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The long-term effects of rapamycin-based immunosuppressive protocols on the expression of renal aquaporins 1, 2, 3 and 4 water channels in rats

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Summary. Background. To this day, the effect of multi-drug immunosuppressive protocols on renal expression of AQPs is unknown. This study aimed to determine the influence of rapamycin-based multi-drug immunosuppressive regimens on the expression of aquaporins (AQPs) 1, 2, 3, and 4 in the rat kidney.

Methods. For 6 months, 24 male Wistar rats were administered immunosuppressants, according to the three-drug protocols used in patients after organ transplantation. The rats were divided into four groups: the control group, the TRP group (tacrolimus, rapamycin, prednisone), the CRP group (cyclosporine A, rapamycin, prednisone), and the MRP group (mycophenolate mofetil, rapamycin, prednisone). Selected red cell indices and total calcium were measured in the blood of rats and quantitative analysis of AQP1, AQP2, AQP3 and AQP4 immunoexpression in the kidneys were performed.

Results. In the TRP and CRP groups, a mild increase of mean corpuscular hemoglobin concentration, hematocrit and total calcium were observed. Moreover, decreased expression of AQP1-4 was found in all experimental groups, with the highest decrease in the CRP group.

Conclusions. The long-term immunosuppressive treatment using multi-drug protocols decreased AQP1-4 expressions in renal tubules, possibly leading to impaired urine-concentrating ability in rat.

Key words: Kidney, Aquaporins, Immunosuppressants, Immunohistochemistry

Introduction

Over 30 years ago Benga (1988) for the first time demonstrated the characteristic protein in the membrane of the red blood cells (RBC) among the polypeptides migrating in the region with the molecular weight of 35-60 kDa. This became the beginning of a great discovery that has shed new light on membrane water transport. A few years after this event, independent research conducted by Agre and co-workers (Preston et al., 1992; Agre and Chrispeels, 1993), honored with the Nobel Prize in 2003, clearly showed that previously observed protein, later called aquaporin 1 (AQP1), is a water channel that had been sought for many years. Today, it is

Abbreviations. AQP, aquaporin; AVP, arginine vasopressin; BKV, BK virus; cAMP, cyclic adenosine monophosphate; CaN, calcineurin; CD, collecting duct; CMV, cytomegalovirus ; CnA, catalic A subunit of calcineurin; CnB, regulatory B subunit of calcineurin; CNI, calcineurin inhibitors; CREB, cAMP-responsive element binding protein; CRP, rats received cyclosporine A, rapamycin, and prednisone; CsA, cyclosporine A; EGTA, egtazic acid; FK-506, tacrolimus; FKBP12, FK506-binding protein of 12 kDa; FRB, FKBP-binding domain; Gsk3b, glycogen synthase kinase type 3b; HCT, hematocrit; HL, Henle's loop; IMCD, inner medullary collecting duct; MCHC, mean corpuscular hemoglobin concentration; MMF, mycophenolate mofetil; MRP, rats received mycophenolate mofetil, rapamycin, and prednisone; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; Na+/K+-ATPase, sodium-potassium pump; NFATc, nuclear factor of activated T-cells; NKCC2, sodium/potassium/chloride transporter type 2; PT, proximal tubules; RBC, red blood cells; TonEBP, tonicity-responsive enhancer binding protein; TRP, rats received tacrolimus, rapamycin, and prednisone.

known that aquaporins (AQPs) are a family of small transmembrane proteins selectively permeable to water and other small molecules. The discovery and definition of their role, primarily in the rapid water flow across the cell plasma membranes, have allowed us to understand many processes related to the proper maintenance of the water and electrolyte balance of the whole organism.

