Annals of Biomedical and Clinical Research

Original article

ABCR No 1; Vol 1; 2022;4-11

Changes in Expression of the Connective Tissue Growth Factor in the Kidneys of Diabetic Rats During Aging

Leonora Bedeković[1], Katarina Vukojević[1]

[1]School of Medicine, University of Mostar

ABSTRACT

Background: Diabetic nephropathy (DN) is the most common cause of chronic renal failure in the world. There appears to be different pathogenetic, hemodynamic and metabolic processes leading to the pathologic mechanisms in DN. We studied the expression of connective tissue growth factor (CTGF) in the kidneys of diabetic rats over time.

Methods: Male Sprague-Dawley rats were injected with 55mg/kg streptozotocin (STZ) (diabetes mellitus (DM) group) or with citrate buffer (control group). The rat kidneys were harvested 2 weeks, 2 months, 6 months and 12 months following the STZ challenge, and sectioned into three different kidney structures: glomeruli, proximal convoluted tubule (PCT) and distal convoluted tubule (DCT). Sections were immunohistochemically stained to monitor the expression of CTGF.

Results: Significant differences in CTGF expression were observed 2 weeks and 2 months after the STZ challenge, with higher CTGF expression in diabetic rats. Two weeks post DM onset, high CTGF expression was demonstrated in the distal tubules of the diabetic rats. The expression of CTGF in the glomeruli of diabetic rats was highest 12 months after diabetes induction, with a complete absence of CTGF expression in the control groups throughout the study.

Conclusions: The major change in expression of CTGF occurs within the first 2 months of DM, particularly in the DTC, implying an early onset of pathophysiological changes in diabetic kidneys, which would normally occur with aging. These findings help to contribute to our understanding of changes associated with DN and guide towards potentially appropriate treatment modalities.

Key words: diabetes mellitus (DM), nephropathy, chronic renal failure, connective tissue growth factor (CTGF)

Article processing history:
Received March 22, 2021
Revised May 16, 2021
Accepted June 21, 2021

ORCID IDs of the authors:
L.B. 0000-0001-5786-0384
K.V. 0000-0003-2182-2890

Corresponding author:
Katarina Vukojević;
E-mail: katarina.vukojevic@mefst.hr, kvukojev@gmail.com,
Tel: +385-21-557-807
Fax: +385-21-557-811

Cite this article as: Bedeković L, Vukojević K. Changes in expression of CTGF in kidneys of diabetic rats during ageing. Annals of Biomedical and Clinical Research. 2022;1:4-11.

https://doi.org/10.47960/2744-2470.2022.1.1.4

INTRODUCTION

Diabetic nephropathy (DN) is the most common cause of chronic renal failure in the world. Poor glycemic control, hyperlipidemia, smoking, oxidative stress, environmental, genetic and epigenetic factors play an important role in the pathophysiological development of DN (1). DN, also called Kimmelsteil-Wilson syndrome, is a clinical syndrome involving the occurrence of pathological albuminuria (>300 mg/day or >200 mcg/min-confirmed in at least two measurements over a period of 3 to 6 months) and a permanent and irreversible decrease in glomerular filtration and arterial hypertension (2).

DN is mainly associated with glomerular dysfunction, particularly causing nodular and diffuse mesangial proliferation and thickening of the glomerular basement membrane, due to excessive extracellular matrix formation. These changes ultimately lead to the occlusion of glomerular capillaries and progressive impairment of the integrity of the glomerular function and hence the kidney itself (5). Additionally, podocyte damage can occur, manifested by shortening, thinning and detachment of the podocyte foot processes from the glomerular basement membrane (6). Moreover, the fenestrated area of the endothelial cells is reduced, the glycocalyx is weakened and the communication between the glomerular endothelial cells and the adjacent glomerular cells is distorted (7).

