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Effect of Ramipril Treatment on Proteinuria and Advanced Glycation End Products in Type 2 Diabetes Mellitus Patients with Nephropathy: One Year Follow up Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AKT, OPK, BDB, SVM and VKA were involved in planning and designing of the research work. Authors PKK, NA, PV, RG and NS were involved in biochemical investigations and samples collection. Author PKK carried out data analysis and wrote the manuscript. Author AKT, the corresponding author was involved in overall supervision and critically revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study was carried out to evaluate the effect of angiotensin converting enzyme inhibitor (ACEI) therapy on proteinuria and serum advanced glycation end products (AGEs) level in type 2 diabetes mellitus (T2DM) patients with nephropathy.
Study Design: Single-arm prospective longitudinal study.

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Duration and Place of Study: The study subjects were enrolled from Diabetic and Nephrology clinic at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India, between September 2013 and July 2014.
Methodology: The study subjects comprised of clinically diagnosed T2DM patients (n = 75) with evidence of persistent micro-albuminuria (ACR; 30-299 mg/g creatinine) or overt albuminuria (ACR; ≥300 mg/g creatinine) tested on two separate occasions. These patients were treated with ACE inhibitor; ramipril (5 mg to 20 mg /day) for 12 months. Effectiveness was assessed based on change in urinary albumin/creatinine ratio (ACR) and serum AGEs level.
Results: Ramipril treatment produced significant fall in log urinary ACR (P<0.001) and significant reduction in serum AGEs level (P<0.001) during 12 months follow up period as compared to baseline values. Also significant positive correlation between serum AGEs level and urinary ACR was observed at baseline. However, after one year follow up the serum AGEs level and urinary ACR did not correlate significantly. No significant change in serum creatinine and estimated glomerular filtration rate (eGFR) were observed after one year follow up.
Conclusions: Apart from antiproteinuric action, ramipril treatment has been found to lower serum AGEs level that may eventually arrest vascular complications in T2DM patients with nephropathy.

Keywords: Advanced glycation end products; type 2 diabetes mellitus; diabetic nephropathy; angiotensin converting enzyme inhibitor; albumin/creatinine ratio.

ABBREVIATIONS

T2DM: Type 2 diabetes mellitus; DN: Diabetic nephropathy; AGEs: Advanced glycation end products; RAGE: Receptor for advanced glycation end products; ACE: Angiotensin converting enzyme; ACEI: Angiotensin converting enzyme inhibitor; ARBs: Angiotensin-II receptor blockers; ACR: Albumin/creatinine ratio.

1. INTRODUCTION

Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD) worldwide and affects nearly 40% of diabetes mellitus patients [1,2]. The renin-angiotensin system (RAS) has been strongly implicated in the pathogenesis of nephropathy in diabetes mellitus and current therapies include treatment with inhibitors and blockers of RAS system like; angiotensin converting enzyme inhibitor (ACEI), angiotensin-II receptor blockers (ARBs) [3]. These drugs have been shown to exert antiproteinuric effect along with the potential to improve renal medullary perfusion in DN patients [4]. Diabetes mellitus triggers hyperglycemia and chronic hyperglycemia facilitates advanced glycation end products (AGEs) formation leading to diabetic complications including nephropathy [5]. Non-enzymatic Maillard reaction occurs between reducing sugar and free amino groups of proteins, lipids and nucleic acid forming Schiff base and biochemical modification of Schiff base form Amadori product which further rearrange to form of AGE [6]. In diabetes mellitus, persistent hyperglycemia causes an increase in the AGEs formation and induction of receptor for advanced glycation end products (RAGE) expression. AGE-RAGE interaction triggers various intra cellular signaling mechanisms leading to diabetic micro-vascular complications including DN [7]. It has been reported that ACE inhibitors have the ability to prevent the formation of AGEs *in vitro* [8]. An early study has shown the reduction of advanced glycation end products by ACE inhibitor in experimental diabetic nephropathy [9]. However, no data is available on ACE inhibitor-mediated change in AGEs level in DN patients. Therefore, the present study was designed to evaluate the effect of ramipril therapy on proteinuria and serum AGEs level in type 2 diabetes mellitus patients with nephropathy.

