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Renoprotective effects of tropisetron through regulation of the TGF-β1, p53 and matrix metalloproteinases in streptozotocin-induced diabetic rats

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ABSTRACT

Renal fibrosis is a major cause of renal failure in diabetic nephropathy. Tropisetron is an antagonist of the 5HT3 receptor that exhibits anti-fibrosis effects. The present research aimed to investigate the protected role of tropisetron against renal fibrosis of diabetic nephropathy and its molecular mechanisms. For this purpose, male Wistar rats were allocated into 5 groups of control, tropisetron, diabetes, tropisetron + diabetes, and glibenclamide + diabetes (n = 7). After induction of type 1 diabetes with a single injection of STZ, tropisetron (3 mg/kg) and glibenclamide (1 mg/kg) were given to the rats daily by intraperitoneal injection for 2 weeks. The obtained data revealed that the treatment of diabetic rats with tropisetron led to a significant decrease in the elevated blood glucose, serum cystatin c, and urinary total protein (UTP) level, indicating the improvement of the impaired kidney function. Moreover, the results of Masson's trichrome staining showed that fibrosis attenuated in the kidney of diabetic rats after tropisetron treatment. RT-PCR and Western blotting revealed that TGF-β1, the apoptotic mediator, and p53 were considerably declined in the kidney of diabetic rats in response to tropisetron treatment. Meanwhile, the expressions of matrix metalloproteinase-9 (MMP-9) and matrix metalloproteinase-2 (MMP-2) were increased. These notable effects were equipotent with glibenclamide, as a standard drug, suggesting that tropisetron can alleviate renal fibrosis in diabetic nephropathy. Our data indicate that tropisetron could improve kidney function and attenuate renal fibrosis through regulation of TGF-β1, p53, and expression of extracellular matrix metalloproteinases.

1. Introduction

It has been well documented that diabetic nephropathy (DN) is the major cause of end-stage renal disease (ESRD) in almost 30%–40% of diabetic patients [1]. Chronic exposure of the kidney to hyperglycemia leads to the development of progressive structural and functional damage [2]. This issue is characterized by proteinuria, a progressive mesangial expansion, glomerular basement membrane (GBM) thickening, tubulointerstitial fibrosis, and impaired renal function [3]. Renal fibrosis is an unfavorable remodeling alteration that occurs in the final stages of the disease. This type of fibrosis is characterized by the activation of transforming growth factor-β1 (TGF-β1) signaling as a major pro-fibrotic cytokine. TGF-β1, as a factor that is increased in the kidney of diabetic patients, is closely related to the degree of albuminuria [4]. Structural disturbances in DN may be a result of an imbalance between synthetic and degrading ECM enzymes [5]. Matrix metalloproteinases (MMPs), as zinc-dependent endopeptidases, are the main physiologic modulators of glomerular ECM degeneration [6] that are usually regulated by TGF-β [7]. Among these peptides, Matrix metalloproteinase-2 (MMP-2) and Matrix metalloproteinase-9 (MMP-9) have been considered important enzymes in the pathogenesis of diabetic nephropathy. Several studies reported that MMP-2 and MMP-9 decreased in the diabetic kidney at both transcription and translation levels [8–11]. However, there are some conflicting results in the literature regarding MMPs change within the last few decades [12–14]. Recently, it has been reported that a cross-talk of p53 and TGF-β genomic clusters in cell growth

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regulation and ECM reconstruction leads to progressive fibrotic disorder in the kidney [15]. Abdel Aziz et al. documented that TGF-β, p53, and extracellular matrix metalloproteinases alterations participate in the development of diabetic renal damage [16].

Nevertheless, existing drug therapy has not been completely successful in treating or preventing fibrosis nephropathy. Thus, there is a pressing need to develop a drug candidate that is both effective and safe [17].

