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DEPARTMENT OF BIOMEDICINE AND BIOTECHNOLOGY

Study of the variations in the expression of different adhesion and cytoskeletal proteins of podocytes (E-Cadherin, Podocin, Vimentin) due to Bisphenol A

DIPLOMA THESIS

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Hradec Králové, 2019 **Zuzana Chvojanová**

DECLARATION

Hereby I declare that this thesis is my own work. All literature and sources of information I used are listed in the list of used literature and they are properly cited. This work has not been used to gain an equal or different degree.

Tímto prohlašuji, že tato práce je mou vlastní prací. Veškerá použitá literatura a zdroje informací jsou uvedeny v seznamu použité literatury a jsou řádně citovány. Tato práce nebyla použita k získání stejného nebo jiného titulu.

Hradec Králové Zuzana Chvojanová

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CONTENT

ABSTRACT

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Title of the diploma thesis: Study of the variations in the expression of different adhesion and cytoskeletal proteins of podocytes (E-Cadherin, Podocin, Vimentin) due to Bisphenol A

Bisphenol A (BPA) is one of the most widespread compounds in the world, producing over 6 billion metric tons per year. It is widely used as part of polycarbonate plastics and epoxy resins, from which reusable plastic bottles, food boxes and some medical equipment are made. It is also used to coat the inner layer of the cans.

Previous studies have shown that BPA contributes to many chronic diseases in the human body, such as kidney disease - diabetic nephropathy. Podocytes - terminally differentiated cells of the Bowman's capsule in glomerulus - are an integral part of the filtration barrier, where they play an important role in preventing the plasmatic proteins from penetrating to the urine. Therefore, in this study, we looked at the effect of BPA on these cells and their particular proteins, using both *in vivo* and *in vitro* methods.

First, we demonstrated the harmful effect of BPA 100 nM on mice kidneys by immunohistochemistry (IHC) method. This was manifested mainly by the enlargement of the distal and proximal convoluted tubules of the glomerulus and Bowman's capsule. In the samples with induced diabetes mellitus, besides the above-mentioned manifestations, a significant mesangial expansion occurred. Using IHC, we also observed a decreased density of podocytes in the glomerulus, and decrease in their size and function, resulting in hypertrophic stress of the podocytes and glomerular collapse.

A previous study by R. Bosch’s laboratory dealing with BPA toxicity to the mice podocytes has shown a clear association between BPA exposure and proteinuria in

mice. Therefore, we investigated the effect of BPA on human podocytes by examining the toxicity of BPA on individual proteins of these cells. In particular, we dealt with E-Cadherin, an adhesive type of protein whose function is primarily to ensure the integrity and polarity of renal epithelial cells. The decrease in protein expression was induced by BPA exposure at all used concentrations (1 nM, 10 nM, 100 nM) in the Western Blot (WB) method. The decrease of E-Cadherin was also manifested in immunocytochemical (ICC) and IHC methods. E-cadherin deficiency is a signal of epithelial and mesenchymal transition, a process in which cells lose cell-cell contact and delaminate from the epithelium. As a result, cell adhesion is impaired. Another protein of interest was Podocin, a cytoskeletal type protein specific for mature podocytes, located in the slit diaphragms. Its function is to regulate the glomerular permeability. In this case, the trend of decreasing protein expression was demonstrated at all of the above-mentioned BPA concentrations used in WB. The ICC method showed a significant decrease of Podocin due to BPA 100 nM treatment on the podocytes. The lack of this protein results in decreased function of podocytes, leading to increased proteinuria. The last studied protein was Vimentin, representing the group of cytoskeletal proteins found in all eukaryotic cells with the mesenchymal origin. The function of this protein is mainly to maintain the stability and shape of the podocyte. Here again, BPA toxicity was manifested by a decrease in Vimentin levels in the cells. The greatest decrease occurred with BPA exposure of 100 nM. The lack of this protein disrupts the already mentioned cell stability, including their shape.

All the measured results confirm the original assumption of a harmful effect of BPA onto human podocytes. Not all of these results were statically significant, but the trend of decreasing values for all proteins was visible.

ABSTRAKT

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Název diplomové práce: Studie změn v expresi různých adhezivních a cytoskeletálních proteinů podocytů (E-kadherin, Podocin, Vimentin) v důsledku Bisfenolu A

Bisfenol A (BPA) patří mezi nejrozšířenější sloučeniny na světě, jehož produkce činí ročně vice než 6 miliard tun. Je široce používaný jako součást polykarbonátových plastů a epoxidových pryskyřic, z nichž jsou vyráběny znovu použitelné plastové lahve, potravinové boxy a některé lékařské vybavení. Také se používá k potažení vnitřní vrstvy plechovek.

V dříve provedených studiích bylo zjištěno, že BPA v lidském organismu přispívá ke vzniku mnoha chronických onemocnění jako například onemocnění ledvin – diabetická nefropatie. Podocyty – terminálně diferenciované buňky Bowmanova pouzdra glomerulu – jsou nedílnou součástí filtrační bariéry, kde hrají důležitou roli v zabránění průniku plasmatických proteinů do moči. Proto jsme se v této studii zaměřili na efekt BPA právě na tyto buňky a jejich konkrétní proteiny, a to pomocí *in vivo* a *in vitro* metod.

Nejdříve jsme pomocí imunohistochemické metody (IHC), prokázali negativní efekt 100 nM BPA na myší ledviny. To se projevilo zejména rozšířením distálních a proximálních stočených tubulů glomerulu a Bowmanova pouzdra. U vzorků s navozeným diabetem mellitem se mimo zmíněné projevy objevila i výrazná mesangiální expanze. Pomocí IHC jsme pozorovali i sníženou hustotu podocytů v glomerulu, k poklesu došlo i v ohledu na jejich velikost a funkci, což vedlo k hypertrofickému stresu podocytů a ke glomerulárnímu kolapsu.

Díky předešlé studii laboratoře R.Bosch zabývající se toxicitou BPA na myší podocyty, byla prokázána jasná souvislost mezi expozicí BPA a proteinurií u myší.

Proto jsme se v této studii zabývali efektem BPA na lidské podocyty a to formou zkoumání toxicity BPA na jednotlivé proteiny těchto buněk. Zabývali jsme se konkrétně E-kadherinem, což je adhezivní typ proteinu, jehož funkcí je zejména zajištění integrity a polarity renálních epiteliálních buněk. Pokles exprese tohoto proteinu vyvolala expozice BPA ve všech použitých koncentracích (1 nM, 10 nM, 100 nM), které byly použity v metodě Western Blot (WB). Pokles E-kadherinu se projevil i při použití imunocytochemické (ICC) a IHC metody. Nedostatek E-kadherinu je signálem epiteliálního a mezenchymálního přechodu, což je proces, při kterém buňky ztrácejí kontakt buňka-buňka a delaminují z epitelu. V důsledku toho je narušena adheze buněk. Dalším zkoumaným proteinem byl Podocin, což je cytoskeletární typ proteinu, specifický pro zralé podocyty, lokalizovaný ve štěrbinové membraně. Jeho funkcí je regulace glomerulární permeability. I v tomto případě byl trend poklesu exprese proteinů prokázán ve všech již výše zmíněných koncentracích BPA použitých při WB. I v ICC metodě se ukázal výrazný pokles Podocinu díky působení 100 nM BPA na podocyty. Nedostatek tohoto proteinu má za následek sníženou funkci podocytů, což vede ke zvýšené proteinurii. Posledním zkoumaným proteinem byl Vimentin zastupující skupinu cytoskeletárních proteinů vyskytujících se ve všech eukaryotických buňkách s mesenchymálním původem. Funkce tohoto proteinu spočívá zejména v udržení stability a tvaru podocytu. I v tomto případě se toxicita BPA projevila poklesem hladin Vimentinu v buňce. K největšímu poklesu došlo při expozi 100 nM BPA. Nedostatkem tohoto proteinu se narušuje již zmíněná stabilita buněk včetně jejich tvaru.

Všechny naměřené výsledky potvrzují původní předpoklad škodlivého účinku BPA na lidské podocyty. Ne všechny tyto výsledky byly staticky významné, ale trend klesajících hodnot byl u všech proteinů viditelný.

1 INTRODUCTION

1.1 Bisphenol A

Bisphenol A (BPA) by chemical name 4,4'-(propane-2,2-diyl)diphenol was first synthesized in 1891 by Russian chemist Alexander Pavlovich Dianin and during the 1930s it was examined by a British scientist Edward Charles Dodds who identified the estrogenic properties of BPA but never found use as a drug. During the next research and testing, he discovered a stronger estrogenic substance called diethylstilbestrol which displaced BPA (Vogel, 2009).

1.1.1 Using

Currently, BPA is a component of polycarbonate plastics and epoxy resins which are used in the production of reusable plastic bottles and as the inner coating of cans and other food and drink containers. Likewise, they are extended into other uses of life such as sunglasses, thermal papers, CD-Roms, medical devices, dental materials and many more. BPA adds them hardness, clarity, lightweight and resistance to the temperature. BPA is also included in polysulfones and polyether ketones as an antioxidant in some plasticizers and as a polymerization inhibitor in polyvinylchloride (PVC) (Rezg et al., 2014).

1.1.2 Preparation

BPA is prepared by condensation of phenol and acetone in acidic medium caused by hydrochloric acid (See Fig. 1).

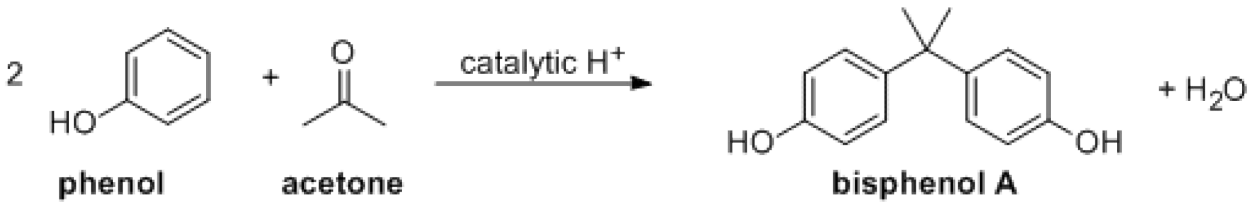

Figure 1 - Preparation of Bisphenol A

1.1.3 Metabolism

BPA gets to the organism mainly via the oral route but also from dental sealants, through the skin and by inhalation of cleaning products (Bosch et al., 2016). Conversion of BPA to more water-soluble substances takes place in the gastrointestinal tract and the liver of the humans and nonhumans primates. The largest

amount of inactive metabolite is formed by conjugation with glucuronic acid to form glucuronide, the other metabolite is sulfate. Most of the administered dose (84-97%) is absorbed and eliminated by urine over the 5-7 hours following oral administration and rising to 100% after 24 hours. Exposure of this substance is measurable by the concentration of BPA in the urine (Teeguarden et al., 2011). Metabolism of BPA is summarized in Fig. 2.

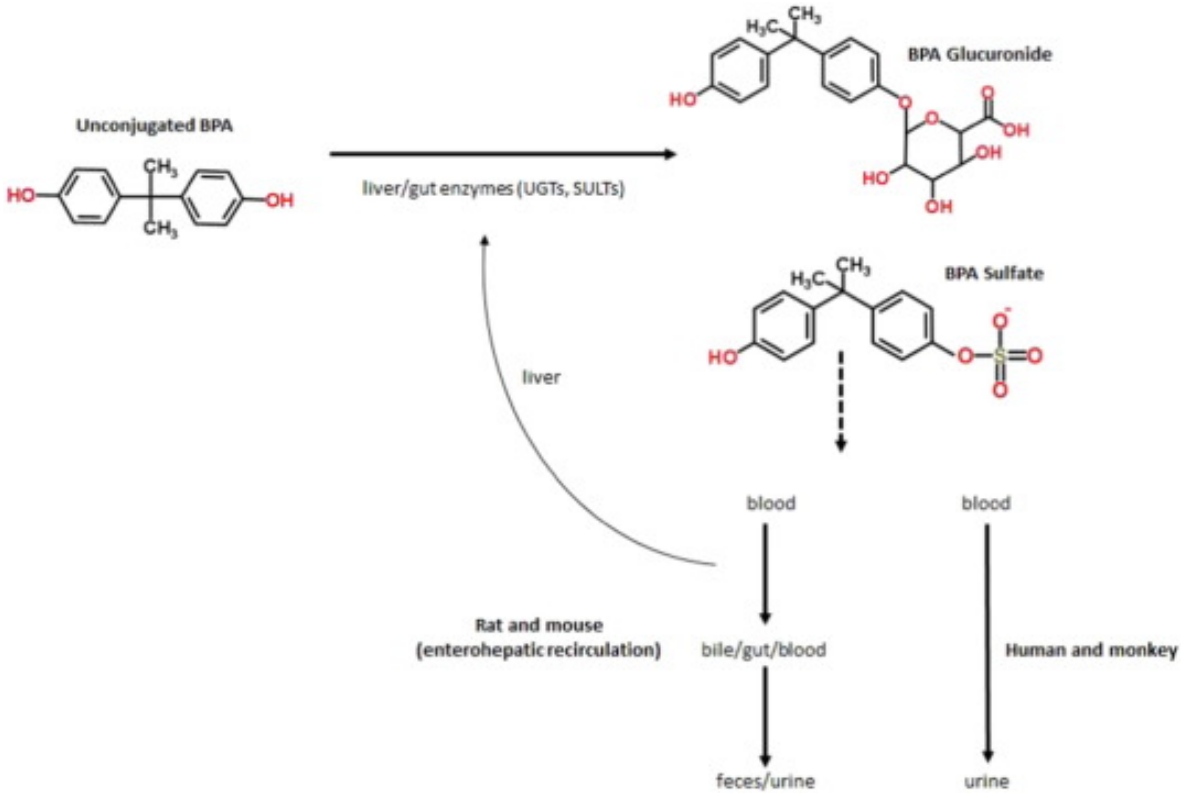

Figure 2 - Metabolism of Bisphenol A (Thayer et al., 2015)
(The abbreviations in the figure mean: BPA=Bisphenol A; UGTs=Uridine diphosphate-glucuronosyltransferases; SULTs=Sulfotransferases)

1.1.4 Bisphenol A and association with diseases

BPA is one of the world's most widely produced substances. Current estimates show that more than 6 billion pounds of BPA are expended per year in the production of polymers, polyvinylchloride plastics and flame-retardant Tetrabromobisphenol A (Rezg et al., 2014). Many studies have shown that more than 97% of the population has a detectable BPA concentration in the urine. Therefore, a question that needs to be asked is: How much is BPA harmful to the human body?

Thanks to previous animal studies, it has been found that higher BPA concentration in the urine may be associated with adverse health effects in humans,

especially in the liver in relation to insulin, diabetes mellitus 2 and obesity (Lang et al., 2009). As well as experimental studies have identified a potential mechanism of BPA nephrotoxicity via injury of glomerular podocytes, oxidative stress inflammation and induction of arterial hypertension (Bosch-Panadero et al., 2016). Both of these problems are related to the cardiovascular system where is increased risk of cardiovascular diseases including coronary heart disease, heart attack and angina pectoris. Also, BPA induces hypertension via increased production of oxygen free radicals instead of nitric oxide. Hypertension can participate in the vascular damage mechanisms and the progression of atherosclerotic lesions (Saura et al., 2014).

But several studies have shown that BPA may be associated with more diseases than those mentioned earlier, for example, chronic respiratory disease, thyroid disorders, breast cancer, behavioral disorders, reproductive disorders in both sexes (Eladak et al., 2015) (See the effects of BPA in Fig. 3).

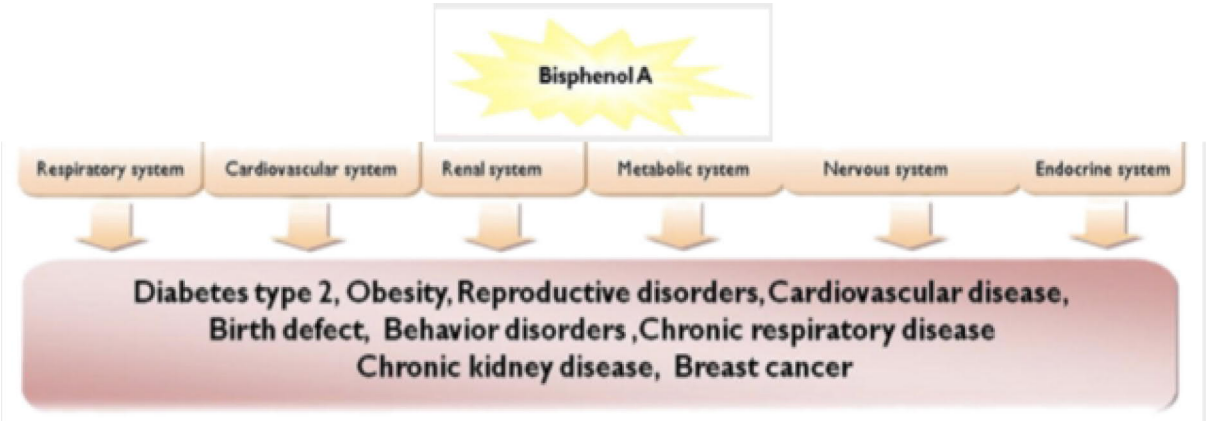

Figure 3 – Bisphenol A association with diseases (Rezg et al. 2014)

1.2 Kidneys

The kidneys are paired organ of bean-like form and weight about 150 grams. They are placed on both side of the lumbar spine, in the retroperitoneal space, between the parietal peritoneum and the posterior abdominal wall. The left kidney is located in the area of the T12-L3 vertebrae and it is usually a little bit bigger than the right one which is placed lower due to slight displacement by the liver. The kidneys are well protected by eleventh and twelfth rib, fat and muscle. This organ is the first one of the urinary system. The other parts of the system are the ureters, the bladder and the urethra (OpenStax College 2013).

