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Ellagic Acid Inhibits TGFβ1/Smad-Induced Renal Fibrosis in Diabetic Kidney Injury

Elajik Asit, Diyabetik Böbrek Hasarında TGFβ1/Smad Kaynaklı Böbrek Fibrozisini İnhibe Eder

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ABSTRACT

Aim: Free radical formation increases due to hyperglycemia occurring in the pathogenesis of diabetes mellitus (DM), and as a result, oxidative stress occurs. Hyperglycemia-mediated oxidative stress plays an important role in the pathogenesis of diabetic nephropathy. The antihyperglycemic, antioxidative, anti-apoptotic, and anti-inflammatory effects of ellagic acid (EA) have been demonstrated by many studies. In this study, it was aimed to demonstrate the antifibrotic effect of EA on TGFβ1/Smad signaling in rats with streptozotocin induced diabetic nephropathy.
Material and Methods: A total of 24 male Sprague Dawley rats, weighing 200-250 g, were used in this study. The animals were divided into four groups as control, EA, DM, and DM+EA. The kidney tissues were used for histological and immunohistochemical procedures. While the collagen density in kidney tissues was revealed by Masson's trichrome staining, the expression levels of fibrotic markers TGFβ1, p-Smad3, and αSMA were determined by the immunocytochemical method.
Results: It was shown that the collagen density in the renal tissue of the DM group increased significantly in the intertubular area, while the collagen density in the EA-treated DM group was statistically significantly decreased. When TGFβ1, p-Smad3, and αSMA immunopositivity in kidney tissue sections of all groups were evaluated, the highest staining intensity was in the DM group, while the intensity of staining was close to the control group in the treatment group. It was observed that αSMA, TGFβ1, and p-Smad3 protein expression were down-regulated with EA treatment.
Conclusion: EA reduced fibrosis in diabetic nephropathy by returning profibrotic parameters to normal levels.
Keywords: Diabetes mellitus; ellagic acid; fibrosis; kidney; TGFβ/smad.

ÖZ

Amaç: Diabetes mellitus (DM) patogenezinde meydana gelen hiperglisemi nedeniyle serbest radikal oluşumu artar ve bunun sonucunda da oksidatif stres meydana gelir. Hiperglisemi aracılı oksidatif stres, diyabetik nefropatinin patogenezinde önemli bir rol oynar. Elajik asit (EA)'in antihiperglisemik, antioksidatif, antiapoptotik ve antiinflamatuar etkileri birçok çalışma ile gösterilmiştir. Bu çalışmada, streptozotosin ile indüklenen diyabetik nefropatili ratlarda EA'nın TGFβ1/Smad sinyalizasyonu üzerine antifibrotik etkisinin gösterilmesi amaçlandı.
Gereç ve Yöntemler: Bu çalışmada, ağırlığı 200-250 g arasında olan toplam 24 adet erkek Sprague Dawley cinsi sıçan kullanıldı. Hayvanlar kontrol, EA, DM ve DM+EA grupları olmak üzere dört gruba ayrıldı. Böbrek dokuları histolojik ve immünohistokimyasal prosedürler için kullanıldı. Masson trikrom boyaması ile böbrek dokularındaki kollajen yoğunluğu ortaya koyulurken, fibrotik belirteçler olan TGFβ1, p-Smad3 ve αSMA'nın ekspresyon seviyeleri ise immünositokimyasal yöntem ile belirlendi.
Bulgular: DM grubunun böbrek dokusundaki kollajen yoğunluğunun intertübüler alanda önemli bir ölçüde arttığı gösterilirken, EA ile tedavi edilen DM grubunda ise kollajen yoğunluğunun istatistiksel olarak anlamlı bir derecede azaldığı gösterildi. Tüm grupların böbrek doku kesitlerinde TGFβ1, p-Smad3 ve αSMA immünopozitifliği değerlendirildiğinde ise en yüksek boyanma yoğunluğu DM grubunda olurken, tedavi grubunda boyanma yoğunluğu ise kontrol grubuna yakındı. αSMA, TGFβ1 ve p-Smad3 protein ekspresyonunun EA tedavisi ile aşağı regüle edildiği gözlendi.
Sonuç: Elajik asit, diyabetik nefropatide profibrotik parametreleri normal seviyelere döndürerek fibrozu azaltmıştır.
Anahtar kelimeler: Diabetes mellitus; elajik asit; fibrozis; böbrek; TGFβ/smad

