Expression pattern of apoptosis-inducing factor in the kidneys of streptozotocin-induced diabetic rats

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Tim Hauke

EXPRESSION PATTERN OF APOPTOSIS-INDUCING FACTOR IN THE KIDNEYS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

Diploma thesis

Academic year:
2017/2018

Mentor:
Assoc. Prof. Katarina Vukojević, MD, PhD, MSc

Split, July 2018.

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1. INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease and a worldwide epidemic. Its hallmark is the presence of hyperglycemia. The characteristic pathologically elevated blood glucose can be caused by impaired insulin secretion, impaired action of insulin on the target tissue or both. The long-term consequences of this, at the beginning silent proceeding illness is damage that occurs to virtually every organ system particularly in the eyes, kidneys, blood vessels, nerves and heart. The principle of this disease is an autoimmune destruction of the beta cells of the pancreas which we call DM Type 1 (absolute insulin deficiency) or a rising insulin insensitivity of the body cells called DM Type 2 (relative insulin deficiency). The result is the same, an insufficient action of insulin. Constant hyperglycemia is the aftermath and leads to characteristic symptoms such as polyuria, polydipsia, weight loss, polyphagia, blurred vision, impaired growth and susceptibility to infections. Acute dramatic consequences like hyperglycemia with ketoacidosis or nonketotic hyperosmolar syndrome which can lead to death are also possible.

Diabetic patients have to struggle with potential loss of vision; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction; increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease; hypertension and abnormalities of lipoprotein metabolism and finally nephropathy (today known as diabetic kidney disease (DKD)) leading to renal failure (1).

1.1. Diabetic kidney disease

1.1.1 Definition

DKD is the chronic loss of kidney function and the major cause of End Stage Renal Disease (ESRD) in developed and developing countries (2). It is a progressive illness and is defined as diabetes with albuminuria (ratio of urine albumin to creatinine $\geq$ 30 mg/g) and impaired glomerular filtration rate (GFR) (<60 mL/min/1.73 m^2) (3).

1.1.2. Etiology

1.1.2.1. Pathogenesis and pathophysiology of diabetic kidney disease

The pathogenesis of DKD is multifactorial and mainly based on pathologic metabolic (hyperglycemia) and hemodynamic (glomerular hypertension) changes in the kidney (4).

The hemodynamic pathway reads as follows. Blood pressure in the glomeruli is under control of the efferent and afferent arterioles and their interplay of constriction or dilation. Hyperglycemia results in the accumulation of metabolic intermediate succinate which in turn acts on the vascular endothelium and triggers renin release from the juxtaglomerular apparatus by paracrine signaling (5). Activation of the renin angiotensin system (RAS) leads to production of angiotensin II and efferent arteriolar vasoconstriction which results in glomerular capillary hypertension and hyperfiltration. Similar to RAS is endothelin-1 (ET-1), which is also induced and is a potent vasoconstrictor and additionally plays a role in hypertension, endothelial dysfunction, inflammation and fibrosis (3). On top of that ET-1 triggers mesangial cell hypertrophy, proliferation and extra cellular matrix (ECM) production (3).

The metabolic pathway describes that a hyperglycemic environment blocks normal glycolysis. This results in an accumulation of glycolysis precursors which then upregulate four distinct pathways, the polyol pathway, hexosamine pathway, production of advanced glycation end products (AGEs), and activation of protein kinase C (PKC) (3). Additionally there is the so called inflammatory pathway because DKD is not entirely the result of hyperglycemia and changed hemodynamics (6). The theory is, that there is a chronic low activation of the innate immune system mediated by NF- κB. NF- κB is a transcription factor that regulates genes for inflammation, immune response, apoptosis and several cytokines. Hyperglycemia, proteinuria and several other factors like viruses, bacteria, oxygen radicals and cytokines induce NF-κB release. NF- κB is essential in the interplay among the different factors leading to structural and functional alterations which are characteristic for DKD. Further there is a correlation between NF-κB and proteinuria and interstitial cell infiltration (6). There is also a greater production of inflammatory cytokines like TNF-α, IL 1, 6, 18 in diabetic patients. Other contributing factors are for example that podocytes in the presents of hyperglycemia have reduced autophagic activity and thus suffer additional damage (3).

1.1.2.2. Molecular basis of glomerular injury

The polyol pathway describes the conversion of glucose to fructose. The side effects of this cascade is that there is a consumption of intracellular NADPH which is needed for regeneration of antioxidants (3). This will lead to additional cell stress and contribute to apoptosis. Additionally fructose is now known to be nephrotoxic and causes proteinuria and reduced GFR (3). The hexosamine pathway increases production of transcription factors like TNF-α and TGF-β1 (3). AGEs are created by irreversible glycation of proteins that occur in the presence of intracellular hyperglycemia. They damage the cells by modifying laminin, I and IV collagen and cause glomerular basement membrane (GBM) permeability (3). The alteration of collagen causes expansion of the ECM. Additionally AGEs can activate downstream signaling of IL 1, 6, TNF-α, TGF-β1, vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF) and on top generate reactive oxidant species (ROS) (3). Hyperglycemia produces diacylglycerol (DAG) which is a cofactor for PKC activation hence it is constantly upregulated. PKC leads to prostaglandin E2 activation and nitric oxide release causing vasodilation of the afferent arterioles resulting also in glomerular hypertension (3).

TGF-β1 is the major fibrogenic growth factor responsible for of glomerulosclerosis, renal cell hypertrophy, increase of mesangial matrix components and interstitial fibrosis (3,7). It regulates differentiation, proliferation, apoptosis, adhesion and migration of various cell types. The main inducer of TGF-β1 is PKC. It is mainly produced by mesangial, proximal tubular cells and infiltrating macrophages (7). TGF-β1 induces in the glomerulus cellular hypertrophy, deposition of ECM biosynthesis in the form of collagen I, IV, V, VI, fibronectin and laminin (8). TGF-β1 and IL6 promote the differentiation of naive T lymphocytes into proinflammatory T helper cells and they in turn cause inflammation and renal scarring. Those events block additionally ECM degradation and increase the vessel permeability and albumin leakage progress.

1.1.2.3. Other pathological changes present in the glomerulus

The renal corpuscle includes the glomerulus and the Bowman's capsule and is the primary filtration unit of the kidney. The glomerulus is the beginning of the nephron and is composed of an afferent and an efferent arteriole, capillaries and mesangial cells who act as a scaffold. The blood passes through a filtration barrier. This barrier has three subunits. At first the blood has to pass through the pores in the capillaries, next through the GBM and finally through foot processes of the podocytes. Diabetic kidney disease can be divided in four types of glomerular lesions. Type one is GBM thickening, type two a is mild mesangial expansion, type two b severe mesangial expansion, type three nodular sclerosis and type four is global glomerulosclerosis of more than 50% (9). Glycosaminoglycans (GAG) which coats the podocytes play an important part of the negatively charged barrier of the glomerulus. Hyperglycemia affects GAG synthesis and thus also contributes to glomerular hyperfiltration and proteinuria (9). The podocytes are also an important part of the glomerulus and play a main role in blood filtration. In DKD there is a loss of podocytes, they detachment from the GBM and their processes are shortened (9). Finally mesangial expansion causes collapsing of the lumen of the capillaries and the weight of the kidney increases (9).

1.1.2.4. Diabetic kidney disease as a tubulointerstitial disease

The tubulointerstitial part of the kidney is composed of the tubular system, interstitial cells and vascular system. Progression of DKD leads to tubulointerstitial fibrosis and atrophy (9). It is now believed that the condition of the tubulointerstitium is in direct correlation to the state and progression of DKD especially in the advanced stages of DKD and that pathologic changes occur even before changes in the glomeruli are present (9).

