Annals of Biomedical and Clinical Research

Original article

ABCR No 1; Vol 1; 2022;19-27

Changes in Expression of the Vascular Endothelial Growth Factor in the Kidneys of Diabetic Rats During Aging

Jelena Faletar, Katarina Vukojević

School of Medicine, University of Mostar

ABSTRACT

Background. Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in most developed countries. A number of specific factors may contribute to the onset and progression of diabetic nephropathy. One of those specific factors is vascular endothelial growth factor (VEGF). We studied the expression of VEGF in the kidneys of diabetic rats during aging.

Methods. Male Sprague-Dawley rats were injected with 55mg/kg streptozotocin (STZ) (DM group) or with citrate buffer (control group). Kidneys were collected after 2 weeks, 2 months, 6 months and 12 months, and were analyzed in three different kidney structures: glomeruli, proximal (PCT) and distal convoluted tubules (DCT). Sections were stained immunohistochemically, using VEGF.

Results. Significant differences in marker expression were observed after 2 months, with higher VEGF expression in diabetic rats. Positive VEGF cells were detected exclusively in the glomeruli of diabetic rats after 2 months. After 2 weeks of DM onset, high VEGF expression was demonstrated in the distal tubules of diabetic rats, as well as a complete absence of VEGF expression in the distal tubules of the control group.

Conclusions. The major change in expression of VEGF occurs after 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.

Key words: diabetes mellitus, kidneys, nephropathy, VEGF

Article processing history:
Received March 22, 2021
Revised June 2, 2021
Accepted September 21, 2021

ORCID IDs of the authors:
J.F. 0000-0002-5655-4622
K.V. 0000-0003-2182-289

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: Faletar J, Vukojević K. Changes in expression of VEGF in kidneys of diabetic rats during ageing. Annals of Biomedical and Clinical Research. 2022;1:19-27.

https://doi.org/10.47960/2744-2470.2022.1.1.19

INTRODUCTION

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in most developed countries (1). Diabetic nephropathy is a chronic microvascular complication of diabetes, characterized by proteinuria and progressive loss of renal function (2). DN is mainly related to glomerular dysfunction (3). Other associated features include nodular and diffuse mesangial cell proliferation and thickening of the glomerular basement membrane (4). Extraglomerular changes such as tubular atrophy, interstitial inflammation, and tubulointerstitial fibrosis also play an important role in the pathogenesis of diabetic nephropathy (5). Vascular changes of the diabetic kidney are arteriosclerosis and hyalinosis of the renal vessels (6). Furthermore, numerous studies have shown an association of glomerular endothelial damage (7), podocyte loss (8) and glomerulotubular junction abnormalities (9) with the development of diabetic nephropathy. Numerous studies have been recently conducted to investigate a number of endogenous factors, activated or inhibited in DM, which could provide protection against diabetic vasculopathy (10). Within the VEGF family, we distinguish five glycoproteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF). The most studied is VEGF-A, and unless otherwise noted, the name VEGF refers to VEGF-A. VEGF165 is the most abundant isoform of VEGF-A in humans, while VEGF164 is the most abundant isoform in rodents (11). VEGF-A promotes endothelial cell differentiation, proliferation and survival, facilitates vasodilation and increases vascular permeability (12). VEGF also plays a crucial role in both normal and pathological angiogenesis (13). Cooper et al. showed that VEGF expression in the kidneys of diabetic rats was increased as early as 3 weeks after the induction of diabetes by streptozotocin (STZ) (14). Many other experimental studies have also found increased expression of VEGF in the kidneys of diabetic rodents (15-16). Accordingly, the application of anti-VEGF therapy in diabetic rats caused a reduction in albuminuria and an improvement in kidney disease (17). In contrast to diabetic rodents in humans with diabetic nephropathy, VEGF expression in the kidneys is reduced (18) which could be due to a decrease in the number of podocytes in the diabetic kidney (19). On the other side, Hohenstein et al. showed overexpression of VEGF in humans with type 2 diabetes (20). Current understanding of the role of VEGF in diabetic kidneys is significantly clearer, and it is known that a number of biological effects contribute to this role in different ways. The amount of VEGF, the type of VEGF, the pharmacological inhibition or genetic manipulation of VEGF, the kind of species (rodents, humans), the site of action of VEGF (paracrine or autocrine action) and the metabolic milieu has an influence on these biological effects (11). Too much or too little VEGF can be harmful and cause glomerular changes (11). In addition to the amount of VEGF, the type of VEGF also plays a significant role in glomerular homeostasis. In their study, Oltean et al. confirmed that early-stage diabetic kidney disease correlates well with high levels of the VEGF165b isoform, but not with advanced nephropathy. Overexpression of VEGF165b in diabetic mice was associated with a reduction in albuminuria (21). Pharmacological inhibition of VEGF improves renal disease in certain diabetic mice, while specific genetic manipulations on podocytes worsen this. In humans, anti-VEGF therapy can cause adverse effects, such as hypertension, proteinuria and thrombotic microangiopathy (11). Therefore, the aim of our study was to investigate the expression pattern of VEGF in diabetic kidneys during aging.