1.2.1 Function

Besides excretory function including the formation of the urine, excretion of the nitrogen compounds and elimination of the final products of the metabolism of xenobiotics (e.g. drugs or toxins), the kidneys hold several others as well very important functions in the human organism. The kidneys keep the proper volume of extracellular fluid and also participate in it via the renin-angiotensin-aldosterone system, because renin, which is on the beginning of this system, is produced in the juxtaglomerular cells of the renal cortex. Another important function is the regulation of pH. It is role shared with the lungs and the buffers in the blood. This function is done by the regulating of the acid-base balance. Another one is the regulation of electrolyte concentration, which helps to correct the osmolality of the extracellular fluid. Endocrine function is also equally important as the others. There are several indispensable substances as the erythropoietin, renin and dihydrocalciferol, active form of vitamin D, which are produced by the kidneys. All of these functions contribute to maintaining the homeostasis of the organism (Eroschenko, 2013).

1.2.2 Macroscopic structure

The kidney surface is made up of the *capsula fibrosa* which is firmly fixed to the kidney in the *sinus renalis*. Under this layer, there is the *cortex renalis*. This layer has granular character due to the presence of the nephrons. To be more precise, all the renal corpuscles and as well as both the proximal convoluted tubules and distal convoluted tubules are located here. Some nephrons have a shorter loop of Henle that does not pass into the *medulla*. These nephrons are called cortical nephrons. The others which have a long loop of Henle and extend to deep into the medulla are known as juxtaglomedullary nephrons. Inner region of the kidney is *medulla renalis* which structure is annealed. This layer consists of the *pyramides renalis* whose pyramidal bases are located on the border of the medulla and cortex. Pyramid spikes (*papillae renalis*) head to *hilum renalis* which is the point of the entry and exit for the blood vessels, nerves, lymphatic vessels and ureters. *Hilum renalis* is part of the *pelvis renalis* which connects the kidneys with the rest of the body. The pelvis is the point where the mayor calyces join together. The whole organ is wrapped in the lipoid layer called *capsula adiposa* (OpenStax College, 2013) (Macroscopic structure of the kidneys you can see in Fig. 4).

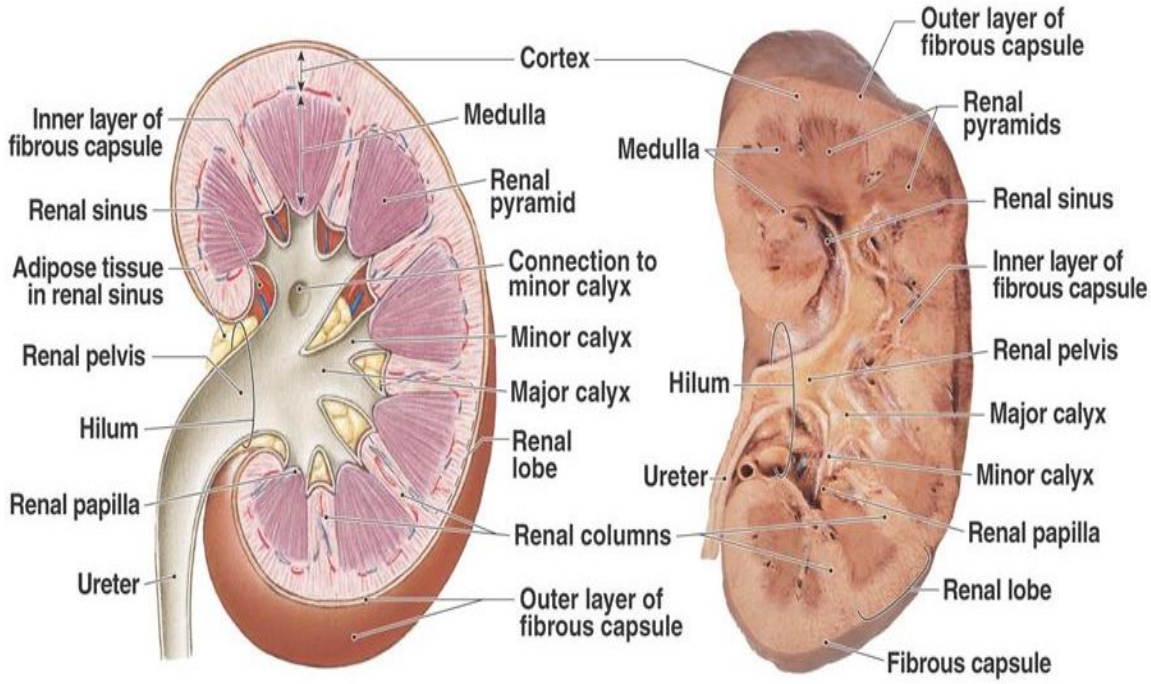

Figure 4 -Structure of the kidney (https://polishedpathology.com/wp-content/uploads/2014/10/kidney.png)

1.2.3 Microscopic structure

The nephron is the functional unit of the kidney. There are approximately 1.2 million of the nephrons in each kidney. It consists of a few parts which are located in the *medulla* or *cortex renalis*.

First of them is the glomerulus formed by the tuft of the afferent arterioles. It is high pressure (~48 mmHg) bed between the afferent and efferent arterioles. The glomerulus is largely surrounded by the Bowman's capsule. This cover is made by two layers and between them, urinary space is located. The first one is the parietal layer simply squamous epithelium which borders the glomerulus. The second sheet is the visceral layer. It consists of singularly shaped cells, which are called podocytes, extending finger-like arms (pedicels) to cover the glomerular capillaries. These projections interconnect to form filtration slots, leaving small gaps between the particular cells to form the sieve. The glomerulus is the place where urinary formation begins as the blood flows through it (Look at the Fig. 5). Approximately 20% of plasma is filters by the sieve of the podocytes and the filtrate is garnered in the Bowman's capsule from where it funnels to the proximal convoluted tubule (PCT). Where the fenestrations in the glomerular capillaries match the spaces between the pedicels the

only thing separating capillary lumen and lumen of the Bowman's capsule is their shared basement membrane. These features comprise the filtration membrane. Only substances that are smaller than 10 nm, their molecular weight is less than the weight of albumin (69 kDa) and do not have a negative charge pass through this membrane. The glomerulus together with the Bowman's capsule make up the renal corpuscle (Eroschenko, 2013; (OpenStax College, 2013).

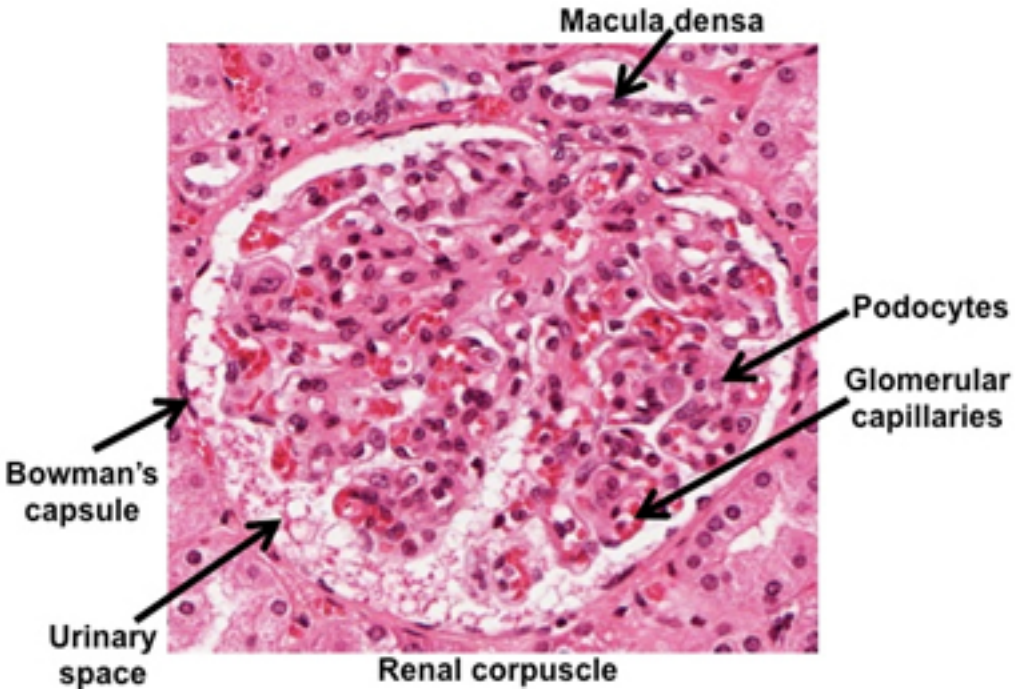

Figure 5 - Renal corpuscle
(https://vmicro.iusm.iu.edu/hs_vm/docs/lab11_3.htm)

Just outside of the renal corpuscle, at the junction where the afferent and efferent arterioles enter and leave Bowman's capsule, the juxtaglomerular apparatus is lying. It is made up by the distal convoluted tubule (DCT) and the glomerular afferent arteriole. The wall of DCT forms a part known as the *macula densa* (See Fig. 5). This cluster of the cuboidal epithelial cells monitors sodium concentration of the fluid in the tubule. In response to increased sodium cells of *macula densa* cause the contraction of afferent arteriole resulting in the reduction of the blood flow through the glomerulus thereby reducing the glomerular filtration rate. The second cell type of this apparatus is the juxtaglomerular cell which is modified smooth muscle cell of the afferent arteriole. These cells produce renin when the blood pressure is reduced. The other type of cells are mesangial cells located near to *macula densa* but their function remains unclear (OpenStax College, 2013).

Next part of the nephron is formed by several types of canals: PCT, DCT, Henle loop and collecting tubule. Filtrate fluid from the Bowman's capsule enters into PCT which is the most active part of the nephron in reabsorption and secretion of solutes (Na^+, Cl^-, glucose, amino acids, etc.). This process serves to maintain the proper volume of the extracellular fluid in the organism. The Henle loop has a descending and ascending part which are just continuations of PCT. Here is made hypotonic fluid that can be facultatively resorbed in other sections of the nephron. It is followed by DCT into which this fluid flows. This tubule enters to the collective duct which is continuous with the nephron but not technically part of it. Each collecting tubule collects the filtrate from several nephrons. These ducts join as they descend to the *medulla renalis* and make up about 30 terminal ducts which empty at the *papilla renalis* (Eroschenko, 2013). Both types of nephron and its structure is summarized in Fig. 6.

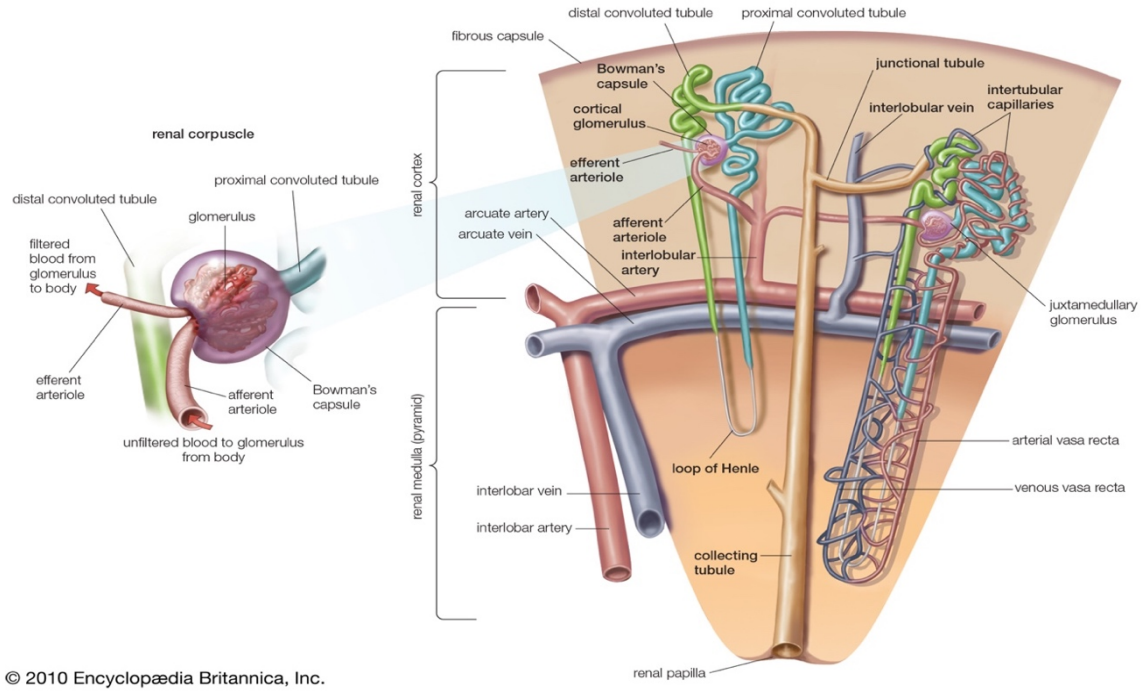

Figure 6 - Nephron structure (Encyclopaedia Britannica, 2015)

1.2.4 Podocytes

Podocytes are highly specialized and terminally differentiated cells of the visceral layer of the Bowman's capsule, wrapped around the glomerular capillaries (Structure of podocyte you can see in Fig. 7). These cells are essential to the integrity of the glomerular filtration membrane and responsible for the maintenance of stability and functionality of the basement membrane of the glomerulus. They play an important role in the prevention of the entrance plasmatic proteins to the urinary ultrafiltrate. Podocytes have the central body from which the primary protrusions crop out generating the secondary arms known as pedicels. Individual pedicels are separated by filtration slots through which filtrate is moved further (Reiser and Altintas, 2016) (See the structure of filtration membrane in Fig. 8).