1.1.2.5. Molecular basis of tubulointerstitial injury

The tubulointerstitium is affected in similar fashion as the glomeruli. The main mediator is TGF- β1. It is induced by proteinuria and its components like AGEs, transferrin, albumin and fatty acids (10). It leads to tubular inflammation, fibrosis and oxidative stress. There is a complex pathophysiologic interaction between the proximal tubule, tubulointerstitium and diabetic milieu (9). The proximal tubule is the principal kidney unit of glucose reabsorption. To block the reabsorption of glucose in the proximal tubule could be one important step in the future prevention of DKD. Another important aspect of tubulointerstitial damage is chronic hypoxia mediated by angiotensin II, nitric oxide and ET1 by impairing peritubular flow and decreased oxygen delivery to the tubules (9). Decreased erythropoietin production in the kidneys later on leads to anemia and intensifies tubular hypoxia (10).

1.1.2.6. Pathological changes present in the tubulointerstitium

The following changes can be observed in DKD. After chronic hyperglycemia the tubular basement membrane thickens at the same time as the GBM (11). Over time the tubules atrophy, interstitial fibrosis occur and late and severe manifestations are the detachment of the glomerulus from the proximal convoluted tubule (11).

1.2 Apoptosis

1.2.1. Definition

Apoptosis is the process of programmed cell death. It occurs constantly and is important during development and aging and overall maintains homeostasis and cell population (12). Apoptosis has to be differentiated from necrosis (oncosis). Necrosis is a toxic process where the cell is passive and follows an energy independent death (12). Usually many cells are affected. In contrast apoptosis is a controlled, active process and energy dependent (12). It can affect only one cell or few cells. Necrosis is characterized by cell swelling, karyolysis, pyknosis, karyorrhexis, disrupted cell membranes, release of cytoplasm and

usually inflammation (12). In comparison apoptosis has very different characteristics like cell shrinkage and convolution, pyknosis and karyorrhexis, intact cell membrane, cytoplasm is retained in apoptotic bodies and usually happens without inflammation because there is no release of chemotactic signals (12). The stimulus which inducts apoptosis can be the same which inducts necrosis only in a different intensity. Sometimes the apoptotic process can switch to necrotic process if the cell runs out of caspases or ATP (12).

1.2.2. Molecular basis of Apoptosis

Apoptosis is mediated by several energy dependent steps. There are two main pathways. The extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (12). There is an additional pathway called granzyme B which is T cell mediated (12). All three pathways converge on the same execution pathway which starts with the cleavage of caspase 3 (12). The following events are DNA fragmentation; cytoskeletal and nuclear protein degradation; cross linking of proteins; formation of apoptotic body and finally phagocytosis (12). Caspases are the most important apoptotic enzymes. There are 10 caspases and they are divided into initiator, effector and inflammatory caspases (12). Their activation is irreversible and will lead to cell death. The extrinsic pathway is activated by a transmembrane receptor. They belong to the family of TNF receptors. Further downstream the receptor will induce death inducing signaling complex (disc) formation following caspase 8 activation and finally caspase 3 (12). The intrinsic pathway is the most interesting for us, since AIF is part of it.

This pathway is without receptors and the stimuli act directly on mitochondria (12). Stimuli are for example lack of growth factors or hormones, radiation, toxins, hyperthermia or viral infection (12). These stimuli change the inner mitochondrial membrane and this in turn results in opening of the mitochondrial permeability transition pore (12). There is a loss of transmembrane potential and subsequent release of pro apoptotic proteins like cytochrome c which will from together with procaspase 9 an apoptosome (12). There is a second group of proteins released sometime later like endonuclease G and caspase activated DNase and finally AIF. Endonuclease G acts similar like AIF and is involved in DNA cleavage (12). The tumor suppressor gene p53 regulates Bcl-2 protein which in turn controls the mitochondrial membrane permeability and thus the apoptotic processes (12). This could be used in future for cancer research. All pathways lead to caspase 3 activation which will initiate the so called

execution pathway (12). Together with caspase 6 and 7 they are called execution caspases and act as intracellular cleavers which degrade plasma membrane, cytoskeletal and nuclear proteins (12).

1.2.3. The dual role of AIF

AIF is a flavin adenine dinucleotide (coenzyme, flavoprotein) containing, NADH dependent oxidoreductase residing in the mitochondrial intermembrane space whose specific enzymatic activity remains unknown (13). AIF has a dual task. At first scientists believed that it played only a role in caspase independent programmed cell death but today we know that it is also an important independent (from apoptotic function) factor for the cell survival in a manner of oxidative phosphorylation (OXPHOS) and thus for the generation of energy (14). Under normal circumstances AIF is located behind the outer mitochondrial membrane. If there is an apoptotic trigger AIF undergoes proteolysis and translocate to the cytosol and nucleus and causes chromatin condensation and large-scale DNA degradation. Cell death induced by AIF is through its proapoptotic activity once it is translocated to the nucleus not due to the loss of AIF from the mitochondria (15). AIFs role in apoptosis was discovered in 1996 as it could maintain the apoptogenic ability in the presence of a pan caspase inhibitor (13).

The cell surviving activity of AIF and its role in DKD is under current investigation. The ideal research outcome would be AIF as a tool to prevent DKD in diabetic patients. AIF has an important role to maintain mitochondrial and therefore kidney homeostasis. Deficiency in AIF results in defective OXPHOS by loss of complex I (part of the electron transport chain) activity (14). This deficiency alone was not enough to cause chronic kidney disease (CKD) but if diabetes was superimposed there were extensive changes in mitochondrial function which augmented the renal lesion (14). The conclusion is that AIF deficiency at least in the renal tubules is a risk factor for DKD (14).

2. OBJECTIVES

AIM:

The aim of our current study is to investigate the significance of AIF expression in the development of DKD by observing the expression of AIF in 2 weeks and 2 months' diabetic kidney samples and then comparing them to healthy rat kidneys of the same age.

HYPOTHESIS:

AIF is deeply involved in programmed cell death. In diabetic kidney disease programmed cell death is a major event, so we expect AIF expression to be increased between 2 weeks and 2 months in the diabetic rat kidney samples while in the control groups we presume no significant changes to be present.

3. MATERIALS AND METHODS

3.1. Ethical background

The experimental protocol was allowed by The Ethics Committee of the University of Split, School of Medicine. All performed procedures were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration.

3.2. 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.

3.3. Induction and validation of diabetes

Experiments were performed using a type I diabetes rat model. Diabetes was introduced into the rats via an intraperitoneal injection of 55 mg/kg streptozotocin (STZ) dissolved in citrate buffer, at a pH of 4.5 after overnight fasting (16). Rats were given standard laboratory food, ad libitum, which is made up of 27% proteins, 9% fat and 64% carbohydrates (4RF21 GLP, Mucedola, Settimo Milanese, Italy).

In order to verify diabetes within the rat models, the blood glucose and body weights of the rats were taken. Blood glucose level was measured in the morning at 8 am after overnight fasting. A One Touch Vita instrument (LifeScan, High Wycombe, UK) measured the plasma glucose of the rats by accessing tail vein blood, and a standard scale measured body weights. Rats with a glucose level above 16.5 mmol/L were considered diabetic and were used in subsequent experimentation. Success rate of diabetic induction was about 80%.

The rats were separated into 2 groups based on the duration of diabetes as measured from the point of injection to the termination of the experiment (2 weeks, 2 months). Each experimental group was matched with a control group consisting of non-diabetic rats raised

over the same time period. Control group rats were given intraperitoneal injections with just citrate buffer. 6 animals were raised for each of the 2 control groups and the 2 experimental groups, totaling 24 rat models.

3.4. Tissue collection and immunohistochemistry

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. Kidney samples were removed and post fixed in the same fixative solution 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. Samples were cooled at room temperature before being incubated with primary antibody.