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease associated with glucose intolerance and hyperglycemia. The most common complication of DM is diabetic nephropathy (1,2). Among the pathological changes of diabetic nephropathy, glomerulosclerosis, tubular inflammation, tubular atrophy, and inter-tubular fibrosis are common (3). Renal fibrosis seen in diabetic nephropathy may damage tissue and thus organ function and finally lead to kidney failure (4,5). Renal fibrosis is associated with transforming growth factor β (TGFβ) / suppressor of mothers against decapentaplegic (Smad) signaling. In diabetic conditions, elevated glucose and its metabolites induce TGFβ1 expression (6,7). The interaction of TGFβ1 with its receptor leads to the uptake of Smad2/3 (8,9). Subsequently, the phosphorylated Smad2/3 complex is translocated to the nucleus, enabling the expression of genes such as fibronectin and α-smooth muscle actin (αSMA) (10,11). Accumulation of fibronectin and αSMA proteins in the glomerular and inter-tubular parts cause interstitial fibrosis. Blocking TGFβ/Smad signaling has been reported to be effective in preventing the progression of diabetic kidney disease (12,13).

Although the etiology of complications of DM is variable, it has not been fully elucidated. However, it is widely accepted that diabetes is a source of oxidant stress (14,15). Ellagic acid (EA) is a polyphenolic compound found in many foods, including raspberries, strawberries, blackberries, pomegranates, cranberries, and walnuts. EA has been shown to have strong antioxidant, anti-aging, anti-proliferative, anti-atherosclerotic, anti-cancer, and anti-mutagenic properties in-vivo and in-vitro studies (16-19). In addition, some experimental studies have shown that EA has antidiabetic and antihyperglycemic effects that reduce glucose intolerance (20,21).

This study aimed to contribute to the elucidation of the effect of EA on the renal fibrosis pathway by investigating the effect of EA on diabetic kidney damage. For this purpose, the expressions of the proteins involved in the fibrosis pathway were revealed by histological and immunohistochemical methods.

MATERIAL AND METHODS

Animals and Experimental Groups

In this study, a total of 24 male Sprague Dawley rats, weighing 200-250 g, were used. This experimental study was approved by the Animal Experiments Local Ethics Committee of Gazi University (G.Ü.ET-22.083). The animals were fed at standard temperature (~24° C) on a 12 h light/dark cycle. Diabetes was induced in animals with a single dose (55 mg/kg) of streptozotocin (STZ) (CAS 18883-66-4, Santa Cruz, CA) injection. Blood glucose level was measured 48 hours after STZ administration. Animals with blood glucose levels of 250 mg/dl were included in the DM group. Diabetic status was confirmed for 8 weeks. The animals were divided into four groups of six animals each: Control, EA, DM, and DM+EA (treatment). EA (sc-202598A, Santa Cruz, CA) was prepared by dissolving in 0.2% dimethyl sulfoxide (DMSO). EA and DM+EA groups were given 100 mg/kg/day orally for 35 days (21,22). After the experiments, all rats were euthanized. The kidneys were used for histological and immunohistochemical procedures.

Histological Analysis

After routine histological procedures, kidney tissues were fixed in 10% formalin and embedded in paraffin. Then, 4 µm thick sections taken from the paraffin block were stained with Masson's trichrome (GBL, 5022, Turkey) for histological evidence of fibrosis. The fibrotic area ratios in the x200 magnification images obtained with the light microscope were calculated with the Image J program.

