Effects of Keto-Analogs in the Pathologic Findings of Diabetic Nephropathic Rats

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Abstract

Objective: Some studies have shown that keto amino acids (KA) reduce proteinuria and improve renal function as well as nutritional status in patients with diabetic nephropathy. We aimed to compare the pathologic findings of kidneys of rats that were given protein-restricted diets enriched with KA with those of rats given protein-restricted diet alone.

Materials and Methods: The study included 22 16-week-old Sprague–Dawley rats and was conducted at the research center. The rats were randomly divided into two equal groups. Group I (study) received KA (35-70 mg/kg) with gavage along with low-protein diet (10% protein), and Group II (control group) received low-protein diet alone. The treatment was continued for 2 weeks, after which the study ended. Blood samples were obtained in the 5th and 7th weeks of treatment for measuring albumin and creatinine levels; bilateral nephrectomy was performed at the end of the study.

Results: All rats had minimal thickness of the basement membrane. The serum albumin levels were significantly higher in the study group (p<0.05). The study group rats had greater thickness of the basement membranes.

Conclusion: Our study may inspire further studies in this particular field with the question whether KA can slow the progression of diabetic nephropathy.

Keywords: Diabetic nephropathy, keto amino acids, kidney failure

INTRODUCTION

End-stage renal failure (ESRD) represents an important health concern due to its increasing incidence and prevalence (1, 2). One of the leading causes of ESRD globally is diabetic nephropathy (DNP), which is also the most common cause of ESRD in Turkey (3, 4). Therefore, preventing this complication of diabetes, the mechanism of which is not fully understood, will be an important step in preventing ESRD development.

DNP is a clinical condition characterized by proteinuria or albuminuria (300 mg/day or 200 mcg/min, respectively), decreased glomerular filtration rate, and increased arterial blood pressure, demonstrated in two different urine assays for 3-6 months (5). Genetic factors may be present in the pathophysiology of DNP; however, environmental factors play a role in the progression of the disease (2).

The amount of protein in the diet is important in all patients with chronic kidney disease (CKD) (6). It has been shown that a low-protein (0.6 mg/kg/day) diet in patients with CKD, including DNP, has been shown to slow the progression to ESRD (7). Low-protein dietary intake causes a decrease in uremic symptoms and levels of phosphate, sulfate, organic acid, and amine, which occurs as a result of protein catabolism in patients (8). However, the fact that patients cannot receive essential amino acids, while they are limited from taking protein causes nutritional deficiency (8, 9). Malnutrition develops in these patients due to protein-restricted diet. In

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addition, a very low-protein diet has been associated with increased mortality in CKD (9).

Keto amino acids (KA) are composed of nitrogen-poor analogs of essential amino acids. KA neutralize the excess nitrogen load by inhibiting the urea cycle and preventing urea production. In addition, it helps in maintaining the nutritional status (10).

In this study, the effects of protein-poor diet and KA given in addition to the protein-poor diet on the kidney of rats with DNP were examined comparatively under light microscopy.

MATERIALS AND METHODS

The study included 22 16-week-old Sprague–Dawley rats and was conducted at the research center. The rats were kept on a 12-hour day/night cycle with a controlled temperature of 23°C±2°C and free access to water and food. At the beginning of the study, 65 mg/kg intraperitoneal streptozocin injection was administered to the rats. At 48 hours, venous blood glucose was measured with glucostrip (Abbott Medical, IL, USA), and rats with blood glucose levels of ≥300 mg/dL were considered diabetic. Rats were fed with a standard rat feed containing 20% protein up to the 5th week. The rats were placed in metabolic cabinets in the 5th week, and 24-hour urine was collected. On the day of collection, anesthesia was performed with propofol and ketamine, and 1 cc of blood was collected from the subclavian artery. It was centrifuged, and the serum was stored at −80°C. The amount of protein in the 24-hour urine was measured using the rat kit (Vettest UPC Urine Protein/Creatinine 24 test).