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 (ab150129, Cambridge, UK) for one hour in a humidified chamber. For nuclear staining 4',6-diamidino-2-phenylindole (DAPI) was applied. Slides were mounted with the mounting media and coverslipped. Sections were observed and images were captured using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71digital camera (Olympus, Tokyo, Japan).

Images were processed with CellA Imaging Software for Life Sciences Microscopy (Olympus Tokyo, Japan). Hematoxylin and eosin (HE) and Mallory staining were also performed. Kidney sections were analyzed focusing on two areas: cortex and medulla. For each of the listed areas, 5 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). The percentage of the surface covered with positive cells (threshold area percent) were analyzed in six randomly

selected fields from each picture and compared between the groups. Quantitative analyses were performed for the following region of interests (glomeruli, proximal tubules, distal tubules and collecting ducts. In each region of interest percent of AIF positive cells was calculated and expressed as a mean±SD.

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 (Figure 8.). Two researchers independently analyzed the staining intensity. The amount of positive cells within each area (glomerulus, proximal convoluted tubule, distal convoluted tubule, collecting duct) were compared between the experimental diabetic groups and control groups. Distinct analyses were conducted for the 4 sections at each time point, and then the data was aggregated for all areas of the control and diabetic rats and evaluated.

3.5. Statistics

Mann-Whitney 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 was expressed as a mean ± standard deviation, with $p<0.05$ serving as the marker of statistical significance.

4. RESULTS

HE staining reveals normal kidney morphology in 2 weeks' control and 2 weeks' diabetic groups and there are no obvious differences (Figure 1 and 2). However, there are characteristic morphologic differences between the 2 month control and 2 month diabetic groups. Those changes are mainly present in the glomeruli (Figure 1) and are related to glomerular hypertrophy and podocyte loss, which can lead to mesangial expansion and glomerular sclerosis.

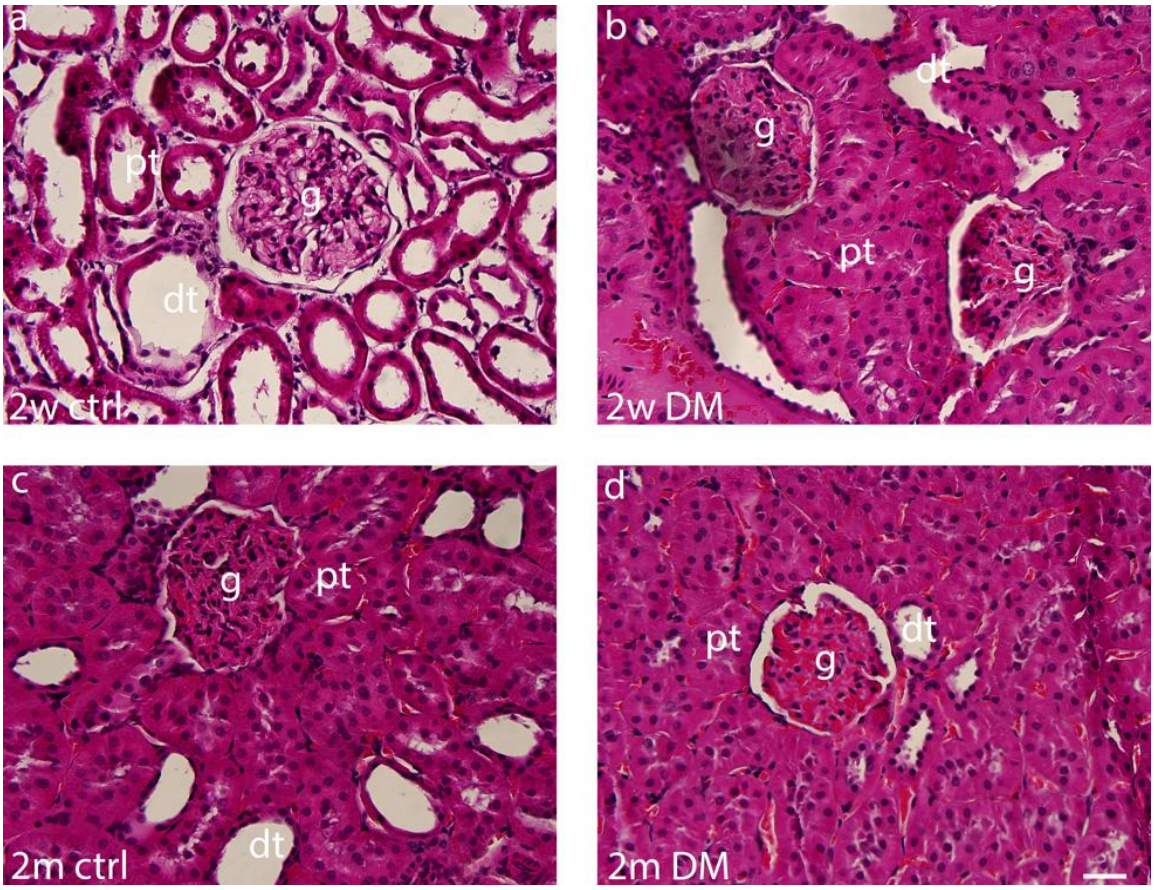


Fig. 1. Transversal section through the kidney tissue with the areas of interest: kidney cortex at 2 weeks in control (a) and diabetes (b); at 2 months in control (c) and diabetes (d). Scale bar 25µm. Legend: dt- distal tubule; g- glomerulus; pt- proximal tubule; ctrl- control; DM- diabetes mellitus type 1.