Immunohistochemistry

Deparaffinized sections were retrieved at high temperatures with citrate buffer (pH, 6.0). Sections were incubated first with 3% hydrogen peroxide (TA-125-HP, Thermo Scientific, USA) and then with Ultra V block (TP-125-HL, Thermo Scientific, USA). Then, the sections were incubated with TGFβ1 primer antibody (bs-0086R, rabbit polyclonal, Bioss Inc., USA), p-Smad3 primer antibody (bs-3425R, rabbit polyclonal, Bioss Inc., USA), and αSMA primer antibody (bsm-52392R, rabbit monoclonal, Bioss Inc., USA) at 1:200 dilution for overnight at 4°C. Sections were then processed according to the secondary antibody kit (TP-125-HL, Thermo Scientific, USA) protocol. The coloration results from the reaction with the aminoethyl carbazole (AEC) (TA-125-HA, Thermo Scientific, USA) chromogen became visible. Background staining was done with Mayer's hematoxylin and the sections were evaluated under a light microscope. In each section, 6 areas were randomly determined at x200 magnification. Immunopositivity values in the areas were determined as a percentage using the Image J program. Two methods were used for negative controls. The first was primary antibody exclusion, and the other was the use of normal rabbit IgG (bs-0295 P, Bioss Inc., USA) as the negative reagent control (NRC).

Statistical Analysis

Mean, standard deviation, median, minimum and maximum values were used to define the variables. Data distribution was evaluated by the Shapiro-Wilk test. The Kruskal-Wallis test was used for univariate analyzes of the variables in the study. Pairwise comparisons of groups with significant differences were made using the Mann-Whitney U test and evaluated by applying Bonferroni correction (0.05/group number). Statistical analyzes were performed using IBM SPSS Statistics 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Armonk, NY), and $p<0.05$ was considered statistically significant.

RESULTS

Histological Results

According to Masson's trichrome staining results, while dense collagen structure was remarkable in the DM group, this density decreased in the treatment group. Collagen density in the EA group was statistically significantly decreased compared to the control group ($p=0.003$). The DM group showed a statistically significant increase in areas of fibrosis compared to the control and EA groups (both $p<0.001$). In addition, the fibrotic area value in the treatment group was statistically significantly decreased compared to the DM group ($p<0.001$, Table 1, Figure 1, 3A).

Immunohistochemical Results

When TGFβ1, p-Smad3, and αSMA immunopositivity in kidney tissue sections of all groups were evaluated, it was observed that the highest staining was in the DM group, while the intensity of staining was close to the control group in the treatment group (Table 2, Figure 2).

Table 1. The descriptive values of the fibrotic area of all groups (%)

		Control	EA	DM	DM+EA	p
Fibrotic Area	mean±SD	4.04±1.18	3.11±1.52	14.08±4.11	8.33±2.43	**<0.001**
	median [min-max]	4.10 [1.62-6.56]	2.78 [0.46-6.51]	15.32 [6.25-20.64]	8.25 [5.09-14.66]	