Connective Tissue Growth Factor (CTFG) is a member of the CCN (Cyr61/CTGF/Nov) family of matricellular proteins. The CCN2 subset is widely distributed in human tissues and organs, such as the connective tissue, heart, brain, lungs, liver, muscles, placenta and in particular, the kidneys (8). These proteins are responsible for facilitating a range of signaling pathways, important for cell adhesion and migration, angiogenesis, myofibroblast activation, extracellular matrix deposition, tissue remodeling and fibrosis. CTGF specifically plays an important role in the development of diabetic glomerulosclerosis by promoting transient cytoskeletal actin breakdown in mesangial cells, high fibronectin production, type I and IV collagen and mesangial cell hypertrophy (9). CTGF have four domains, each of which can bind several ligands capable of modifying their function. CTGF can interact with a wide range of cellular receptors including integrins, tyrosine receptor kinase A, heparan sulfate proteoglycans, extracellular matrix proteins, fibronectin, VEGF, TGF-β, etc. (10). With so many potential interactions, it is predictable that CTGF affects a multitude of different biological events. For example, Mason et al. (12) have demonstrated that CTGF stimulates the profibrotic effects of TGF-β, by amplifying the TGF-β-Smad2/3 signaling pathway (12).

CTGF expression in healthy glomeruli is low, but CTGF levels in the early stages of diabetes increase and continue as the disease progresses (12). Roestenberg et al. (2006) concluded that significant changes occur after 1 week in the form of increased urinary albumin excretion and increased CTGF expression in mesangial cells. The increased expression of CTGF in renal glomeruli is present in podocytes and parietal epithelial cells (13). Distal to the glomeruli, CTGF expression is also increased in the proximal and distal tubular epithelial cells of diabetic rodents (12). Numerous other studies confirm these findings (8). In contrast, Baelde et al. (15) showed a decrease in renal CTGF expression in people with type 2 diabetes, in association with a reduced number of podocytes in DN. Studies in other organ systems suggest that the inhibition of CTGF may not only prevent but also inhibit fibrosis (10). Additionally, investigations into the potential utility of CTGF quantification as a clinical biomarker of early DN and targeted anti-CTGF therapy for the prevention of chronic renal failure are underway (2,8).

Therefore, the aim of this study is to investigate the expression pattern of CTGF in the kidneys of diabetic rats during aging, in order to elucidate its relevance in potential chronic renal failure.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats were acquired from the University of Split, each of them weighing between 160 and 180 grams. The rats were raised under controlled conditions, consisting of an environment temperature of 22±1°C and a 12-hour light/12-hour dark lighting schedule.

Induction and validation of diabetes

Experiments were conducted using a type 1 diabetes mellitus (DM1) rat model (14). In summary, DM was induced by an intraperitoneal injection of 55mg/kg STZ, freshly dissolved in citrate buffer, pH 4.5, after fasting overnight. The rats were fed with unrestricted, standard laboratory chow, made up of 27% proteins, 9% fat and 64% carbohydrates (4RF21 GLP, Mucedola, Settimo Milanese, Italy).

Plasma glucose levels and body weights were used to validate the induction of DM. Plasma glucose levels using tail vein blood was measured using a OneTouch Vita instrument (LifeScan, High Wycombe, UK). The rats were considered diabetic if they had a glucose level >16.5 mmol/L and were, therefore, included in further experiments.

Diabetic rats were allocated into four groups, based on the duration of diabetes from the injection of STZ until the end of the experiments: 2 weeks (DM-2 w), 2 months (DM-2 m), 6 months (DM-6 m) and 12 months (DM-12 m). For each diabetic group, there was a matched control group, which was kept in the experiment for the same respective period (C- 2 w, C-2 m, C- 6 m, C-12 m). The control rats were injected intraperitoneally with citrate buffer only. In each diabetic and control group, there were six animals for each required age, totaling 48 models of experimental animals.

Tissue collection and immunohistochemistry

The experimental rats were euthanized with isoflurane (Forane, Abbott Laboratories, Queenborough, UK). Then, 300 mL of Zamboni's fixative at pH 4 (4% paraformaldehyde and 15% picric acid in 0.1 M phosphate-buffered saline) was perfused intracardially (14).

Kidney samples were subsequently acquired and post fixed in 300 mL of Zamboni's fixative at pH 4 (4% paraformaldehyde and 15% picric acid in 0.1 M phosphate-buffered saline) for further analysis. These were then processed with transverse cuts and embedded in paraffin blocks, which were cut into 7 μm thick sections. After deparaffinization, tissue sections were rehydrated using alcohol and water, thoroughly rinsed in distilled water and heated in a microwave oven with sodium citrate buffer (pH 6.0) at 95°C for 12 minutes. Samples were cooled down to room temperature before being incubated with primary antibodies (14).

