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Original Article

Internal Medicine Section

Reduced sRAGE Production and ADAM10 Gene Expression in Peripheral Blood Samples of Diabetic Nephropathy Patients

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ABSTRACT

Introduction: Excessive signalling via the Receptor for Advanced Glycation End products (RAGE) is implicated in inflammatory renal damage in Diabetic Nephropathy (DN). RAGE signaling is modulated by its soluble form (sRAGE) that arises due to ectodomain cleavage catalysed by A Disintegrin and Metalloproteinase 10 (ADAM10). The sRAGE functions as a decoy and competes with RAGE for binding to the cognate ligand.

Aim: The aim of this study was to evaluate sRAGE and ADAM10 gene levels in peripheral blood samples of Diabetic Nephropathy patients.

Materials and Methods: The present observational study was conducted in the Department of General Medicine, RL Jalappa Hospital and Research Centre, Kolar, Karnataka, India, between January 2019 to April 2020. Study comprised of three groups: group 1 of 30 DN patients; group 2 of 28 Type II Diabetis Mellitus (T2DM) patients without microvascular complications and group 3 comprised of 30 healthy volunteers. Blood samples obtained from the study participants were cultured for 24 hours along with insulin treatment or a suitable control. sRAGE levels were measured in the conditioned media by Enzyme Linked Immunosorbent Assay (ELISA) technique. Expression of the ADAM10 gene was measured in the cell pellet by using the quantitative real-time Polymerase Chain Reaction (PCR) technique.

Results: A total of 88 subjects were included in the study, with 30 patients in group 1 (DN, mean age 55.34±7.76 years), 28 patients in group 2 (T2DM, mean age: 55.07±7.7 years) and 30 subjects in group 3 (healthy individuals, mean age: 55±8.05 years). sRAGE levels were significantly lower in nephropathy patients when compared to healthy volunteers ($p=2.5\times10^{-9}$). Likewise, ADAM10 expression levels were also significantly lower in nephropathy patients when compared to healthy volunteers ($p=1.3\times10^{-4}$). Insulin treatment led to significantly higher sRAGE production in healthy volunteers as compared to T2DM patients (p-value 3.3×10^{-11}). Insulin treatment leads to significant upregulation of the ADAM10 gene expression in healthy volunteers as compared to DN and T2DM.

Conclusion: Abnormal RAGE signaling in DN may arise due to diminished sRAGE production as a consequence of reduced ADAM10 expression.

Keywords: A disintegrin and metalloproteinase 10, Insulin, Soluble receptor for advanced glycation end-products, Type II diabetes mellitus

INTRODUCTION

The Diabetic Nephropathy (DN) is a common complication of Type II Diabetes Mellitus (T2DM) [1,2]. It is characterised by histological changes in the nephron such as tubulointerstitial fibrosis, basement membrane expansion, podocytopathy, and glomerulosclerosis [3]. Local inflammatory stress plays a crucial role in promoting the development and progression of DN [4-6]. Advanced Glycation End products (AGE) are the major drivers of inflammation in DN [7,8]. AGEs are formed due to glycation and subsequent oxidation of proteins and lipids [9].

AGEs activate the RAGE-products which then results in the upregulation of Nuclear factor kappa B (NF-κB) and inflammatory cytokine genes [10,11]. RAGE activation is regulated by its soluble form referred to as sRAGE. RAGE is a type I transmembrane protein comprising of a large ligand-binding ectodomain, the middle transmembrane stem, and the intracellular tail [12]. Proteolytic cleavage of the receptor at the membrane surface results in the shedding of the ectodomain as sRAGE [13]. ADAM10 is the main enzyme involved in the shedding process [14]. sRAGE regulates RAGE activation by competing with the cell surface receptor for binding to AGE ligand [15].

The inverse relationship between sRAGE and inflammatory signaling via RAGE implies that sRAGE deficiency may lead to abnormal RAGE activation. Furthermore, such a deficiency may arise due to the paucity of the ADAM10 enzyme. We checked this hypothesis by using blood sample as the surrogate specimen. RAGE is expressed in leucocytes in addition to podocyte and other cells [16]. Furthermore, the study parameters encompassed intrinsic physiological properties and not disease induced phenomenon. The objectives of this study were to check whether sRAGE production is reduced in DN or T2DM patients and its association to reduced expression of ADAM10 gene.