Eighteen rats with DNP diagnosis were randomly divided into two equal groups. Group 1 (study group) received a low-protein diet (10% protein) and 35-70 mg/kg KA with gavage (11), and Group 2 (control group) was given a low-protein diet alone. The rats received subcutaneous long-acting insulin (insulin glargine-Sanofi) three times a week. This treatment was continued for 2 weeks. At the end of these 2 weeks, the animals were anesthetized with propofol and ketamine, and their blood was collected, centrifuged, and stored at −80°C. The rats were weighed at the beginning and at the end of the experiment, and their weights were recorded. Then, nephrectomy was performed, and all animals were sacrificed. Nephrectomy content was put into formol and sent to pathology laboratory. During the study, the care and evaluation of the data of the animals were conducted in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. This study was approved by the University Ethics Committee for Experimental Animals and supported by the University Scientific Research Projects Unit.

Biochemical Method

Rat albumin Enzyme Linked Immunosorbent Assay (ELISA)

Following the thawing of the serum and urine samples, an albumin ELISA kit (elabscience, USA, lot no: E-EL-R0362) was used to measure the albumin beta value. Samples and standards were added to the plates coated with antihuman monoclonal antibodies prior to incubation. After incubation, the plate was washed to remove the unbound enzyme. Then, the 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was added to change the color of the liquid to blue. With the effect of the acid, the final color was yellow. The optical density was evaluated at 450 nm with a standard plate reader (Thermo Scientific Microplate Reader). The detection limits of the kit used were between 1.25 and 80 µg/mL.

Rat serum creatinine ELISA

Following the thawing of the serum and urine samples, a serum creatinine ELISA kit (elabscience, USA, lot no: E-EL-R0058) was used to measure the serum creatinine beta value. Samples and standards were added to the plates coated with antihuman monoclonal antibodies prior to incubation. After the incubation, the plate was washed to remove the unbound enzyme. Then, the TMB solution was added to change the color of the liquid to blue. With the effect of the acid, the final color was yellow. The optical density was evaluated at 450 nm with a standard layer reader (Thermo Scientific Microplate Reader). The detection limits of the kit were between 3.13 and 200 µg/mL.

Evaluation of urine protein

The protein amounts in the samples were evaluated using the Bradford assay kit (Thermo scientific Pierce BCA) and were monitored with a microplate Reader at a wavelength of 595 nm (Thermo Scientific Multiskan FC, 2011-06, USA).

Pathological evaluation

For histological evaluation, bilateral nephrectomy materials were fixed with 10% formol. These tissues were recorded and transferred to a Thermo closed system, a fully automatic tissue tracking device. The tissue was embedded in paraffin blocks after the tissue follow-up was completed; 3-µm-thick sections were taken with microtome. All sections were stained with hematoxylin–eosin and periodic acid Schiff stains. The histopathological examination was evaluated by light microscopy. The pathological classification was performed according to the criteria published by Tervaert et al. in 2010 (12). Glomerular lesions (thickening of basement membranes, mesangial enlargement, nodular sclerosis, and advanced diabetic glomerulosclerosis) and tubulointerstitial lesions (tubular atrophy and interstitial fibrosis) were evaluated as none: 0, <25%: 1, 25%-50%: 2, and >50%: 3; interstitial inflammation was evaluated as none: 0, if around fibrotic interstitium and atrophic tubule: 1, and if in other areas: 2; arteriolar hyalinosis was evaluated as none: 0, one arteriole: 1, and multiple arterioles: 2.

Statistical Analysis

Data are presented as average±standard deviation. The comparison of the averages of variables that do not show normal distribution was performed using the variance analysis and Mann–Whitney U tests. Two-tailed p<0.05 were considered sta-

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tistically significant. Statistical analyses were performed using The Statistical Package for the Social Sciences (SPSS) version 19.0 (IBM Corp.; Armonk, NY, USA).

RESULTS

The characteristics of the rats at the beginning of the experiment are shown in Table 1. There was no significant difference between their weights and blood sugar levels on the 1st day of the study (p=0.161). In all rats, the blood glucose level was >300 mg/dL 48 h after streptozocin injection. Polyuria and weight loss in the animals continued throughout the experiment. The average proteinuria in the 5th week was 19.67±9.31 μg/mL. When the rats were divided into two groups, this value was found to be 19.38 μg/mL in the study group and 19.96 μg/mL in the control group. There was no significant difference between the groups (p=1.00).