A goat anti-CTGF antibody (L-20) (SC-14939, Santa Cruz Biotechnology, SAD) was diluted at 1:200 ratios in Dako REAL antibody diluent (Dako Denmark A\S, Glostrup, Denmark) and then applied to the sample tissue. Following the application of the primary antibody, the tissue sample was kept overnight in a humidified chamber at room temperature. Sections were rinsed with PBS and incubated with the secondary antibody, donkey anti-goat from Abcam (ab150123, Cambridge, UK) for 1 hour in a humidified chamber. The final stained kidney samples were observed and imaged using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus, Tokyo, Japan). Following imaging, the samples were processed with Cell A Imaging Software for Life Sciences Microscopy (Olympus Tokyo, Japan); 4',6-diamidino-2-phenylindole (DAPI), hematoxylin and eosin (H&E) and Mallory staining were also performed.

Kidney sections were analyzed focusing on two areas: cortex and medulla. For each of the listed areas, five non-overlapping fields were captured for analysis using 40× objective magnification, each field representing one image. Microphotographs were examined using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Kidney sections were semi-quantitatively analyzed and described as four categories in regard to the staining intensity: (0) indicating the absence of any reactivity, (1) a mild reactivity, (2) moderate reactivity, (3) strong reactivity. Two researchers analyzed the staining intensity independently. The number of positive cells within each area (glomerulus, PCT, DCT) were compared between the experimental diabetic groups and control groups. After separate analyses were conducted for the different kidney structures for each group and period, the data were pooled for all kidney structures of the control and diabetic rats and re-analyzed.

Statistics

The chi-square test was used for statistical analysis to examine the differences between the control groups and the diabetic groups. Data analysis was conducted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The data were expressed as a mean ± standard deviation, with $p<0.05$ serving as the marker of statistical significance.

RESULTS

Immunofluorescence staining showed different signal intensity between individual kidney structures in the control and diabetic groups during aging (Figures 1- 4).

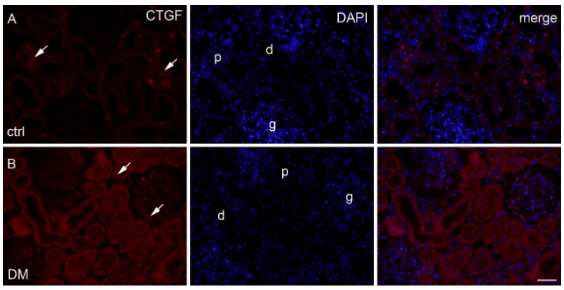

Figure 1. Immunofluorescence imaging of the kidney cortex, isolated from the control and DM mice at 2 weeks. CTGF positive cells were seen as red staining of cytoplasm (arrows) within different areas of the kidney cortex. Co-localization of CTGF and DAPI nuclear stain is shown in the far-right column (merge). Scale bar 20 µm. Legend: d- distal tubule; p- proximal tubule; g- glomerulus; ctrl- control, DM- diabetes mellitus type I.

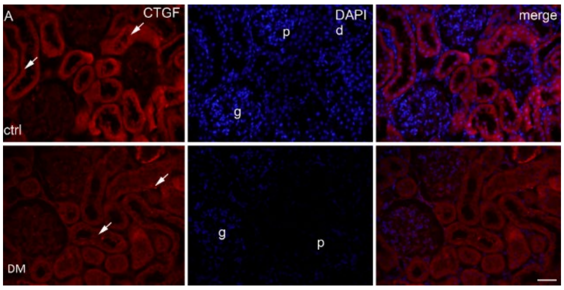

Figure 2. CTGF positive cells were seen as red staining of cytoplasm (arrows) within different areas of the cortex of the kidneys. Co-localization of CTGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 2 months. Scale bar 20 µm. Legend: d- distal tubule; p- proximal tubule; g- glomerulus; ctrl- control, DM- diabetes mellitus type I.

