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Expression Of Type Iv Collagen And Connective Tissue Growth Factor In Diabetic Nephropathy Treated With Fenugreek In Strptoztocin Induced Rat Models

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ABSTRACT:
Background: Diabetes nephropathy (DN) refers to the unique pathological, functional, and structural changes that occur in the kidneys of people with type 1 and type 2 diabetes. It is marked by a steady decrease in kidney function and persistent albuminuria. Extra cellular mesangial accumulation (ECM) and oxidative stress plays important role in progression of diabetic nephropathy. Expression of type IV collagen and connective tissues growth factor are the important predictors of ECM accumulation and oxidative stress among DN patients. Fenugreek have been identified with anti-diabetic, hypocholesterolemic, and antioxidant effects. Hence this study was conducted with the aim of studying the impact of fenugreek on these two predictors. **Methods:** Wister albino rats have been used to elicit diabetes using streptozotocin. A blood glucose level of more than 250 mg./dl were marked as diabetic rats. Control group, diabetic control rats and diabetic rats treated with as dose of 9 g seed powder/kg were made with each group containing 4 rats. The study period was 12 weeks. Blood glucose was measured using one touch test strip, kidney weight/body weight was measured, histological examination by electron microscopy and immunohistochemistry was used for identifying the expression of type iv collagen and CTGF both quantitatively and qualitatively. **Results:** A total of 12 rats were included in the study, 4 rats were taken as control group. Among the established diabetes rats, 4 rats were taken as diabetic control rats and another 4 rats were taken fenugreek treated diabetic rats. There was a significant regulation in the expression of type iv collagen and CTGF in both histological examination and immunohistochemistry. **Conclusion:** The present study has proven that fenugreek has its properties of controlling both ECM accumulation and also the development of oxidative stress in glomeruli.

Conclusion: Diabetic nephropathy, Wister albino rats, Type IV collagen, Connective tissue growth factor.

Introduction:

Chronic kidney disease and end-stage renal disease (ESRD) are emerging health issues in individuals with diabetes on a global scale. The global prevalence of chronic kidney disease (CKD) was 697.5 million cases in 2017. India was the second most affected country, with 115.1 million cases. [1] The total magnitude and pattern of CKD in India have been reported inconsistently. The kidneys of patients with diabetes (types 1 and 2) undergo specific pathological, functional, and structural modifications, which are referred to as diabetic nephropathy (DN). In the clinical setting, DN is characterized by persistent albuminuria and a continuous decline in kidney function.[2] Diabetic nephropathy (DN), which is now more commonly referred to as diabetic kidney disease (DKD), is a substantial cause of morbidity and mortality in both type I and type II diabetes.[3] Globally, the prevalence of DN is significantly elevated due to the substantial rise in the prevalence of diabetes. The total number of individuals affected worldwide has increased by 46.0% to approximately 783 million, as reported by the IDF 2021.[4] This has resulted in a significant socioeconomic burden on the nation, healthcare professionals, and patients. The prevalence of DN in India varies from 0.9% to 62.3%.[5] The patient's quality of life is significantly impacted by the significant economic burden that DN imposes. As the disease advances to the advanced stages of CKD, the cost of DN increases exponentially. Type 2 diabetes mellitus patients are at an elevated risk of disease progression.[6]

The clinical symptoms of the disease are consistent with a variety of histopathological alterations that affect all kidney compartments. These changes in the kidney's function are indicative of the presence of DN. The thickening of the glomerular basement membrane (GBM) is one of the first quantifiable modifications in DN. The thickening is a consequence of the development of extracellular matrix (ECM) components in the internal lamina rare of the GBM, such as type IV collagen, laminins, and nidogen/entactin.[7,8] Collagen IV is a protein trimeric extracellular matrix that is rich in Gly-X-Y amino acid triplet repeats. It is composed of alpha chains. The triple helix of collagen is formed by the interlocking of three alpha-chains. Gly is required in each third position, as it is the sole amino acid with a side chain that is sufficiently long to accommodate the helix's center.[9] In the mature structure of GBM, collagen IV a3, a4, and a5 are the predominant components and are exclusively derived from podocytes. GBM thickening is a common early alteration in both Types I and II DN, and it increases in proportion to the length of the disease[10] The accumulation of extracellular matrix is the cause of the thickening of GBM, which is characterized by an increased deposition of standard extracellular matrix components, including laminin, fibronectin, and collagen IV and VI. [11] These accumulations are the result of either increased development, decreased degradation of these proteins, or a combination of the two. GBM thickening is not merely an early indicator of DKD; it can also predict which diabetes patients will ultimately develop progressive nephropathy.[12]