Tropisetron as an antagonist of the 5HT3 receptor has been used clinically in chemotherapy-induced emesis [18]. Previous studies identified many beneficial properties of tropisetron including anti-oxidative stress, anti-inflammatory, anti-apoptotic, anti-diabetic, and anti-fibrotic effects [18–21]. Stegemann et al. reported antifibrogenic and anti-fibrotic properties of tropisetron observed in a mouse model of scleroderma mediated by TGF-β1 [22]. Tropisetron in protecting UVA-induced skin aging by modulating MMPs levels leads to collagen degradation and has a potential therapeutic effect in fibrotic events [23]. Despite these studies, the effect of tropisetron on the diabetic fibrotic kidney has not been clarified yet. Based on the above information, in the current study, the therapeutic effect of tropisetron against diabetic fibrotic kidney was investigated by determination of TGF-β1, p53, and extracellular matrix metalloproteinases expressions.

2. Materials and methods

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Urmia Medical University. The rats were housed in an air-conditioned room at 24 °C with 12 h light/dark cycles. A total of 35 male Wistar rats weighing 250 ± 20 g and aged 3–4 months were assigned into 5 groups (n = 7 in each group): Control group, Tropisetron group, Diabetes group, Tropisetron + diabetes group, and Glibenclamide + diabetes group. The control group was treated only with saline (ip) for 2 weeks. Diabetes was induced by injecting streptozotocin (STZ, 50 mg/kg, ip; Sigma Chemical Co., St Louis, MO) in rats. Rats in the tropisetron group received tropisetron with a dose of 3 mg/kg dissolved in saline (Cayman Chemical Co, USA) intraperitoneally (ip) for 2 weeks [18,19,24]. Tropisetron in Tropisetron + diabetes group was injected ip for 2 weeks after diabetes induction. Also, glibenclamide (standard drug, 1 mg/kg, ip; Sigma, India) was injected for 2 weeks after diabetes induction.

2.1. Induction of type 1 diabetes

STZ as a standard drug was used in a single dose (50 mg/kg) to induce diabetes. Seventy-two hours after injection of STZ, fasting blood glucose (FBS) was checked in a drop of blood obtained using a fine needle with a standard glucometer (Elegance, CT-X10, Frankenberg, Germany). The blood glucose level above 300 mg/dl was selected as diabetic.

After two weeks of treatment (tropisetron 3 mg/kg, ip), rats were housed in metabolic cages for 24 h to collect urine for subsequent measurements of urinary total protein (UTP). FBS was measured in blood samples obtained from the tip of the tail by using a glucometer device. Then, the rats were euthanized by ketamine (60 mg/kg) and xylazine (4 mg/kg). After anesthetizing the rats, their abdominal cavity was exposed. The blood was taken from the heart by a narrow heparinized needle, centrifuged at 4000×*g* for 20 min to isolate plasma, and then stored at - 80 °C for later analysis. Both kidneys were isolated and then a piece of the left kidneys was homogenized by Ultra Turrax (T10B, IKA, and Germany) in an RNAase-containing solution to measure the expression levels of MMP-2, MMP-9, and TGF-β1 using the Real Time-PCR method. Also, the other part of the left kidney was frozen at −80 °C for Western Blot analysis (p53). Right kidneys were fixed in buffered formalin and embedded in paraffin for analyzing the histopathological changes.

2.2. ELISA method

The plasma cystatin C levels were detected using the quantitative sandwich enzyme immunoassay method with a commercial Elisa kit (Cusabio, China). In brief, a specific cystatin C antibody was placed into micro-plates. Standards and samples were put into the wells to react with present cystatin C by an immobilized antibody in the wells. Afterward, all unbound substances were cleared and a biotin-conjugated Horseradish Peroxidase (HRP) was added to the wells. In the next step, a substrate solution was added to the relevant wells and color change appeared in proportion to the cystatin C level. Finally, the color intensity was measured to report the data [25].