MATERIALS AND METHODS

The present observational study was conducted at Department of General Medicine, RL Jalappa Hospital and Research Centre, Kolar, Karnataka, India, between January 2019 to April 2020. The study was approved by Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India (Approval letter number SDUMC/KLR/IEC/218/2018-19 dated 02/01/2019). Informed consent was obtained from the study participants in writing before enrolment in the study.

Sample size calculation: Sample size was calculated based on the prevalence rate (2.5%) of DN in South India using OpenEpi software version 3.01. The number of participants required for the study to achieve a power of 90% was found to be 27 [17].

Inclusion criteria:

- Patients of stage 4 and 5 DN, fasting plasma glucose (>126 mg/dL), glycated haemoglobin HbA1c (>6.5%), creatinine (>1.2 mg/dL), estimated Glomerular Filtration Rate (eGFR <125 mL/min 180L/day and 2 mL/sec) and Blood Urea Nitrogen (BUN >24 mg/dL) were included in the DN group. Staging of DN was based on the guidelines of the Joint Committee on DN [18].

- Patients with type II diabetes with fasting plasma glucose (>126 mg/dL), glycated haemoglobin (>6.5%) and creatinine (<1.2 mg/dL) were included in group 2.
- The healthy volunteers, age and gender matched participants with fasting plasma glucose (<100 mg/dL) and no history of any chronic illness were included in group 3.

Exclusion criteria: Patients with stage 1 to 3 DN and suffering from chronic co-morbidities, those patients with history of microvascular complications were excluded from the study.

The study population of 88 participants comprised of 3 groups:

1. Group 1 comprised of 30 DN patients.
2. Group 2 comprised of 28 T2DM patients without microvascular complications.
3. Group 3 comprised of 30 healthy volunteers.

Procedure

Blood culture set-up: Blood samples (3 mL) were collected from the study participants in Ethylene Diamine Tetra-acetic Acid (EDTA) vacutainer and used for cell culture. The culture was set-up by adding 500 µL of whole blood to 497µL of Rosewell Park Memorial Institute 1640 medium (supplemented with 1% antibiotic and 10% foetal bovine serum). The cultures were incubated for 24 hours at 37°C in 5% CO_2 atmosphere. For each sample collected, two cultures were set-up. The first culture was treated with insulin (Cat. No.12585014; Thermo Scientific, United States of America) to a final concentration of 25 mIU/mL [19] and the second culture was treated with phosphate buffer saline (vehicle control).

Transcript preparation: The blood cultures were subjected to centrifugation at 3000 rpm after the incubation was completed. The conditioned media was separated out from the cell pellet and were aliquoted and stored at -80°C. The cell pellet was subjected to isolation of total Ribonucleic Acid (RNA) by the trizol method (Cat. No. 15596018 Thermo Scientific, USA). cDNA was then prepared from the isolated total RNA (Cat. No. 1708891 iscript cDNA synthesis kit; BioRad, USA). The synthesised cDNA were stored at -20° C till further analysis.

Gene expression analysis: Gene expression was assessed by qPCR using the (Cat. No. 1725271 SsoAdvancedUniversal SYBR Green; BioRad, USA). The housekeeping gene used to normalise the ADAM10 gene expression in this study was Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). ADAM10 gene expression was analysed using the following primer pair 5' ACT GCT GAT GAG AAG GAC CC 3' and 3' CCA GAC CAA GTA CGC CAT CA 5'. The thermal cycling program for ADAM10 gene expression comprised of an initial denaturation at 95°C for three minutes, followed by 39 cycles at 95°C for 10 seconds and 59°C for 30 seconds. PCR primers were designed using Primer3 software version 0.4.0. Optimum thermal cycling condition was determined by carrying out gradient PCR. Condition that did not produce primer-dimer in melt-curve analysis and gave primer efficiency of 90-110% was selected.

The method of comparative threshold cycle (Ct) was used to quantify the relative gene expression normalised to the house keeping gene GAPDH. The fold change in ADAM10 gene expression was determined by calculating 2-ΔΔCT, where ΔCT=Ct (ADAM10)-Ct (GAPDH) and ΔΔCT=ΔCt (treated)-ΔCt (untreated). The ΔCt values were used for statistical comparison between treated and untreated samples within each study group. The ΔΔCT values were used for statistical comparison between study groups.