EA: ellagic acid, DM: diabetes mellitus, SD: standard deviation

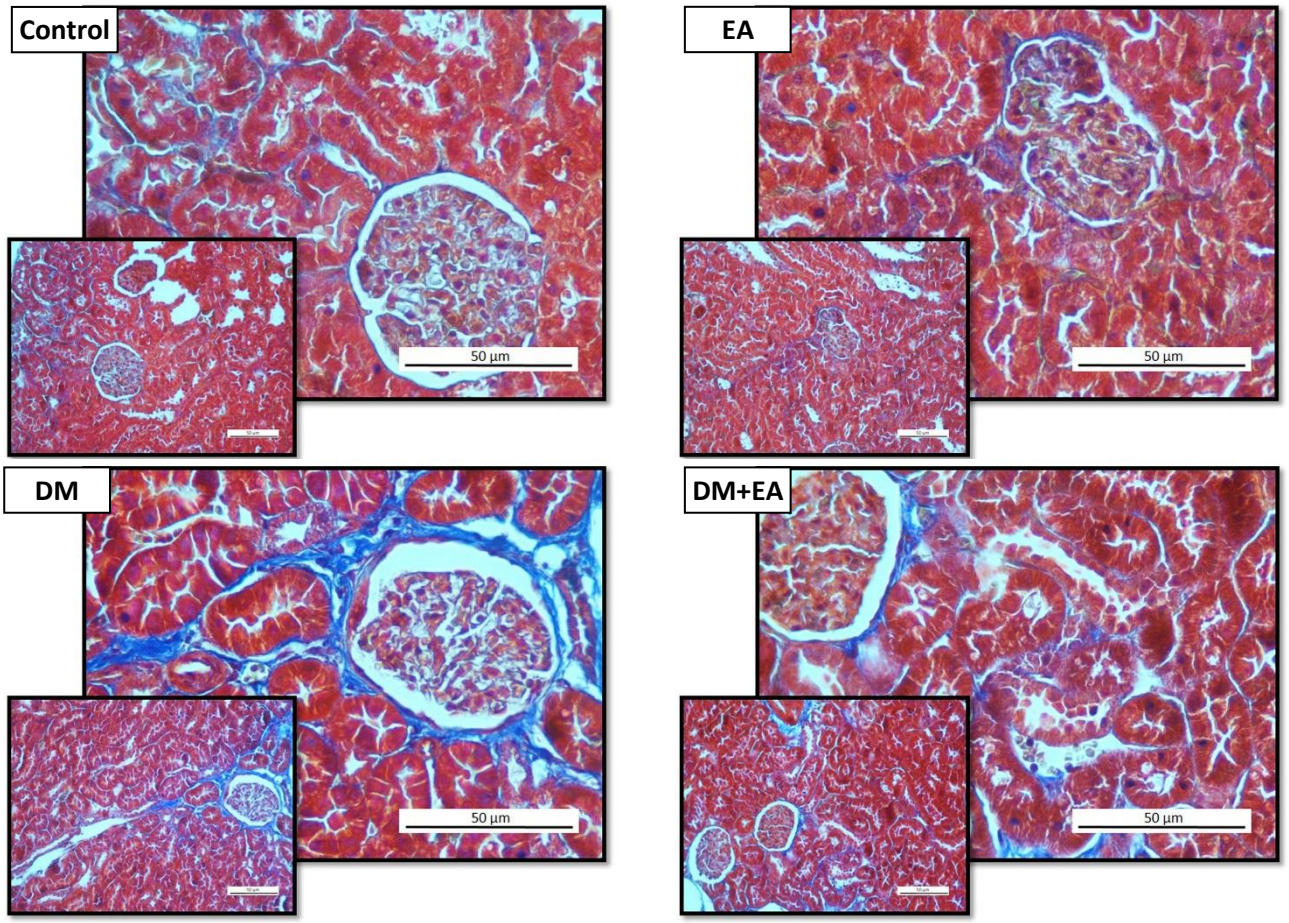

Figure 1. Representative microphotographs showing the kidney. Collagen deposition is highlighted in blue on kidney tissues of control, EA, DM, and DM+EA groups by Masson's trichrome staining (x400; thumbnail x200). EA: ellagic acid, DM: diabetes mellitus

When the TGFβ1 immunopositivity density was evaluated statistically, the values of the EA group showed a statistically significant decrease compared to the control group (p=0.006). The immunostaining intensity of the DM group increased significantly compared to the control and EA groups (both p<0.001). The decrease in staining intensity in the treatment group was statistically significant compared to the DM group (p<0.001) (Figure 2, 3B).

When the p-Smad3 immunopositivity density was evaluated statistically, there was no significant difference between the control and EA group (p=0.180), while the immunostaining density of the DM group increased statistically significantly compared to the control and EA groups (both p<0.001). The decrease in staining intensity in the treatment group was statistically significant compared to the DM group (p<0.001) (Figure 2, 3C).

When the αSMA immunopositivity density was evaluated statistically, there was no significant difference between the control and EA group (p=0.173), while the immunostaining density of the DM group increased significantly compared to the control and EA groups (both p<0.001). The decrease in staining intensity in the treatment group was statistically significant compared to the DM group (p<0.001). In addition, when the treatment group was compared with the control and EA groups, the difference was statistically insignificant (p=1.000, p=0.898, respectively) (Figure 2, 3D).

Table 2. Immunopositivity descriptive values of TGFβ1, p-Smad3, and αSMA of all groups (%)

		Control	EA	DM	DM+EA	p
TGFβ1	mean±SD	7.17±2.39	5.75±2.97	27.96±6.49	12.57±2.83	**<0.001**
	median [min-max]	6.66 [3.64-14.80]	4.97 [2.46-13.26]	28.52 [17.94-42.16]	12.13 [8.94-20.56]	
p-Smad3	mean±SD	4.42±2.05	5.21±2.40	21.42±6.98	10.99±2.26	**<0.001**
	median [min-max]	4.10 [1.51-8.69]	4.77 [1.98-10.25]	21.00 [10.25-38.16]	10.67 [7.49-16.25]	
αSMA	mean±SD	9.21±3.64	8.01±2.75	24.08±4.39	9.49±3.43	**<0.001**
	median [min-max]	8.66 [3.26-18.26]	7.63 [4.59-15.36]	25.08 [13.26-32.16]	8.76 [5.05-20.23]	