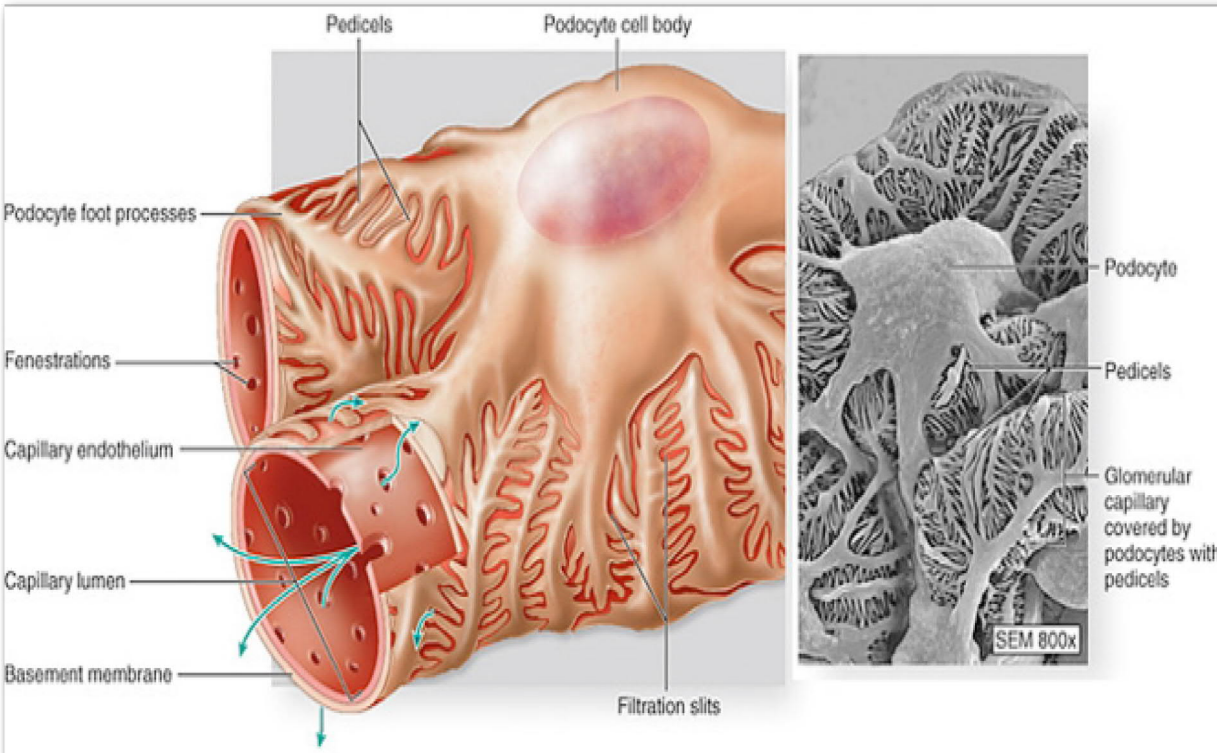

Figure 7 – Podocytes (https://www.westmont.edu/_academics/pages/departments/kinesiology/kns011/Lectures/UrinarySystem.pdf)

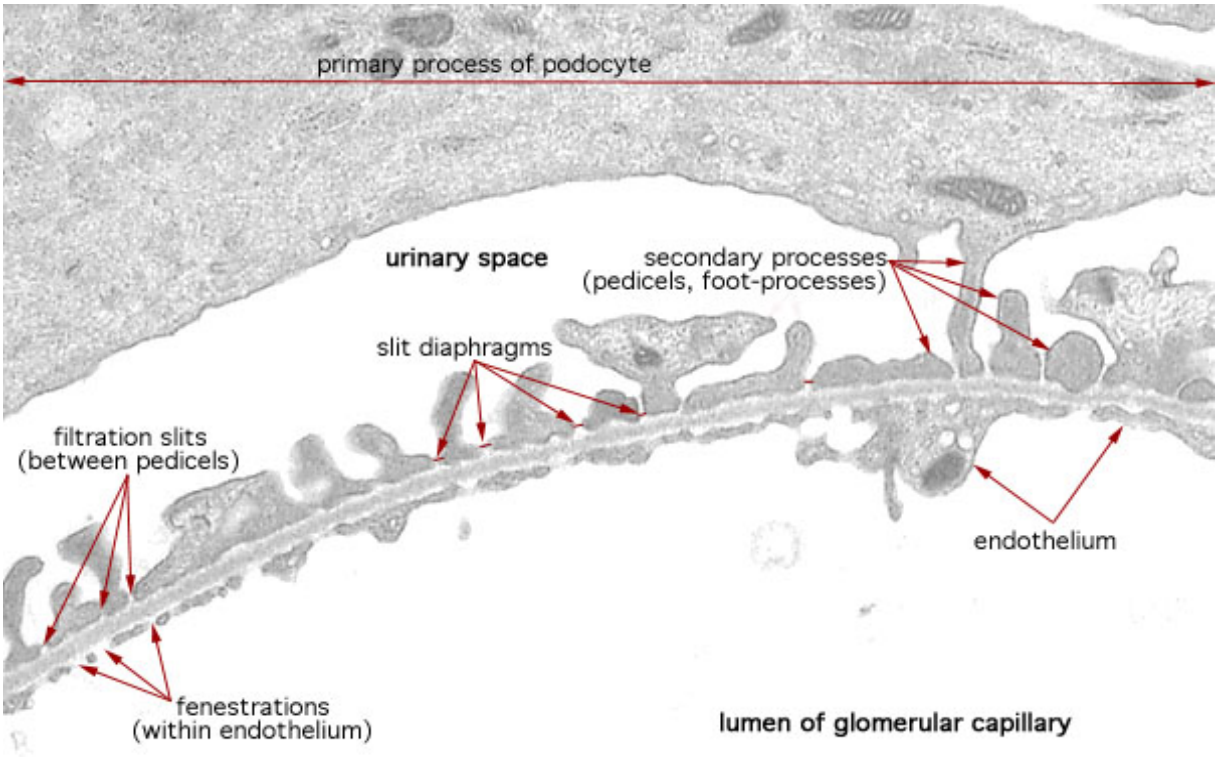

Figure 8 - Filtration membrane
(http://medlib.bu.edu/histology/p/22402lba.htm)

1.2.5 Kidney diseases

Renal diseases can be divided into acute and chronic, and untreated acute illnesses can lead to chronicity. For example, glomerulonephritis along with glomerulonephropathies (e.g. diabetic nephropathy) are among the most common kidney disorders and can often lead to chronic kidney failure (Sabljar Matovinović, 2009). Other kidney disorders include nephritic syndrome for which hematuria and hypertension are typical. In contrast, nephrotic syndrome is characterized by its high proteinuria (more than 3.5 g /24 hours) and related swelling and hyperlipidemia (Elie et al., 2012). Another disease can be kidney failure, which is divided into the acute and chronic form. Acute failure results in a sudden deterioration in renal function accompanied by oliguria to anuria. This failure is reversible. Chronic renal failure is irreversible renal impairment, which is mainly shown as a decrease in glomerular filtration and can end up with the necessary dialysis or renal transplantation (Sabljar Matovinović, 2009).

1.3 Diabetes Mellitus

Diabetes mellitus is a heterogeneous disorder of the metabolism for which is typically an increase of blood glucose level. The cause of mentioned hyperglycemia is impaired insulin secretion, impaired insulin action or both (Kerner, 2014).

For diabetes mellitus four types are distinguished, the majority of cases is classified as type 1 or 2. Diabetes mellitus type 1 (DM1) is characterized by the fact that it is primarily the result of pancreatic beta-cell destruction followed by the deficiency of the insulin susceptible to ketoacidosis. This type usually occurs until the age of approximately 25 years. But latent autoimmune diabetes in adults (LADA), which appears at abnormally high age, is included in this type as well. The type 2 diabetes (DM2) may range from predominant insulin resistance with a relative insulin deficiency to the predominant secretory defect with the insulin resistance. It is associated with other problems so-called Metabolic syndrome in many cases (Punthakee et al., 2018). Another type is gestational diabetes mellitus in which glucose tolerance disorders appear for the first time or are diagnosed during pregnancy. The last group includes other specific types comprising a wide variety of relatively uncommon conditions, primarily specific genetically defined forms of diabetes or diabetes associated with other diseases (e.g. endocrinopathies - Cushing syndrome, diseases of the exocrine pancreas - pancreatitis, genetic defects of the β-cell function or insulin action, etc.) or drug use (e.g. glucocorticoids, neuroleptics) (Kerner, 2014).

Typical symptoms of diabetes mellitus include thirst, polyuria, weight loss or blurred vision. In its most severe cases, ketoacidosis or a non-ketotic hyperosmolar state can occur and lead to stupor, coma and, in absence of effective treatment, death (Alberti and Zimmet, 1998). The chronic hyperglycemia is associated with the long-term effects of diabetes mellitus. They include microvascular complications affecting the development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and neuropathy with risk of foot ulcers or amputation. Other complications can be a feature of autonomic dysfunction, including sexual dysfunction as well as to increase of the risk for cardiovascular disease due to atherosclerotic complications (Punthakee et al., 2018).

1.3.1 Diabetic nephropathy

With the increasing incidence of DM2, diabetic nephropathy (DN) is becoming a worldwide health problem. It is the main complication of long-standing uncontrolled DM which can lead to renal failure. DN is characterized by clinical symptoms as progressing proteinuria, hypertension and consecutive renal failure (Bose et al., 2017). The major vascular pathology of the kidneys is characterized by the glomerular dysfunction caused by hyperglycemia. The occurrence of proteins in the urine indicates the damage to the glomerular filtration barrier (Wang et al., 2017). Structural changes in the glomerulus, such as the increase of thickness of the basal membrane, mesangial expansion due to the accumulation of the extracellular matrix and cellular hypertrophy, leading to fibrosis and glomerular sclerosis (Sharma et al., 2003).

Depending upon the functional and structural changes that the progress of DN can be divided into five phases:

1. This stage is characterized by renal hypertrophy and glomerular hyperfiltration. The increase in filtration is usually 20-40%.
2. This phase is called latent because it is without any clinical symptoms. But increased glomerular filtration persists. The histological changes are also beginning to develop.
3. The incipient DN is characterized by albuminuria about 30-300 mg/24 hours and it is accompanied by increased blood pressure. The structural changes of kidney continue.
4. The manifest phase of DN is followed by albuminuria higher than 300 mg/24 hours. The velocity of glomerular filtration starts to decrease. Gradual impairment of renal function results in dialysis treatment.
5. Chronic renal failure is characterized by the decrease of velocity of glomerular filtration bellow 15 ml/min. Gradual impairment of renal function results in dialysis treatment (Tesař et al., 2002).

1.3.1.1 Glomerulus and diabetic nephropathy

During DN, several changes may occur in the glomerulus. For example, glomerular hypertrophy is probably caused by changes in glomerular hemodynamics in glomerular hyperfiltration (Wang et al., 2017). Another change is the increase in the thickness of the basal membrane which is a consequence of extracellular matrix accumulation and mesangial cell expansion (See the differences between healthy and

diabetic glomerulus in Figs. 9-10). These changes can lead to nodular sclerosis or even to glomerulosclerosis (Tervaert et al., 2010).

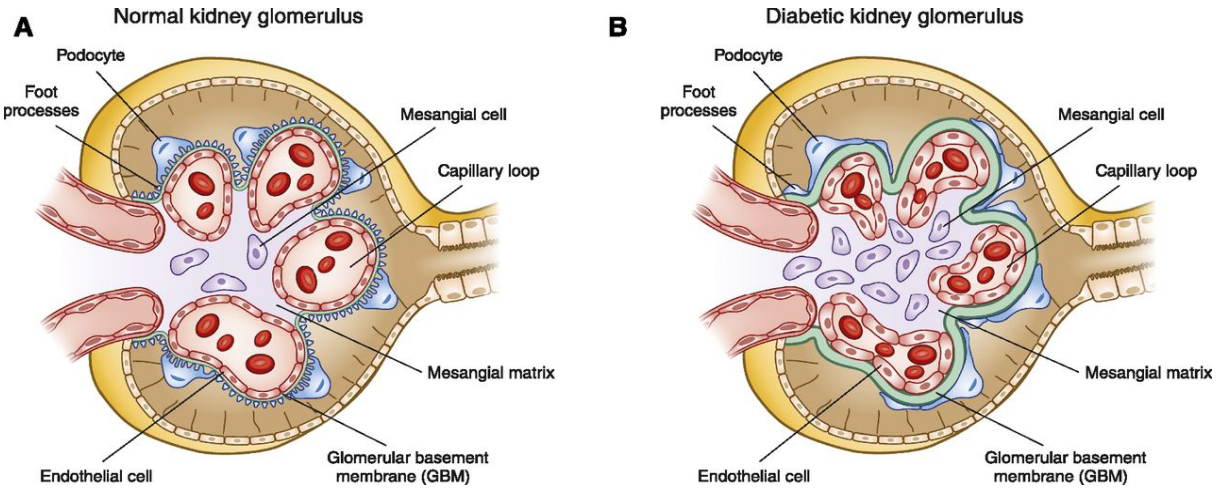

Figure 9 - Structure of glomerulus in diabetic nephropathy (Alicic et al. 2017)

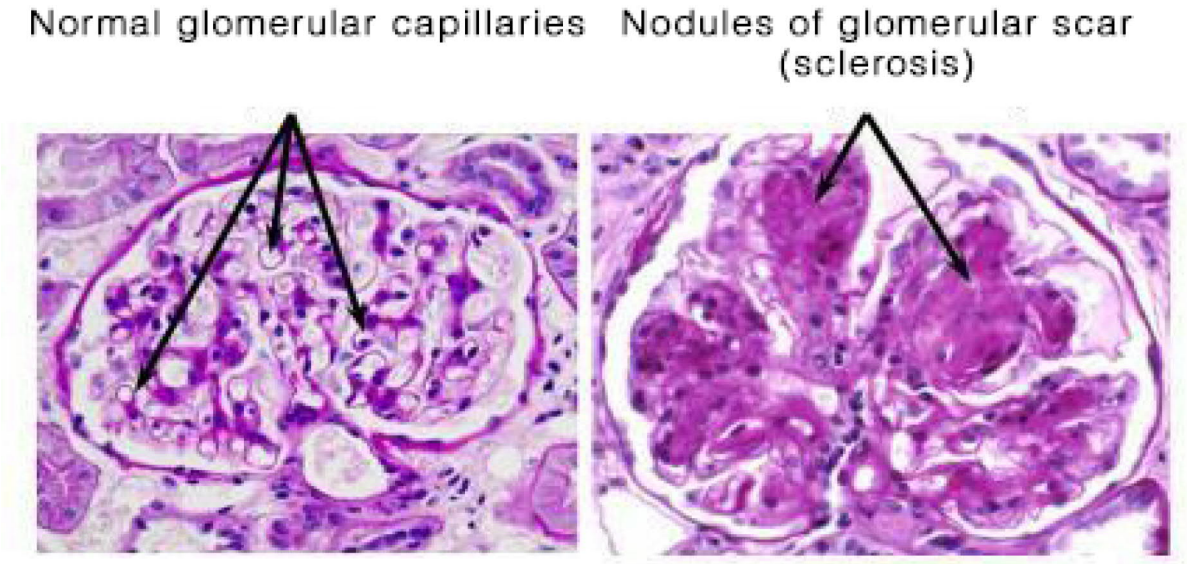

Figure 10 - Structure of glomerulus in diabetic nephropathy by light microscope (https://unckidneycenter.org/kidneyhealthlibrary/glomerular-disease/diabetes/)

1.3.1.2 Podocytes and diabetic nephropathy

Podocytes and their injury (podocytopathy) play an important role in the DN. The cell changes contribute to the glomerular hypertrophy, glomerulosclerosis and foot process effacement in DN. As a part of the glomerulus, podocytes enlarge their size as well. There is also a decrease in their number known as podocytopenia. It is caused primarily due to detachment and apoptosis of podocytes because then podocytes do not proliferate even in normal number. The detachment is associated with disruption

in integrin interaction which leads just to it. The apoptosis is induced by hyperglycemia and the lack of podocyte also leads to nodular and glomerular sclerosis at the later stages of DN. The foot process effacement is a phenomenon characterized by the loss of normal interdigitating pattern of foot process between the neighboring podocytes. It leads to the leakage of macromolecules such as albumin through the glomerular filtration barrier. These changes are directly partaking in the development of proteinuria and glomerulosclerosis (Bose et al., 2017).

1.4 E-Cadherin

E-Cadherin is predominant protein from the Cadherin group, which is localized in epithelial cells, where it plays an important role in maintaining renal epithelial polarity and integrity (Van Roy et al., 2008). It is transmembrane adhesion protein which forms the base of the adherens junction complex. The function of E-Cadherin is mediating the adhesion of neighboring cells via calcium-dependent interactions between their ectodomains. The cytoplasmic domain of Cadherin also interacts with the series of other regulatory proteins. Together, these Cadherin-interacting proteins mediate tissue morphogenesis (Jia et al., 2011). Also, E-Cadherin interacts with cytoskeleton via association with cytoplasmatic Catenin proteins (Reshetnikova et al., 2007).

1.5 Podocin

Podocin is a protein of the cytoskeleton which belongs to the Stomatin protein family. For this group is typical NH_2- and COOH- terminal intracellular domains of their chain and hairpin-like structure. Its molecular weight is approximately 42 kDa (Sellin et al., 2002). It is specific for mature podocytes. It is localized onto the glomerular podocyte foot process membrane at the insertion site of the slit diaphragm and regulates the function of the glomerular permeability (Schwarz et al., 2001). As well Podocin interacts with the COOH-terminal, cytoplasmic domain of Nephrin, and significantly elevates Nephrin-induced signaling (Nakatsue et al., 2005).

1.6 Vimentin

Vimentin is one of the cytoplasmic intermediate filaments in the cytoskeleton of the eukaryotic cell. It contributes to building a dynamic filament network and provides mechanical stability and upkeep of the cell shape (Buchmaier et al., 2013). Its molecular weight is about 57 kDa (Kusinska et al., 2009). It is a general marker of the cells of mesenchymal origin and vascular smooth muscle (Stamenkovic et al., 1986).

2 HYPOTHESES AND OBJECTIVES

From previous proteomic studies of the R. Bosch's laboratory group, BPA has been found to affect some proteins associated with cytoskeleton and cell adhesion. These studies were carried out on animal models of mice CD1. In this work, we focused on selected proteins and wanted to find out and check changes in their expression. We performed these tests on *in vivo* models (mice kidneys) and on *in vitro* models (human podocytes). Both of these models were compared to observe possible changes in selected proteins.

Objectives of this work:

- Review literature about Bisphenol A, E-Cadherin, Podocin, Vimentin - all associated with kidneys
- Practice the methods: Western Blot, Immunocytochemistry and Immunohistochemistry
- Detect the effect of BPA exposure on E-Cadherin, Podocin, Vimentin
- Compare the results of *in vivo* and *in vitro* models

3 MATERIALS AND METHODS

3.1 Biological material

3.1.1 Animal models

For the experiments, CD1 mice (25-30 g) were used. All studies were carried out in concordance with guidelines established by Institutional Animal Care and Use Committees at the University of Alcala (Olea-Herrero et al., 2014).