2.3. Primer design and Real Time-PCR

The RNA was first extracted by the special kit (GENEALL South Korea, Cat no 305–101) according to the manufacturer's protocol. RNA concentration was confirmed by spectrophotometer at the absorbance of 260–280 nm and evaluated by a combination of Tris base, acetic acid, and EDTA (TAE)-agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized from total RNA extracts using a universal cDNA synthesis kit. Briefly, total RNA was reversed to cDNA using a poly (T) primer with a 3 degenerate anchor and a 5 universal tag (Exiqon, Vedbaek, Denmark). Each cDNA was used as a template for RNA quantitative real-time PCR using the SYBR Green master mix (Exiqon, Vedbaek, Denmark). The specific primers of the target genes and the glyceraldehyde dehydrogenase (GAPDH) gene, as a housekeeping gene, were examined on the NCBI site using the Generunner software (Table 1). Relative quantitative expressions of the genes were evaluated through the $2^{-(\Delta\Delta Ct)}$ method. The data were expressed as the fold-difference to the reference gene.

2.4. Western blot

Western blot method was applied to determine the p53 protein level in the kidney tissue. In summary, the Bradford assay kit (Sigma Aldrich. USA) was applied to estimate the protein concentration of the supernatant. Then, a 20-μg protein was loaded in each well made by a special comb after combined with a-2X sample loading buffer. After isolation of proteins in 10% SDS-gels, they were transferred to Polyvinylidene fluoride (PVDF) membranes in an hour. The blocking of the membranes was done and then applied with primary antibodies against p53 and β-actin. Washing with a Tris-buffered solution was done for about 4 × 5 min and then the secondary antibody was added to the membranes. After 1 h of incubation in the shaker, the membranes were washed for 3 × 5 min with wash buffer and then membranes were incubated with the enhanced chemiluminescence (ECL, Amersham) reagents in the dark-room. Finally, with exposure of the membrane with X-ray film, the intensity of the bands was measured using Image J software (IJ 1.46r version, NIH, USA) and normalized to the bands of the reference gene (β-Actin). Catalog numbers and companies of the antibodies are presented in Table 2.

Table 1
Sequences of primers used for evaluating of MMP-2, MMP-9 and TGF-β1 gen expressions.

	Sequence of primer	Product size	Tm
MMP-2	Forward:CTGTCTCCTGCTCTGTAGTTAATC Reverse: GATACGGTCAGCACCTTTCTT	104	59
MMP-9	Forward: GCTGCTCCAACTGCTGTATAA Reverse: TGGTGTCCTCCGATGTAAGA	93	59
TGF-β1	Forward: CCGCAACAACGCAATCTATG Reverse: CTTCCCGAATGTCTGACGTATT	83	59
GAPDH	Forward: AGACAGCCGCATCTTCTTGT Reverse: CTTGCCGTGGGTAGAGTCAT	207	59

Table 2
The p53 antibody used in Western blotting assays.

Primary antibody	Company	Dilution	Catalog number
P53	SANTA CRUZ	1:500	sc-126
β-Actin	SANTA CRUZ	1:300	sc-130657

2.5. Histopathological examination

To evaluate kidney tissue fibrosis, 5 μm kidney tissue sections were stained by Masson's Trichrome staining following the manufacturer's instructions (Asiapajohesh, Amol, Iran). The intensity of kidney fibrosis was assessed by adopting a semiquantitative method as described in Ref. [26]. The intensity level was scored in the range of 0 (normal kidney) to 8 (total fibrosis). The criteria set for scoring kidney fibrosis were as follows: grade 0 = normal kidney, grade 1 = minimal fibrosis thickening of kidney tissue, grades 2 and 3 = moderate thickening of kidney tissue without obvious damage to the structure of kidney tissue, grades 4 and 5 = increased fibrosis with complete damage to the structure of the kidney and production of fibrosis bands or small fibrosis masses, grades 6 and 7 = severe disturbances of structure and wide fibrosis areas, and grade 8 = total fibrotic obliteration [25].

2.6. Statistical analysis

Kolmogorov Smirnov test was performed to examine data normality. Statistical analysis was done using SPSS version 16.0 (IBM Corp., Armonk, NY, USA). The significance between groups was found using the ANOVA test followed by Tukey's post-hoc test. Results were expressed as mean ± SEM. The P values less than 0.05 were considered significant.