Estimation of sRAGE levels: The conditioned media was used to assess the levels of sRAGE on induction with insulin and compared to the uninduced conditioned media. Commercially, available kits were used to estimate sRAGE (#SEA645Hu, Cloud-Clone Corp., USA). Protocol was carried out according to the manufacturer's guidelines. sRAGE arises due to proteolytic shedding of membrane RAGE and also due to an alternate splice variant that lacks the membrane anchoring domain (called as endogenously secreted RAGE).

STATISTICAL ANALYSIS

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) Statistics version 20.0 (International Business Machine Corporation, Armonk, New York). Shapiro-Wilk test was performed with Q-Q plots and normality plots to evaluate the normality of the data. Mean and standard deviation were determined, if the data showed normal distribution. Median and interquartile range was calculated, if the data was not normally distributed. The data obtained from the three groups followed normal distribution. Therefore, mean and standard deviation were calculated and parametric tests were used for comparison. The p-value less than 0.05 were considered statistically significant.

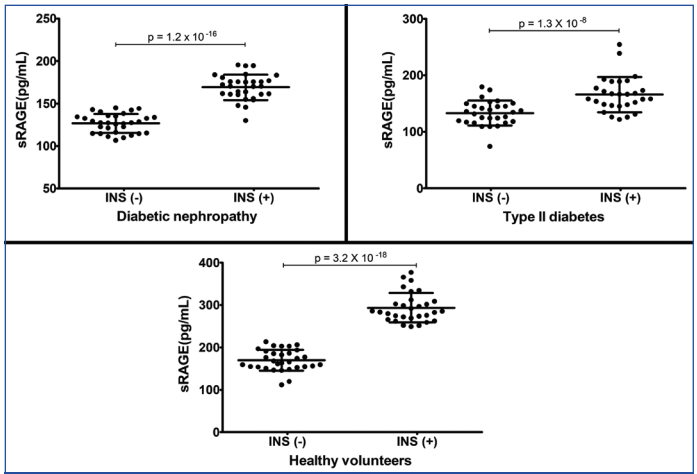
RESULTS

A total of 88 participants were included in this study among which 30 were DN patients, 28 were T2DM patients and 30 healthy volunteers. The clinical and demographic profile of the study participants is summarised in [Table/Fig-1].

Effect of insulin treatment on sRAGE levels: First, the effect of insulin treatment on sRAGE production was compared in each study group. Insulin treatment resulted in higher levels of sRAGE levels in all the three groups. The average fold increase was 1.29±0.10 pg/mL ($p=1.2\times10^{-16}$, paired t-test) in the DN, 1.25±0.11 pg/mL ($p=1.3\times10^{-8}$, paired t-test) in the T2DM and 1.74±0.19 pg/mL ($p=3.2\times10^{-18}$, paired t-test) in the healthy volunteers. The highest fold change was observed in the case of healthy volunteers when compared to the DN and T2DM. The results are shown in [Table/Fig-2].

Parameters	Diabetic nephropathy	Type II diabetes	Healthy volunteers
Number of subjects (n)	30	28	30
Age (years)	55.34±7.76	55.07±7.7	55±8.05
Gender (male/female%)	14/16	15/13	14/16
FPG (mg/dL)	148.82±22.50	151.92±19.84	86.88± 5.84
HbA1c (%)	6.81±0.78	6.91±0.68	4.52±0.9
Serum creatinine (mg/dL)	1.62 ±0.13	0.985±1.37	-
eGFR (mL/min/1.73 m^3)	18.68±6.36	-	-
Blood urea nitrogen (mg/dL)	48.86±16.24	12.66± 6.12	-