In the biochemical analysis performed, the average serum creatinine level in the study group was 45.26±29.87 μg/mL in the 5th week and 86.20±19.36 μg/mL in the 7th week (p=0.79). In the control group, the average serum creatinine level was 51.27±14.98 μg/mL in the 5th week and 92.26±23.04 μg/mL in the 7th week (p=0.79).

The average serum albumin level of the rats was 16.03±3.89 μg/mL in the 5th week and 10.12±4.58 μg/mL in the 7th week. The average serum albumin level in the 5th week was 16.11±3.49 μg/mL in the study group and 15.95±4.45 μg/mL in the control group. The average serum albumin level in the 7th week was 13.15±4.53 mg/dL in the study group and 7.09±1.83mg/dL in the control group. In both groups, the serum albumin levels in the 7th week were lower than those in the 5th week. The decrease in serum albumin level was significantly lower in the study group than in the control group (p=0.008).

Table 1. Evaluation of Study Variables Using the Mann–Whitney U test

|	Study Group (n=9)	Control Group (n=9)	p
First weight, gr	339.11±54.92	317.00±29.82	0.387
Weight in the 7th week, gr	225.44±50.46	232.44±22.24	0.931
Blood glucose level in the 5th week, mg/dL	95.88±14.49	103.00±13.78	0.161
Serum creatinine in the 5th week, μg/mL	45.26±29.87	51.27±14.98	0.796
Serum creatinine in the 7th week, μg/mL	86.21±19.37	92.26±23.04	0.546
Proteinuria in the 5th week, μg/mL	19.38±8.01	19.96±10.93	1.00
Albumin in the 5th week, μg/mL	16.11±3.49	15.95±4.45	0.863
Albumin in the 7th week, μg/mL	13.15±4.53	7.09±1.83	0.008
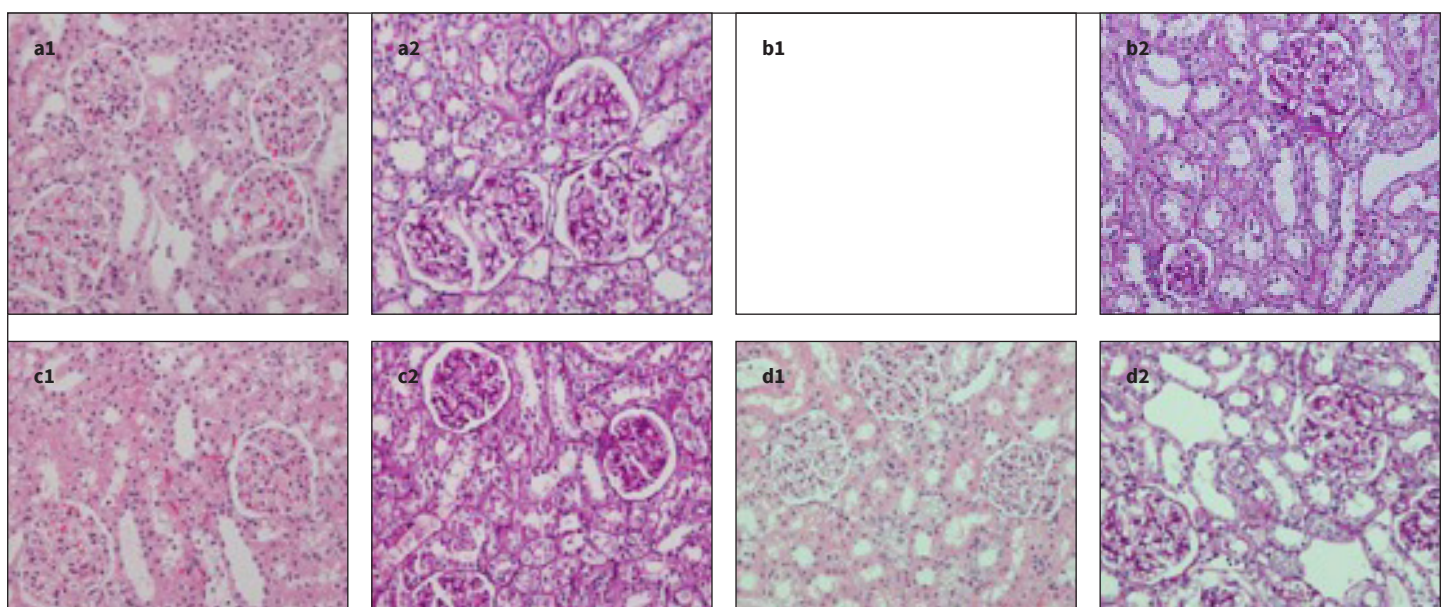
Figure 1. a-d. Pathological samples from rats; Groups a and b: study groups, Groups c and d: not receiving keto-amino acid. In Groups a) and b), HEX200 and PASX200 dyes show segmental thickening of the basement membranes and regular tubulus structures. In Groups c) and d), normal tubulus cells draw attention in HEX200 and PASX200 dyes.