2. MATERIALS AND METHODS

2.1 Study Design

The study subjects comprised of clinically diagnosed T2DM patients (n = 80) with evidence of persistent micro-albuminuria (ACR; 30-299 mg/g creatinine) or overt albuminuria (ACR; ≥300 mg/g creatinine) on two separate occasions (6 weeks interval) were enrolled between September 2013 and July 2014 from Diabetic and Nephrology clinic at University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India. Diabetes diagnosis was based upon American Diabetes Association

(ADA) guideline 2012. Out of eighty patients, 75 patients completed the 12 months follow up. Patients having age between 30 to 65 years, duration of diabetes ≥5 years, with the evidence of diabetic retinopathy and CKD stage 1 to 3 were recruited. Patients who were unable to tolerate ACE inhibitor and patients taking any non-steroidal anti-inflammatory drugs (NSAID) were excluded from the study. Patients were treated initially with ACE inhibitor (ramipril) 5 mg/day along with anti-diabetic treatment including oral hypoglycemic drugs; metformin, glimepiride etc, and/or insulin. The dose was up-titrated to a maximum of 20 mg/day. All enrolled patients were under adequate controlled blood pressure; (systolic blood pressure (SBP) <140 mm Hg and diastolic blood pressure (DBP) <90 mm Hg) and satisfactory glycemic control (fasting blood glucose; 3.88–7.21 mmol/L and post-prandial blood glucose; 7.77–9.99 mmol/L) during the study. The initial follow up was carried out within 2-4 weeks to look for any untoward effects like hyperkalemia (<5.2 meq/L), fall of eGFR (>25% decline) or fall of blood pressure (>30 mm Hg in systolic blood pressure). The study was approved by Institutional Ethics Committee-Human Research (IEC-HR) of University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi.

2.2 Biochemical Parameters Estimation

5 ml blood sample was collected for biochemical analysis. Blood was centrifuged at 1000g for 15 minute for plasma and serum separation. All parameters were determined within a month after sample collection. The plasma glucose level was measured by glucose oxidase-peroxidase method and quantified spectrophotometrically at 500 nm. Glycated hemoglobin (HbA1c) was estimated by micro-column based technique and quantified spectrophotometrically at 500 nm. Total cholesterol, serum sodium, potassium and hemoglobin were estimated using Olympus Autoanlyzer and Star-21 Semi-autanalyzer at our Hospital Laboratory Services.

2.3 Estimation of Urinary Albumin/ Creatinine Ratio (ACR) and Estimated Glomerular Filtration Rate (eGFR)

Morning spot urine samples were collected for urine albumin and urine creatinine test. Serum and urine creatinine were carried out by alkaline picrate Jaffee´s kinetic method [10]. Urine albumin was estimated by turbidometric method by using nephelometer (Nephstar®, Goldsite Diagnostics Inc.). The sensitivity limit is 10 mg/L. Albumin /creatinine ratio was calculated and expressed in mg/g creatinine. Estimated glomerular filtration rate was calculated by following Modification of Diet in Renal Disease (MDRD) equation [11] and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [12].

2.4 Estimation of Serum Advanced Glycation End Products

Serum AGEs level was determined following the method described by Kalousova et al. [13]. Briefly, serum was diluted 1:50 with phosphate buffer saline (pH =7.4). Fluorescence intensity was measured by a Multimode reader (Synergy H1 Hybrid Reader, BioTek, USA) using emission maximum at 440 nm and excitation maximum at 350 nm. The results were expressed in arbitrary unit (AU).

2.5 Clinical Response Point

The decrease in urinary ACR was calculated by; decrease in urinary ACR% = (baseline value - follow up value) X100 / baseline value. Patients were classified as active responders (decrease in urinary ACR ≥50%), partial responders (decrease in urinary ACR ≥30% to <50%), and non-responders (reduction in urinary ACR <30%) at the end of 12 months follow up.

