involved in the pathogenesis of podocyte injury and kidney disease [6-8], and abnormal mitochondrial morphology and number changes have been found in patients with mitochondrial related nephropathy [4, 9]. However, the relationship between mitochondrial morphological changes and mitochondrial number changes and non hereditary nephropathy or proteinuria is not clear, and there is still a lack of stereological research evidence. Adriamycin induced nephropathy in rats is a classic model of nephropathy. This study used this model to explore the relationship between the changes of mitochondrial morphology and number and the occurrence and development of proteinuria.

In this study, single blind random sampling and single blind analysis were used to reduce bias in the measurement of mitochondria. Electron microscopy and stereology were used to analyze the changes of mitochondrial morphology and number. Similar to that reported by imasawa et al. [11], this study shows that the mitochondria in the control group are mostly oval and distributed in the podocyte body and podocyte process. This study found that

mitochondrial morphological variation was involved in the occurrence of proteinuria in adriamycin rats. When there was obvious proteinuria in adriamycin induced nephropathy model in rats, i.e. 4 and 6 weeks after adriamycin injection, the morphology of podocyte mitochondria changed significantly, showing different sizes and shapes, from oval to various shapes, such as round and irregular oval, and some mitochondria were significantly enlarged and swollen. This is similar to the morphological changes of mitochondria observed in podocytes of patients with mitochondrial associated nephropathy. Hotta et al. [4] observed the ultrathin sections of the renal tissues of 4 patients with focal segmental glomerulosclerosis with mitochondrial A3243G mutation by electron microscope, and found that the damaged podocytes had abnormal mitochondria, which were diverse in size and shape, irregular in outline, and increased in mitochondrial cristae. The swollen mitochondrial cristae were disordered. Dinour et al. [12] also found an increase in abnormal mitochondria in damaged podocytes in 2 patients with glomerulopathy related to A3243G mutation who showed proteinuria and progressed to terminal kidney. Barisoni et al. [13] also observed that the morphological changes of podocyte mitochondria were accompanied by proteinuria and glomerular segmental sclerosis in the mouse model of collapse glomerulopathy. These abnormal mitochondrial morphology may be involved in podocyte injury by affecting mitochondrial energy metabolism. This study also further measured the mitochondrial morphological indicators, including the area, perimeter, shape factor and maximum aspect ratio of mitochondria, and compared the differences of various indicators between the adriamycin group at different time points and the control group at the same time point. The results showed that no statistical difference was found. The reason may be related to the large variation among adriamycin rats and the large variation of mitochondrial morphology in the podocytes of the same rat, Hotta et al. [4] also observed that in patients with mitochondrial associated nephropathy, abnormal mitochondrial aggregation in different glomeruli and podocytes is different.

It has been previously reported that the number of mitochondria in podocytes of patients with mitochondrial associated nephropathy increased. Markowitz et al. [14] found that the number of mitochondria in podocytes increased in patients with collapse type focal segmental glomerulosclerosis caused by extensive application of pamidronate disodium. However, the correlation between the increase of mitochondrial number and proteinuria is not clear, and there is no objective basis of stereology. In this study, the quantitative analysis of the number of mitochondria in the process of protein emergence, occurrence and progression of adriamycin induced nephropathy model in rats was carried out by stereological method. The results showed that the area number density of mitochondria in podocytes of renal tissue was significantly higher than that of the control group at the same time point when adriamycin was injected for 2 weeks. Similar results can be found in the model of type 2 diabetes related nephropathy studied by Coimbra et al. [15]. This study found that the number of podocyte mitochondria in the renal tissue of the model rats increased significantly at the age of 10 weeks before the occurrence of proteinuria. The results of this study and Coimbra et al. Suggest that the increase in the number of podocyte mitochondria is not only seen in patients with mitochondrial associated

nephropathy associated with mitochondrial gene mutation, but also an early phenomenon in the process of acquired podocyte injury and proteinuria. This phenomenon may be used as a pathological phenomenon for clinical evaluation of podocyte injury in patients with nephrotic syndrome or proteinuria, and is worthy of further study on mitochondrial stereology in patients with nephrotic syndrome.

The reason for the increase in the number of mitochondria and the exact mechanism involved in proteinuria and podocyte damage are not clear. The increase in the number of mitochondria may lead to the increase in the area of mitochondrial membrane, which is an important place for mitochondria to conduct energy metabolism and produce ATP [16]. Therefore, this study further discussed the changes of mitochondrial membrane area. The results showed that the surface area density of podocyte mitochondria relative to podocyte body increased when adriamycin was injected for 2 weeks (P = 0.065). The lack of statistical significance may be related to the small number of rats. It is speculated that the increase in the number of mitochondria before proteinuria in the early stage of adriamycin induced nephropathy in rats may be a compensatory phenomenon of podocytes to injury stimulation.