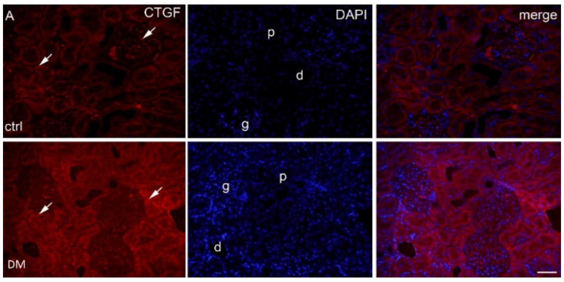

Figure 3. CTGF positive cells were seen as red staining of cytoplasm (arrows) within different areas of the cortex of the kidneys. Co-localization of CTGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 6 months. Scale bar 20 µm. Legend: d- distal tubule; p- proximal tubule; g- glomerulus; ctrl- control, DM- diabetes mellitus type I.

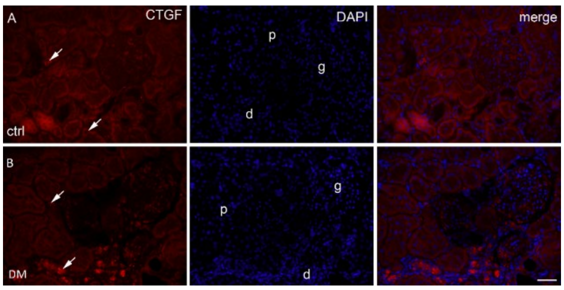

Figure 4. CTGF positive cells were seen as red staining of cytoplasm (arrows) within different areas of the cortex of kidneys. Co-localization of CTGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 12 months. Scale bar 20 µm. Legend: d- distal tubule; p- proximal tubule; g- glomerulus; ctrl- control, DM- diabetes mellitus type I.

We used the immunofluorescence data to analyze the total number of CTGF- positive cells between different experimental groups and within all three kidney cortex structures separately (PCT, DCT and glomeruli). There was a significant difference in the total number of CTGF-positive cells in diabetic rats 2 weeks and 2 months post-induction, compared to the control groups (p<0.001). Moreover, there was a significant difference between the diabetic rats and control groups at 6 months and 12 months after diabetes induction (p<0.01) (Figure 5).

The highest number of CTGF-positive cells in the control groups was observed at 6 months, while the highest number of CTGF-positive cells in the diabetic groups was observed 2 weeks after diabetes induction (Figure 5).

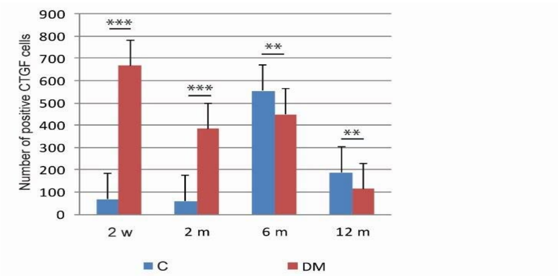

Figure 5. Number of CTGF-positive cells at 2 weeks (2 w), 2 months (2 m), 6 months (6 m) and 12 months (12 m) in the control (C) and diabetes (DM) groups as quantified from the immunofluorescence data. Data presented as a mean ± standard deviation, t-test. Asterisks denote significant difference. **p<0.01, ***p<0.001.

At 2 weeks, there was a complete absence of CTGF-positive cells in the glomeruli and proximal tubules of the control group, while CTGF was highly expressed in diabetic rats (Figure 6.a).

The percentage of CTGF-positive cells in the control and diabetic groups decreased 2 months after the induction of diabetes in the proximal and distal tubules (Figure 6.b).

Six months (after) post-induction there was a significant difference in the percentage of CTGF-positive cells between the control and diabetes groups in the PCT (p<0.001). There was a complete absence of CTGF expression in the glomeruli of both groups (C and DM). A slightly smaller difference in the percentage of CTGF-positive cells was observed in the distal

tubules between the control group (81.32%) and the diabetic group of rats (81.99%) (Figure 6.c).