2.6 Statistical Analysis

Data was expressed as mean ± SD, median and (IQR) or percentage (%) as applicable. The Kolmogorov-Smirnov test was used to determine the distribution of data. As the urinary albumin and albumin/creatinine ratio data were not normally distributed, log transformation was done to normalize the data. Student's paired t-test was used for comparison between baseline and follow up data. Correlation between serum level of AGE and urinary ACR was analyzed by using Pearson′s correlation coefficient analysis. $P<0.05$ was considered as the level of significance.

3. RESULTS

3.1 The Demographic Characteristics of Study Subjects

The demographic characteristics of enrolled study subjects are summarized in Table 1. The age of the patients ranged between 30 to 65 years. The duration of diabetes ranged between

5 years to 20 years and median of duration of diabetes was 7 years. 48% of enrolled patients had family history of diabetes and 17% had family history of hypertension.

Table 1. Demographic characteristics of study subjects

Parameters	T2DM with nephropathy
Numbers of patients (n)	75
Male/Female (n)	40/35
Age (years)	50.53 ± 7.29*
Duration of diabetes (years)	7 (5 - 10)**
Family history of diabetes (Yes/No)	36/ 39
Family history of hypertension (Yes/No)	13/ 62

**mean ± SD, **median (interqurtile range)*

3.2 Biochemical and Clinical Parameters at Baseline and after 12 Months Follow up

The biochemical and clinical parameters at baseline and after 12 months treatment with ramipril are listed in Table 2. At the time of recruitment baseline values of serum sodium, potassium, total cholesterol and urinary albumin, urinary creatinine were found elevated in enrolled patients as compared to standard reference values and the serum sodium, potassium, hemoglobin, total cholesterol, urinary albumin and urinary creatinine level of these patients decreased significantly after the treatment with ramipril at 12 months follow up as compared to baseline values. However, no significant change in fasting blood glucose, post prandial blood glucose, HbA1c, serum creatinine, eGFR (MDRD), eGFR (EPI), SBP and DBP was observed after one year follow up. Estimation of urine albumin was carried out by immuno-turbidometry and the result varied widely. Due to large skewed values of albumin, urinary ACR was expressed as log ACR. Serum AGEs level was found to be 2.52 ± 0.80 x 10^5 AU at baseline and significant decrease (P<0.001) was observed at 12 months follow up. On ramipril treatment for 12 months, significant decrease in log urinary ACR (P<0.001) was also observed.

3.3 Correlation Analysis of Serum AGEs Levels with Urinary ACR

Correlation of serum AGEs with urinary ACR at baseline, and after one year follow up is presented in Table 3. Significant positive correlation was observed between baseline values of serum AGEs and log urinary ACR. However, at one year follow up after introduction of ramipril therapy the serum level of AGEs and urinary ACR did not correlate significantly, Table 3A. After adjustment for age, sex, blood pressure and renal function, the correlation of serum AGEs with urinary ACR at baseline remained significant but was found insignificant after one year follow up, Table 3B.

Table 2. Biochemical and clinical parameters at baseline and after follow up

Parameters	Baseline n = 75	Follow up n = 75	*P* value
Serum sodium (mmol/L)	138.94 ± 5.22	134.11 ± 6.65	0.001
Serum potassium (mmol/L)	4.29 ± 0.61	4.04 ± 0.78	0.014
Fasting plasma glucose (mmol/L)	7.15 ± 0.60	6.57 ± 0.80	0.091
Post-prandial plasma glucose (mmol/L)	9.25 ± 1.71	8.39 ± 1.35	0.071
HbA1c (%)	6.12 ± 0.25	5.73 ± 0.90	0.062
Hemoglobin (gram /L)	119.2 ± 21.4	113.3 ± 17.4	0.023
Total cholesterol (mmol/L)	4.96 ± 1.33	4.48 ± 0.88	0.010
Serum creatinine (µmol/L)	92.82 ± 34.47	95.47 ± 36.24	0.392
eGFR (MDRD) ml/min/1.73 m^2	75.4 ± 30.33	68.85 ± 20.53	0.044
eGFR (EPI) ml/min/1.73 m^2	78.52 ± 25.02	75.4 ± 21.31	0.243
SBP (mmHg)	145.69 ± 10.18	140.36 ± 9.14	0.067
DBP (mmHg)	85.95 ± 11.56	82.78 ± 10.43	0.062
Log urinary albumin (mg/L)	2.54 ± 0.61	2.37 ± 0.71	0.002
Urinary creatinine (g/L)	0.95 (0.64 -1.44)	1.20 (1.00 - 1.52)	0.003
Log urinary ACR (mg/g creatinine)	2.55 ± 0.65	2.30 ± 0.69	0.001
Serum AGEs (AU)	2.52 ± 0.80 x 10^5	1.78 ± 0.57 x 10^5	0.001

