Original Article

Alteration of Inflammation Marker Levels with Alfa Keto Analogs in Diabetic Rats

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ABSTRACT

Background: Diabetes mellitus is the most well-known and common cause of end-stage renal disease. Excessive inflammatory processes were hypothesized to be one of the reasons for progression to end-stage disease. Even though progression to end stage disease tried to be prevented with some dietary measures such as lowering nitrogen in diet, none of the methods tried were successful enough. **Aims:** In our study, we aimed to determine the effects of alfa keto analog use in altering levels of inflammatory markers when added to dietary program in a diabetic rat model. **Patients and Methods:** The study was performed on 22 male Sprague Dawley rats with streptozocine induced diabetic nephropathy. Both groups were fed with low protein diet except for study group with added alfa keto analogs. Biochemical values and inflammatory markers were studied with ELISA assay. **Results:** Significant difference in serum albumin was found between study group and control group following administration of alfa keto analogs ($p < .001$). Also mentioned dietary modification made a significant difference in suppression of inflammatory reactions for interleukin (IL)-1, IL-6, IL-10, IL-18 and tumor necrosis factor-α. **Conclusion:** Adding keto amino acids to diets that are already low on protein, can slow progression to end-stage renal disease by reducing inflammation and protein loss in an animal model.

Keywords: *Alfa keto analogs, diabetic nefropathy, interleukin, rat*

Introduction

Diabetes mellitus (DM) is the most common cause of end-stage renal disease (ESRD) worldwide, causing diabetic nephropathy (DNP) which occurs in one-third of patients with type 1 and 25% of patients suffering from type 2 DM.[1,2] According to some authors, kidney failure can be prevented by monitoring all risk factors contributing to DNP such as old age, male gender, hypertension, smoking and hyperuricemia with their interaction with each other.[2,3]

Being on either low protein (0.6 g/kg/day) or very low protein (0.3 gr/kg/day) diet has been proven to be effective on prevention of progression from chronic kidney disease (CKD) to ESRD by reducing the accumulation of nitrogen waste products.[4,5] Alfa keto analogs of keto amino acids (KAA) are nitrogen-free analogs of essential amino acids which are shown to decrease urea production and prevent malnutrition, which is an important issue for patients on low protein diets, in clinical studies.[6] In a study by Chang *et al*.,[7] 120 diabetic patients were divided into two groups, where both were provided with low protein diets but only one with KAA supplementation, it was shown that, KAA supplementation delayed the progression to CKD without making a big impact on nutritional status.

Studies show chronic inflammation elements such as cytokines, growth factors, chemokines, nuclear factors and adhesion factors taking part in pathogenesis of progression to DNP.[1,2,8,9]

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Although there are diverse options for DM treatment, none of them is proved to prevent kidney damage so far. Suppressing inflammation can be a milestone in prevention of progression towards ESRD. In our study, we aimed to find out the effects of KAA in altering levels of inflammatory markers when added to dietary program in a diabetic rat model.

Material and Methods

The study was performed on 22 male Sprague Dawley rats in Research Center. All animal procedures were done according to the guidelines of the European Council for Animal Care and the Animal Ethics Review Committee of the University (protocol number 2017/39).

Rats stayed at the experimental animal center in a controlled environment at a temperature of 22°C ± 1°C with a 12-hour light/dark cycle. The animals were fed with distilled water ad libitum and standard chow diet. Their weights were monitored, and blood sugar was measured from venous sample of rats' foot (Free Style Optimum Glucometer ®). Then they were given streptozocine intraperitoneally at 65 mg/kg. Twenty rats with blood sugar level equal to or above 300 mg/dl at 48th hours were accepted as diabetic. Insulin glargine (Lantus® Sanofi-Aventis Deutschland GmbH) was administered intraperitoneally 0.1–0.2 units/kg intraperitoneally every day. Rats were fed with standard diet until 5th week of the study where DNP was determined by 24 hours urine analysis. Eighteen of twenty rats were diagnosed with DNP and were randomly divided into two interventional groups each group containing 9 rats. Group I (study group) was given KAA (35-70 mg/kg) by gavage along with low protein diet, group II (control group) was fed with only low protein diet (10% protein). The treatment continued for 2 weeks. Rats were weighed at the end of the study. Blood samples were obtained at 5th and 7th weeks of treatment for measurement of albumin, creatinine, interleukin (IL)-1, IL-6, IL-10, IL-18 and tumor necrosis factor (TNF)-α.

Sample collection and preparation

Blood samples were collected in tubes containing heparin and serum samples were removed by centrifugation for 10 minutes at 3000 x rpm. The samples were maintained at −80°C before performing assays. Urine samples were collected in tubes and maintained at −80°C before performing assays.