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. DM was induced by an intraperitoneal injection of 55mg/kg STZ, freshly dissolved in citrate buffer, pH 4.5, after fasting overnight. Rats were fed with standard laboratory chow ad libitum, 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 OneTouch Vita instrument (LifeScan, High Wycombe, UK). Rats were considered diabetic with 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). 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 anesthetized 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.

The kidney samples were removed 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. The kidney samples were then processed with transverse cuts and then embedded in paraffin blocks. These blocks were then cut into 7 µm thick sections and investigated under immunofluorescence. After deparaffinization, tissue sections were rehydrated using alcohol and water. The samples were then thoroughly rinsed in distilled water and heated in a microwave oven with sodium citrate buffer (pH 6.0) at 95°C for 12 minutes. The samples were cooled at room temperature before being incubated with primary antibody.

Goat anti-VEGF antibody from Abcam (ab46154, Cambridge, UK) was diluted at 1:200 ratios in Dako REAL antibody diluent (Dako Denmark A\S, Glostrup, Denmark) 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 they 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 with regard to the staining intensity: (0) indicating

the absence of any reactivity, (1) a mild reactivity, (2) a moderate reactivity, (3) a strong reactivity. Two researchers independently analyzed the staining intensity. The number of positive cells within each area (glomerulus, proximal convoluted tubule, distal convoluted tubule) 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, data were pooled for all kidney structures of the control and diabetic rats and re-analyzed.

Statistical analysis

A 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). Data were expressed as a mean ± standard deviation, with $p<0.05$ serving as the marker of statistical significance.

RESULTS

The VEGF marker used in immunofluorescence staining showed different staining intensities in the observed structures of the proximal and distal convoluted tubules and the glomeruli in the control and diabetic groups during aging (Figures 1-4).

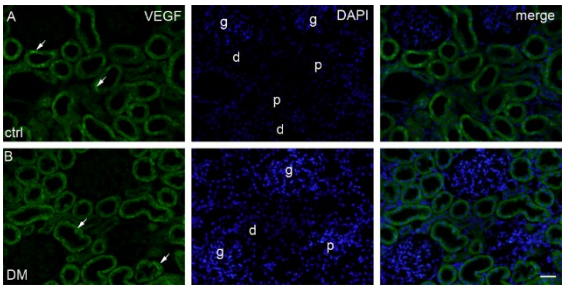

Figure 1. VEGF positive cells were seen as green staining of the cytoplasm (arrows) within different areas of the cortex of the kidneys. Co-localization of VEGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 2 weeks. Scale bar 20 µm. Legend: d- distal tubule; g- glomerulus; p- proximal tubule; ctrl- control, DM- diabetes mellitus type 1.

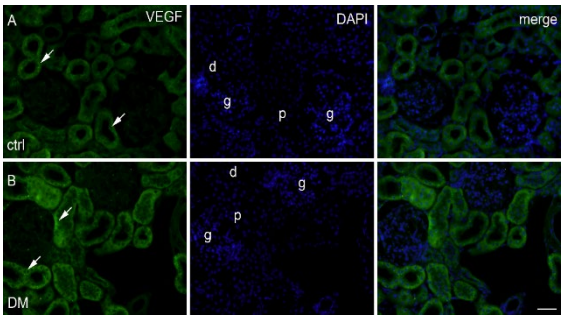

Figure 2. VEGF positive cells were seen as green staining of cytoplasm (arrows) within different areas of the cortex of the kidneys. Co-localization of VEGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 2 months. Scale bar 20 µm. Legend: d- distal tubule; g- glomerulus; p- proximal tubule; ctrl- control, DM- diabetes mellitus type 1.