TGFβ1: transforming growth factor β1, p-Smad3: phosphorylated suppressor of mothers against decapentaplegic 3, and αSMA: α-smooth muscle actin, EA: ellagic acid, DM: diabetes mellitus

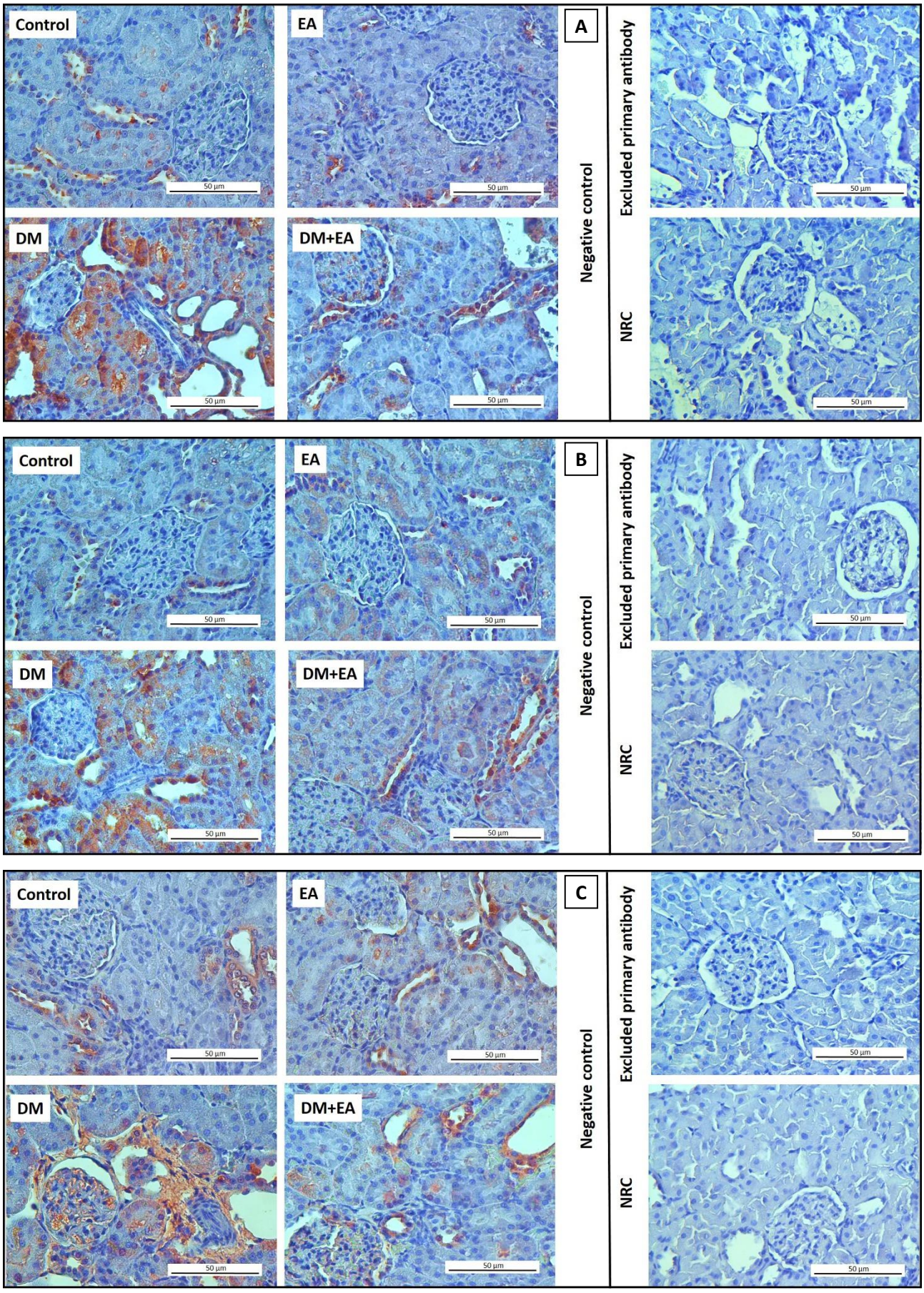

Figure 2. Representative microphotographs for the negative control and immunostaining with TGFβ1 (A), p-Smad3 (B), αSMA (C) primary antibodies in kidney tissues of all the groups. No staining occurred in controls with nonspecific IgG of the same isotype as primary antibodies (x400; AEC-hematoxylin). TGFβ1: transforming growth factor β1, p-Smad3: phosphorylated suppressor of mothers against decapentaplegic 3, and αSMA: α-smooth muscle actin, EA: ellagic acid, DM: diabetes mellitus, NRC: negative reagent control

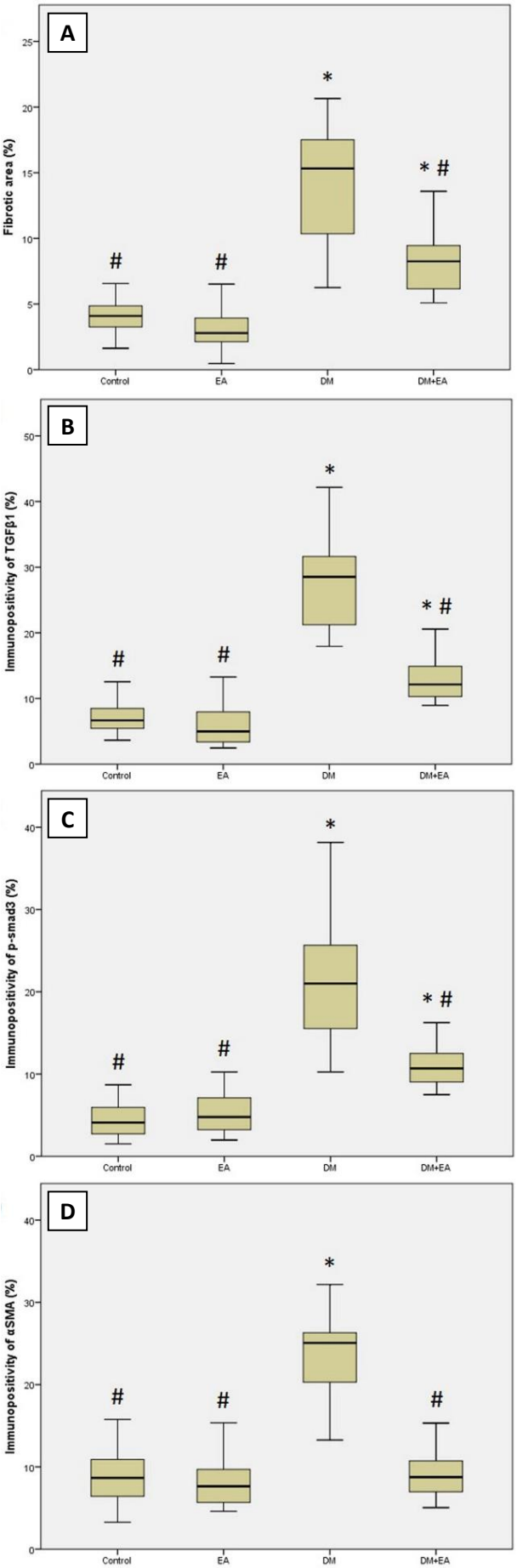

Figure 3. Quantitative summary of fibrotic area staining ratios (A), and immunopositive staining ratios (B-D).
*: p<0.05 vs. control, #: p<0.05 vs. DM (Bonferroni correction Mann-Whitney U tests)

Briefly, evidence was provided to show that elevated glucose increases TGFβ1 protein expression in the kidney. Increased TGFβ1 protein levels in diabetic kidneys triggered phosphorylated Smad3 translocated to the nucleus, activating TGFβ/Smad signaling. As a result of this activation, αSMA expression was induced in the intertubular area. In conclusion, renal fibrosis was induced in rats with diabetic kidney injury. TGFβ/Smad signal inhibition was observed with EA treatment. As a result, it was determined that fibrosis decreased with decreased collagen density and decreased αSMA expression.