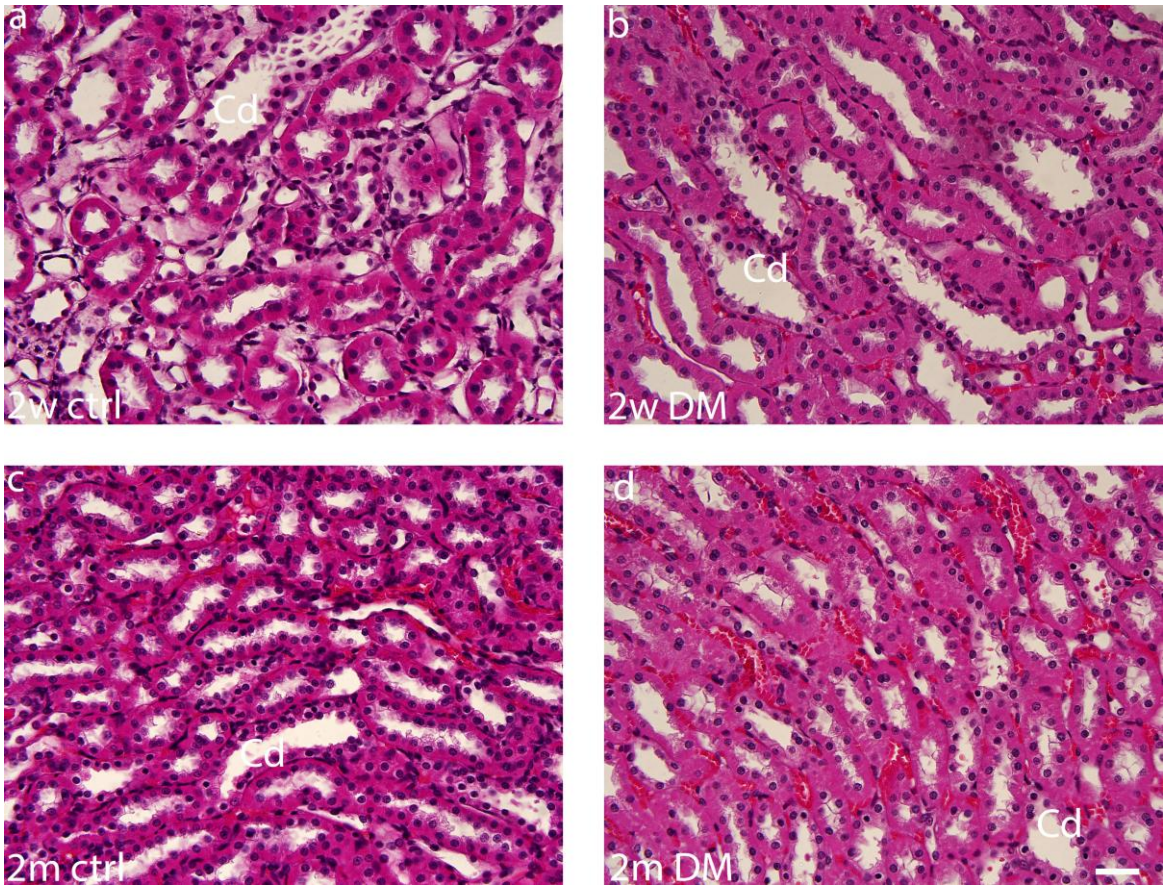


Fig. 2. Transversal section through the kidney tissue with the areas of interest: kidney medulla at 2 weeks in control (a) and diabetes (b); at 2 months in control (c) and diabetes (d). Scale bar 25µm. Legend: Cd- collecting ducts; ctrl- control; DM- diabetes mellitus type 1.

Mallory staining in the 2 weeks' groups showed no obvious difference and normal kidney structures are present in both 2 weeks' diabetic and 2 weeks control groups (Figure 3 and 4 a, b). In the 2 months' groups we can see changes in terms of extent of damage through replacement of podocytes with connective tissue cells (Figure 3 and 4 c, d).

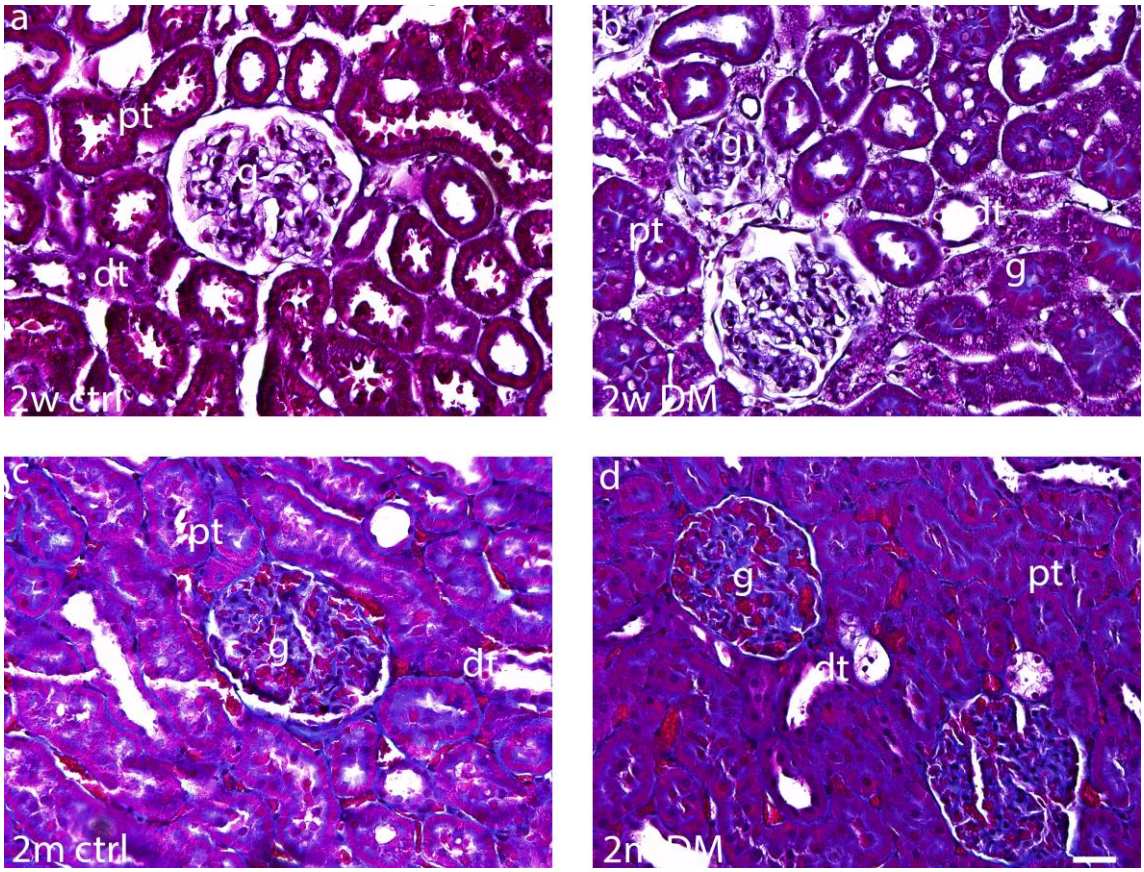


Fig. 3. Transversal section through the kidney tissue with the areas of interest: kidney cortex at 2 weeks in control (a) and diabetes (b); at 2 months in control (c) and diabetes (d). Scale bar 25µm. Legend: dt- distal tubule; g- glomerulus; pt- proximal tubule; ctrl- control; DM- diabetes mellitus type 1