The pathogenic mechanisms have been recognized to involve oxidative stress and several growth factors, including connective growth factor (CTGF) and transforming growth factor-1 (TGF-β1). Hyperglycemia-induced oxidative stress increases TGF-β1, which stimulates ECM production and suppresses degradation in diabetic kidneys. TGF-β1 is a key mediator of ECM accumulation in diabetic kidneys. [13,14] CTGF, a key mediator, transmits signals from TGF-β1 to induce ECM synthesis and renal fibrosis in DN development [11, 12]. To improve DN treatment, an agent that reduces renal oxidative stress and blocks the TGF-β1/CTGF signaling pathway may be effective.[15] Fenugreek (Trigonella foenum-graceum L.) is a popular food ingredient and herbal medicine. The most processed part is the seed. Fenugreek seed is

commonly used in Indonesian and Indian cuisine for curry seasonings. In China and India, fenugreek is used as a traditional medicine for lower limb edema and lactation stimulation.[16] Fenugreek seed extracts have been shown to have anti-diabetic, hypocholesterolemic, and antioxidant effects in animal and human studies. Diabetes research has shown that fenugreek has pharmacological effects on peripheral glucose utilization, insulin secretion, and intestinal gum fiber. Fenugreek is an ideal choice for DN therapy.[17] The present study was conducted with the aim of studying the effect of fenugreek on type iv collagen and connective tissue factor which are the important predictors of Diabetic nephropathy.

Methods:

An experimental study was conducted in animal research center of Pharmacology department belonging to a Dhanalakshmi Srinivasan medical college and hospital situated in Tamil Nadu, South India. This study was conducted for a period of three months between November 2022 and January 2023. Before the research was conducted, approval was obtained from the Institutional Animal Ethics committee (IACE) of the study center. The CPCSEA guidelines was followed throughout the conduct of the study.[18] One of the commonly used condiments in India is fenugreek seed. We purchased the fenugreek seeds from National institute of Siddha which is located in Chennai, Tamil Nadu. These seeds were made into powdered form. A 200g of this grounded fenugreek powder was mixed with two liters of distilled water and it was made into decoction by means of boiling for 30 minutes. After cooling of this decoction for 60 minutes at room temperature, it was filtered twice through a coarse sieve to obtain the fenugreek filtrate.

Wister albino rats, each ageing 8 weeks and weighing around 180-200 grams were acquired from Mass biotech institute, situated in Chengalpattu, South India. these rats were housed in pathogen free areas in Animal house of the study center. Access for the standard diet and water was ensured in the animal house and it was followed throughout the entire study duration. These rats were acclimatized for about seven days. Streptozotocin which was obtained from Sisco Research Labarotories pvt ltd was injected peritoneally at a dose of 60mg/kg in citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5) to all rats after 12 hours of fasting. After three days, blood glucose levels of these rats were detected using One-Touch strip. Those rats with blood glucose levels of more than 250mg/dl were regarded as diabetic rats. Four diabetic rats were randomly administered fenugreek (9 g seed powder/kg) daily for 12 weeks (DNF group) and four diabetic rats were treated with a vehicle control for 12 weeks (DN group) one week later. For a period of 12 weeks, four normal rats were treated with citrate buffer alone and treated with vehicle control (Control group).

For the purpose of a light microscopic examination, a portion of kidney tissues was fixed in 10% buffered formalin and embedded in paraffin. A small section of renal cortex tissues was cut into pieces, prefixed in 2.5% glutaraldehyde (0.2 mol/L cacodylate buffer, pH 7.4) for 4 hours, postfixed in 1% buffered sodium tetroxide for 1 hour, and embedded in Epon. The JEM-1200 EX electron microscope was employed to examine the ultrathin renal cortex sections. The protein levels of type IV collagen (Col IV) and CTGF in renal tissue sections were analyzed using immunochemistry. In summary, the renal tissue sections were treated with polyclonal rabbit anti-rat Col IV (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-rat CTGF .HRP-anti-rabbit IgG and diaminobenzidine (DAB) were employed to detect the bound antibodies, which were subsequently counterstained with hematoxylin. PBS was used to incubate the negative controls. The computer imaging analysis system was employed to determine the semi-quantitative percentages of positive staining areas in the glomerulus. Data was presented as mean± standard deviation. The difference between two groups was analyzed using student t test and between three groups was identified using ANOVA. A p value of less

than 0.05 was considered as statistical significance.