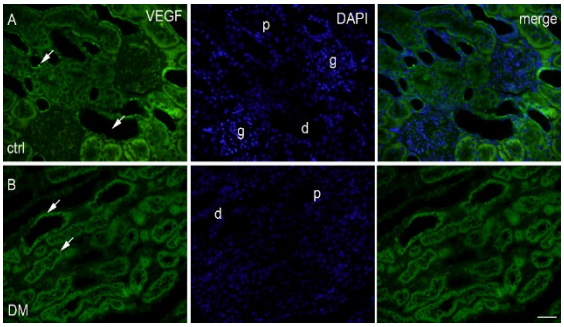

Figure 3. VEGF positive cells were seen as green staining of the cytoplasm (arrows) within different areas of the cortex of the kidneys. Co-localization of VEGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 6 months. Scale bar 20 µm. Legend: d- distal tubule; g- glomerulus; p- proximal tubule; ctrl- control, DM- diabetes mellitus type 1.

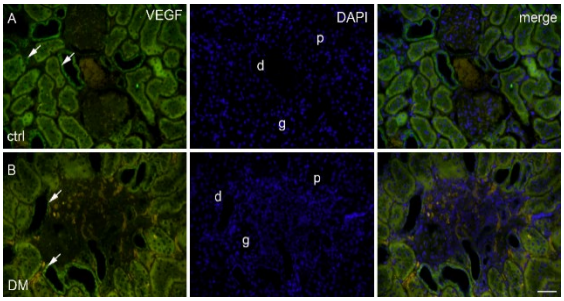

Figure 4. VEGF positive cells were seen as green staining of the cytoplasm (arrows) within different areas of the cortex of the kidneys. Co-localization of VEGF and DAPI nuclear stain are shown in the far-right column (merge). Kidney cortex in the control and DM groups at 12 months. Scale bar 20 µm. Legend: d- distal tubule; g- glomerulus; p- proximal tubule; ctrl- control, DM- diabetes mellitus type 1.

Total number of VEGF-positive cells in groups of diabetic and control rat kidneys

We first analyzed the total number of VEGF-positive cells between groups, including all three structures of the kidney cortex (PCT, DCT and glomeruli). In the control groups (C-2 weeks, C-2 months, C-6 months, C-12 months) VEGF expression was variable. VEGF expression in groups of diabetic rats (DM-2 weeks, DM-2 months, DM-6 months, DM-12 months) was also variable. There was a significant difference in the total number of VEGF-positive cells in diabetic rats 2 months post-induction, compared to the 2-month control group ($p<0.001$). Furthermore, there was no significant difference between the diabetic rat and control groups after 2 weeks and 6 months ($p<0.01$), and 12 months after diabetes induction ($p<0.001$).

The highest number of VEGF-positive cells in the control groups was observed 12 months after diabetes induction, while the highest number of VEGF-positive cells in the diabetic groups was observed 6 months after diabetes induction (Figure 5).

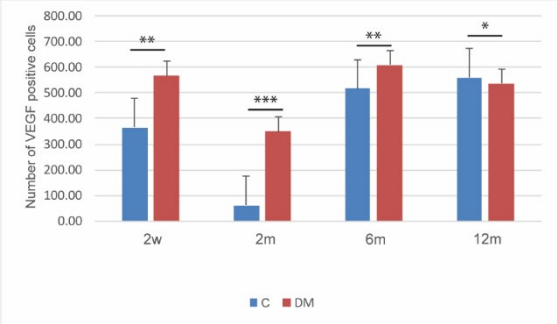

Figure 5. Number of VEGF-positive cells at 2 weeks (2 w), 2 months (2 m), 6 months (6 m) and 12 months (12 m) in the control (C) and diabetes (DM) groups. Data presented as a mean ± standard deviation, chi-square test. Asterisks denote significant difference. *p<0.05, **p<0.01, ***p<0.001.

Percentage of VEGF-positive cells in three different kidney structures (PCT, DCT and glomeruli) in the control and diabetes groups

Two weeks after DM induction, there was complete absence of VEGF-positive cells in the glomeruli of the control group and diabetic rats. In the distal tubules, the percentage of VEGF-positive cells was 86.3%, while the expression of VEGF in the control group of the same renal structure was completely absent. A slightly smaller difference in the percentage of VEGF-positive cells was observed in the proximal tubules between the control group (85.5%) and the diabetic rats (85%) (Figure 6.a).