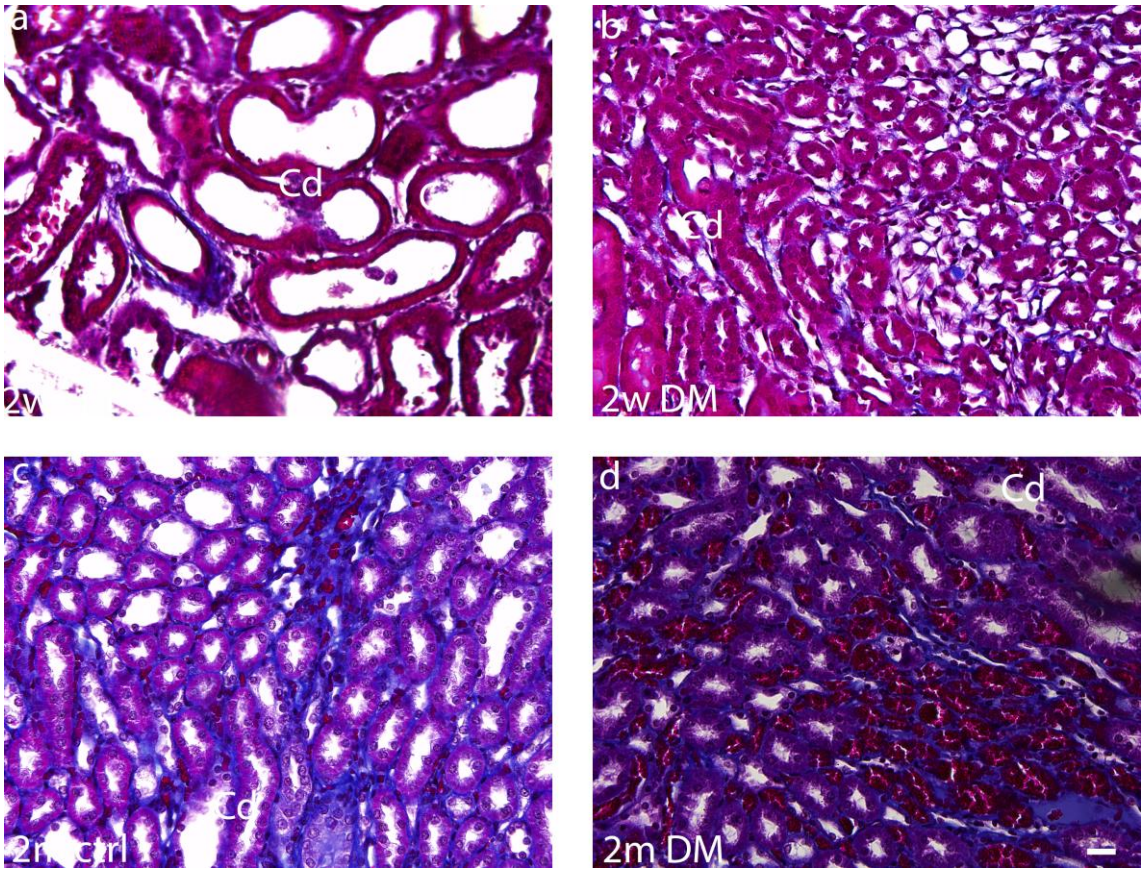


Fig. 4. Transversal section through the kidney tissue with the areas of interest: kidney medulla at 2 weeks in control (a) and diabetes (b); at 2 months in control (c) and diabetes (d). Scale bar 15µm. Legend: Cd- collecting ducts; ctrl- control; DM- diabetes mellitus type 1.

AIF positive cells are displayed as red staining of cytoplasm within different areas of the cortex and medulla of kidneys during 2 weeks and 2 months after induction of diabetes mellitus (Figure 5 and 6). Cytoplasmic staining is better observed when images were merged with DAPI nuclear stain as shown on Figure 5 and 6.

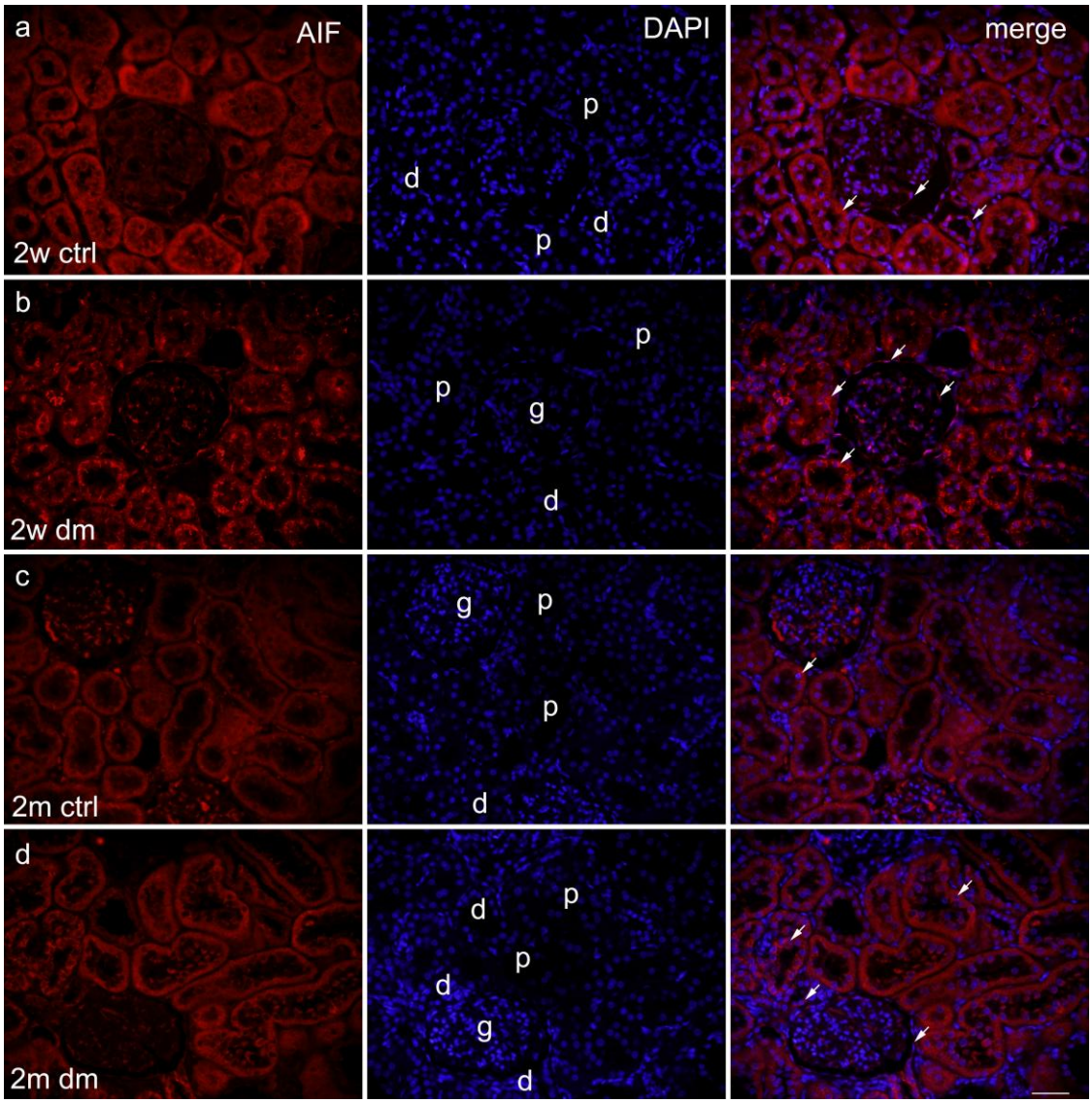


Fig. 5. AIF positive cells were seen as red staining of cytoplasm (arrows) within different areas of the cortex of kidneys. Co-localization of AIF and DAPI nuclear stain are displayed in the right column (merge). Kidney cortex in control and DM at 2 weeks (a, b) and 2 months (c, d). Scale bar 25µm. Legend: d- distal tubule; g- glomerulus; p- proximal tubule; ctrl- control; DM- diabetes mellitus type 1