To date, 13 AQPs have been identified in mammals (AQP0 - AQP12), of which 9 (AQP1 - AQP8 and AQP11) are located in kidneys (Michałek, 2016). It is widely known that AQP1, AQP2, AQP3 and AQP4 play a key role in renal water reabsorption (Kortenoeven and Fenton, 2014). Any disturbance in their location or expression directly affects renal water excretion and the proper maintenance of the whole body water balance (Fenton and Knepper, 2007). A number of factors that may affect the normal course of renal water retention via AQPs have been studied, and many related disorders have been described (Kortenoeven and Fenton, 2014). Unfortunately, to date, very few studies have been conducted on the potential impact of immunosuppressive therapies on changes in AQP expression in the kidneys. Moreover, available data comes from experiments in which the effects of only individual drugs were analyzed.

Immunosuppressive drugs are conventionally applied in clinical practice to prevent immune-borne rejection of vascularized transplanted organs. The development of long-term immunosuppressive regimens based on multi-drug combinations aims to reduce the risk of damage and graft rejection in post-transplantation patients and to minimize single drug toxicity (Baroja-Mazo et al., 2016). In kidney transplant patients, the standard combination therapy usually includes: calcineurin inhibitors (CNI), such as cyclosporine A (CsA) and tacrolimus (FK-506); antimetabolite purine antagonists, including mycophenolate mofetil (MMF); corticosteroids, including prednisone; and less often used mammalian target of rapamycin (mTOR) inhibitors, including rapamycin. The use of immunosuppressive drugs from other classes allows modulation of the immune response through various mechanisms of action (Tsai et al., 2018).

It should be highlighted that rapamycin has a favorable nephrotoxicity profile and a different mechanism of action compared to CNI. Therefore, rapamycin-containing multi-drug protocols have been developed to minimize the adverse effects associated with the use of CsA or FK-506. It is also suggested that combination therapy might delay the appearance of drug resistance (Li et al., 2014; Baroja-Mazo et al., 2016). Moreover, due to the unique antiproliferative properties of rapamycin, it is used in immunosuppressive protocols in patients after organ transplantation with cancer - CNI is replaced by rapamycin. Drugs from the group of mTOR inhibitors in patients with a history of cancer can be used de novo or included in therapy several months after transplantation (pre-therapy). Drugs from this group can also be implemented many years after

transplantation, after cancer diagnosis (late conversion) (Hoogendijk-van den Akker et al., 2013). Additionally, the mTOR inhibitors in immunosuppressive schemes are usually also applied in patients with the cytomegalovirus (CMV) or the Polyoma BK virus (BKV) infection (Tedesco Silva Jr et al., 2010). Cytomegalovirus and BKV still remain the most important infectious pathogens in renal transplant recipients, and they affect the survival of recipients and transplants. It was revealed that mTOR inhibitors both inhibit mTOR viral kinase and may affect the function of host immune cells involved in the antiviral response. Clinical trials on the efficacy and safety of treatment with the mTOR inhibitors compared to other immunosuppressants - most commonly CNI or mycophenolic acid - have shown a lower incidence of the CMV infection. The benefits of treatment with mTOR inhibitors can be found primarily by seronegative recipients receiving a seropositive organ, recipients not subjected to anti-CMV prophylaxis, and patients not responding to antiviral therapy. Interestingly, immunosuppressive regimens containing mTOR inhibitors in combination with CsA are associated with a lower incidence of BKV infection compared to regimens involving CNI + MMF (Tedesco Silva Jr et al., 2010).

The mechanism of action of immunosuppressive drugs within individual renal tubular sections is not fully explained. The multidirectional effect of immuno-suppressants associated with activation of various intracellular pathways that causes that the final effect depends on many factors, including the duration of the therapy and also the type and dose of drugs. Studies concerning the influence of single immunosuppressive drugs on the water channels in kidneys have shown that long-term administration of CsA, FK-506 and rapamycin causes a decrease in the expression of renal aquaporins, especially AQP2, which is directly associated with increased urinary excretion of water (Lim et al., 2004; Li et al., 2007; da Silva et al., 2009; Rinschen et al., 2011; Gao et al., 2013; Chen et al., 2014). In turn, the use of glucocorticoids, including prednisone, causes a decrease in the secretion of arginine vasopressin (AVP) (Erkut et al., 1998). To this day, the effect of mycophenolate mofetil on renal expression of AQPs is unknown. So, will the multi-drug regimens deepen the effect of particular drugs and increase renal water loss via AQPs, although they minimize the nephrotoxicity? Searching for the answer to this question, we undertook research aimed to determine the influence of rapamycin-based multi-drug immunosuppressive protocols on the expression of aquaporins 1, 2, 3, and 4 in the rat kidney.