The percentage of VEGF-positive cells in the control and diabetic groups decreased 2 months after the induction of diabetes in the proximal and distal tubules. An increase in the percentage of VEGF-positive cells (34%) was observed in the glomeruli of diabetic rats, compared with the glomeruli of diabetic rats 2 weeks after induction of diabetes (0%). A difference in the percentage of VEGF-positive cells in the proximal tubules was found between the control group (22%) and diabetic rats (37%) ($p<0.01$). After 2 months of DM onset, relatively high VEGF expression was demonstrated in the distal tubules of the diabetic rats (49.5%), as well as a complete absence of VEGF expression in the distal tubules of the control group (Figure 6.b).

Six months post-induction, there was no significant difference between the control and diabetes groups in the PCT ($p<0.05$) and DCT. There was a complete absence of VEGF expression in the glomeruli of both groups (C and DM) (Figure 6.c).

Twelve months post-induction there was almost no difference in the percentage of VEGF-positive cells between the control group (72.3%) and diabetic rats (72%) in the proximal tubules. In the distal tubules, there was no significant difference between the control and diabetic rats ($p<0.05$). There was a complete absence of VEGF expression in the glomeruli of both groups (C and DM) (Figure 6.d).

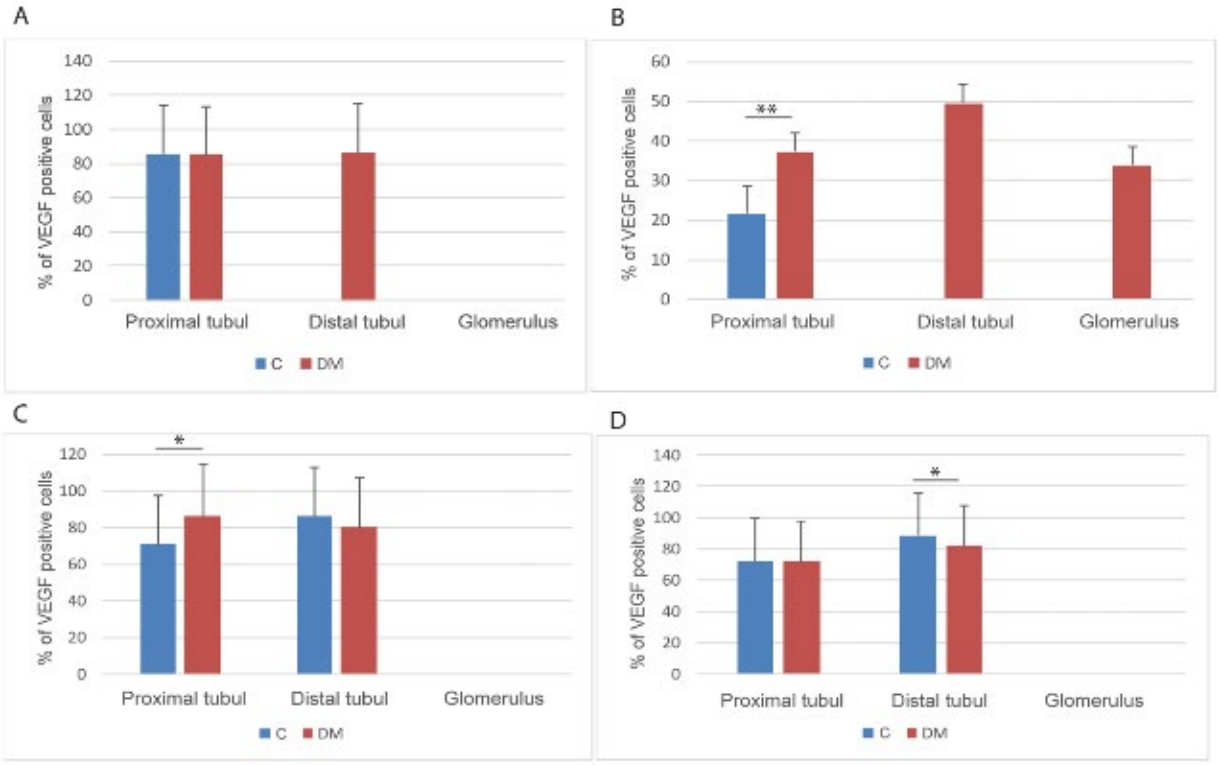

Figure 6. Distribution of the percentage of VEGF-positive cells in different renal structures (PCT, DCT and glomerulus) between the control (C) and diabetes (DM) groups (a) 2 weeks, (b) 2 months, (c) 6 months and (d) 12 months after DM induction. Data are presented as a mean ± standard deviation, chi-square test. Asterisks denote significant difference. *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Analyzing the total number of VEGF-positive cells in the three cortical structures (PCT, DCT and glomeruli) of the control and diabetes group indicated a significant difference 2 months post-induction. In that period, VEGF expression was around five and a half times higher among diabetic rats, compared with the controls. Although VEGF expression in diabetic rats was highest 6 months post-induction, there was no significant difference compared to the controls. Twelve months post-induction, there was a reversal in VEGF expression, which was higher in the control group, compared to diabetic rats. It is important to note that VEGF expression in diabetic rats was increased as early as 2 weeks after diabetes induction compared with the controls. The previously described results could determine a positive correlation between increased VEGF expression and early diabetic nephropathy in experimental animals. Moreover, VEGF could play a role in the early pathogenesis of the disease, and targeted anti-VEGF therapy could slow the progression of the disease. One of the most influential studies describing the expression patterns of VEGF in diabetic kidneys was published by Cooper et al. In that study, the investigators showed increased VEGF expression as early as 3 weeks after the induction of diabetes with STZ in rats, which lasted at least 8 months post-induction (14). Other studies have also confirmed increased VEGF expression in early diabetic nephropathy (15,16). The question that arises here is whether this constitutes overexpression of VEGF compensatory mechanism or an integral part of the disease pathogenesis. For a better understanding of the role of VEGF in DN, genetic manipulations and gene deletions of VEGF genes were investigated. Lavoz et al. demonstrated that pharmacological inhibition of VEGFR signaling in diabetic mice leads to a significant reduction in albuminuria and renal impairment, and a reduction in the activity of signaling pathways essential for VEGF activation (22).