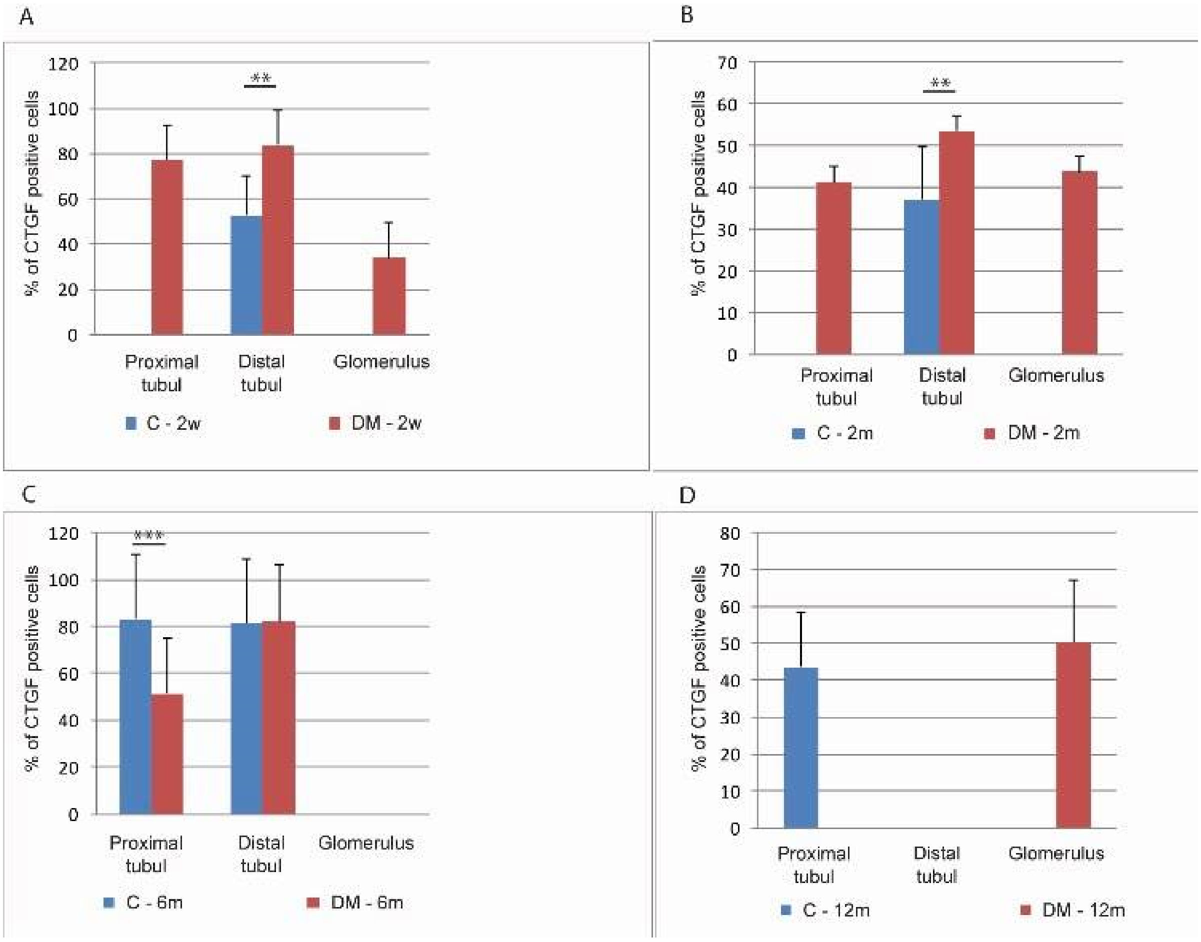

Figure 6. Distribution of the percentage of CTGF-positive cells in different renal structures (PCT, DCT and glomerulus) between the control (C) and diabetes (DM) groups (a) 2 weeks, (b) 2 months, (c) 6 months and (d) 12 months after DM induction. Data presented as a mean ± standard deviation, t-test. Asterisks denote significant difference. **$p<0.01$, ***$p<0.001$

There was a difference in the percentage of CTGF-positive cells in the proximal tubules between the control group (43.56%) and the diabetic group of rats (0%) (Figure 6.d).