DISCUSSION

DM is an endocrine metabolic disease with many side effects. Oxidative stress plays an important role in the onset and progression of diabetes and its complications. The most common complication of DM is diabetic nephropathy. Therefore, oxidative stress due to hyperglycemia plays an important role in the development of diabetic nephropathy (1,2). Among the pathological changes of diabetic nephropathy, glomerular and tubulointerstitial damage are the leading ones (3). Renal fibrosis seen in diabetic nephropathy is the accumulation of fibrotic matrix and scar formation in response to serious injury (4,5). Reducing oxidative stress remains an important goal in the treatment of diabetic nephropathy. Phytochemicals with antioxidant properties and free radical scavengers are frequently used in recent studies (23-26). In experimental diabetes studies, antioxidant applications have been shown to reduce hyperglycemia caused by STZ (23,27).

Gallic acid-derived EA has been shown to reduce myocardial infarction areas and suppress cardiac fibrosis in myocardial infarction and regulate the expression of antiapoptotic genes and mitochondrial respiratory enzyme activities (25,26). In a study investigating the effect of EA on testis by creating diabetes with STZ, it was found that EA reduced the expression of Nrf-2, which is a marker of oxidative stress, and the apoptotic index (22).

Smad proteins are important in the TGFβ signaling pathway. Different components of the Smad signaling pathway interact with regions on Smad proteins, inducing phosphorylation of Smad proteins and signal initiation. Smad2 and Smad3 are transported to the nucleus along with other transcription factors. Smad7 has a negative effect on TGFβ signaling, leading to polyubiquitination and degradation of TGFβ receptors. This shows that Smad factors are the main mediators of the fibrinogenic effects of TGFβ (28,29). In a study by Shu et al. (28) in which they investigated the hepatoprotective effects of limonin, a natural tetracyclic triterpenoid compound, it was shown that in a liver fibrosis model, limonin suppressed TGFβ-induced Smad2/3 phosphorylation and subsequent nuclear translocation, and increased Smad7 expression. In the diabetes study of Meng et al. (24), it was shown that silymarin application in myocardial fibrosis reduced collagen and fibrous structure, downregulated TGFβ1 and Smad2/3 levels, and upregulated Smad7 levels.

In our study, when we evaluated the effects of EA on TGFβ1/Smad signaling in diabetic kidney tissues, it was

observed that TGFβ1 protein, which plays an important role in the fibrotic pathway in diabetic kidney tissues, increased significantly and decreased with EA treatment. It was observed that high glucose up-regulated the expression of p-Smad3, the activated form of Smad3, which causes the activation of TGFβ1 expression and TGFβ1 transcription in the kidney, and down-regulated these levels with treatment.

In a myocardial infarction study with EA, it was reported that EA reduces cardiac fibrosis, suppresses HDACs expressions and expression of fibrous-related genes, decreases cardiac fibroblast proliferation, and is a phytochemical with anti-fibrotic properties (26). In the study of Li et al. (9), in which they created renal fibrosis in mice, it was shown that collagen accumulation and α-SMA expression increased. Collagen deposition was visualized by Masson's trichrome staining and a significant increase was detected. In the study, TGFBR1 induction by lentivirus injection method was regulated by MicroRNA-10a/b post-transcriptional mechanism; collagen deposition, fibrotic gene expressions, and α-SMA expression were down-regulated.

In our study, a significant increase in collagen density in the intertubular area of the kidney tissue of the diabetic injury group was demonstrated by Masson's trichrome staining. Collagen density was found to be statistically significantly decreased in the DM group treated with EA. While the expression level of the profibrotic marker αSMA increased in DM groups in line with fibrosis, its expression level was significantly decreased in the treatment groups. In particular, it was noteworthy that the αSMA expression levels of the control and treatment groups were similar.

CONCLUSION

In conclusion, TGFβ1 and p-Smad3 expression levels in the treatment group were statistically significantly decreased compared to the DM group. While the expression level of the profibrotic marker αSMA increased in DM groups in line with fibrosis, its expression level was significantly decreased in the treatment groups. It is thought that this study will shed light on studies investigating different signaling pathway mechanisms in the relationship between diabetes and EA.

Ethics Committee Approval: The study was approved by the Animal Experiments Local Ethics Committee of Gazi University (21.07.2022, G.Ü.ET-22.083).

Conflict of Interest: None declared by the authors.

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