Urine protein assay

Protein amount in samples were determined by Bradford assay kit (Thermo Scientific Pierce BCA) and was observed at 595 nm wavelength with plate reader (Thermo Scientific Multi-scan FC, 2011-06, USA).

Rat IL-10 ELISA assay

Samples were thawed and IL-10 ELISA kit (Elabscience, USA, lot no: E-EL-R0016) was used for the quantitative measurement of IL-10 in serum and urine samples. Samples and standards were added to appropriate wells which were pre-coated with anti-human monoclonal antibody before incubation. After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 31.5 pg/ml and 2000 pg/ml.

Rat IL-18 ELISA assay

Samples were thawed and IL-18 ELISA kit (Elabscience, USA, lot no: E-EL-R0567) was used for the quantitative measurement of IL-18 in serum and urine samples. Samples and standards were added to appropriate wells that were pre-coated with anti-human monoclonal antibody before incubation. After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 15.68 pg/ml and 1000 pg/ml.

Rat IL-6 ELISA assay

Samples were thawed and IL-6 ELISA kit (Elabscience, USA, lot no: E-EL-R0015) was used for the quantitative measurement of IL-6 in serum and urine samples. Samples and standards were added to appropriate wells, which were pre-coated with anti-human monoclonal antibody before incubation. After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 62.5 pg/ml and 4000 pg/ml.

Rat IL-1 beta ELISA assay

Samples were thawed and IL-1 beta ELISA kit (Elabscience, USA, lot no: E-EL-R0012) was used for the quantitative measurement of IL-1 beta in serum and urine samples. Samples and standards were added to appropriate wells, which were pre-coated with anti-human monoclonal antibody before incubation.

After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 31.5 pg/ml and 2000 pg/ml.

Rat TNF-alpha ELISA assay

Samples were thawed and TNF-alpha ELISA kit (Elabscience, USA, lot no: E-EL-R0019) was used for the quantitative measurement of TNF-Alpha beta in serum and urine samples. Samples and standards were added to appropriate wells, which were pre-coated with anti-human monoclonal antibody before incubation. After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 78.13 pg/ml and 5000 pg/ml.

Rat albumin ELISA assay

Samples were thawed and albumin ELISA kit (Elabscience, USA, lot no: E-EL-R0362) was used for the quantitative measurement of albumin beta in serum and urine samples. Samples and standards were added to appropriate wells which were pre-coated with anti-human monoclonal antibody before incubation. After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 1.25 μg/ml and 80 μg/ml.

Rat creatinine ELISA assay

Samples were thawed and creatinine ELISA kit (Elabscience, USA, lot no: E-EL-R0058) was used for the quantitative measurement of Creatinine beta in serum and urine samples. Samples and standards were added to appropriate wells, which were pre-coated with anti-human monoclonal antibody before incubation. After incubation, washing the plate to remove the uncombined enzyme. Then TMB solution was added for the color of the liquid changes into the blue. At the effect of acid, the color finally becomes yellow. Optical density was read on a standard automated plate reader at 450 nm (Thermo Scientific Microplate Reader). The detection range of kit was between 3.13 μg/ml and 200 μg/ml.

Results

Rats enrolled in this study were initially weighted between 280 g and 424 g and their blood sugar levels were between 87 mg/dl and 92 mg/dl. On the day of group formation and on the day of sacrifice, weights and blood sugar levels of the rats were similar between groups. Evaluation on the 5th week revealed similar rate of proteinuria for both groups (19.38 ± 8.01 μg/ml, 19.96 ± 10.93 μg/ml for study and control groups, respectively) and all rats were diagnosed with DNP.

At the 7th week, a significant difference in serum albumin was found between study and control group as 13.15 ± 4.53 μg/ml to 7.09 ± 1.83 μg/ml (p <.001).

Table 1: Comparison of group characteristics on 5th and 7th weeks of study

	Study group (KAA)	Control group (Non-KAA)	*P*
Blood glucose at 5th week	95.88±14.49	103±13.78	0.302
Proteinuria at 5th week	19.38±8.01	19.96±10.93	0.901
Weight at 5th week	339.11±54.92	317±29.82	0.304
Weight at 7th week	225.44±50.46	232.44±22.24	0.708
Serum creatinine at 5th week	45.26±29.87	51.27±14.98	0.796*
Serum creatinine at 7th week	86.21±19.37	92.27±23.04	0.555
Albumin at 5th week	16.11±3.49	15.95±4.45	0.931
Albumin at 7th week	13.15±4.53	7.09±1.83	**0.002**

Analyses performed with independent samples *t*-test for parametric values. Significant values with a significance level of 0.05 are bolded. *Non-parametric value comparison performed with Mann-Whitney *U* test

Table 2: Comparison of inflammatory responses on 5th and 7th weeks of study with their differences between weeks (Δ-value)