Materials and methods

Animals

The studies were performed on 24 sexually mature three-month-old male Wistar rats, which were obtained from a licensed breeder (the Institute of Occupational

Medicine, Lodz, Poland). The animals used in experiment had health and genetic certificates approved by a veterinarian. Before starting the experiment, all animals survived an adaptation period (two weeks) and were weighed (mean weight of 305 g). All animals received water ad libitum and were fed with high-quality specialized laboratory diet of LSM type (Agropol Motycz, Lublin, Poland) with the energy value 1474 kJ/100 g, including 17.6% protein. The rats were housed in ventilated rooms maintained at 21°C and humidity of 50±5% in standard cages (six rats per cage) and subjected to a 12 h light/12 h dark cycle. The rats were randomly divided into four groups (six rats in each group): the control group and three experimental groups (TRP, CRP and MRP). Animals in the control group were administered bread balls without any drug. Rats in the experimental groups received immunosuppressants, according to the three-drug protocols used in patients after organ transplantation: 1) in the TRP group rats received FK-506, rapamycin, and prednisone, 2) in the CRP group rats received CsA, rapamycin, and prednisone, and 3) in the MRP group rats received MMF, rapamycin, and prednisone. Immunosuppressive agents were orally administered to animals in their pharmaceutical form in bread balls every 24 hours for 6 months. The drug doses (Table 1) were based on literature data (Van Westrhenen et al., 2007; Schmitz et al., 2009), and were adjusted to body mass of rats and properly calculated considering metabolic differences between rat and human. It should be noted that FK-506 dosing was based on its target blood levels. In clinical practice, it allows for better clinical outcomes, to avoid drug-induced adverse effects and the maintenance of efficacy (Kikuchi et al., 2019). In our experiment the applied doses of immunosuppressants allowed us to obtain drug concentrations in the blood of the animals within the therapeutic range (Kędzierska et al., 2015).

The protocol of the experiment and animal welfare procedures were approved by the Local Ethical Committee for Experiments on Animals of the Pomeranian Medical University in Szczecin, Poland (No. 06/08 and No. 24/08).

Collection of material for the study

Twenty two rats completed the study (two rats in the CRP group died in the fourth month of the experiment). All animals were anesthetized intraperitoneally with a ketamine hydrochloride (50 mg/kg of body mass), next they were sacrificed, and the blood samples were collected for hematologic and biochemical analyzes. Subsequently, during the section, kidneys of rats were obtained. For immunohistochemical evaluation kidneys were fixed in 4% buffered paraformaldehyde and embedded in paraffin blocks.

Haematological and biochemical analysis

The blood samples were collected in tubes with a 10% (w/v) sodium ethylenediamine-tetraacetic acid in distilled water. The hematological tests were performed using a Cobas m 511 integrated hematology analyzer (Roche Diagnostics Hematology, Westborough, MA). Red cell indices included mean corpuscular hemoglobin concentration (MCHC) and hematocrit (HCT). The total calcium concentration in the blood serum was determined using a colorimetric method using a commercial reagent kit (Aqua Med, Łódź, Poland) and following standard procedures recommended by the producer.

Immunohistochemistry

In the present study, the following primary antibodies were used to examine the detailed location

Table 1. Drug doses in the control and experimental groups.