DISCUSSION

In this study, we observed changes in CTGF expression in the kidneys of diabetic and control rats as they aged. Roestenberg et al. previously showed that significant renal changes in diabetic rats occur after 1 week in the form of increased urinary albumin excretion and increased expression of CTGF in the renal cortex (13). Usually, CTGF expression in healthy kidneys is low, however, prolonged hyperglycemia results in the increased expression of various proinflammatory cells, including CTGF. Numerous animal and in vitro experiments have shown that CTGF plays a key role in the development of renal fibrotic changes (16). Moreover, several studies suggest that CTGF could become a clinical biomarker of early DN in the future (2) and that targeted anti-CTGF therapy could slow the development and progression of the disease and prevent the development of chronic renal failure (8). Although CTGF expression in diabetic rats was high 6 months after diabetes induction, there was no statistically significant difference compared to the control group. This finding might be associated with a decrease in the number of podocytes that are known in DN (15).

In our study, we also compared CTGF expression in the different renal structures (proximal tubule, distal tubule, glomerulus) of the control groups and diabetic rats after 2 weeks, 2 months, 6 months and 12 months. Noticeably, a high expression of CTGF was observed in the distal tubules of the diabetic rats at 2 weeks. However, we also observed a temporal decrease in the CTGF expression in the distal tubules of diabetic rats, which suggests that changes distal to the glomeruli develop early in the DN. Additionally, 6 months after diabetes induction, CTGF expression in the proximal tubules was significantly higher in the control group by comparison with the diabetic rats. Therefore, tubular damage occurs in early DN, given that excess glucose in the glomerular filtrate leads to increased glucose reabsorption in the tubules, synthesis of profibrotic mediators and tubular damage (17). In glomeruli, CTGF-positive cells were detected in the diabetic group of rats 2 weeks after diabetes induction, followed by gradual growth over 12 months (except in the 6th month) with a complete absence of expression in the control groups. Hyperglycemia, especially if severe and prolonged, leads to inflammatory and profibrotic changes in the glomeruli, ultimately causing glomerulosclerosis (18). Wu et al. showed that, among the rats sacrificed after 1, 4 and 8 weeks, the autophagic disorder can contribute to early DN and autophagy is important for the regulation of cellular homeostasis in kidneys (19). Menini et al. showed that there was a higher rate of glomerular cell apoptosis in diabetic rats, compared with the controls after 4 and 6 months and concluded that glomerular cell apoptosis, as the last feature, is preceded by glomerular and podocyte hypertrophy and proteinuria, which can lead to mesangial expansion and glomerular sclerosis (20).

In conclusion, our results demonstrate that the most significant changes in the expression of CTGF occur within 2 months of DM, implying an early onset of pathophysiological changes in diabetic kidneys, which normally occur with aging. Additionally, the most noticeable changes occur in the distal tubules 2 weeks after the induction of diabetes. These findings would suggest that DN originates in the DCT, in contrast to the current theory describing the functional impairment of podocytes as the initial stage of DN. Further molecular studies to examine changes occurring in the kidney during aging will allow insights into the physiology of healthy and diabetic kidneys and will help elucidate the mechanisms underlying these changes as well as plan treatment aimed at kidney damage control.

CONCLUSIONS

The major change in the expression of CTGF occurs within 2 months of DM onset, particularly in the DTC, implying an early onset of pathophysiological changes in diabetic kidneys, which would usually occur with aging. These findings help to contribute to our understanding of changes associated with DN and guide towards potentially appropriate treatment modalities.

FUNDING
This research was funded by the Institutional Grant for Excellence 2018. Awarded to Prof. Vukojević.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS
Katarina Vukojević - conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, resources, validation, visualization, software, writing of original draft. Leonora Bedeković wrote the manuscript with support from Katarina Vukojević. All authors have read and agreed upon the published version of the manuscript.

ETHICAL BACKGROUND

Institutional review board statement: The experimental protocol was approved by the Ethics Committee of the University of Split, School of Medicine. All the experiments are performed in accordance with the principles set out in the World Medical Association Declaration of Helsinki. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board No 910-08-17-02-0009 ur.br. 2181-198-01-01-17-0003 from 10.02.2017.

Informed consent statement: Non applicable.

Data availability statement: The work did not form databases.