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Results:

Table 1 shows the levels of blood glucose, body weight, and kidney weight and body weight/kidney weight ratio. The diabetic model was well established in such as way that the blood glucose levels are higher in the diabetes control group rats on comparing the control rats at p value <0.05.. The blood glucose level was low among diabetic rats treated with fenugreek when compared to diabetic control group rats. The above finding was statistically significant at p value <0.05. Similarly, it was observed that ratio of kidney weight to body weight was higher for DN group on comparison to control group. And the kidney weight to body weight ratio was lower among the DNF group when compared to DN group. The above findings were statistically significant at p value <0.05. These observations proves that fenugreek has protective effect on diabetic nephropathy by reducing the blood glucose levels as well as the controlling the renal cellular hypertrophy.

Expression of type IV collagen and connective tissue growth factor:

In the present study, the expression of type IV collagen and connective tissue growth factor was assessed by both using histological examination and immunohistochemistry. The histological features of diabetic nephropathy were established in such a way these levels of type iv collagen and CTGF were higher in DN group on compared to control group. Also, these levels were higher were comparatively higher lower in DNF group on comparing with DN group. The above findings are statistically significant at p value <0.05.(table 2 and Fig 1 &2) The pathological changes are distinctly more up regulated in DN group whereas in DNF its very minimal shown on fig 3.

Table 1- Effect of fenugreek on Blood glucose level, body weight, kidney weight and kidney weight/body weight ratio among rat model(N=12)

Parameters	**Control group (n=4)**	**STZ induced Diabetic nephropathy(n=4)**	**Fenugreek treated diabetic group (n=4)**
Blood glucose(mg/dl)	173± 21	561± 54*	184 ± 38#
Body weight(g)	529± 21	350 ± 25*	340 ± 53#
Kidney weight(mg)	1.77 ± 0.02	1.84 ± 0.12*	1. 25± 0.25#
Kidney weight/body weight (g/mg)	3.44 ± 0.26	5.23 ± 0.06*	4.11± 0.02#

*p value < 0.05 (DN vs Control), # p value <0.05 (DNF vs DN)

Table 2- Differences in the quantitative expression of type IV collagen and CTGF among the study subjects (N=12)

Parameters	**Control group (n=4)**	**STZ induced Diabetic nephropathy(n=4)**	**Fenugreek treated diabetic group (n=4)**	**P value**
Type IV Collagen(%)	6.35± 2.31	22.43 ±7.23	12.23 ±4.47	<0.05
Connective	1.17 ±0.06	6.5 ±2.46	3.42 ±1.05	<0.05

tissue growth factor (%)				

Fig2- Scatter plot showing the differences in expression of type IV collagen among the study subjects(N=12)