Data are presented as mean ± SD, median (IQR), P<0.05; Significant level, AU = Arbitrary unit, eGFR: Estimated glomerular filtration rate, SBP; Systolic blood pressure, DBP; Diastolic blood pressure, ACR; Albumin/creatinine ratio, AGEs; Advanced glycation end products

Table 3A. Correlation of AGEs level with urinary ACR at baseline and after follow up

N =75	Urinary ACR at baseline (*P* value)	Urinary ACR after follow up (*P* value)
Serum AGEs at baseline	r = 0.391 (0.001)	
Serum AGEs after follow up		r = 0.225 (0.057)

r = Pearson's correlation coefficient, P<0.05; significant level

Table 3B. Correlation of AGEs level with urinary ACR after adjustment of confounding factors

N =75	Urinary ACR at baseline (*P* value)	Urinary ACR after follow up (*P* value)
Serum AGEs at baseline	r = 0.380* (0.001)	
Serum AGEs after follow up		r = 0.218* (0.060)

*r = Pearson's correlation coefficient, P<0.05; significant level; *after adjustment of age, sex, blood pressure and renal function*

3.4 Patient's Response to ACE Inhibitor Therapy and Their Serum AGEs Level

Distribution of responder and non-responder with regard to antiproteinuric effect of ramipril treatment and serum AGEs level are listed in Table 4. At the end of the 12 months follow up, out of 75 patients, 44% (n=33) patients were found active responders while 16% (n=12) patients responded partially and 40% (n=30) patients did not respond to antiproteinuric effect of ramipril therapy. In contrast, serum AGEs level reduced significantly in all the patients on ramipril therapy,–irrespective of antiproteinuric response of ACE inhibitor therapy.

4. DISCUSSION

Diabetic nephropathy is currently the leading cause of chronic kidney disease. It is also one of the most significant long-term complication in terms of morbidity and mortality for patients with diabetes [14]. In the present report the antiproteinuric efficacy of angiotensin converting enzyme inhibitor therapy in diabetic nephropathy patients and corresponding change in serum AGEs level have been evaluated. In recent time measurement of urinary ACR has been considered as a better method for assessment of micro-albuminuria in diabetic nephropathy [15]. Micro-albuminuria is an indicator of diabetic nephropathy particularly at the early stage when significant change in serum creatinine level is not observed. In the present study we evaluated urinary ACR, serum creatinine and eGFR as the measures of renal function. Due to large skewed values of urine albumin, urinary ACR was expressed as log ACR. Significant decrease in log urinary ACR on ramipril treatment were found in these patients as compared to baseline data. This clearly indicate that ramipril exert antiproteinuric effect in type 2 diabetes mellitus patients with nephropathy, however, no significant change in serum creatinine and eGFR were observed during one year follow up.