REFERENCES

1. Papadopoulou-Marketou N, Chrousos G, Kanaka-Gantenbein C. Diabetic nephropathy in type 1 diabetes: a review of early natural history, pathogenesis, and diagnosis. Diabetes Metab Res Rev. 2016;33:2841.
2. Vujičić B, Turk T, Crnčević-Orlić Ž, Đorđević G, Rački S. Dijabetička nefropatija. Medicina Fluminensis. 2010;46:360-75.
3. Marco G, Colucci J, Fernandes F, Vio C, Schor N, Casarini D. Diabetes induces changes of catecholamines in primary mesangial cells. Int J Biochem Cell Biol. 2008;40:747-754.
4. Najafian B, Alpers C, Fogo A. Pathology of Human Diabetic Nephropathy. Contrib Nephrol. 2011;170:36-47.
5. Zheng S, Noonan WT, Metreveli NS, Coventry S, Kralik PM, Carlson EC, et al. Development of late-stage diabetic nephropathy in OVE26 diabetic mice. Diabetes. 2004;53:3248-57.
6. Lin J, Susztak K. Podocytes: the Weakest Link in Diabetic Kidney Disease? Curr Diab Rep. 2016;16.
7. Fu J, Lee K, Chuang PY et al. Glomerular endothelial cell injury and cross talk in diabetic kidney disease. Am J Physiol Renal Physiol 2015; 308:287-297.
8. Wang S, Li B, Li C, Cui W, Miao L. Potential Renoprotective Agents through Inhibiting CTGF/CCN2 in Diabetic Nephropathy. J. Diabetes Res. 2015;2015:1-11.
9. Connolly S. Transcriptome Profiling and the Pathogenesis of Diabetic Complications. J. Am. Soc. Nephrol. 2003;14:279-83.
10. Lipson K, Wong C, Teng Y, Spong S. CTGF is a central mediator of tissue remodeling and fibrosis and its inhibition can reverse the process of fibrosis. Fibrogenesis Tissue Repair. 2012;5:1-8. 8.
11. Leask A, Abraham D. All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J. Cell Sci. 2006;119:4803-10.
12. Mason R. Connective tissue growth factor (CCN2), a pathogenic factor in diabetic nephropathy. What does it do? How does it do it? JCCS. 2009;3:95-104.
13. Roestenberg P, van Nieuwenhoven F, Joles J, Trischberger C, Martens P, Oliver N et al. Temporal expression profile and distribution pattern indicate a role of connective tissue growth factor (CTGF/CCN-2) in diabetic nephropathy in mice. Am J Physiol Renal Physiol. 2006;290:1344-54.
14. Bakovic M, Juric Paic M, Zdrilic E, Vukojevic K, Ferhatovic L, Marin A, Filipovic N, Grkovic I, Puljak L. Changes in cardiac innervation during maturation in long-term diabetes. Exp Gerontol. 2013;48(12):1473-8.
15. Baelde H, Eikmans M, Lappin D, Doran P, Hohenadel D, Brinkkoetter P et al. Reduction of VEGF-A and CTGF expression in diabetic nephropathy is associated with podocyte loss.KI. 2007;71:637-45.
16. Toda N, Mukoyama M, Yanagita M, Yokoi H. CTGF in kidney fibrosis and glomerulonephritis. Inflamm Regen. 2018;38.
17. Morić V. Kontinuirano mjerenje arterijskoga tlaka i biljezi bubrežnoga oštećenja u ranom otkrivanju dijabetičke nefropatije u djece. Zagreb: Sveučilište u Zagrebu, Medicinski fakultet; 2020.
18. Najafian B, Crosson J, Kim Y, Mauer M. Glomerulotubular Junction Abnormalities Are Associated with Proteinuria in Type 1 Diabetes. J Am Soc Nephrol. 2006;17:S53-S60.
19. Wu WH, Zhang F et al. The role of programmed cell death in streptozotoci-induced early diabetic nephropathy. J Endocrinol Invest.2011;34:296-301.
20. Menini S, Iacobini C, Oddi G et al. Increased glomerular cell (podocyte) apoptosis in rats with streptozotocin-induced diabetes mellitus: role in the development of diabetic glomerular disease. Diabetologia. 2007;50:2591-2599.