3. Results

3.1. FBS, UTP, and cystatin C

As shown in Table 3, two weeks after induction of diabetes, fasting blood glucose increased significantly ($P < 0.001$) compared to the control and tropisetron group. Besides cystatin C, as an early marker of nephropathy elevated in diabetic rats ($P < 0.001$). To predict kidney damage in animals with type 1 diabetes, we measured UTP in diabetic rats that were significantly higher than those in the control and tropisetron group ($P < 0.001$). At the end of the experiment, tropisetron treated diabetic rats revealed a remarkable decrease ($P < 0.01$) in the blood glucose, cystatin C and UTP ($P < 0.05$) levels. The same findings were achieved in the glibenclamide treated group.

3.2. MMP-2, MMP-9, and TGF-β1 gene expression

Two weeks after the induction of diabetes, we examined MMP-2, MMP-9, and TGF-β1 gene expressions in the renal tissues by real-time PCR. We found that MMP-2 (0.45 ± 0.06) and MMP-9 (0.5 ± 0.03) as endopeptidases that degrade extracellular matrix proteins had significantly lower expression in diabetic animals. Moreover, TGF-β1 (1.79 ± 0.12) induced renal fibrosis by suppressing the activity of matrix metalloproteinases genes in the diabetic group ($P < 0.001$), compared with the control and tropisetron groups.

Furthermore, tropisetron treatment for 2 weeks increased MMP-2 (0.86 ± 0.04) and MMP-9 (0.81 ± 0.04) while it decreased TGF-β1 (1.4 ± 0.1) mRNA levels significantly ($p < 0.05$) in the diabetic rats. Similar results were developed in the animals treated with the standard drug, glibenclamide (Fig. 1a,b,1c).