The mice were kept in the room of the controlled temperature at 21°C (+/- 2°C) with the cycle of 14h/10h - light/dark under the pathogen-free conditions. Mice were given *ad libitum* access to food and water. There were 4 groups of mice: control, treated with BPA, diabetic and diabetic treated with BPA. Each group was represented by 10 mice. The dose of BPA was 150 μg/ml and it was administered via oral way by the water. Diabetes was made up by three consequent daily intraperitoneal injections of Streptozotocin (Sigma, USA) in the dose of 65 mg/kg body weight solute in citrate buffer pH 4.5. The mice with glucose level in blood more than 300 mg/dl were included in the study. Animals were sacrificed under ether anesthesia with isoflurane (IsoFlo, Abbott Animal Health, UK) 6 weeks following the development of diabetes. The kidney of each mice was fixed in 10% formaldehyde and used for Immunohistochemistry experiments (Izquierdo et al., 2006).

3.1.2 Cell culture

All the work regarding of the cell culture was practiced in the laminar flow hood for securing a sterile environment. All the material was sprayed by 70% solution of ethanol before using to prevent any contamination.

The human podocytes which were used during the study were stored in the liquid nitrogen, therefore, it was necessary to thaw and resuspend them in the medium RPMI 1640 warmed up to 37°C. This immortalized cell line was first grown under the conditions contributing to the cell proliferation to reach the higher cell number. It means that the cells were stored in the 75 cm^2 cell culture flasks with standard RPMI 1640 medium supplemented by 10% FBS (fetal bovine serum) and 0.1% antibiotics, in the incubator at 33°C and 5% CO_2. The growth process and possible contamination were checked by light microscope. After the cell propagation reached approximately 80% confluence, the cells were trypsinized and shifted to the conditions supporting the

terminal differentiation for 15 days. These conditions are the growth in the same medium as previously but in the incubator at 37°C and 5% CO_2.

All the reagents which were used in this process were warmed up to the temperature of 37°C by the water bath.

For complete information about the reagents used during cell culture see Table 1.

Table 1 - Material used in cell culture

• **Complete RPMI 1640 medium (Sigma - life science, USA)**	500 ml RPMI 1640 medium 10 % FBS 0.1 % antibiotics (10 000 units penicillin, 10 mg streptomycin, 25 μg amphotericin B per 1 ml)
• **Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich, USA)**	
• **Trypsin/EDTA Solution for Primary Cells (Sigma-Aldrich, USA)**	

3.1.2.1 Cell line

The human podocytes used in this study were obtained using a nephrectomy of a 3-year-old child with minimal functional kidney, but the glomerular function was not damaged. Glomeruli were isolated and cultured from it. Grown epithelial cells were cultured for 10-14 days and then passaged and transfected with the tsSV40 gene construct (Saleem et al., 2002).

3.2 Biochemical methods

3.2.1 Western blot

Western blot (WB) is the analytical method widely used for the separation and detection of the proteins.

3.2.1.1 Seeding and treatment

Cultivated differentiated cells were equally distributed in the Petri dishes and medium added to a total volume of 8 ml. We prepared from each future sample 4 Petri dishes - in our case: Control, BPA 1 nM, BPA 10 nM, BPA 100 nM. These prepared cells were stored into the incubator at 37°C, 5% CO_2 for 3-5 days until they were ready

for the experiment. Their status was checked by the light microscope. It followed by cell treatment.

We prepared individual sample solutions using a solution of 43.8 mM BPA in absolute ethanol. From this solution, BPA 50 μM in DPBS was prepared and stepwise solutions of 1, 10 and 100 nM of BPA from it. The final sample's solutions were prepared with the medium. The volume of 8 ml from each sample was put to single Petri dishes for 24 hours into the incubator at 37°C, 5% CO_2. Then the liquid was removed, Petri dishes were washed with DPBS and all the liquid was removed from them.

The work in this phase of the experiment was practiced under the same conditions as the above-mentioned cell culture. All the material is summarized in Table 2.

Table 2 – Material for the seeding and treatment

• **Complete RPMI 1640 medium (Sigma - life science, USA)**	500 ml RPMI 1640 medium 0.5 % FBS 0.1 % antibiotics (10 000 units penicillin, 10 mg streptomycin, 25 μg amphotericin B per 1 ml)
• **Bisphenol A (BPA) (Aldrich, USA)**	1 nM BPA 10 nM BPA 100 nM BPA 50 μM BPA
• **Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich, USA)**	
• **Ethanol absolute PA (Panreac Quimica, Spain)**	

3.2.1.2 Cell lysis and protein extraction

The Cytobuster™ - Protein Extraction Reagent, which causes cell lysis, was added to each Petri dish in the volume of 60 μl. The cells were scraped by a scraper and transferred to the Eppendorf tube®. This lysate was centrifuged at 4°C, at 16000 rpm for 30 minutes. After centrifugation, the only supernatant was carefully transferred to the new Eppendorf and the samples were used immediately for protein quantification by Bradford test or kept at -20 °C for later use.

This whole step was done on the ice to protect the proteins.

3.2.1.3 Bradford assay

The Bradford assay (BA) was used to quantify the proteins in the sample. It is a spectrophotometric analytical method based on the binding of Coomassie Brilliant Blue to proteins, resulting in a visible change in color (from brown to blue). The intensity of the color depends on the amount of proteins.

For this test, we used the samples obtained from the previous extraction in 3 different concentrations: pure sample, dilution with water in a ratio of 1:4 and 1:9. A series of bovine serum albumin at different concentrations (Bio-Rad, USA) was used to prepare the calibration curve. The samples in the volume of 1 μl and 200 μl of Quick Start™ Bradford 1x Dye Reagent (Bio-Rad, USA) were added to each well. All the samples were prepared in triplicates and the absorbance was measured on the spectrophotometer at the wavelengths of 595 nm (See in Fig. 11).

Based on the BA results, samples of the same protein concentration were prepared, which are then used for the WB itself.

All the chemical reagents which were used in these steps, you can see in Table 3.

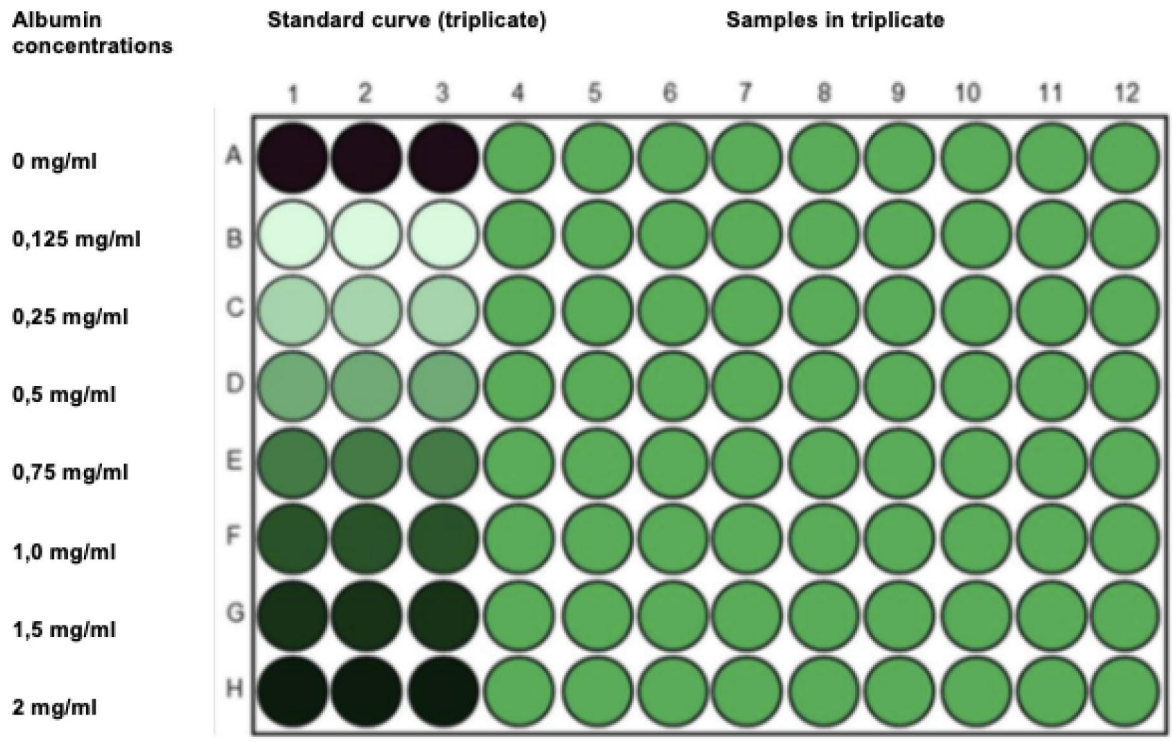

Figure 11 - Bradford assay

Table 3 - Chemical reagents used in cell lysis, extraction and Bradford assay

• **Cytobuster™ - protein extraction reagent (Novagen, USA)**	
• **Series of Bovine serum albumin (BSA) (Bio-rad, USA)**	BSA 0.125 mg/ml BSA 0.25 mg/ml BSA 0.5 mg/ml BSA 0.75 mg/ml BSA 1 mg/ml BSA 1.5 mg/ml BSA 2 mg/ml
• **Quick Start™ Bradford 1x Dye reagent (Bio-Rad, USA)**	

3.2.1.4 SDS-PAGE electrophoresis

SDS-PAGE electrophoresis was used to isolate proteins based on molecular weight. In this case, a 12% separating gel was used due to the molecular weight of 57 kDa proteins (Vimentin) and 42 kDa (Podocin) and for WB with E-Cadherin (120 kDa) we prepared 10% separating gel. The freshly prepared gels were inserted into the an electrophoretic chamber with electrophoretic buffer. The samples, consisting of 20 μg of the proteins supplemented with distilled water and the solution of β-mercaptoethanol (Sigma, USA) and 4x Laemmli sample buffer (Bio-Rad, USA) in the ratio of 1:9, were added to each slot of the stacking gel. As molecular weight size marker was used Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA) in a volume of 6 μl. These samples were denatured in a dry bath at 100°C for 5 minutes before use. The electrophoresis itself took place in 2 parts under different conditions. The polyacrylamide gel was placed in a chamber containing electrophoresis buffer. This buffer contained 25 mM Tris; 192 mM glycine; 0.1% (w/v) SDS (Sodium dodecyl sulfate); pH 8.3. In the first part when the samples were going from the stacking gel into the separating gel the voltage was 90 mV, approximately 20 minutes. After the samples passed into the separating gel, the voltage was changed to 120 mV. The separation proceeded until the samples pass through the whole gel. It took approximately 1 hour (The process of SDS-PAGE electrophoresis is depicted in Fig. 12).

All the chemical components used in the electrophoresis are summarized in Table 4.

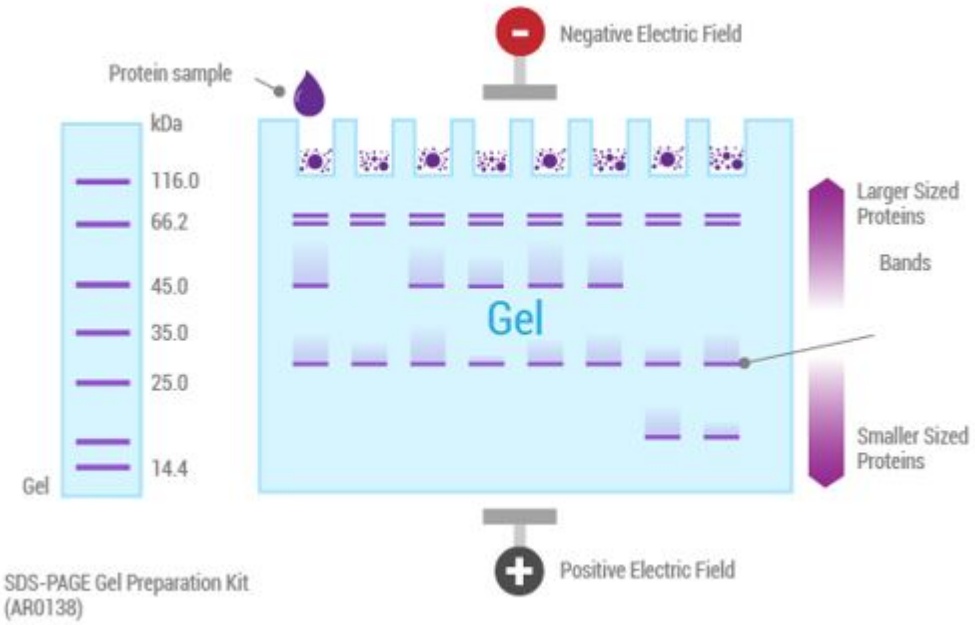

Figure 12 - Electrophoresis process (https://www.bosterbio.com/images/wb-sds-page.JPG)

Table 4 - Reagents used in electrophoresis

• **Separating gel (10 %)**	2.24 ml Separating gel buffer (Tris HCl 1.5 M, pH 8.8) 6.224 ml Acrylamide 30% 84.4 μl SDS 20% (Sodium dodecyl sulfate) (Panreac Quimica SA, Spain - Barcelona) 120 μl APS 10% (Ammonium persulfate) (Bio-Rad, Japan) 3.4 μl TEMED (Tetramethylethylenediamine) (Sigma - life science, USA) 8.444 ml Distilled water
• **Separating gel (12%)**	2.24 ml Separating gel buffer 7.98 ml Acrylamide 30% 84.4 μl SDS 20% 120 μl APS 10% 3.4 μl TEMED 7.19 ml Distilled water
• **Stacking gel**	1.2 ml Stack gel buffer (Tris HCl 0.5 M, pH 6.8) 1.2 ml Acrylamide 30% 134.4 μl APS 10% 3.44 μl TEMED 6.72 ml Distilled water
• **Sample buffer**	4x Laemmli sample buffer (Bio-Rad, USA) β-mercaptoethanol (Sigma-Aldrich, USA) in ratio 9:1
• **Electrophoresis buffer**	10x TGS (25 mM Tris HCl; 192 mM Glycine; 0.1% (w/v) SDS; pH 8.3) (Bio-Rad, Germany) Distilled water in ratio 1:9

3.2.1.5 Transfer to the membrane

The PVDF membrane was activated in methanol and distilled water. Then 4 pieces of Whatman's paper, activated membrane, gel with proteins (only the part with separating gel) and another 4 pieces of Whatman's paper were gradually stacked in the transfer machine (See the process of the transfer in Fig. 13), a semidry method was used. All the layers were soaked in the transfer buffer and placed on top of each other so that bubbles are not between the layers.

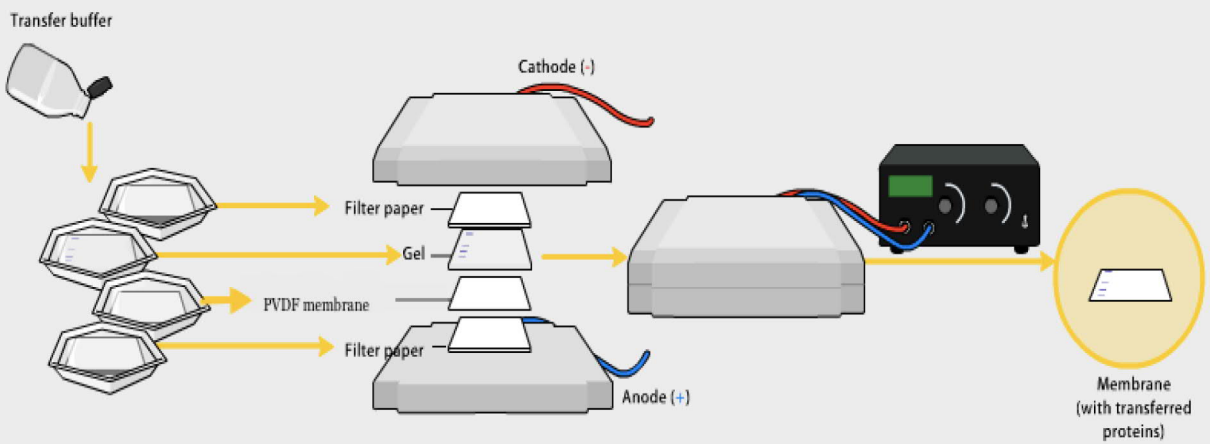

Figure 13 - Transfer to the membrane (https://en.wikibooks.org/wiki/Molecular_Cloning/western_blot)

3.2.1.6 Blocking

The membrane with transposed proteins was incubated in 5% non-fat milk solution in Tween - Tris buffered saline (TTBS) to block places where the proteins did not bind. After addition of the antibody, its molecules bind specifically only to blotted proteins.