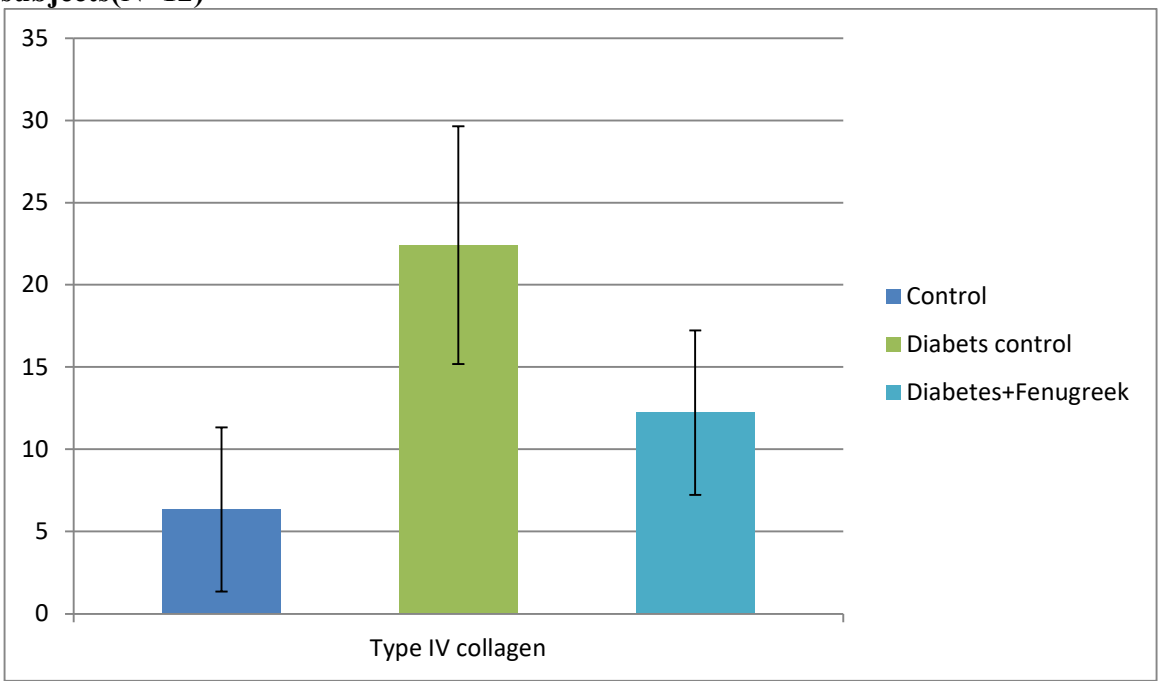


Fig2- Scatter plot showing the differences in expression of CTGF among the study subjects(N=12)

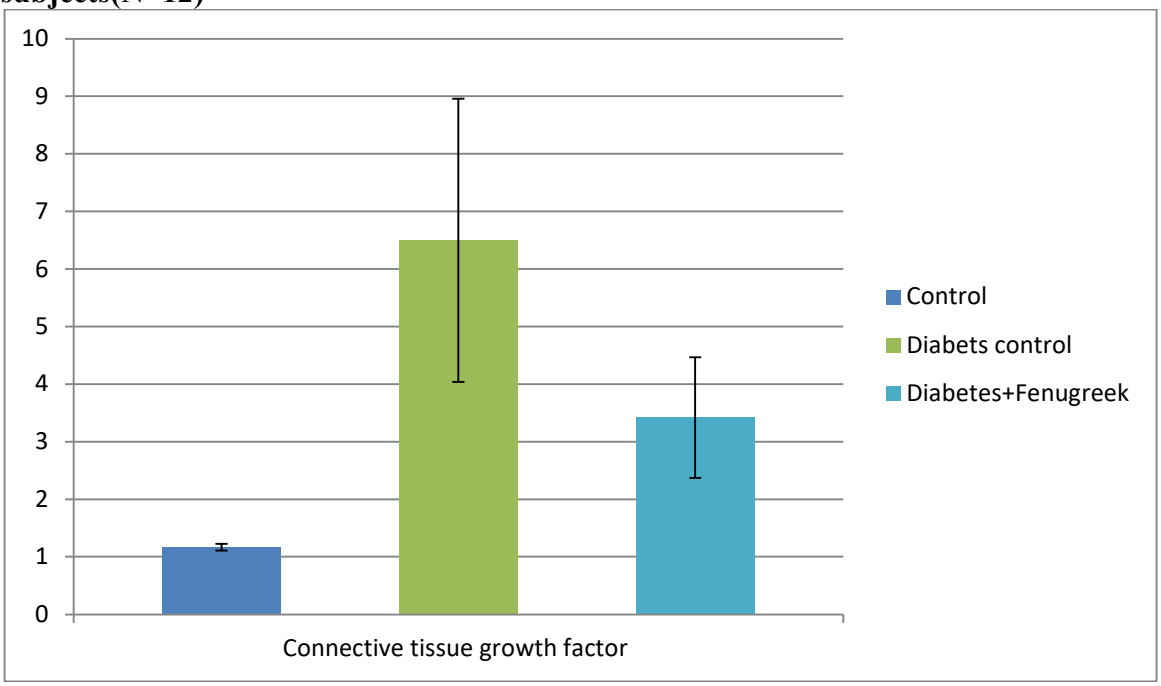


Fig 3- Expression of Type IV collagen and CTGF among the study subjects (N=12)

Control (n=4)	Diabetic rats (n=4)	Diabetic rats treated with fenugreek(n=4)
Fig 3-a) type IV collagen in glomeruli		