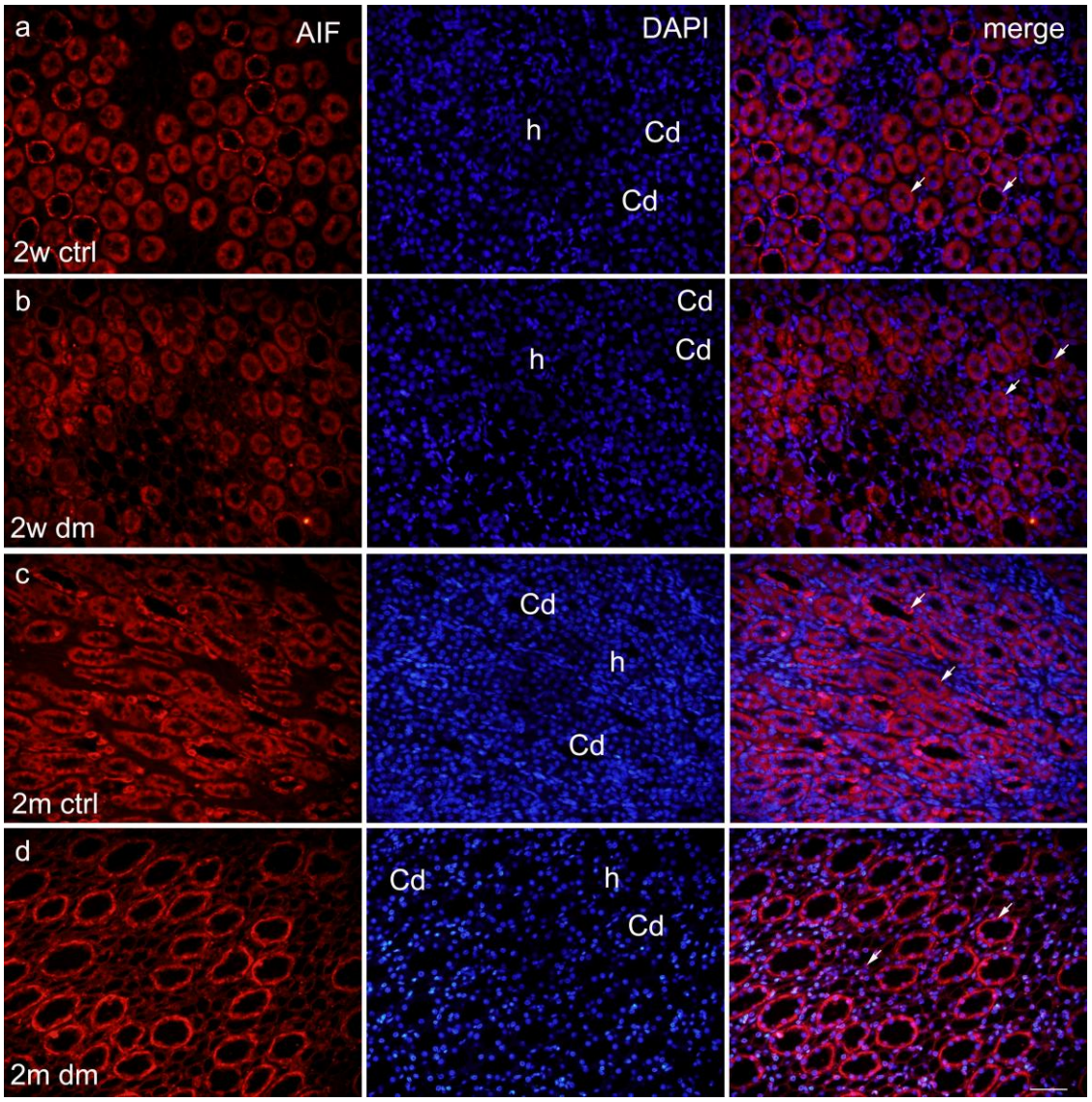


Fig. 6. AIF positive cells were seen as red staining of cytoplasm (arrows) within different areas of the medulla of kidneys. Co-localization of AIF and DAPI nuclear stain are shown in the far-right column (merge). Kidney medulla in control and DM at 2 weeks (a, b) and 2 months (c, d). Scale bar 25µm. Legend: cd- collecting duct; h- loop of Henle; ctrl- control; DM- diabetes mellitus type 1

Threshold area percent (TAP) of AIF positive cells in 2 weeks and 2 months of control and diabetic rat groups revealed statistically significant difference between control and diabetic group with higher threshold area percent of AIF in diabetic groups (Figure 7).

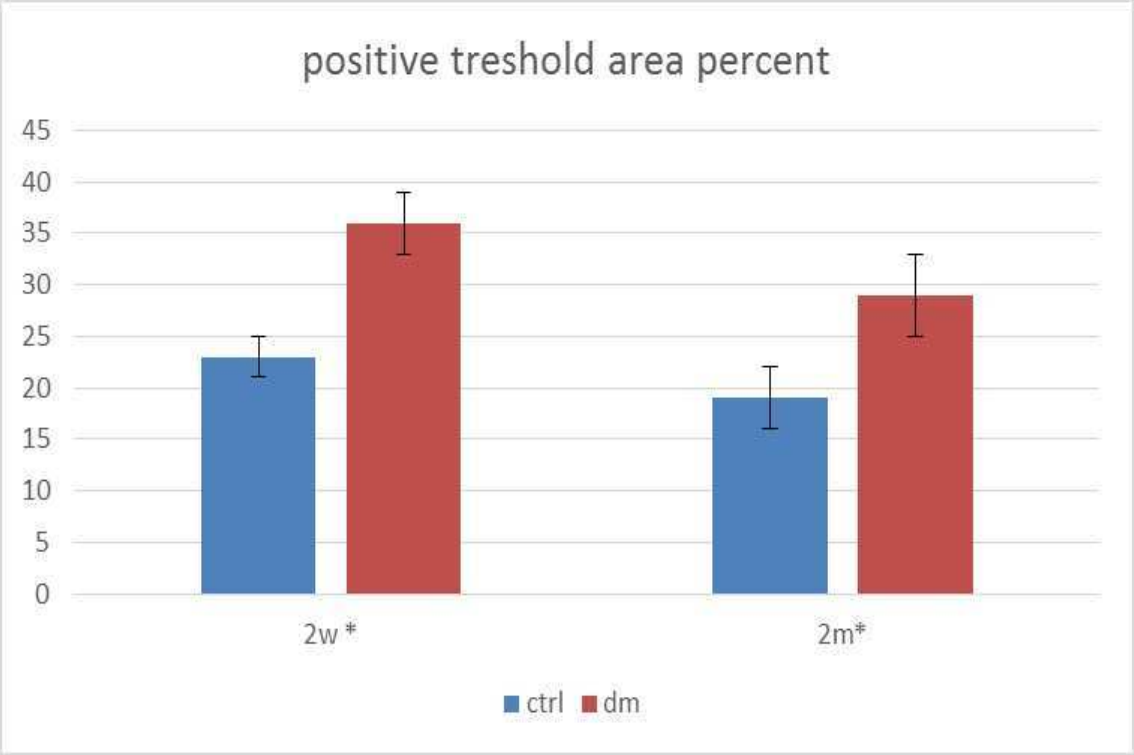


Fig. 7. TAP of AIF positive cells in 2 weeks (2w) and 2 months (2m) of control and diabetic rat groups. Asterisk denotes significant difference: *$p<0.05$. Data presented as M±SD. Legend: ctrl- control; dm- diabetes mellitus type 1

AIF expression is located in the cortex (mainly present in the proximal and distal tubules but also in the glomeruli) and medulla (in the collecting ducts). Strong intensity of AIF expression can be seen in proximal and distal tubules in diabetic in both 2 weeks and 2 months' groups but also in 2-month control group in collecting ducts. AIF staining intensity was mild in both 2 weeks and 2-month control groups. In the control groups the glomeruli show no staining at all but they are moderately stained after 2 weeks and 2 months in the diabetic groups. There was no difference in AIF intensity between the control and diabetic groups in the collecting ducts (Figure 8.).