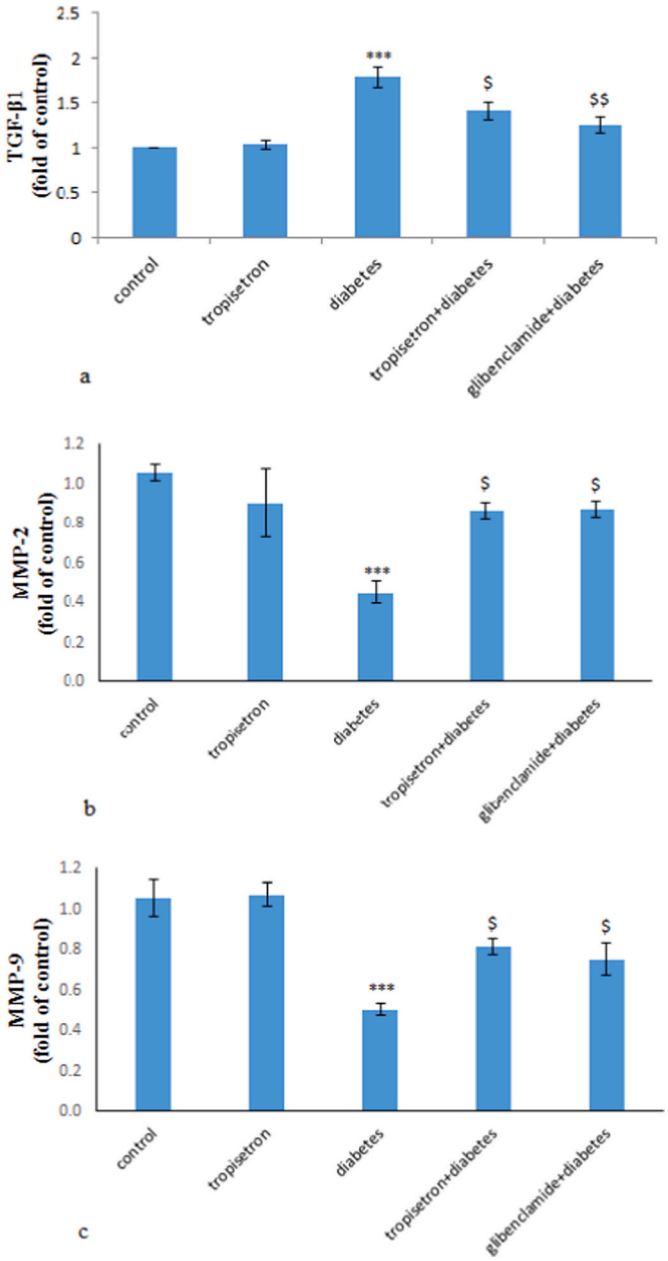

Fig. 1. Effect of tropisetron treatment on the renal TGF-β1 (a), MMP-2 (b) and MMP-9 (c) gene expressions in different groups at the end of experiment. ***P < 0.001 vs control group. $ P < 0.05, $$ P < 0.01 vs diabetic group. All data are expressed as the means ± SEM (n = 7).

Table 3
Effect of tropisetron treatment on FBS, UTP and cystatin c in different groups at the end of experiment. FBS: fasting blood glucose, UTP: urinary total protein excretion.

Groups	control	tropisetron	diabetes	tropisetron +diabetes	glibenclamide + diabetes
FBS (mg/dl)	83±4.1	88±10.8	515±29.6***	308±33.7$$	339±38.3$
Cystatin c (ng/ml)	1.08±0.09	1.71±0.2	5.96±0.42***	3.75±0.55$$	3.35±0.38$$$
UTP (mg/dl)	0.5±0.12	0.61±0.18	2.5±0.53***	1.54±0.22$	1.31±0.28$

3.3. P53 protein expression

To evaluate p53 protein expression as a proapoptotic molecule in this study, Western Blotting was performed in the kidney of different groups. The data analysis exhibited a significant increase ($P < 0.001$) in p53 (2.24 ± 0.05) protein level in the diabetic group compared to the control (1 ± 0.0) and tropisetron (1.07 ± 0.04) groups. Tropisetron co-treatment markedly ($p < 0.05$) attenuated p53 (1.99 ± 0.09) level in the kidney of diabetic rats. The same result was observed in the diabetic kidney tissue of the rats treated with glibenclamide (1.62 ± 0.06) ($p < 0.001$) (Fig. 2a and b).

3.4. Histopathological results

According to the results of the present study, microscopic fibrosis scores in the kidney of STZ induced diabetes in the rats at the end of the experiment. There was no lesion score in the control and tropisetron groups (grade 0). Also, the microscopic lesion score in the kidney of diabetic animals was 4–5, which is a manifestation of increased fibrosis with apparent damage to the kidney' s structure and development of fibrosis bands. Administration of tropisetron and glibenclamide for 2 weeks attenuated fibrotic changes in the diabetic kidney; however, it is more prominent in the tropisetron + diabetes group (Fig. 3a and b).

4. Discussion

The renoprotective effect of tropisetron has been demonstrated by evaluating various parameters such as renal function markers, histopathological features, and inflammatory cytokines and found that these effects are independent of the 5HT3 receptor [19]. As reported in this study and our previous studies, tropisetron can decline blood glucose levels [18] by stimulating β-cell regeneration and insulin secretion [24],