In addition, this study also found that the number of mitochondria in podocytes did not decrease significantly, but the surface area density of mitochondria relative to the cytoplasm of podocytes decreased significantly after 6 weeks of adriamycin injection, which has not been reported. The decrease of mitochondrial surface area density can affect the production of ATP, thus affecting the function of podocytes, suggesting that the decrease of mitochondrial surface area density of podocytes is involved in the progress of podocyte disease, so it may be used as a morphological index for the progress of podocyte disease in clinic.

In conclusion, this study explored the dynamic changes of podocyte mitochondrial morphology and number during the development of proteinuria in adriamycin induced nephropathy model through stereological analysis for the first time, revealing that podocyte mitochondrial morphology polymorphism is involved in the occurrence of adriamycin induced proteinuria, and the increase of podocyte mitochondrial number is an early event before the occurrence of proteinuria in adriamycin induced nephropathy model, The decrease of podocyte mitochondrial surface density and a large amount of proteinuria occurred at the same time. It is speculated that the increase of mitochondrial number is an early compensatory event of adriamycin induced nephropathy in rats. The decrease of mitochondrial surface density is involved in the progression of podocyte induced nephropathy.

Thank:

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References

[1] Guan Na, Ding Jie, Yang Jiyun, et al. Chinical charac-teristics and prognosis analysis of children with idiopathic nephrotic syndrome with different responses to steroid therapy from a single center in 20 years[J]. Chinese Journal of Applied Clinical Pediatric, 2014, 29 (17) : 1291-1295. (in Chinese)

[2] Deng Jianghong, Ding Jie, Guan Na, et al. Morphomet-ric analysis of of glomerular

foot process in puromycin aminonucleoside nephropathy in rat[J]. Chinese Journal of Sterology and Image Analysis, 2003, 8 (1) : 9-15. (in Chinese)

[3] Tharaux P L, Huber T B. How many ways can a podo-cyte die? [J]. Seminars in Nephrology, 2012 , 32(4) : 394-404.

[4] Hotta O, Inoue C N, Miyabayashi S, et al. Clinical and pathologic features of focal segmental glomerulosclerosis with mitochondrial trna Leu (UUR) gene mutation [J]. Kidney International, 2001, 59(4) : 1236-1243.

[5] Guan Na. Progress in diagnosis and treatment of mito-chondrial nephropathy[J]. Chinese Journal of Pediatric, 2014, 52(7) : 503-505. (in Chinese)

[6] Xie Kewei, Gu Leyi. Mitochondria injury and glomerular dis-ease[J]. Chiese Journal of Integrated Traditional and West-ern Nephrology, 2013, 14(3) : 360-362. (in Chinese)

[7] He Weichun, Yang Junwei. The study progression of mi-tochondria and podocyte[J]. Chinese Journal of Neph-rology, 2007, 23(1) : 61-63. (in Chinese)

[8] Zhu C, Xuan X, Che R, et al. Dysfunction of the PGC-1 α-mitochondria axis confers adriamycin-in-duced podocyte injury[J]. American Journal of Physiology-Renal Physiology, 2014, 306(12) : 1410-1417.

[9] GüCer S, Talim B, Asan E, et al. Focal segmental glomer-ulosclerosis associated with mitochondrial cytopathy: report of two cases with special emphasis on podocytes [J]. Pedi-atric Developmnetal Pathology, 2005, 8(6) : 710-717.

[10] Yang Zhengwei. The basic tools for the study of quantita-tive morphology of biological tissue: The utility of stereo-logical method[M]. Beijing: Science Press, 2012. (in Chinese)

[11] Imasawa T, Rossignol R. Podocyte energy metabolism and glomerular diseases[J]. The International Journal of Biochemistry and Cell, 2013, 45(9) : 2109-2118.

[12] Dinour D,mini S, Polak-Charcon S, et al. Progressive nephropathy associated with mitochondrial trna gene mu-tation[J]. Clinical Nephrology, 2004, 62(2) : 149-154.

[13] Barisoni L, Madaio M P, Eraso M, et al. The kd/kd mouse is a model of collapsing glomerulopathy [J]. Jour-nal of the American Society of Nephrology, 2005, 16 (10) : 2847-2851.

[14] Markowitz G S, Appel G B, Fine P L, et al. Collapsing focal segmental glomerulosclerosis following treatment with high-dose pamidronate[J]. Journal of the American Society Nephrology, 2001, 12(6) : 1164-1172.

[15] Coimbra T M, Janssen U, Gr□ne H J, et al. Early events leading to renal injury in obese Zucker (fatty) rats with type II diabetes[J]. Kidney International, 2000, 57(1) : 167-182.

[16] Mannella C A, Lederer W J, Jafri M S. The connection between inner membrane topology and mitochondrial function[J]. Journal of Molecular and Cellular Cardi-olgy, 2013, 62: 51-57.