In our study, we also compared VEGF expression in different renal structures between controls and diabetic rats after 2 weeks, 2 months, 6 months, and 12 months of diabetes duration. The most noticeable result was 2 weeks after diabetes induction and was presented by a high expression of VEGF in the distal tubules of diabetic rats, and the complete absence of VEGF expression in the distal tubules of the control group. After that period (6 and 12 months after diabetes induction), VEGF expression increased in both groups in the distal tubules, being higher in the control groups than among the diabetic rats. These results suggest that the most significant pathological changes in the distal tubules occur within 2 months of diabetes duration. Observing glomeruli, VEGF-positive cells were detected exclusively in the glomeruli of diabetic rats 2 months after diabetes induction. In all other groups, the complete absence of VEGF expression in glomeruli was recorded. Such a pattern of VEGF expression in glomeruli suggests that glomerular damage associated with diabetic nephropathy occurs somewhat later than pathological changes in distal tubules, which distances us from the fact that DN is primarily associated with glomerular damage (17). Other studies have confirmed that overexpression of VEGF-A on podocytes contributes to glomerular decay (23), which could explain the complete absence of cells 6 and 12 months after diabetes induction, as the podocytes are part of the glomeruli. Smaller differences in VEGF expression during aging between control groups and diabetic rats were observed in the proximal tubules. VEGF expression in the proximal tubules was almost equalized 12 months after the induction of diabetes in both groups.

CONCLUSIONS

In conclusion, our results showed that the most significant changes in expression of VEGF occurs within 2 months of DM, implying an early onset of pathophysiological changes in diabetic kidneys, which usually occur with

aging. We also proved that the most noticeable changes occur in the distal tubules 2 weeks after the induction of diabetes. These findings indicate that DN originates in the DCT, in contrast to the previous belief that podocytes exhibiting functional impairment are the initial site of damage. These findings help to contribute to our understanding of changes associated with DN and guide towards potentially appropriate treatment modalities

Further 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. After researching the available literature, it has been established that there was no significant research in the changing of VEGF expression in the kidneys of diabetic rats during aging, notwithstanding the extensive research into the impact of VEGF on DN in the context of other parameters. Consequently, the results of this study could serve as a solid basis for understanding pathogenesis and new strategies in treating DN, but also as a solid foundation for new research in this field.

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. Jelena Faletar 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 urbr 2181-198-01-01-17-0003 from 10.02.2017.

Informed Consent Statement: Non applicable.

Data Availability Statement: The work did not form databases

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