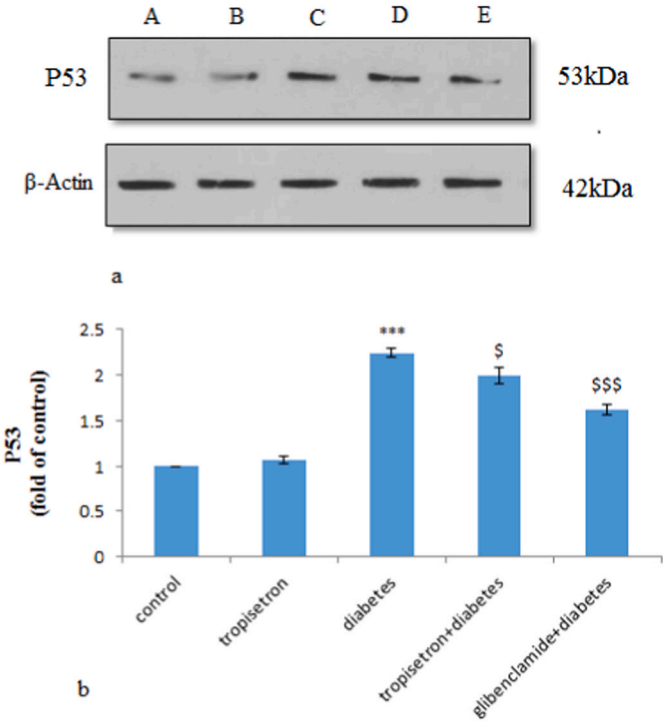

Fig. 2. Effect of tropisetron treatment on the renal protein levels. The blotting images of p53. The bar charts represent the quantitative analysis of p53 normalized against β-actin. ***$P < 0.001$ vs control group. $ $P < 0.05$, $$$ $P < 0.001$ vs diabetic group. A: control, B: tropisetron, C: diabetes, D: tropisetron + diabetes, E: glibenclamide + diabetes. All data are expressed as the means ± SEM ($n = 7$).

suggesting its protective role in diabetic renal impairment. However, there is little information to delineate the mechanistic pathway responsible for its nephroprotection [27]. The current study evidenced that tropisetron could ameliorate kidney function by mitigating renal fibrosis, which is manifested by decreasing urine albumin excretion and cystatin c level. This was mediated through several molecular targets including TGF-β1, p53, MMP-2, and MMP-9 expressions in diabetic nephropathy.

The appearance of urinary albumin is an essential indicator for the diagnosis of DN [28]. Moreover, the serum creatinine level is the most common marker that has been used as an endogenous filtration index in clinical practice. However, it is well known that serum cystatin c, as a protease inhibitor, serves as a better predictor of GFR, especially in the early stage of renal failure. Also, this marker is a more precise test for renal function compared to the ubiquitously used serum creatinine [29]. Notably, cystatin c is a small protein that is affected less by age, gender, and also muscle mass than creatinine and is freely filtered by the glomerulus [30]. Elevated levels of cystatin C have been detected in different models of renal injury and DN [31]. This novel first study displays a direct effect of tropisetron on serum cystatin c levels as a protective drug on diabetic nephrotoxicity.

Renal fibrosis, which is expressed by excessive deposition of the extracellular matrix, is suggested to be a major pathological insult that leads to glomerular and tubular dysfunction and deterioration in diabetic nephropathy [32]. The present study illustrated that tropisetron could alleviate renal tubulointerstitial fibrosis by TGF-β1-dependent signaling in DN. Previously, it was known that the TGF-β1 gene and protein expressions were elevated in both clinical and experimental studies in diabetic nephropathy [33,34]. As one of the important cytokines belonging to the TGF-β superfamily, it plays a key role in diabetic renal interstitial fibrosis. This effect is mediated by enhancing renal apoptosis and affecting the synthesis and degradation of ECM proteins, especially by upregulating collagen and fibronectin formation [35–37]. Hyperglycemia and several molecular signaling such as angiotensin II, and oxidative stress pathways in DN have been documented to trigger TGF-β1 related molecules probably by activation of Protein kinase C (PKC) pathway [38,39]. In line with our study, Stegemann et al. reported that tropisetron suppressed collagen synthesis through inducing TGF-β1 and also could prevent skin fibrosis and reduced the collagen fibers in dermal fibrosis induced by bleomycin in vivo and in vitro [22].