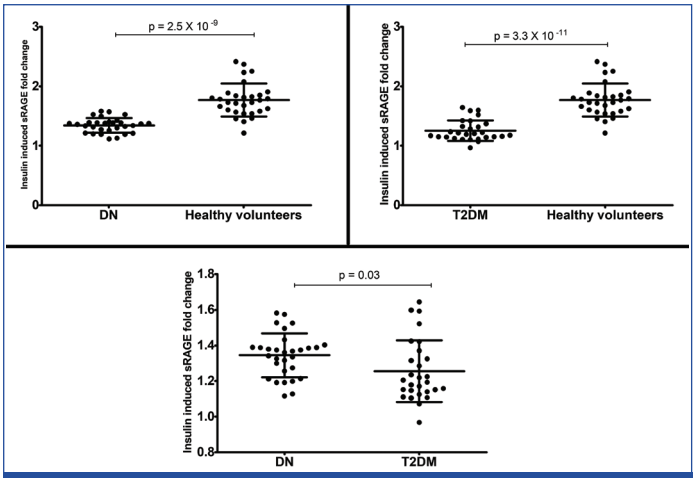
[Table/Fig-1]: Demographic and biochemical characteristics (Data represented as Mean±SD).
N=88 subjects; FPG: Fasting plasma glucose; HbA1c: Glycated haemoglobin; eGFR: Estimated glomerular filtration rate



[Table/Fig-2]: Effect of insulin on sRAGE production in the study groups: a) sRAGE levels in the treated and untreated conditioned media in Diabetic nephropathy; b) sRAGE levels in the treated and untreated conditioned media in Type II diabetes; c) sRAGE levels in the treated and untreated conditioned media in Healthy volunteers.

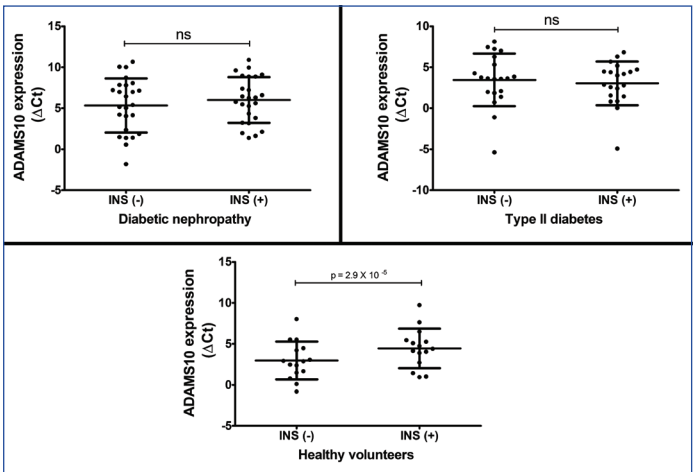
Second, the fold change in insulin-induced sRAGE production was compared between the three study groups. When compared

to the healthy volunteers the fold change was significantly lower in the nephropathy ($p=2.5\times10^{-9}$, unpaired t-test) and T2DM group ($p=3.3\times10^{-11}$, unpaired t-test). The results are shown in [Table/Fig-3]. These results indicate that insulin treatment leads to significantly higher sRAGE production in healthy volunteers.



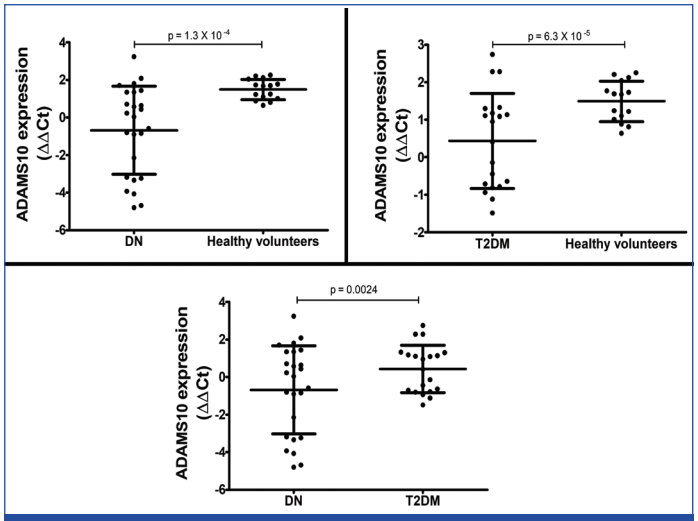
[Table/Fig-3]: Comparison of insulin-induced sRAGE production between the study groups: a) sRAGE levels between diabetic nephropathy and healthy volunteers; b) sRAGE levels between the type II diabetes and healthy volunteers; and c) sRAGE levels between the diabetic nephropathy and type II diabetes groups.