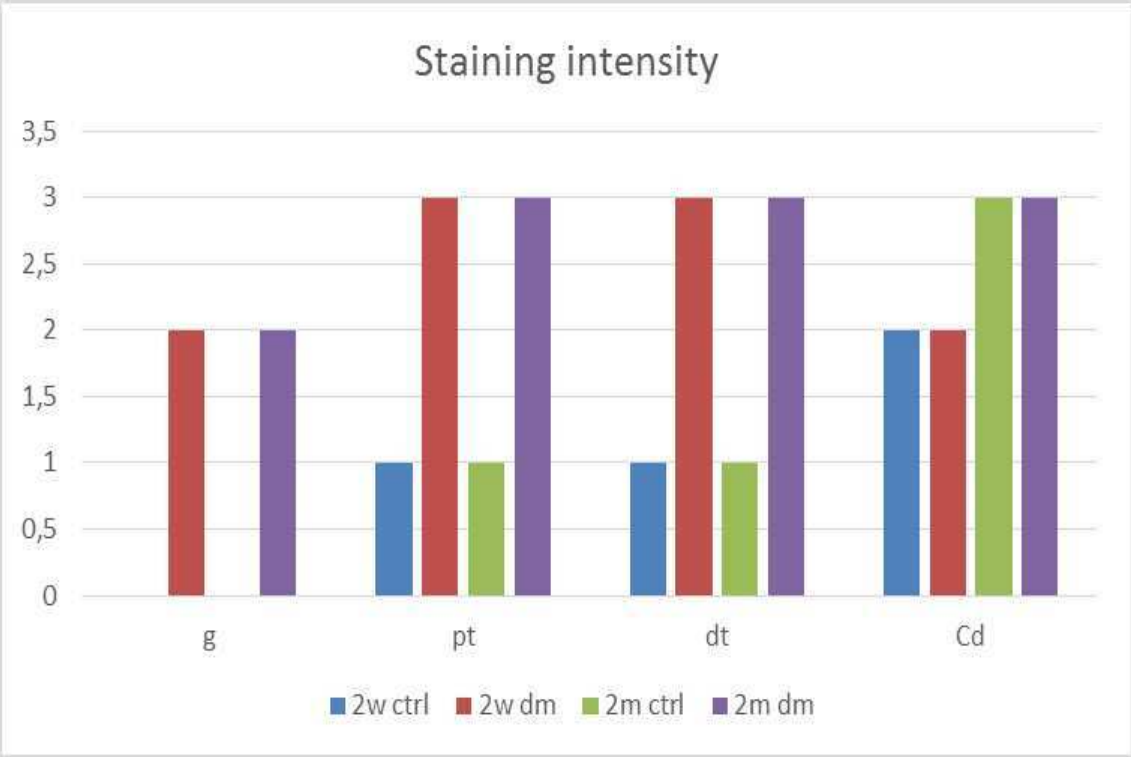


Fig. 8. Staining intensity of AIF in glomeruli (g) proximal tubules (pt) distal tubules (dt) and in collecting ducts (Cd) in 2 weeks (2w) and 2 months (2m) of control and diabetic rat groups. Legend: ctrl- control; dm- diabetes mellitus type 1.

The percentage of AIF positive cells in the glomeruli is over all the lowest. Both control groups show low AIF expression (4% in 2 weeks ctrl; 7% in 2 month ctrl). In proportion the greatest rise in cell positivity is displayed from the 2 weeks control group to 2 weeks diabetes group (38%) in glomeruli. The cell positivity of the 2 weeks diabetic group is cut nearly in half to 18% in the 2 month control group in glomeruli. A similar pattern even though not as drastic can be seen in the proximal convoluted tubular cells (92% positivity 2 weeks DM; 89% positivity 2 month DM). The only decrease in AIF cell positivity from 2 weeks control group (83%) to 2 month control group (80%) is seen in the proximal convoluted tubules. The distal convoluted tubules and collecting ducts show identical patterns in terms of a consistent rise of cell positivity from 2 weeks control to 2 month diabetic groups but not a great difference between the groups. The percentage of positive cells in the distal convoluted tubules are 24% in 2 weeks control; 36% in 2 weeks diabetic; 37% in 2 month control and 42% in 2 month diabetic of AIF cell positivity. The highest cell positivity is shown in the collecting ducts. Every group has more than 80% cell positivity.

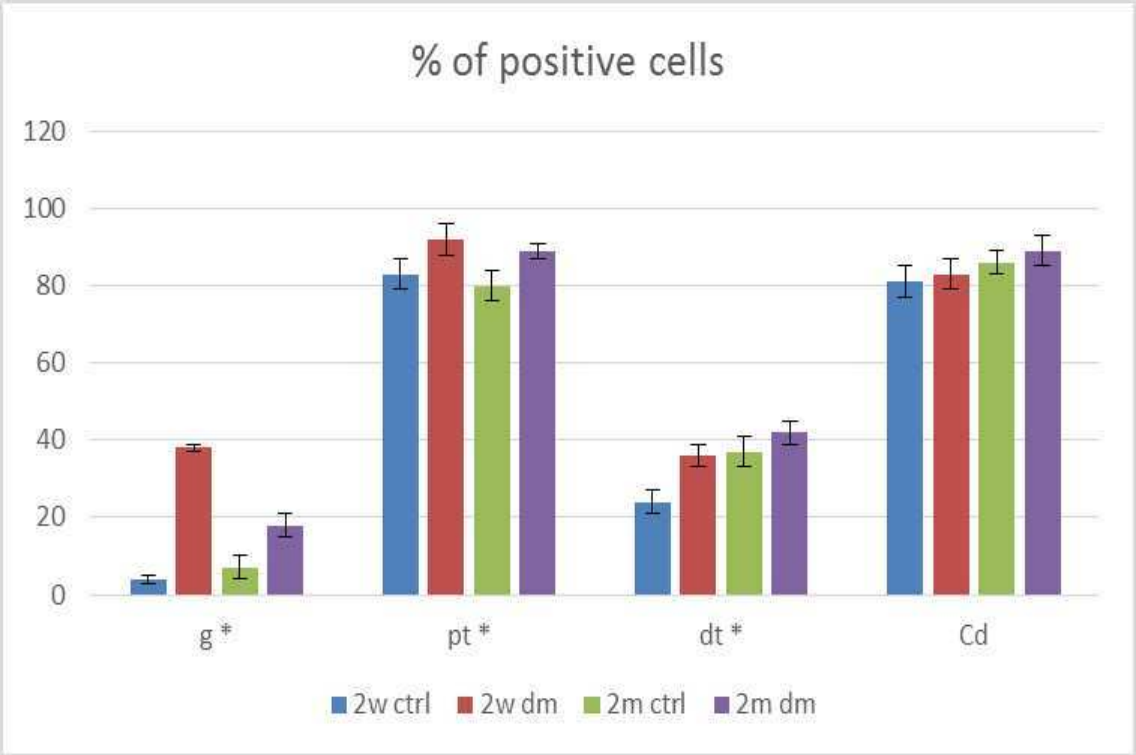


Fig. 9. Percentage of AIF positive cells in glomeruli (g) proximal tubules (pt) distal tubules (dt) and in collecting ducts (Cd) of control and diabetic rat groups. Legend: 2w (2 weeks); 2m (2 months); ctrl- control; dm- diabetes mellitus type 1.

5. DISCUSSION

In our experimental study we demonstrated with HE and Mallory staining that there are pathologic changes in diabetic rat kidney already 2 month after diabetes induction. There are additional contributing factors to renal sclerosis besides hypertension and hyperglycemia. According to Stadler *et al*. dyslipidemia is an important factor (17). At a baseline level renal epithelial cells use fatty acids as their energy source (17). The fact is, that DKD leads to abnormal fatty acid oxidation and therefore causes renal cell energy depletion and subsequently apoptosis, cell differentiation and sclerosis (17). Isermann *et al*. showed with their mice model that activated protein C (APC) is reduced in diabetic mice (18). APC modulates the mitochondrial apoptosis pathway and has thus an important role in maintaining normal kidney function by preventing glucose induced apoptosis in endothelial cell and podocytes which are part of the filtration barrier (18).

The limitation of our study is that we did not gather data about kidney parameters like creatinine, albumin and blood level urea to further quantify DKD. Regarding AIF and its specific role in DKD, there are currently only a very limited number of studies available. Also, the sample size in the next studies could be increased. Further similar studies should try to find other factors responsible for apoptosis which have a stronger connection to diabetic kidney disease. The ultimate goal would be a factor which is the main mediator in apoptosis which in turn could be blocked by physicians and thus could terminate the development/progression of diabetic nephropathy.

Regarding AIF we hypothecated that AIF and programmed cell death are strongly correlated. The ideal outcome of our experiment would have been that there are no significant changes in AIF expression in our 2 weeks control group in comparison to the 2 month control group. On top of that it would have been great if there would have been a strong rise in AIF in the diabetic groups compared to the control groups and that AIF would have been much more expressed in the 2 month diabetic group compared to the 2 weeks diabetic group. Such an ideal result is unfortunately not present. We achieve the closest result in the distal convoluted tubules and collecting ducts nevertheless the AIF cell positivity increased in the 2 month control groups.