	Study group (KAA)	Control group (Non-KAA)	*P*
IL-1 5th week	8.9±7.66	7.24±3.30	0.558
IL-1 7th week	13.12±4.41	6.46±4.85	**0.008***
IL-6 5th week	9.75±3.70	14.82±5.98	**0.046**
IL-6 7th week	9.93±4.12	22.71±3.55	**0.000**
IL-10 5th week	7.68±3.25	9.71±3.37	0.212
IL-10 7th week	5.98±2.24	13.32±4.21	**0.000**
IL-18 5th week	6.72±3.47	12.29±4.08	**0.007**
IL-18 7th week	4.92±2.38	17.82±2.76	**0.000**
TNF-α 5th week	11.45±4.44	19.31±7.35	**0.014**
TNF-α 7th week	11.67±4.94	29.25±4.63	**0.000**
Δ IL-1	4.22±9.77	-0.78±4.92	0.190
Δ IL-6	0.17±5.08	7.89±6.96	**0.016**
Δ IL-10	-1.70±4.17	3.62±6.55	**0.024***
Δ IL-18	-1.80±4.17	5.53±5.14	**0.005**
Δ TNF-α	0.22±6.10	9.94±8.79	**0.015**

Analyses performed with independent samples *t*-test for parametric values. Significant values with a significance level of 0.05 are bolded. *Non-parametric value comparison performed with Mann-Whitney *U* test

Results from other relevant variables are presented in Table 1.

When differences of inflammatory markers between 5th and 7th weeks were analysed, IL-6 and TNF-α had the highest significantly different increases as p: 0.016 and p: 0.015, respecively. Mean comparison of all inflammatory markers studied and their differences are listed in Table 2 for further understanding.

Discussion

The Modification of Diet in Renal Disease (MDRD) Study is the largest randomised prospective study that claimed low protein diets and low phosphorus diets can slow progression to ESRD.[10] Alfa keto analogs of KAA decrease the formation of urea by taking nitrogen from non-essential aminoacids, reusing the amino group.[11] In recent studies, it was shown that ESRD progression and proteinuria can be prevented by using low protein diet combined with KAA.[12,13]

Some studies suggest that IL-6 levels are associated with early detections of DN like glomerular basal membrane thickening also with renal hyperthrophy and increased proteinuria.[14-16] IL-6 mRNA was found in mesengial, interstitial and tubular areas of kidney biopsy specimen in diabetic patients proving its role acting as an inflammation marker in DNP.[17] TNF-α is also one of the cytokines which has a fundamental role in pathogenesis of DNP with its ability to reduce glomerular filtration, toxicity to renal cells, induction of apoptosis and causing necrosis.[15,18-20] In clinical studies, serum and urinary concentration of TNF-α were detected higher in patients with DNP than without, with a positive correlation between TNF-α levels and DNP progression.[21-23] Our study showed that within 2 weeks time frame of the study period, IL-6 and TNF-α levels were significantly suppressed with the use of KAA in diet.

IL-18 is the cytokine that is a member of IL-1 superfamily which activates pathways producing other cytokines like IL-1, interferon gamma or TNF-α.[24-26] Araki *et al*.[27] found that elevated IL-18 can be an early marker for DNP in patients with type 2 diabetes. Similar to that study, both in serum and urine samples, elevated IL-18 levels were detected in patients with DNP.[21,28] We think that initial difference in IL-18 levels can be due to a selection bias but on the other hand our study revealed suppression of IL-18, similar to other markers, with use of KAA following completion of 2 weeks study. IL-10 is the cytokine which is known as limiting the cascade of pro-inflammatory cytokines and decreasing T cell mediated immune responses with, as previously shown, a significant elevation in IL-10 levels in diabetic patients positively correlated with albuminurea.[29-31] In our study, IL-10 levels were found to be significantly decreased with the completion of KAA added diet course. We think that KAA worked in lowering proteinurea in conjuction with lowering damage to the kidneys caused by increased cytokines.

Some studies have shown that IL-1 causes the expression of adhesion molecules in glomerular endothelium and other kidney parts and IL-1 has also been shown to be elevated in streptozocine-induced diabetic rat models.[32,33] In our study, even though the differences of changes in levels of IL-1 in the mentioned time period is found to be insignificant, levels of IL-1 increased in study group against our hypothesis of expected improve in inflammatory status. Higher levels of IL-1 in the study group can be due to an unknown KAA suppression effect on an unrecognized pathway, but further studies need to be done to understand the exact effect on these cascades.

Conlcusion

Adding KAA to diets that are already low on protein, can slow progression to ESRD by reducing inflammation and protein loss in an animal model. Further clinical studies are needed to analyse the effects of KAA on human health.

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Conflicts of interest

There are no conflicts of interest.

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