Group	Drugs	Pharmaceutical form (name, manufacturer, city, country)	Dose (mg/kg of body weight/day)	Time of administration (months)
C (control)	None	-	-	-
TRP	tacrolimus	Prograf, Astellas Pharma Inc., Tokyo, Japan	4	
	rapamycin	Rapamune, Pfizer, Inc., New York, USA	0.5	6
	prednisone	Encorton, Polfa, Pabianice, Poland	4	
CRP	cyclosporine A	Sandimmum-Neoral; Novartis International AG, Basel, Switzerland	5	
	rapamycin	Rapamune, Pfizer, Inc., New York, USA	0.5	6
	prednisone	Encorton, Polfa, Pabianice, Poland	4	
MRP	mycophenolate mofetil	CellCept; Hoffman-La Roche Ltd., Basel, Switzerland	20	
	rapamycin	Rapamune, Pfizer, Inc., New York, USA	0.5	6
	prednisone	Encorton, Polfa, Pabianice, Poland	4	

C: control group without any medication; CRP: rats received cyclosporine A, rapamycin, and prednisone; MRP: rats received mycophenolate mofetil, rapamycin, and prednisone; TRP: rats received tacrolimus, rapamycin, and prednisone.

and expression of AQPs in the kidneys of rats: (1) mouse monoclonal anti-Aquaporin 1 (sc-25287, Santa Cruz Biotechnology), (2) rabbit polyclonal anti-Aquaporin 2 (NB110-74682, Novus Biologicals), (3) rabbit polyclonal anti-Aquaporin 3 (NBP1-97927, Novus Biologicals) and (4) rabbit polyclonal anti-Aquaporin 4 (NBP1-87679, Novus Biologicals). Depending on the type of the primary antibody, labeling of the antigen-antibody complexes were visualized with the use of secondary polyclonal goat anti-rabbit (P 0448, Dako) or anti-mouse (sc-516102 Santa Cruz Biotechnology) horseradish peroxidase-conjugated antibodies.

Immunostaining of paraffin-embedded kidney tissue sections from all rats was performed according to the protocol previously described in detail by Michałek et al. (2014). Sections (2-3 µm thick) were deparaffinized and rehydrated with xylene and in a graded ethyl alcohol series, respectively. The activity of endogenous peroxidase was blocked by treating all slides with 0.30% hydrogen peroxide in methanol for 30 minutes. To reveal antigens, sections were incubated in 1 mM Tris solution (pH 9.0) supplemented with 0.5 mM egtazic acid (EGTA) and heated in a microwave oven for 16 min. The non-specific binding of Ig was prevented by incubating the sections in 50 mM NH_4Cl for 30 min, followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Next, the sections were incubated with anti-AQPs primary antibodies diluted in 0.01M PBS with 0.1% BSA and Triton X-100. In the present experiment, kidney sections were incubated all night at 4°C in a humid chamber with primary anti-AQP1 antibodies (dilution 1:100), anti-AQP2 (dilution 1:100), anti-AQP3 (dilution 1:500) and anti-AQP4 (dilution 1:500). Subsequently, the slides were incubated with a complex containing a secondary antibody conjugated with horseradish peroxidase. Next, diaminobenzidine in chromogen solution (Dako, K3468) was used. Mayer's hematoxylin (Sigma-Aldrich Co., St Louis, MO, USA) was used as a counterstain. As the final step, all slides were dehydrated and coverslipped. The obtained sections were analyzed under a light microscope (Olympus BX 41, Hamburg, Germany). Specificity of immunostaining was confirmed by following the above procedures by replacing the primary antibody with IgG from mouse and rabbit serum, respectively. In addition, all reactions using a particular antibody were carried out under the same conditions.

Quantitative analysis of immunohistochemistry

Quantitative analysis of immunohistochemistry was conducted according the protocol recommended by the manufacturer. All slides under immunostaining were scanned at 400× absolute magnification (resolution of 0.25 µm/pixel) with the use a ScanScope AT2 scanner (Leica Microsystems, Wetzlar, Germany). The background illumination levels were calibrated using a prescan procedure. Moreover, the scanner was configured to minimize focus problems. Using an ImageScope viewer (Version 11.2.0.780; Aperio Technologies, Inc., Vista, CA, USA), the obtained digital images of the IHC slides were examined. For the automatic analysis of AQP1-4 expression in particular parts of the kidneys, the following algorithms were used: membrane v9 algorithm (version 9.1; Aperio Technologies, Inc.) for detailed analysis of AQP expression in the apical and basolateral plasma membranes, and cytoplasmic v2 algorithm (version 2.0; Aperio Technologies, Inc.) for detailed analysis of intracellular expression of AQP2. The percentage of renal tubular cells with weak, medium, and strong positive immunostaining in the plasma membranes and intracellular vesicles were determined for individual AQPs (AQP1-4). The percentage of cells with the expression of each AQP for each group was counted in a total of 30 random fields (5 fields from each rat, 20-30 tubules per field).