In our study, patients were classified as active responder (decrease in urinary ACR ≥50% of baseline value), partial responder (decrease in urinary ACR ≥30% to <50% of baseline value), and non-responder (reduction in urinary ACR <30% of baseline value) after treatment with ramipril. Various studies have adopted 30 to 50% reduction in proteinuria as response point in DN patients on ACE inhibitor therapy. In this connection, Bakris et al. [16] have observed 33 ± 8% reduction in proteinuria, while Vleming et al. [17] have reported 45% reduction in proteinuria as the response point. Bedogna et al. [18] have also classified patients as good responder (≥30% reduction in proteinuria) and poor responder (<30% reduction in proteinuria) in their study [16-18]. Heart Outcomes Prevention Evaluation (HOPE) study has also shown significant reduction in albumin/creatinine ratio by ACE inhibitor [19]. However, the beneficial effects of combination of ACE inhibitor and ARBs have been also reported by some studies [20,21]. The mechanism of antiproteinuric effect of ACE inhibitor is yet to be elucidated. Initially it has been postulated that reduction in proteinuria is due to improvement in glomerular permeselectivity by ACEI [22]. Recently it has been suggested that ACE inhibitor ameliorate glomerular membrane size-selective dysfunction which translate into an antiproteinuric effect [23].

Table 4. Serum AGEs level in responders and non-responders

No. of patients (%)	Active responder	Partial responder	Non-responder
	33 (44%)	**12 (16%)**	**30 (40%)**
Serum AGEs at baseline	$2.60 \pm 0.83 \times 10^5$	$2.58 \pm 0.73 \times 10^5$	$2.39 \pm 0.80 \times 10^5$
Serum AGEs after follow up	$1.86 \pm 0.61 \times 10^5$*	$1.82 \pm 0.53 \times 10^5$*	$1.68 \pm 0.54 \times 10^5$*

Serum AGEs level are expressed in arbitrary unit (AU) and presented as mean ± SD.
** P<0.001 vs. values at baseline*

In the present study, serum AGEs level of the enrolled patients was found to be significantly higher as compared to normal reference value established in our laboratory [24]. High level of AGEs has been suggested as one of the predictor for diabetic nephropathy and correlated with the severity of nephropathy in diabetic patients [24,25]. A significant positive correlation of serum AGEs level with urinary ACR was observed at baseline in the present study, however, after one year follow up the serum AGEs level and urinary ACR did not correlate significantly. This indicates that as the level of AGEs increases, proteinuria in DM patients become more pronounced. Adjustment for potential confounding factors i.e. age, sex, blood pressure, and renal function also revealed that AGEs levels remained significantly correlated with urinary ACR but insignificantly correlated with ACR after one year follow up. Similar to our finding, Piwowar et al. [26] have reported significant correlation between plasma AGEs and urinary albumin/creatinine ratio in DN patients.

To the best of our knowledge there is no report available on the effect of ramipril therapy on serum AGEs level in patients with diabetic nephropathy. In the present study, ramipril treatment resulted in significant reduction in AGEs level indicating that ramipril not only reduce protein excretion in DN patients but also reduces AGEs formation. The most important finding of the present study is that ramipril treatment reduced serum AGE level even in those patients where no antiproteinuric effect of ramipril is observed. While ramipril treatment showed significant antiproteinuric effect in 60% of patients, AGEs lowering effect was found in all of them. In an early study, Forbes et al. [9] have shown that ramipril administration reduce accumulation of AGEs in streptozotocin-induced diabetic nephropathy. Also in accordance to our finding, ramipril therapy has been shown to cause significant decline of fluorescent AGE levels in non-diabetic patients with nephropathy [27]. The mechanism leading to decrease in AGEs due to ramipril treatment remained unknown. Based on an *in vitro* study it has been proposed that AGEs lowering effect of ACE inhibitor is possibly through interference with the formation of free radicals and reactive oxygen species that are responsible for AGEs formation and not through trapping of RCO precursor of AGEs [28]. In support to this hypothesis, De Cavanagh et al. [29] have reported treatment with ACEI increases antioxidants defenses. Our findings add a new dimension to ACEI-mediated beneficial effect. Amelioration of AGE-RAGE interaction-mediated signal transduction, a pathway of renal cell fibrosis may be associated with the benefits of ACEI therapy in DN patients through lowering of AGEs level.

5. CONCLUSION

In conclusion, the present study shows that ramipril therapy not only decrease proteinuria but also reduce AGEs formation in type 2 diabetic patients with nephropathy and suggesting its overall beneficial effect on vascular complications associated with diabetes mellitus.

CONSENT

All authors declare that written informed consent was obtained from the patient.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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