Several studies have demonstrated that TGF-β1 is a key regulator of ECM that remodels participation in glomerular mesangial matrix expansion [38,40]. The increasing ECM deposition is a characteristic pathological change in DN that results in progressive glomerular sclerosis [41]. ECM remodeling as a dynamic adhesion complex is regulated by matrix synthesis and degradation. Chronic hyperglycemia causes the imbalance of this network deposition and contributes to the accumulation of ECM and subsequently tissue fibrosis [42,43]. As important components of the ECM regulation system, two major proteinases including MMP-2 and MMP-9 play essential roles in the development of DN [44]. Although recent investigations have reported some alterations of MMPs in the diabetic kidney, the results are controversial [13,38,42,43]. The present study demonstrated that the expression of MMP-2 and MMP-9 decreased in the diabetic nephropathy, while tropisetron treatment could reverse these alterations and lead to a decrease in ECM deposition. This indicates that tropisetron may be an effective drug to protect the kidney from interstitial fibrosis and functional impairments in diabetes.

This finding is consistent with a previous study that showed tropisetron could suppress the accumulation of fibronectin as an ECM protein proposing its protecting role in DN [20]. Moreover, tropisetron can regulate the expression of MMPs in UVA-induced accumulation of collagen degradation and also antagonized the effect of TGF- β1 in human dermal fibroblasts [23]. Stegemann et al. indicated that tropisetron not only attenuated collagen formation induced by TGF-β1 in experimentally skin fibrosis but also diminished the collagen content in