3.2.1.7 Immunodetection

The blocking was followed by the immunodetection. But first, the membrane was washed three times with TTBS for 5 minutes and incubated with the primary antibody overnight at 4 °C on a shaker. After that washing again in the same manner with TTBS and incubating with the secondary antibody for 1 hour at room temperature on a shaker. Afterwards, it was washed again with TTBS and incubated for 1 minute in Luminata™ Crescendo Western HRP Substrate (Millipore Corporation, USA). The protein detection was performed on Fusion Solo S. After the measurement, the membrane was incubated in Coomassie solution for 15 minutes and washed several times with TTBS. It was performed as control of loading the proteins that could not be

done in the usual way - for example employing detection of actin or tubulin because BPA also affects their levels.

The reagents used in the steps of the transfer, blocking and immunodetection are mentioned in Table 5.

Table 5 - Reagents and solution used in the transfer, blocking and immunodetection

• **Transfer buffer**	100 ml 10x TG (25 mM Tris, 192mM Glycine, pH 8.3) (Bio-rad, Germany - Münich) 200 ml Methanol 700 ml Distilled water
• **Immun-Blot® PVDF membranes for Protein Blotting (Bio-Rad, USA)**	
• **5% non-fat milk solution**	0.5 g Milk casein 9.5 ml T-TBS
• **Tween - Tris buffered saline 1x (T-TBS 1x)**	50 ml TBS 10x, pH 7.6 (24.2 g Tris HCl, 80.0 g Sodium chloride, 895.8 g Distilled water) 0.5 ml Tween 20 (Sigma - Aldrich, USA) 450 ml Distilled water
• **Luminata™ Crescendo Western HRP Substrate (Millipore Corporation, USA)**	
• **Methanol Pure (Panreac Quimica, Spain)**	
• **Coomassie solution**	125 mg Brilliant Blue R (Sigma, USA) 250 ml Methanol 25 ml Acetic acid (Montplet & Esteban SA, Spain) 225 ml Distilled water

3.2.1.8 Antibodies used in Western Blot

All the antibodies which we used for WB, are summarized in Table 6.

Table 6 - Antibodies used in Western Blot

Antibody	Molecular weight	Source of antibody	Company	Dilution
E-Cadherin	120 kDa	Mouse, monoclonal	DB Transduction Laboratories™	1:1000 T-TBS
Podocin	42 kDa	Rabbit, polyclonal	Abcam	1: 5000 T-TBS
Vimentin	57 kDa	Mouse, monoclonal	Santa Cruz Biotechnology	1:500 T-TBS
Goat Anti-Rabbit IgG	-	Rabbit, polyclonal	Invitrogen	1:10 000 T-TBS
Goat Anti-mouse IgG	-	Mouse, polyclonal	Invitrogen	1:10 000 T-TBS

3.2.2 Immunocytochemistry

Immunocytochemistry (ICC) is a laboratory technique used to confirm the expression and location of specific peptides or protein antigens in the cells by special combinations of the antibodies and target molecules.

3.2.2.1 Seeding and treatment

The differentiated human podocytes were seeded in the amount of 6000 cells/well (1 ml) to 24 well plate with cover slips. We counted the cells by Neubauer chamber and they were diluted in the RPMI 1640 medium with 0.5% FBS and 0.1% antibiotics. The plate with the cells was stored in the incubator (37 °C, 5% CO_2) approximately 3-5 days. When the cells were ready for the experiment, the treatment was performed with BPA 100 nM for 48 hours. Half of the wells were control samples and the other half was treated by BPA solution. This plate was stored in the incubator under the same conditions which were previously mentioned, during the treatment. After 48 hours, the media was removed, the samples were washed with DPBS solution and the cells were fixed with 4% paraformaldehyde solution for 10 minutes. Then the

fluid was changed to DPBS solution and the plate was stored at 4 °C until the further use.

3.2.2.2 Permeabilization

This step, which involves permeabilization of the cell wall, is important in the expression of proteins within the cells. The 0.5% solution of Triton X-100 in DPBS was used for 10 minutes to achieve permeability. After the time has passed, the samples were washed three times for 5 minutes with DPBS solution.

See Table 7 for the information about all material and chemical reagents which were used in these steps of ICC.

Table 7 - Media, reagents and solutions used in seeding, treatment and permeabilization

• **Complete RPMI 1640 medium**	500 ml RPMI 1640 medium 0.5% FBS 0.1% antibiotics
• **BPA 100 nM**	BPA (Aldrich, USA - St. Louis) RPMI 1640 medium
• **Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich, USA)**	
• **Paraformaldehyde 4% (Panreac Quimica, Spain)**	
• **Triton X 100 0,5%**	50 μl Triton X 100 (Sigma-Aldrich,USA) 10 ml PBS

3.2.2.3 Blocking and incubation

The blocking of non-specific binding of the antibodies was performed with a solution of 5% NDS in DPBS for 30 minutes. It was followed by the incubation with the primary antibody overnight at 4 °C. The concentration of the primary antibody was for Vimentin 1:500 and for Podocin 1:400. Antibodies were diluted in the DPBS containing 5% NDS. The total volume of the primary antibody solution used for one sample was 40 µl. The solution was removed and the cells were washed 3x DPBS for 5 minutes.

Then the incubation with secondary antibody conjugated with fluorochrome was performed if the subsequent detection was done by a confocal microscope. As the fluorochromes were used anti-mouse Alexa 568 (red color) and anti-mouse Alexa 488

(green color). Amount of 1 μl of the antibodies was diluted in 1000 μl of DPBS and NDS 5% solution. To each sample was added 40 μl of the antibody solution and allowed to incubate for 1 hour at room temperature. After that, the samples were washed by DPBS three times for 5 minutes. The prolong gold antifade reagent with DAPI was added to each of them and the coverslips were put onto microscope slides.

If we used the light microscope for the detection, we used the Ultravision Quanto Detection System kit after the incubation with the primary antibody. The primary antibody amplifier was used as the first from the kit for 10 minutes. Thereafter, the cells were washed by DPBS again 3 times for 5 minutes. In the next step, the polymer Quanto-HRP was used for 10 minutes and then washed again with DPBS. After that, the solution of the chromogen DAB concentrate and DAB substrate buffer was added for approximately 3-4 minutes until the cells became brownish. This solution is composed of 1 drop of DAB concentrate per 1 ml of DAB buffer. Distilled water was used to stop the reaction. Then the DAB enhancer was used for 1 minute and the reaction was stopped by the distilled water again. Thereafter the samples were dropped by the Hematoxylin Carrazzi's solution to color cell nuclei to blue. The chemical reaction took only a few seconds and ended again by distilled water. Then samples were washed with lots of ethanol solutions with different concentrations (70, 80, 90, 100º) and xylene. Each washing took 3 minutes. As the last step, we put Entellan® new-rapid mounting microscopy medium onto the microscope slide and enclosed the coverslips with the cells.

For detailed information about used reagents and solutions see Table 8.

Table 8 - Reagents and solutions used in blocking and incubation with antibodies

• **5% Normal donkey serum (NDS) (Millipore Corporation, USA)**	0.5 ml NDS 9.5 ml PBS
• **Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich, USA)**	
• **Ultravision Quanto Detection System (peroxidase) - including DAB chromogen (Master diagnóstica, Spain)**	Primary antibody amplifier Quanto-HRP DAB enhancer DAB Quanto chromogen DAB Quanto substrate (DAB = diaminobenzidine)
• **Prolong gold antifade reagent with DAPI (ThermoFisher Scientific, Spain)**	
• **Hematoxylin Carrazzi's solution**	25 g aluminum sulfate 0.1 g potassium iodate 0.5 g hematoxylin 100 ml glycerin 400 ml distilled water
• **Ethanol in the various concentration (70, 80, 90, 100%)**	Ethanol absolute (Panreac Quimica, Spain) Distilled water
• **Xylene (Panreac Quimica, Spain)**	
• **Entellan® new-rapid mounting microscopy medium (Merck, Germany)**	
• **Distilled water**	

3.2.2.4 Antibodies used in Immunocytochemistry

Information about the antibodies which were used in ICC are summarized in Table 9.

Table 9 - Antibodies used in Immunocytochemistry

Antibody	Molecular weight	Source of antibody	Company	Dilution
E-Cadherin	120 kDa	Mouse, monoclonal	BD Transduction Laboratories™	1:200 NDS 5% in DPBS
Podocin	42 kDa	Rabbit, polyclonal	Abcam	1:400 NDS 5% in DPBS
Vimentin	57 kDa	Mouse, monoclonal	Santa cruz Biotechnology	1:500 NDS 5% in DPBS
Anti-mouse Alexa 568	-	Mouse	Invitrogen, USA	1:1000 NDS 5% in DPBS
Anti-mouse Alexa 488	-	Mouse	Invitrogen, USA	1:1000 NDS 5% in DPBS

3.2.3 Immunohistochemistry

3.2.3.1 Fixation and embedding the tissues, cutting the sections

The samples of kidneys were rinsed twice in DPBS and fixed in 10% solution of formaldehyde in DPBS for 24 hours at room temperature. It was followed by dehydrating the samples and embedding them in paraffin. After that, we cut 5 μm thick sections with a stainless steel blade and mounted them on 3-aminopropyl-triethoxysilane (TEPSA)-coated slides. The samples had to be dewaxed by the series of xylene and ethanol solutions in the various concentrations (70, 80, 90, 100º) which were used from the most concentrated to the most diluted solution. After all of that, the samples were washed by water to rehydrate the sections.

Solutions used in this phase of immunohistochemistry (IHC) are mentioned in Table 10.

Table 10 - Sample preparation

• **Dulbecco's Phosphate buffered saline (DPBS) (Sigma-Aldrich, USA)**	
• **Formaldehyde 10 % (Panreac Quimica, Spain)**	
• **Paraffin (Panreac Quimica, Spain)**	
• **Xylene (Panreac Quimica, Spain)**	
• **Ethanol in the various concentration (70, 80, 90, 100%)**	Ethanol absolute (Panreac Quimica, Spain) Distilled water

3.2.3.2 Antigen retrieval

The samples were put in a conventional pressure cooker with 2l of 0.01M citrate buffer pH 6. It was heated at the highest pressure for 2 minutes and stand for 20 minutes. Afterwards, rinse the samples with water for 5 minutes. Block the endogenous peroxidase activity by incubating the sections in 0.3% H_2O_2 for 15 min at room temperature and then washed in distilled water and TBS. Both of the washing took 5 minutes.

See Table 11 for all the information about used reagents in the step of antigen retrieval.

Table 11 - Reagents and solutions used in retrieving of the antigen

• **Citrate buffer 0.01 M, pH 6**	164 ml 0.1 M Sodium citrate 36 ml 0.1 M Citric acid 1800 ml Distilled water
• **Distilled water**	
• **Hydrogen peroxide 3.3%**	20 ml 33.3% Hydrogen peroxide 180 ml Distilled water
• **0.05M Tris buffered saline pH 7.4**	6.05 g Tris HCl (Merck, Germany) 8.5 g Sodium chloride 1000 ml Distilled water

3.2.3.3 Immunohistochemical staining

The sections were incubated in TBS containing 3% NDS and 0.05% Triton X-100 for 30 minutes.

The primary antibody solution, in ratio 1:400 diluted in TBS with 0.3% NDS and 0.005% Triton X-100, was added to each slide in the amount of 40 μl overnight at 4°C followed by washing in TBS twice for 10 minutes.

Then we used the Ultravision Quanto Detection System kit. The primary antibody amplifier was used for 10 minutes. Thereafter, the slides were washed by TBS again 3 times for 8 minutes. In the next step, the polymer Quanto-HRP was used for 10 minutes and then washed again with TBS in the same manner. After that, the solution of the chromogen DAB concentrate and DAB substrate buffer was added for approximately 3 - 4 minutes until the sections became brownish. The coloration was checked by the light microscope. This solution is composed of 1 drop of DAB concentrate per 1 ml of DAB buffer. Distilled water was used to stop the reaction. Then the DAB enhancer was used for 1 minute and the reaction was stopped by the distilled water again.

Thereafter the samples were counterstained with Carazzi's Hematoxylin, washed with water, dehydrated, cleared in xylene and mounted with Entellan®.

For the information regarding the used reagents and solutions see Table 12.

Table 12 - Reagents and solutions used in immunohistochemical staining

• **TBS containing 5% Normal donkey serum (NDS) and 0.05% Triton X 100 (Tx 100)**	50 μl Triton X 100 0.3 ml NDS 9.7 ml PBS
• **TBS containing 0.5% Normal donkey serum (NDS) and 0.005% Triton X 100 (Tx 100)**	TBS containing 5% NDS and 0.05% Tx 100 and TBS in ratio 1:9
• **Ultravision Quanto Detection System (peroxidase) - including DAB chromogen (Master diagnóstica, Spain -)**	Primary antibody amplifier Quanto-HRP DAB enhancer DAB Quanto chromogen DAB Quanto substrate (DAB = diaminobenzidine)

The rest of the reagents were described previously at the chapter *3.2.2.3 Blocking and incubation.*

3.2.3.4 Antibodies used in immunohistochemical method

The details about the antibodies used in IHC are summarized in Table 13.

Table 13 - Antibodies used in immunohistochemistry

Antibody	**Molecular weight**	**Source of antibody**	**Company**	**Dilution**
E-Cadherin	120 kDa	Mouse, monoclonal	BD Transduction Laboratories™	1:200 0.3% NDS, 0.005% Tx-100 in TBS
Podocin	42 kDa	Rabbit, polyclonal	Abcam	1:500 0.3% NDS, 0.005% Tx-100 in TBS
Vimentin	57 kDa	Mouse, monoclonal	Santa Cruz Biotechnology	1:400 0.3% NDS, 0.005% Tx-100 in TBS

3.3 Laboratory equipment

For the information about used material and laboratory equipment see Table 14.

Table 14 - Laboratory equipment

Name	Company
Laminar box TELSTAR PV - 30/70	TELSTAR
Incubator NUAIRE Autoflow	NUAIRE
Light microscope - Nikon Eclipse TS 100	Nikon
Light microscope - Leica DM 1000 equipped with a digital camera (OptikaM®, Italy)	Leica Microsystem
Confocal microscope LEICA TCS-SL	Leica Microsystem
Water bath Precisterm	P Selecta
Centrifuge Heraeus Sepatech, Megafuge 1.0 R	Heraeus
Centrifuge Biofuge fresco	Heraeus
Power Pac™ Basic	Bio-Rad
Absorbance reader BioTek ELx 800	BioTek Instruments, inc.
WB detection machine Fusion Solo S	Vilber Lourmat
Shaker DRS-12, Sky line	ELMI
Mini - Shaker PSU-2T	Biosan
Shaker Reax 2000	Heidolph
Dry bath Grant Boekel BBA	LAB-CENTER, S.L.
Microm HM 310	Microm
Termofin	P Selecta
Laboratory Hood Flores Valles, 1800 SQ, V 124	Flores Valles

Scales Sartorius BP 121S	Sartorius
Automatic pipetes	Boeco
Pipettes (2, 5, 10 ml)	Deltalab S.L.
Flask	Cellstar®
Flacons	Dismadel
Pasteur pipettes	Dismadel
Petri dishes	Cellstar®
Well plate (24,96)	Cellstar®
Eppendorf	Daslab®
Refrigators (4°C, -20°C,-80°C)	Liebherr

3.4 Software

- ImageJ
- Microsoft Excel
- Vision Capt
- Las X software
- GraphPad Prism 7.0

3.5 Statistics

Data are reported as the mean ± SEM. Normal distribution was assessed by the Kolmogorov-Smirnov test. To determine the effects of BPA, one-way ANOVA or Kruskal-Wallis followed by a Bonferroni or Dunns test, respectively, was carried out. The p values presented in figures and tables corresponded to post hoc test. All statistical analyses were performed using the GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

4 RESULTS

4.1 Kidney's changes

4.1.1 Microscopic structure

In both cases - female, male - there are visible differences by comparison with the control samples. Diabetic mice samples showed an extreme extension of Bowman's capsule and convoluted tubules. BPA treated mice had altered renal corpuscles and dilated convoluted tubules. In case of diabetic mice treated by BPA, the results showed mesangial expansion and also some renal corpuscles were destroyed and the epithelium in the tubules was damaged (See the effect of BPA on the mice kidneys in Fig.14).