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In the pathological examination of the kidneys of the rats, a minimal thickness increase in the basement membranes was detected. It was observed that the basement membrane was thicker in two rats when compared to others, and atrophic tubules with minimal mononuclear infiltration were seen in one rat (Figure 1).

DISCUSSION

In the diabetic rat model, the use of KA in the diet along with a low-protein diet did not lead to an increase in nitrogenemia while maintaining albumin levels. In the study, no significant difference was found between the serum creatinine levels at the end of 7 weeks. In both groups, the serum albumin levels in the 7th week were found to be lower than those in the 5th week in accordance with the progression of DNP. However, when the albumin levels of both groups were analyzed separately, the albumin levels were significantly higher in the group receiving KA. Although the mechanism of this anabolic effect caused by KA has not been fully resolved, in a rat model of CKD, it was shown that apoptosis was suppressed in rats fed very low-protein diet in which KA were given, protein synthesis was increased, and protein degradation was observed (13).

In a prospective, randomized controlled study, 23 patients with Stage IV DNP were given KA with low-protein diet for 1 year, and the other group received diabetic diet only. At the end of the study, proteinuria was found to be significantly lower in the group receiving KA, and the decrease in glomerular filtration rate was found to be slower (14). In our study, our aim was to determine the amount of final proteinuria in rats fed according to our protocol. However, due to the deterioration of the general condition of the rats and the rapid progression of the catabolic process, the study was terminated early, and the control proteinuria could not be evaluated. However, if we consider that the albumin level is significantly higher in the study group than in the control group, we believe that proteinuria is lower in the study group than in the control group. There was no significant difference in the serum creatinine levels between the study and control groups. Because the study was terminated earlier than planned, the positive effect on the serum albumin levels of the study group could possibly not be detected in the serum creatinine levels.

The relationship between structural abnormalities and renal function in DNP is better explained using light and electron microscopies (15). Mesangial dilatation, which can be detected by electron microscopy in Type I diabetes mellitus, is the best demonstrated parameter in relation to renal function (16).

Glomerular basement membrane thickening detected in pathological examination manifests itself more clinically with proteinuria (17). In our study, the prominent pathology detected in the nephrectomy content was also the glomerular basement membrane thickening (Figure 1). Other findings that may be seen in DNP are structural changes that occur in podocytes, renal tubules, interstitium, and arterioles (18). These pathologies

could not be detected in the samples obtained in our study. The failure to show mesangial expansion may be attributed to the lack of electron microscopic examination. In a study by Fioretti et al. (15), it was found that over 15% of overt proteinuria was detected in biopsy materials with normal or near-normal renal structure, and 50% proteinuria developed in cases with DNP. Because the 24-hour proteinuria of the rats in our study is >10 g/day, it can be said that there is severe nephrotic proteinuria in the rats and that DNP is severe when the amount of proteinuria is examined.

We consider that the detection of only glomerular basement membrane thickening in our samples could be correlated with the fact that we could not perform the electron microscope examination and the shortness of the follow-up period. In addition, low-protein diet given after grouping of animals may have a positive effect on the pathological findings. The short follow-up period after rats were divided into two groups may be the reason why we could not see the positive effect of KA on pathology. Also, in another study, it would be possible to form a group of rats receiving a normal protein diet to rule out the likelihood that the present positive effect results from the low-protein diet.

CONCLUSION

DNP is an important renal disease in terms of causing ESRD. Low-protein intake plays an important role in the prevention of this disease. Although KA given in addition to a low-protein diet have been shown to affect serum albumin levels positively, in our study, to determine their effects on renal pathology, there is a need for electron microscopy studies using animal models that may also have long-term life expectancy.

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