We see a different view point in the staining intensity. The results of AIF intensity are closer to our hypothesis with a significant rise in staining intensity in the diabetic groups in comparison to the control groups even though there is still no difference between the diabetic groups. How to interpret those results is speculative. It is important to remember that AIF has a dual role, not only in apoptosis but also in cell function and homeostasis and that there are apparently differences in AIF expression in different parts of kidney like glomerulus, convoluted tubules, collecting ducts, cortex and medulla. Habib *et al*. found out that the renal tubules show pathologic changes earlier in comparison to the glomeruli and that the tubules could be overall more sensitive at the beginning of DKD (19). This could be true and underlined by the fact that in our study the highest amount of AIF cell positivity has been found in diabetic rats already after 2 weeks. If we would conduct the study again it would be interesting to see how the values would change over a longer period of time.

The results of our thesis underline the fact that there is a rise in AIF expression in DKD. This means that AIF is related to kidney sclerosis which is especially obvious in the glomeruli. The amount of positive cells rose from 4% to 38% in the 2 weeks groups and from 7% to 18% in the 2 month groups. This is contradictory to what Coughlan *et al*. found out. In their study they concluded that AIF deficiency is a risk factor for diabetic kidney disease (14). Specifically, they stated that there are extensive changes in mitochondrial function if there is diabetes superimposed on AIF knockout mice (14). Furthermore, they found out that there is a fourfold decrease in renal cortical AIF in humans with DKD in comparison to healthy ones (14).

There is a lack of similar studies but the differences between our study and Coughlan *et al*. could by partially explained by the previously mentioned dual role of AIF. Already in 2004 Jäättelä *et al*. pointed out that there are difficulties in measuring AIF correctly (20). They found out that AIF staining intensity in immunofluorescence increases remarkably upon translocation to the cytosol while a similar rise could not be demonstrated in immunoblot analysis (20). Consequently, it could be possible that we measured only the AIF present in the nucleus in other words the apoptotic AIF while Coughlan *et al*. measured the AIF present in the mitochondria and therefore the AIF responsible for cell survival. Further studies and methods should focus more on additional kidney parameter and it should be clear (if possible) which kind of AIF is examined.

In conclusion, if we measure AIF we have to differentiate which kind of AIF we are currently measuring. When we are sure about it we can further assess the amount of AIF and only then it is possible to compare different studies. However, our study provides additional insight into AIF expression pattern during short term diabetes model and this knowledge might be important for the studies dealing with AIF as potential therapeutic target and marker of advancement of DKD.

6. CONCLUSION

1. AIF is a major contributor in programmed cell death and therefore is an indicator for DKD.
2. AIF expression in healthy kidneys is important to maintain oxidative phosphorylation and thus energy generation.
3. Since AIF has two tasks it is important to differentiate them by all means.
4. There are differences in expression of AIF in different cell populations of the kidney.
5. In the future, it can be helpful in the diagnosis of DKD to focus on different areas of the kidneys besides the glomeruli.

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7. SUMMARY

Title: EXPRESSION PATTERN OF APOPTOSIS-INDUCING FACTOR IN THE KIDNEYS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

Objectives: The aim of this study is to investigate the significance of AIF expression in the development of DKD by observing the expression of AIF in 2 weeks and 2 months diabetic kidney samples and then comparing them to healthy rat kidneys of the same age.

Materials and methods: Diabetes mellitus (DM) was induced by i/p injecting 55 mg/kg streptozotocin (STZ) to male Sprague-Dawley rats, and was validated by measuring blood glucose level. Control group received citrate buffer. Animals were sacrificed after 2 weeks and 2 months. Cortex areas (glomeruli, proximal and distal tubules) and medullary areas (collecting ducts and distal tubules) were analyzed with antibodies raised against AIF.

Results: AIF expression is located in the cortex as well as in the medulla of the kidney. In the cortex AIF is mainly present in the proximal convoluted tubules (pct) and in the medulla mainly present in the collecting ducts (cd). The highest percentage is present in the 2 weeks DM pct group with 92% positive staining. The cd shows a very mild incline in AIF positivity form 81% in the 2 weeks control group to 89% in the 2 month DM group. The staining intensity rose from moderate to strong staining. The pct showed a similar incline and the staining intensity increased stronger from mild to strong. The same rise in intensity was observed in the distal convoluted tubules but the relative increase in AIF positivity was stronger. What is striking is that the incline and absolute AIF staining intensity is roughly the same from the 2 weeks to 2 month in the control group and the DM group, that there is no big difference between the 2 weeks and 2 month DM groups. The absolute amount of AIF cell positivity is also roughly equal in the DM groups and in the DM 2 week group (38%) even twice as big as in the DM 2 month group (18%).

Conclusions: Since AIF has a dual role, for the future it is important, if we want to compare studies to find a method to differentiate AIF in different states. If we manage this, AIF could become a parameter to assess kidney status in DKD and one day becomes a tool to block progression of DKD.

9. CROATIAN SUMMARY

NASLOV: IZRAŽAJ ČIMBENIKA INDUKCIJE APOPTOZE (AIF) U BUBREZIMA ŠTAKORA SA STREPTOZOTOCIN-INDUCIRANIM DIJABETESOM

Ciljevi: Cilj ovog istraživanja bio je istražiti značaj AIF ekspresije u razvoju dijabetička bolest bubrega (DBB) promatranjem ekspresije AIF u 2 tjedna i 2 mjeseca bubrega bubrega, a zatim usporedbom sa zdravih bubrega štakora iste dobi.

Materijali i metode: Diabetes mellitus (DM) je induciran i/p injektiranjem 55 mg/kg streptozotocina (STZ) mužjacima Sprague-Dawley štakora i validiran mjerenjem razine šećera u krvi. Kontrolne životinje su primale citratni pufer. Životinje su žrtvovane nakon 2 tjedna i 2 mjeseca od indukcije dijabetesa ili citratnog pufera. Područja kore (glomeruli, proksimalni i distalni tubuli) i područja srži (sabirni kanali i distalni tubuli) analizirani su protutijelima na AIF.

Rezultati: Izražaj AIF-a nalazi se u kori, kao i u srži bubrega. U kori, AIF je uglavnom prisutan u proksimalnim zavojenim kanalićima (pct) dok je u srži uglavnom prisutan u sabirnim kanalićima (cd). Najveći postotak prisutan je u 2 tjednu DM pct skupine s 92% pozitivnih stanica. Cd pokazuje blago smanjenje pozitivnosti na AIF od 81% u kontrolnoj skupini od 2 tjedna do 89% u 2-mjesečnoj DM skupini. Intenzitet bojenja se povećavao od umjerenog do jakog bojenja. Slično smanjenje presutno je u pct, a intenzitet bojanja povećao se od blagog do jakog. Isti porast intenziteta zabilježen je u distalnim zavijenim kanalićima, ali relativno povećanje AIF pozitivnosti je bilo jače. Ono što je uočljivo je da je smanjenje i apsolutni intenzitet bojenja AIF-a otprilike isti od 2 tjedna do 2 mjeseca u kontrolnoj skupini i DM skupini, te da nema velike razlike između 2 tjedna i 2 mjeseca DM skupine. Apsolutna količina AIF pozitivnih stanica također je približno jednaka u skupinama DM i u dvjema skupinama od dva tjedna (38%), čak dvostruko veća u skupini DM od 2 mjeseca (18%).

Zaključci: Budući da AIF ima dvostruku ulogu, za buduće studije je važno pronaći način razlikovanja AIF-a u različitim statusima, ako ih želimo usporediti. Ako se to može postići, AIF može postati parametar za procjenu stanja bubrega u DBB-a i eventualno ciljni alat za blokiranje napretka DBB-a.

10. CURRICULUM VITAE

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