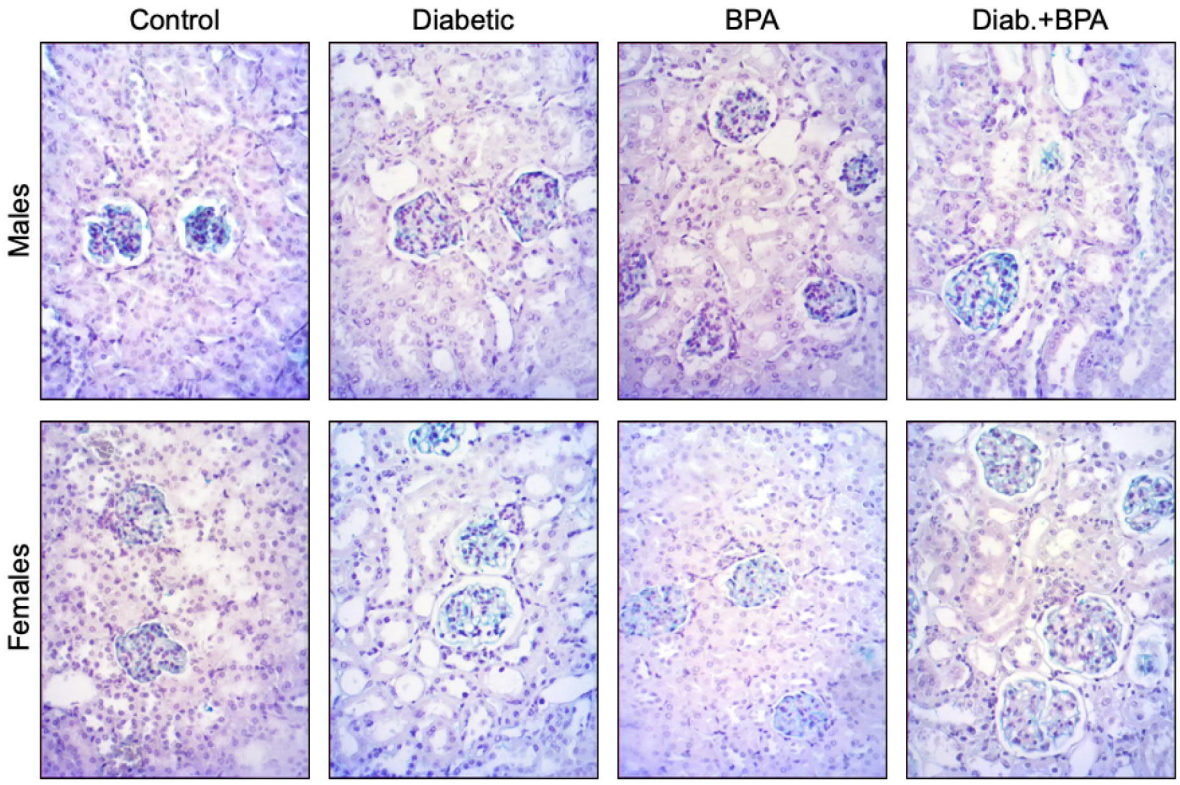

Figure 14 - Immunohistochemistry of mice kidneys (Used zooming 200x)

4.1.2 Podocyte's number

In both diabetic groups, there was a reduction in the number of podocytes and renal corpuscles. Higher damage was shown in the samples of mice treated by BPA. The diabetic group treated by BPA showed a lower number of podocytes as well. From a gender perspective, results in male samples were found to correspond to the above-mentioned facts, but female mice did not have a significant difference between the

BPA-treated group and the diabetic group treated with BPA (For the results look at the Figs. 15 and 16).

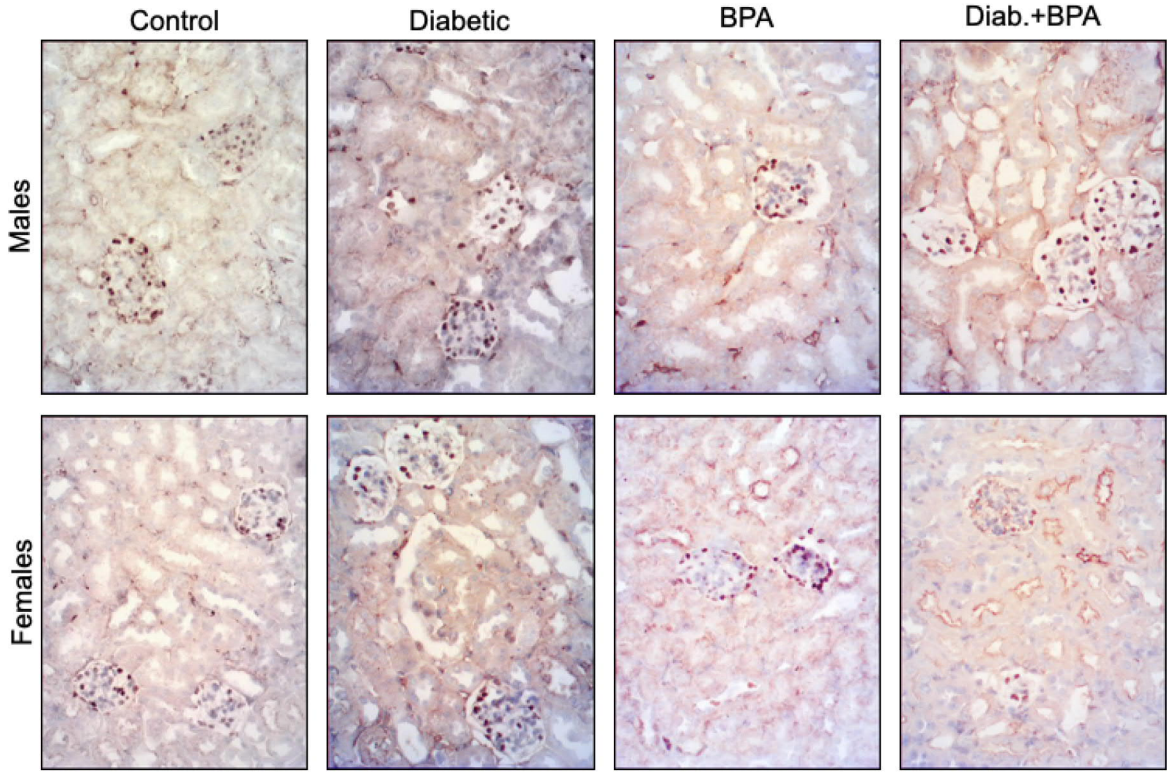

Figure 15 - The number of podocytes (Podocytes were marked by the specific marker WT-1 and the counted at 10 renal corpuscles in 10 sections of kidney in each animal. Used zooming 200x)

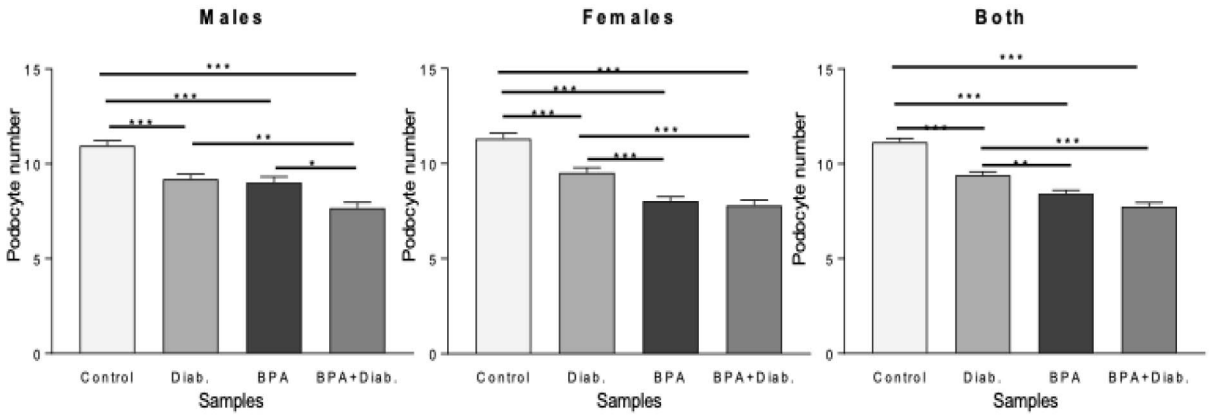

*Figure 16 - The Graphs of podocyte's number (*p<0.05, **p<0.01 and ***p<0.001 using ANOVA test for the comparison between control and BPA-treated cells.)*

The reduction of podocyte number, size and function leads to progressive glomerulosclerosis in glomerular diseases. The decrease of podocytes is associated with hypertrophic podocyte stress and podocyte detachment and glomerular tuft collapse (Kikuchi et al., 2017).

4.2 E-Cadherin

4.2.1 Western Blot

The results of WB have approved an expected decrease of E-Cadherin in all concentrations of BPA. The effect of BPA has not increased exponentially by the dose of BPA (See Fig. 17). This fact is typical for BPA because it is nonmonotonic compound (Vom Saal and Myers, 2008).

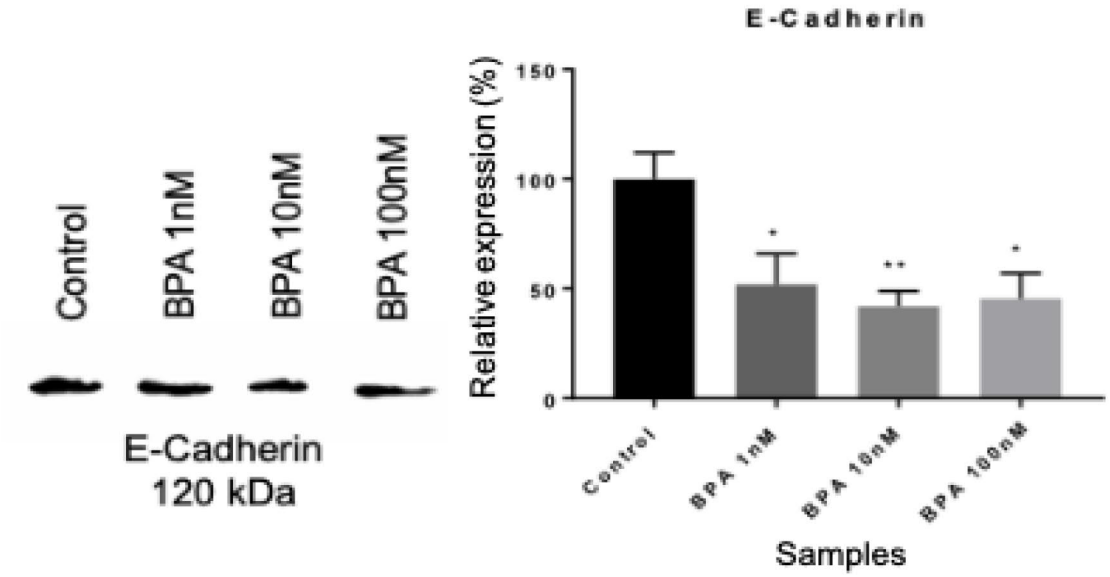

*Figure 17 – Western Blot results of E-Cadherin (Data are the means ± SD of three different experiments, each performed in duplicate. *p<0.05 and **p<0.01 using ANOVA test for the comparison between control and BPA-treated cells.)*

4.2.2 Immunocytochemical method with fluorochrome indicator

By using the ICC method, we expected a decrease in protein and its occurrence in the cytoplasmic membrane because it is an adhesion type of protein. The drop actually occurred, but the location of E-cadherin (green color) was in both the membrane and the cytoplasm and near the nucleus (blue color) (The results are shown in Fig. 18).

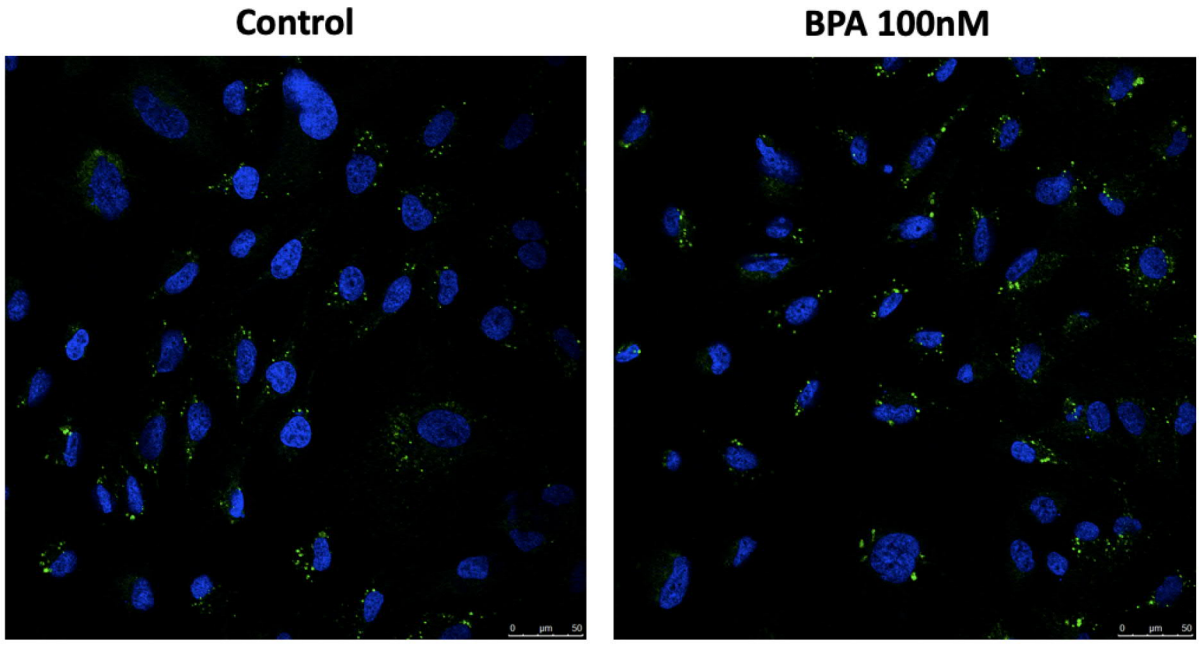

Figure 18 – Immunocytochemistry results of E-Cadherin with the fluorochrome indicator

4.2.3 Immunocytochemistry with DAB indicator

Even when using DAB as an indicator in ICC, there was a decrease in the total amount of E-cadherin, indicating as a lower saturation of the brown color (See Fig. 19).

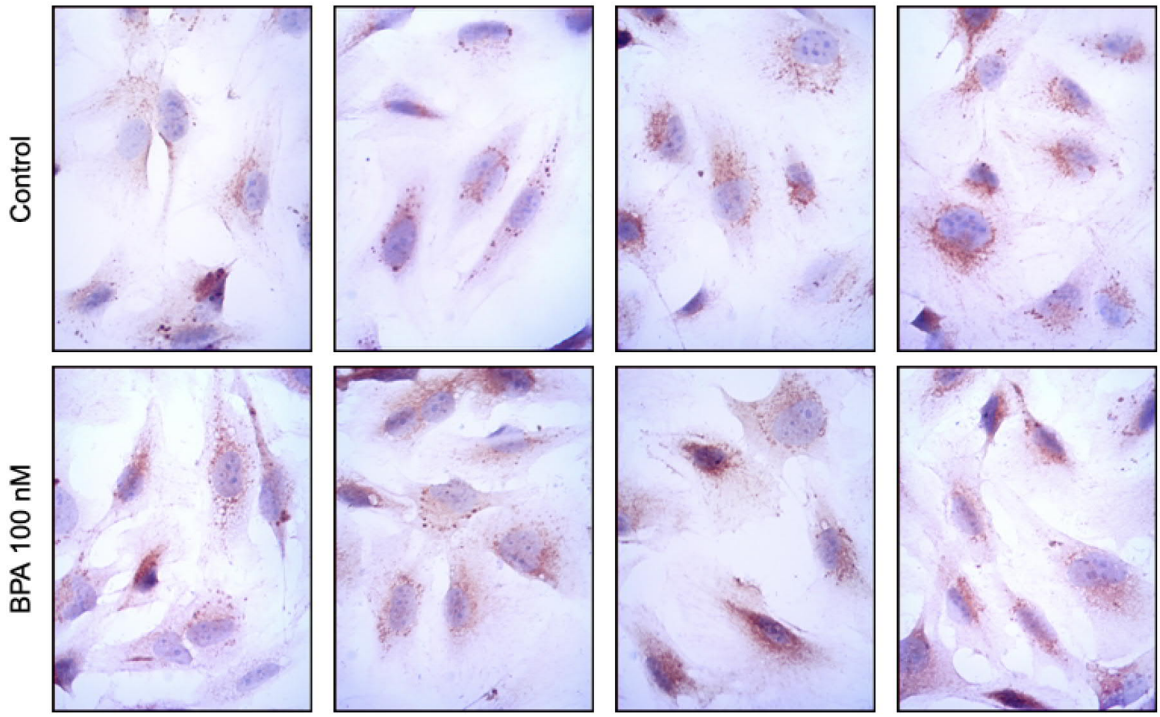

Figure 19 - Immunocytochemistry results of E-Cadherin with DAB indicator (Used zooming 200x)

4.2.4 Immunohistochemistry

When observing the effects of BPA on E-Cadherin, we could see similar results to the results of IHC mentioned above (See Fig. 14). Especially the enlargement of tubules and Bowman's capsule, since by the loss of E-cadherin has caused that cells were not so close together as they should be (The results are depicted in Fig. 20).