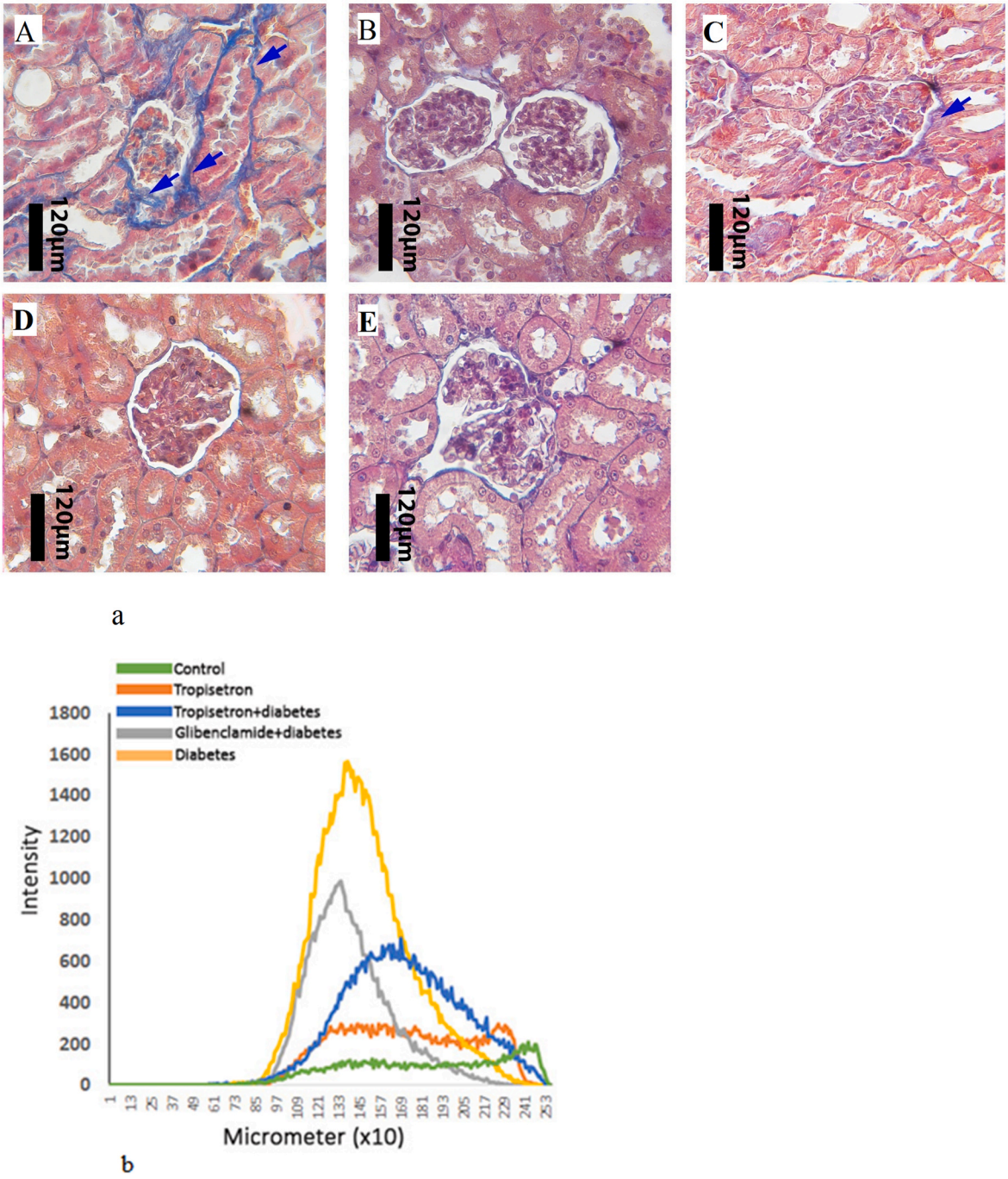

Fig. 3. Photomicrographs of renal tissue by Masson Trichrome staining (a) demonstrate that microscopic lesion score was 4–5 that implied increased fibrosis with definite damage to the kidney structure and formation of fibrosis bands in diabetic animals (black arrow). Tropisetron and glibenclamide treatment decreased fibrosis bands in the kidney of diabetic rats. There is no significant differences between control and tropisetron + diabetes group. Magnification × 400. Scale bars are as indicated. Software analysis (b) for collagen intensity in 2530 × 2530 μm of tissue. A: diabetes.

dermal fibrosis induced by bleomycin [22]. These results are in line with our results for the antifibrotic status of tropisetron established in DN.

Interestingly, our results showed an up-regulation of the pro-apoptotic p53 protein expression as a downstream molecule of TGF-β1 in the renal tissue of STZ induced diabetes that was reversed by tropisetron treatment. P53, as the main co-factor in the TGF-β1-triggered transcription of various pro-fibrotic genes, provides an extensive role in TGF-β1 in tissue injury [45]. Besides, TGF-β1-induced binding of p53 to the target promoter in renal cells results in renal fibrosis [46]. Oxidative stress originating from chronic hyperglycemia can induce phosphorylation of p53, which interferes with mitochondrial bax and bcl-2. This interference leads to the activation of the intrinsic pathway of apoptosis [47]. So, these are notable findings to identify potential mechanisms of tropisetron to rescue kidney tissue against fibrosis in DN.

Glibenclamide exposure also enhanced MMP-2 and MMP-9 mRNA expressions and decreased TGF-β1 and p53 levels in the diabetic kidney in the current research. Glibenclamide is an approved drug in diabetes treatment by inhibition of the ATP-sensitive K+ channels. Here, we showed that glibenclamide might possess an anti-fibrotic effect in DN.

Taken together, this is the first study that declared tropisetron and glibenclamide could regulate the expression of MMP-2 and MMP-9 and p53 protein levels by inhibiting the expression and production of TGF-

β1. This issue may be confirmed through the study of tissue-specific above-mentioned parameters of deficient animals in a follow-up experiment. In further trials, more controlled researches are required to confirm the proposed mechanisms.

5. Conclusion

In the present study, it was found that fibrosis levels in the kidney were markedly increased in DM and were recovered following tropisetron administration as reflected by a decrease in TGF-β1 and p53 levels and an increase in matrix metalloproteinases (MMP-2 and MMP-9) levels. Our study offers new insight into a novel mechanism by which tropisetron could be applied to prevent and treat diabetic renal fibrosis.

CRediT authorship contribution statement

Bagher Pourheydar: Data curation, histopathological examination. Mahrokh Samadi: Data curation, Formal analysis. Parisa Habibi: Data curation, Ahmad Ali Nikibakhsh: Conceptualization, Methodology. Roya Naderi: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

All data are expressed as mean ± SEM (n = 7): ***P < 0.001 vs control and tropisetron groups. $ P < 0.05, $$ P < 0.01, $$$ P < 0.001 vs diabetic group.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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