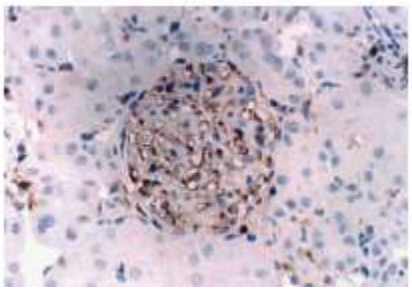
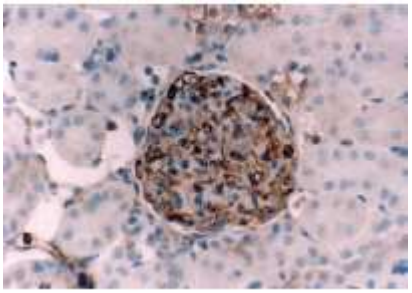
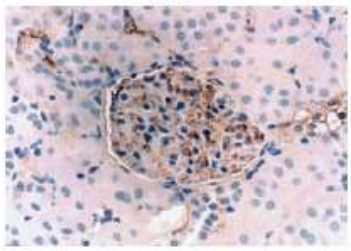
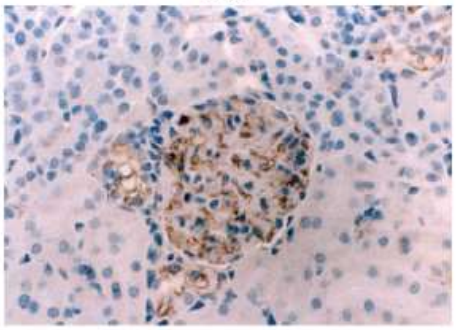
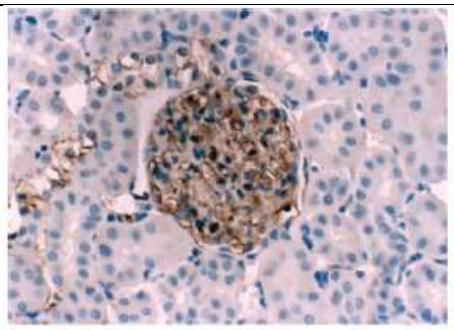
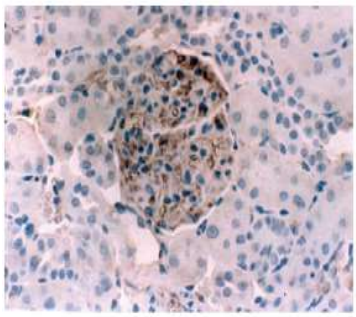

		

Fig 3.b) CTGF expression in glomeruli		
		

Discussion:

The current study evaluated the effect of fenugreek on diabetic nephropathy in terms of blood glucose levels, renal cellular hypertrophy, histological and quantitative differences in type iv collagen and connective tissue expression in the glomeruli. The establishment of diabetic nephropathy was ensured and confirmed by significant differences in blood glucose levels and kidney weight between control rats and diabetic rats. In the presents study, there was significant effect of fenugreek on reducing the blood glucose levels among the diabetic rats when compared to diabetic rats used as control. These findings are consistent with the similar studies conducted by Gong G et al.[17] The kidney weight to body weight ratio was also significantly lower among the diabetic rats treated with fenugreek than the diabetic rats used as control. The above findings are comparable to studies conducted by Sayed AA et al[19] and Hadi A et al.[20] These observations proves that fenugreek had a significant effect on glycemic control and alleviating the renal cellular hypertrophy.

Although many factors are implicated in the development and progression of DN, the ECM accumulation has been recognized as a crucial determiner in DN development. Type IV collagen is the predominant components of the ECM. CTGF, a 349-amino acid cysteine-rich peptide, is another important growth factor involved in DN development. CTGF has been recognized as the downstream mediator of TGF- $\beta 1$ in regulating matrix metabolism during the fibrotic process. To the study the effect of expression these type iv collagen and connective tissue factor , histological examination as well as quantitative assessment using immunohistochemistry was done in which significant

pathological changes in CTGF and type iv collagen were observed. Quantitatively by immune histochemistry also there was significantly low levels of type iv collagen and CTGF among the diabetic rats treated with fenugreek than those diabetic rats used as control rats. Similar findings were observed in a study conducted by Jin Y et al.[21] Al- Also Bag A, et al [22] and Waili N et al[23,] though exclusively not done experiment among diabetic rats but proved the effect of fenugreek on kidney tissues. Thus, the physical, metabolic, and pathological effect of fenugreek had proven to be effective on alleviating the progression of diabetic nephropathy.

Conclusion:

Fenugreek administered to streptozotocin induced rat model in the present study has shown that two crucial indicators of progression of diabetic nephropathy such as type iv collagen and connective tissue growth factor has shown to be significantly up regulated both in terms of pathologically as well as quantitatively. Hence, these findings prove that diabetic nephropathy could be treated effectively by fenugreek. The results of our study also help future researchers on exploring the pharmacological features of fenugreek.

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