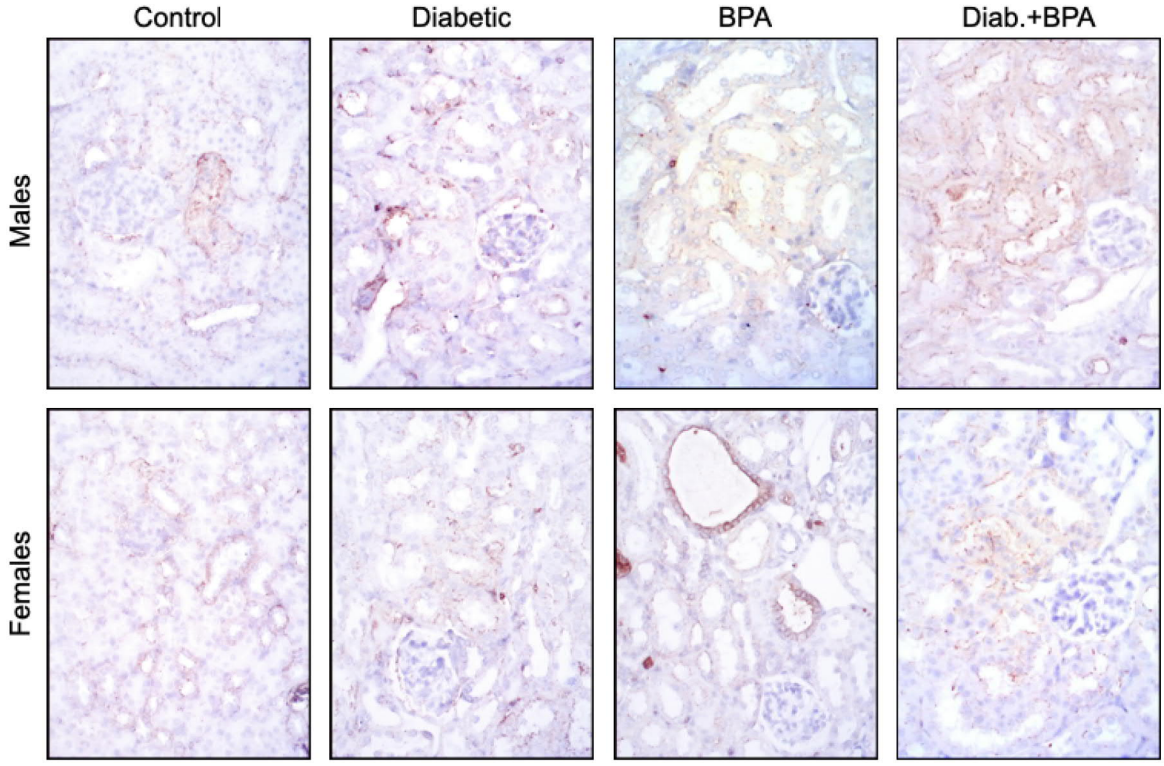

Figure 20 – Immunohistochemistry results of E-Cadherin (Used zooming 200x)

4.3 Podocin

4.3.1 Western Blot

Even in Podocin results, we observed the trend of decrease due to BPA exposure. A more pronounced decline was seen especially in 100 nM BPA solution (Look at the Fig. 21).

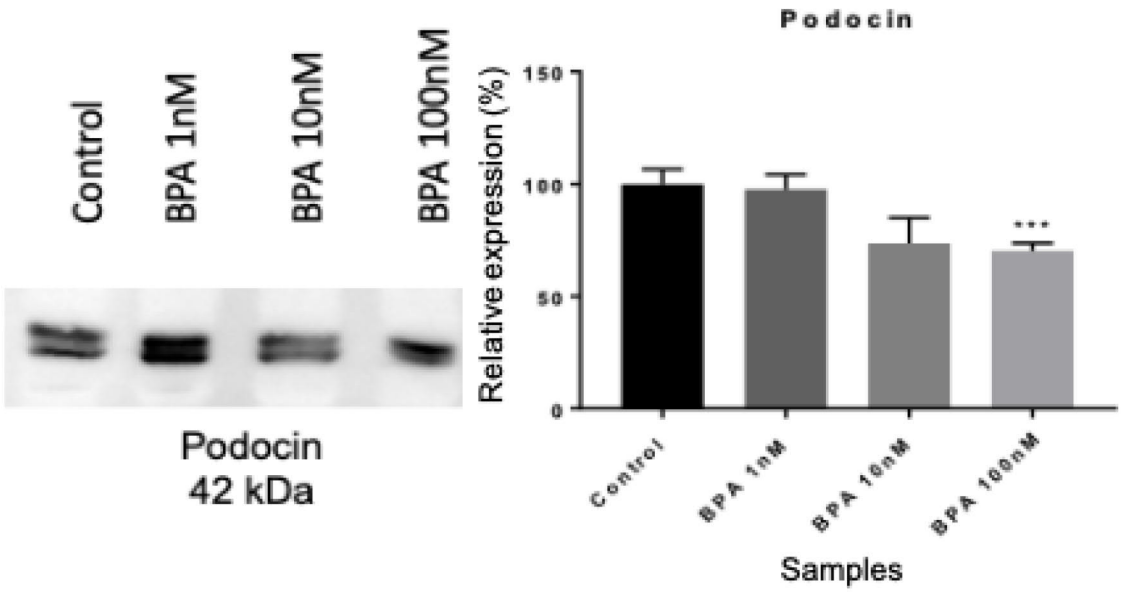

*Figure 21 – Western Blot results of Podocin (Data are the means ± SD of three different experiments, each performed in duplicate. ***p<0.001 using ANOVA test for the comparison between control and BPA-treated cells.)*

4.3.2 Immunocytochemistry with fluorochrome indicator

When using ICC with fluorochrome indicator, the amount of Podocin was reduced after BPA treatment. The occurrence of the protein was also further from the nucleus compared to the control samples (The differences are seen in Fig. 22).

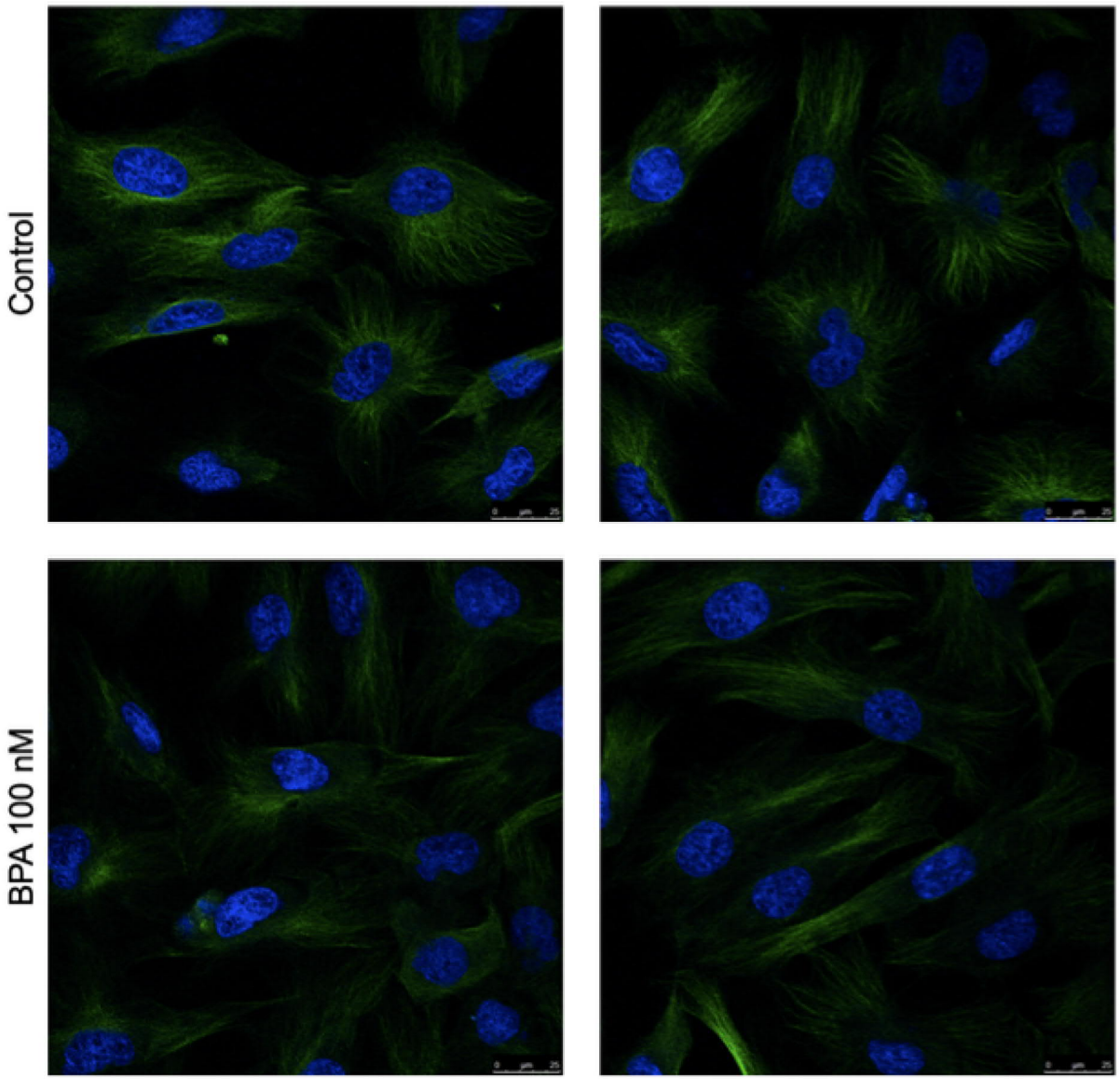

Figure 22 - Podocin results of immunocytochemical method with fluorochrome indicator

4.3.3 *Immunocytochemistry with DAB indicator*

As well as WB results, when using the ICC with the DAB indicator, there was a significant decrease of Podocin caused by BPA 100 nM exposure, resulting in a disruption of the filtration membrane function (See Fig. 23).

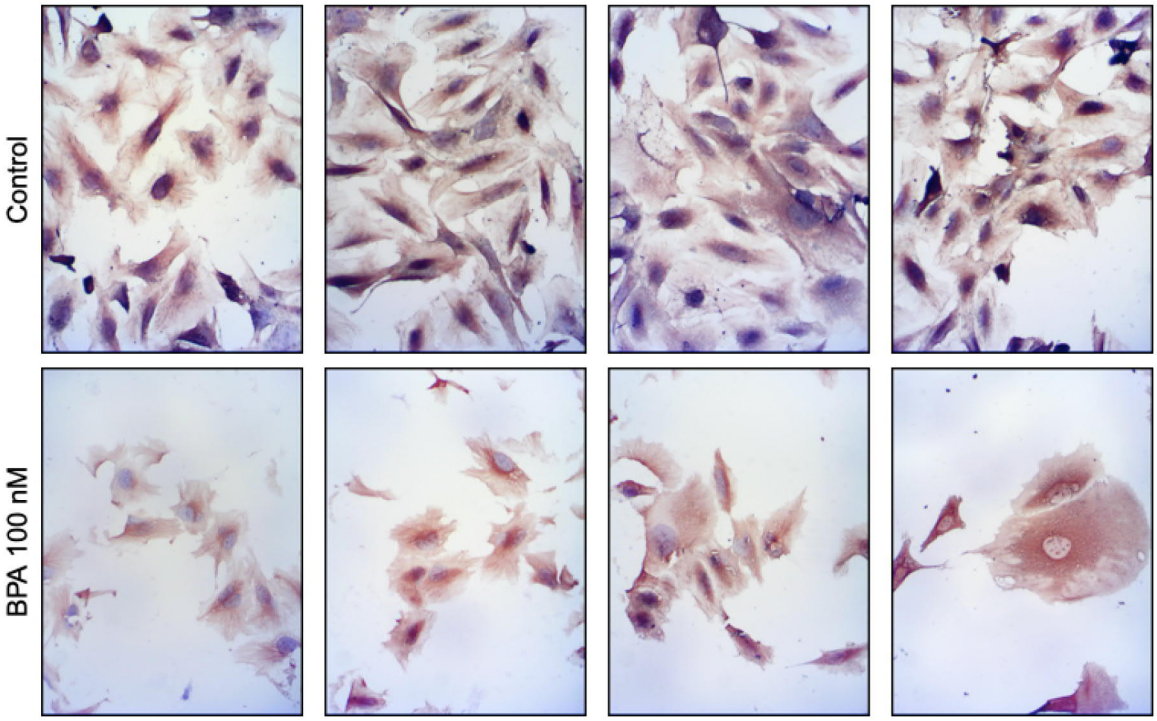

Figure 23 - Immunocytochemistry with DAB indicator - results of Podocin (Used zooming 200x)

4.3.4 *Immunohistochemistry*

The IHC test is not suitable for observing the decrease of Podocin in the podocytes. The negative effect of BPA 100 nM on podocytes, causing their damage and decline, can be observed using WT-1. Podocytes express podocyte-specific markers such as WT-1 or Nephrin which do not appear in other nephron cells. Wilm's tumor suppressor 1 (WT-1) is a transcription factor specifically expressed in podocyte's nuclei (Kreidberg, 2003). It plays an important role in the maintenance of podocyte function, in cell proliferation and mesenchymal-epithelial balance in the normal development of the cells (Saleem, 2011).

4.4 Vimentin

4.4.1 Western Blot

BPA exposure revealed a tendency to decrease protein concentration. At concentrations of 10 nM and 100 nM BPA, there was a demonstrable decrease in Vimentin expression. This is a non-exponential decline as well as for both previous proteins (The results are summarized in Fig. 24).

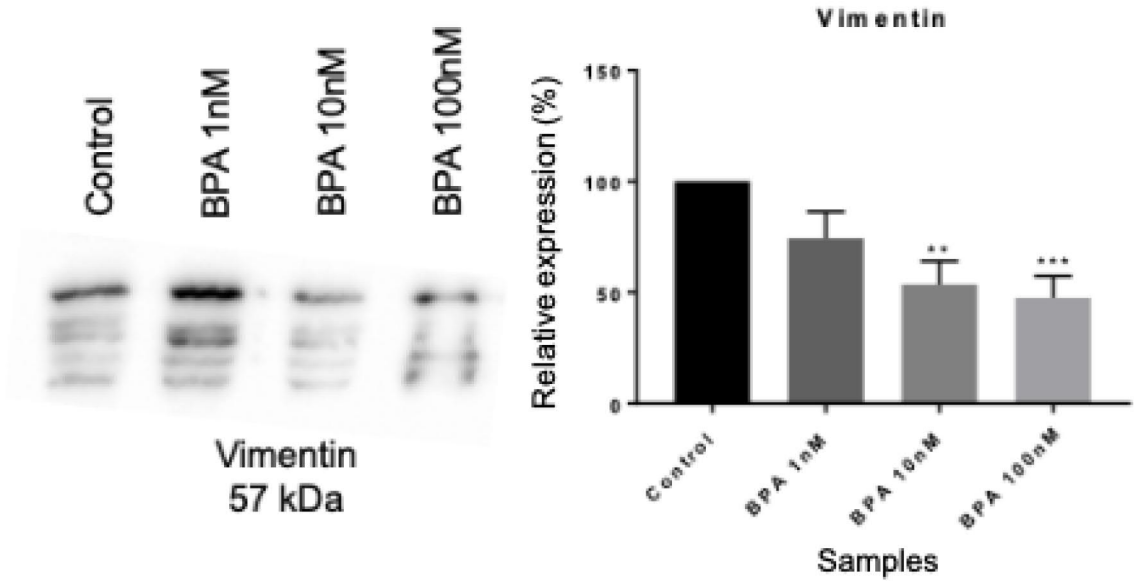

*Figure 24 - Vimentin Westren Blot results (Data are the means ± SD of three different experiments, each performed in duplicate. **p<0.01 and ***p<0.001 using ANOVA test for the comparison between control and BPA-treated cells.)*

4.4.2 Immunocytochemistry with fluorochrome indicator

The figures from the confocal microscope show a decrease of Vimentin compared to the control samples. Vimentin was located further from the nuclei after the BPA 100 nM solution, causing the inability of the cytoskeleton filaments to attach (See in Fig.25).