The present study, compared the effect of insulin treatment on ADAM10 gene expression in each study group. The ΔCt values followed normal distribution. Insulin treatment resulted in higher normalised expression (ΔCt) of the ADAM10 gene in healthy volunteers ($p=2.9\times10^{-5}$; paired t-test) whereas there was no significant change in DN (p=0.159; paired t-test) and T2DM (p=0.143; paired t-test). The results are shown in [Table/Fig-4].



[Table/Fig-4]: Effect of insulin on ADAM10 gene expression in the study groups: a) Normalised gene expression in Diabetic nephropathy; b) Normalised gene expression in Type II diabetes; and c) Normalised gene expression in Healthy volunteers.

The authors in this study compared the relative expression (ΔΔCt) of the ADAM10 gene between the three study groups. The ΔΔCt values showed normal distribution. Compared to healthy volunteers, ΔΔCt was significantly lower in DN ($p=1.3\times10^{-4}$; unpaired t-test) and also T2DM ($p=6.3\times10^{-5}$; unpaired t-test) [Table/Fig-5]. These results indicate that insulin treatment leads to significant upregulation of the ADAM10 gene expression in healthy volunteers but not in DN and T2DM.



[Table/Fig-5]: Comparison of insulin-induced change in ADAM10 gene expression between the study groups: a) Relative gene expression between the diabetic nephropathy and healthy volunteers; b) Relative gene expression between the type II diabetes and healthy volunteers; and c) Relative gene expression between the diabetic nephropathy and type II diabetes group.

DISCUSSION

The status of sRAGE and ADAM10 in DN was evaluated in this study. The observations, analysis and findings of this study indicate that sRAGE production and ADAM10 expression are not responsive to insulin in nephropathy and type II diabetes.

The results of this study are in contrast to the previous reports [20-22]. Grossin N et al., reported that sRAGE levels were significantly lower in patients with DN and diabetic retinopathy compared to diabetic patients without any microvascular complications [19]. The relationship between reduced sRAGE levels and development of microvascular complications in diabetes is further supported by the studies of Farhan SS and Hussain SA [20]. Derosa G et al., showed that sRAGE levels were significantly lower in patients with DN compared to patients with non DN [21]. In contrast to these studies, herein, the authors measured the sRAGE production under the influence of insulin stimulation. In this study, the authors found that there is a significant decrease in sRAGE levels in DN and T2DM when compared to healthy volunteers. However, there was no significant difference between the DN and T2DM groups. This indicates that factors other than insulin may also be involved in sRAGE production, particularly in the DN group.

sRAGE arises due to proteolytic cleavage of ectodomain of membrane bound RAGE. The proteolytic cleavage is mediated by the ADAM10 enzyme. Therefore, defects in ADAM10 may affect sRAGE production. In this study, the authors noticed down regulation of ADAM10 gene expression. Furthermore, a positive correlation was observed between sRAGE and ADAM10 gene expression. These patterns imply a role for ADAM10 in the reduced sRAGE production observed in this study. ADAM10 down regulation has also been reported in the renal biopsies from DN patients [23].

Insulin is indicated to regulate sRAGE production by controlling ADAM10 gene expression. Chen CD and co-workers showed that insulin treatment of COS (CV-1 in Origin with SV40 genes)-7 cells resulted in increased ADAM10 protein levels [24]. Further studies by Hu XT et al., showed that Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) signalling pathway and upstream transcription factor 1 are involved in the insulin mediated upregulation of the ADAM10 gene expression [25]. Also, Lam JK et al., showed that treatment of THP-1 (Tamm-Horsfall Protein 1) macrophages with an ADAM10 inhibitor resulted in decreased sRAGE production [26]. The results of this study show that insulin treatment of blood samples leads to elevated sRAGE production in healthy volunteers but not in DN and T2DM. This indicates that ERK1/2 signalling pathway may be defective in DN and T2DM. Further, studies are required to confirm this relationship.

Limitation(s)

In this study, only the ADAM10 gene expression was estimated without measuring its protein level. Also, total sRAGE was measured in the conditioned media.

CONCLUSION(S)

Overall, this study shows that sRAGE production and ADAM10 gene expression are diminished in DN and T2DM. These results

imply that regulation of RAGE pathway may be abnormal in DN and T2DM.

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