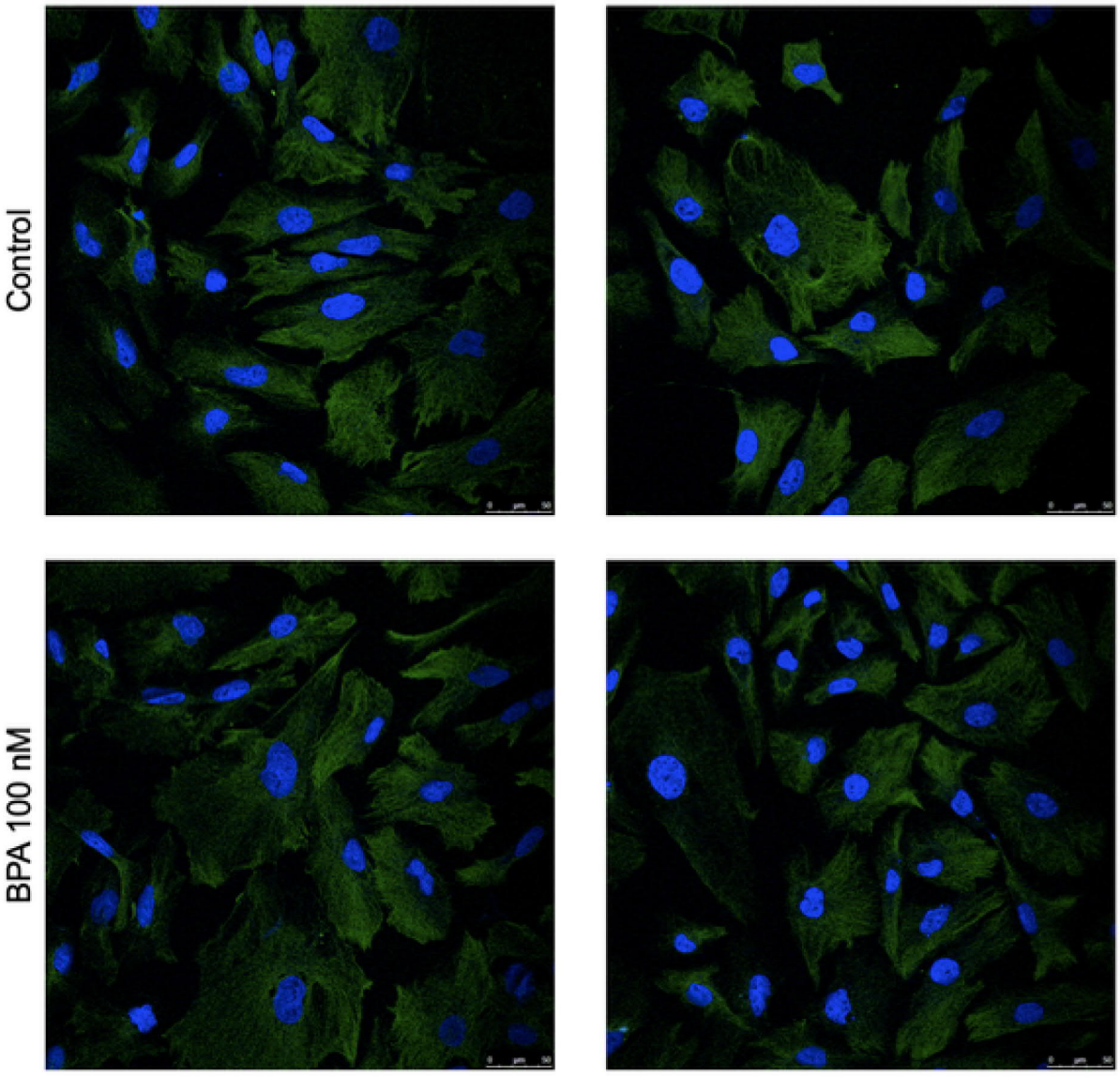

Figure 25 - Vimentin results of immunocytochemical method with fluorochrome indicator

4.4.3 Immunohistochemistry

The decrease of vimentin, which was manifested by the widening of convoluted tubules and the structural changes of renal corpuscles, was significant in all the groups of mice - diabetic, BPA-treated and diabetic treated by BPA – in the comparison with the control samples (See the differences in Fig. 26).

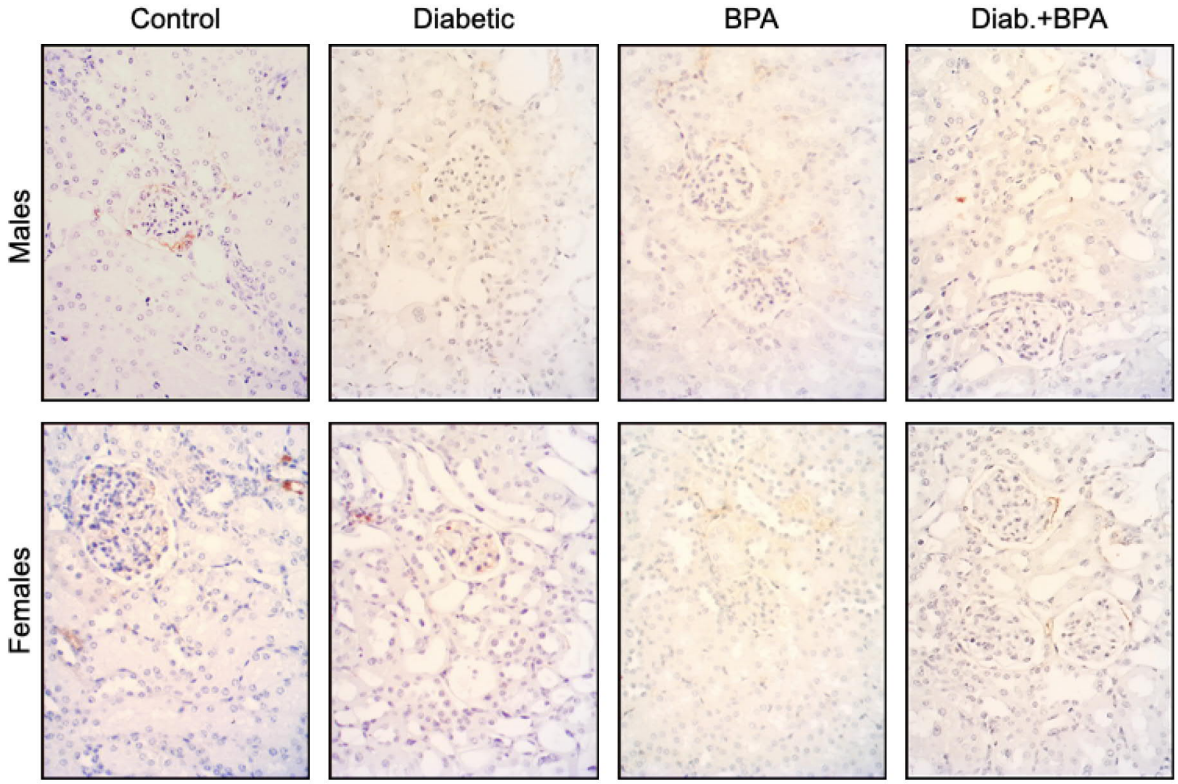

Figure 26 - Immunohistochemistry results of Vimentin (Used zooming 200x)

5 DISCUSSION

The main aim of this study was to verify and compare the toxicity of BPA to the various proteins which are part of the podocytes. In previously performed studies, it was found significant toxicity of BPA to the kidney which was manifested as the various forms of nephropathy. R. Bosch's Laboratory investigated the effect of BPA on the mouse podocytes, they clearly demonstrated that BPA causes podocytopathy with proteinuria in the mice (Olea-Herrero et al., 2014). Based on this study, we focused on BPA toxicity to both mouse podocytes by testing the tissue of mouse kidneys by the IHC and human podocytes using WB and ICC methods. We tested the specific proteins of podocyte cytoskeleton, including Podocin and Vimentin, and the proteins required for the adhesion, represented in this study by E-Cadherin.

First, we tested the toxicity of BPA to the mice kidney. To perform these tests, we used a BPA 100 nM solution. The tests confirmed the toxicity of BPA at this concentration, which was manifested by the decrease in podocyte density (number of podocytes per glomerular tuft) and the damage to the destruction of the structure of individual glomeruli in the kidney. These manifestations lead to a disruption of the glomerular filtration flow into the increased proteinuria, as demonstrated in a previous study by R. Bosch research team (Olea-Herrero et al., 2014). Podocytes are highly specialized, terminally differentiated cells that are in the visceral layer of Bowman's capsule. They are formed by the body, the main processes and the foot processes that are interconnected by slit membranes. They are an important part of the glomerular filtration barrier. The breakdown of this barrier results in proteinuria in various diseases such as Diabetes mellitus (Saleem et al., 2002). Thanks to the association of nephropathy with diabetes, the renal cells of mice suffering from diabetes mellitus have been shown to be more severely damaged by the BPA treatment. These results are consistent with previously performed studies deal with BPA renal toxicity (Krieter et al., 2013; Olea-Herrero et al., 2014; Bosch et al., 2016; Mas et al., 2018). This concentration does not occur in the human body under normal health conditions. The serum BPA concentrations are about 1.49 - 0.11 ng/ml in normal men group (i.e., 6.5 - 0.5 nM), which is much higher than in normal women group: 0.64 - 0.10 ng/ml (i.e., 2.8 - 0.4 nM) (Takeuchi and Tsutsumi, 2017). While the tolerable daily intake (TDI) of BPA for the human body set as a safe threshold is 4 μg/kg. It means that each

individual can be exposed to this dose every day of his/her life without any risk to his/her health (EFSA, 2015).

No one has focused on the study of the harmful effect of BPA on specific proteins of human podocytes yet. Thanks to the results of previous studies of R. Bosch's laboratory, we discussed this topic in this work.

In E-cadherin results, a decline of the protein expression was caused by the treatment with BPA. The loss of E-cadherin was observed in all of the methods used in this study. This again confirmed the previous assumption of the negative effect of BPA on the individual proteins contained in podocytes. The decrease was significant at all concentrations of BPA solutions (1 nM, 10 nM, 100 nM). Using WB, the greatest decrease in E-cadherin was seen at BPA 10 nM exposure. These results confirm the typical property of nonmonotonic compounds for BPA (Vom Saal and Myers, 2008).

Even using other methods - ICC and IHC - the negative effect of BPA at 100 nM was confirmed. Due to this undesirable effect of BPA, E-cadherin decreased significantly in the podocytes. When using the ICC methods, we expected the location of the protein only in the cytoplasmic membrane because it is an adhesive type of protein. However, the images of the confocal microscope showed that E-cadherin was not only in the mentioned membrane but also in the cytoplasm and near the cell nucleus. Using IHC showed that the decrease of E-cadherin due to BPA treatment caused the dilation of convoluted tubules and Bowman's capsule. These extensions were due to the not so close contact of the individual cells in the kidneys.

E-cadherin as one of the adhesive proteins is expressed in all mammalian epithelia. As the key molecule of the cadherin-catenin-cytoskeleton complex, it plays a considerable role in keeping not only renal epithelial polarity and integrity, preserving epithelial cell survival, and controlling proliferation. It is localized in the adherens junction of epithelial cells and interacts with the cytoskeleton via the associated cytoplasmic catenin proteins. These interactions fix the tissue morphogenesis (Van Roy and Berx 2008). The lack of E-cadherin is the signal of epithelial to mesenchymal transition (EMT). It is a process in which cells lose cell-cell contact and delaminate from the epithelium. As a result, cell adhesion is impaired. Also, the loss of function of this protein is associated with increased tumor invasion and metastasis (Jia et al., 2011).

In the case of Podocin, which takes place in slit diaphragms of podocytes, we expected the decrease of the protein expression after the exposure of BPA at all the

using concentrations. Indeed, this decline was seen in previous studies on the mice (Olea-Herrero et al., 2014). For ICC we used BPA of 100 nM because at this concentration BPA toxicity is the most pronounced in all which we used in this study. We also used lower concentrations of BPA in WB, which also showed a negative effect on the podocytes and also on Podocin itself. The testing by WB method showed that the greatest decrease in the Podocin concentration has occurred as well with the BPA 100 nM. The deficiency of this protein results in decreased function of the podocytes, leading to the increased proteinuria (Karin Schwarz, 2001). This result is due to the fact that podocin belongs to the group of cytoskeletal proteins of podocytes and thus affects their stability. This protein is specific for mature podocytes and occurs in the slit diaphragms (Gribouval et al., 2002). Besides its function as a structural protein of the renal filtration unit, Podocin is part of a multiprotein complex. The function of this complex is anchoring the membrane to the actin cytoskeleton, maintaining its stability, and establishing its location in the lateral plasma membrane of the podocyte foot processes. The mentioned complex is consisting of the transmembrane proteins Nephrin, Neph1, Neph2, CD2-associated protein and transient receptor potential channel (Relle et al. 2011). Podocin is involved in these reactions because it is located on the podocyte foot process where its COOH terminus interacts with the transmembrane protein Nephrin and CD2-associated protein (Kriz and Kretzler 2003). Proteinuria is a manifestation of filtration membrane damage in the glomerulus, of which the podocytes are an essential part.

As mentioned above, the IHC method for Podocin is not appropriate. Wilm's tumor suppressor 1 (WT-1) testing is used to determine 100 nM BPA toxicity for podocytes or Podocin. This IHC testing was performed on mice kidneys earlier by R. Bosch laboratory (Olea-Herrero et al., 2014).

The last protein which we tested during this study was Vimentin. It is an essential substance of the eukaryotic cell’s cytoskeleton belonging to the group of the cytoplasmic intermediate filaments. Vimentin is involved in the construction of a filament network in the cells that guarantees the stability and integrity of the cells and their shape (Buchmaier et al., 2013). Besides, this protein also plays an important role in the mechanical stability, migration and mobility of cells (Ivaska et al., 2005). As with the previous proteins, the WB showed the trending of the decrease in the protein expression after BPA exposure at all used concentrations, with the largest decline occurring with 100 nM BPA. Again, there was a nonmonotonic property of BPA. Using

the ICC method with the DAB indicator, tests with Vimentin were unsuccessful, and therefore their results are not published in this study. However, further ICC experiments with fluorescence indicator showed a decrease in Vimentin expression by the effect of BPA. The deficiency of Vimentin in the podocytes manifested itself mainly in the shape of these cells and the stability that was impaired (Buchmaier et al., 2013).

As well as the results of the IHC method showed a decrease in Vimentin concentration, manifested mainly by the enlargement of convoluted tubules and structural damage of the renal corpuscles.

6 CONCLUSION

The main concern of this study was to examine and compare BPA toxicity to the renal podocytes. In the study, we focused on the specific proteins of these cells, namely the cytoskeletal type represented by Podocin, which occurs exclusively in mature podocytes, and Vimentin. Furthermore, the adhesive type represented by E-Cadherin. Due to previously performed studies in R. Bosch's laboratory, in which the negative effect of BPA was tested and confirmed in mice podocytes, our study focused even on human podocytes.

The kidney BPA treatment test showed a negative effect of BPA on this organ, manifested by decreased podocyte density and impaired glomerular structure. As a result of this damage, the function of the glomerular filtration was reduced, leading to increased proteinuria. In mice samples, we compared the effect of BPA on healthy mice kidneys and the kidneys of mice suffering from diabetes mellitus. It is the disintegration of the filter membrane that is associated with DN. Along with the negative effect of BPA treatment, the damage to the kidney structure was more pronounced in diabetic mice.

Thanks to the previous study on the effect of BPA on the mice podocytes, which showed a clear association between BPA and its induced proteinuria, we focused on the effect of BPA on the specific proteins of human podocytes. For all tested proteins (E-Cadherin, Podocin and Vimentin), we used several methods to demonstrate the negative effects of BPA. The results showed that exposure of BPA in higher concentration (100 nM) caused the decrease in all the studied proteins expression, resulting in damage to podocytes leading to increased proteinuria. At lower concentrations of BPA (1 nM, 10 nM), its harmful effect is not so pronounced, however, the trend in decreasing concentrations of individual proteins was visible.

In conclusion, the toxicity of BPA to human podocytes is demonstrable even due to the above findings, however, this topic deserves yet another, more detailed examination of the specific mechanisms causing this damage.

7 LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BA	Bradford assay
BPA	Bisphenol A
BSA	Bovine serum albumin
CO_2	Carbon dioxide
DAB	Diaminobenzidine
DAPI	Diamidinophenylindole dihydrochloride
DCT	Distal convoluted tubule
DM 1	Diabetes mellitus 1
DM 2	Diabetes mellitus 2
DN	Diabetic nephropathy
DPBS	Dulbecco's Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IHC	Immunohistochemistry
LADA	Latent autoimmune diabetes in adults
NDS	Normal donkey serum
PCT	Proximal convoluted tubule
PVC	Polyvinylchloride
PVDF (membrane)	Polyvinylidene difluoride (membrane)
SDS-PAGE (electrophoresis)	Sodium dodecyl sulfate-polyacrylamide gel (electrophoresis)
SEM	Standard error of the mean
SULTs	Sulfotransferases
TBS	Tris buffered saline
TDI	Tolerable daily intake
TEMED	Tetramethylenethylenediamine

TEPSA	Aminopropyltriethoxysilane
TTBS	Tween-Tris buffered saline
Tx 100	Triton X 100
UGTs	Uridine diphosphate-glycuronosyltransferases
WB	Western Blot
WT-1	Wilm’s tumor suppressor 1

8 LIST OF FIGURES

9 LIST OF TABLES

10 REFERENCES

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