Statistical analysis

All statistical analyzes were conducted by using Statistica 8.0 software (StatSoft, Krakow, Poland). The quantitative values were evaluated by the Shapiro-Wilk normality test. Pairwise comparison between control and experimental groups were performed by the Kruskal-Wallis test with Dunn's multiple comparison test for post hoc analysis. The level of statistical significance was $p < 0.05$.

Table 2. Selected red cell indices and total calcium in the blood of rats in the control and experimental groups.

Group	MCHC (mmol/L)		HCT (L/L)		Ca (mmol/L)	
	Median (range)	X±SD	Median (range)	X±SD	Median (range)	X±SD
C (control)	14.0 (13.6-14.2)	13.9±0.2	0.754 (0.739-0.779)	0.756±0.013	1.94 (1.82-2.80)	2.07±0.37
TRP	14.1 (12.6-14.9)	14.0±0.8	0.804 (0.767-0.844)	0.805±0.031	2.16 (1.50-2.52)	2.11±0.36
CRP	14.4 (13.8-15.0)	14.4±0.5	0.769 (0.660-0.835)	0.758±0.090	2.44 (2.36-2.54)	2.45*±0.08
MRP	13.8 (11.5-13.9)	13.4±0.9	0.724 (0.605-0.787)	0.719±0.067	2.17 (2.02-2.27)	2.15±0.09

C: control group without any medication; Ca: calcium; CRP: rats received cyclosporine A, rapamycin, and prednisone; HCT: hematocrit; MCHC: mean corpuscular hemoglobin concentration; MRP: rats received mycophenolate mofetil, rapamycin, and prednisone; TRP: rats received tacrolimus, rapamycin, and prednisone; X±SD: arithmetical mean±standard deviation; *: $p < 0.05$ vs. control (Kruscal-Wallis test).

Results

Selected hematologic and biochemical parameters in the blood of rats

The red cell indices such as mean corpuscular hemoglobin concentration, and hematocrit, and also total calcium were determined in the blood of rats in the control and experimental groups (Table 2). The highest level of MCHC was noted in the CRP group, while the lowest level was noted in the MRP group. Level of MCHC both for the CRP and the MRP group was statistically insignificant in comparison to the control group. The highest HCT was revealed in the TRP group, while the lowest was revealed in the MRP group. Level of HCT both for the TRP and the MRP group was statistically insignificant in comparison to the control group. The highest concentration of total calcium was observed in the CRP group (p<0.015), while the lowest concentration was observed in the control group.

Immunolocalization of AQP1, 2, 3 and 4 in rat kidney

Immunoexpression of AQP1 (Fig. 1) was observed both in the renal cortex and medulla of rat kidney in the control (Fig. 1A,E,I) and all experimental groups - TRP (Fig. 1B,F,J), CRP (Fig. 1C,G,K) and MRP (Fig. 1D,H,L) groups. In the renal cortex AQP1 expression was revealed in the brush border and basolateral membranes of proximal tubular epithelial cells (Fig. 1A-H), and also in the endothelium of glomeruli capillaries (Fig. 1A-D). In proximal tubules (PT) different intensity of labeling of AQP1 was found. Straight PTs exhibited mostly strong intensity of labeling, while different parts of convoluted PTs were characterized by various intensity. In medulla, AQP1 expression was observed in the apical and basolateral membranes of epithelial cells of the descending thin limb of Henle's loop (HL) (Fig. 1I-L). In the inner medulla, strong expression of AQP1 was revealed in tubules characterized by very low epithelium with dilated intercellular spaces.
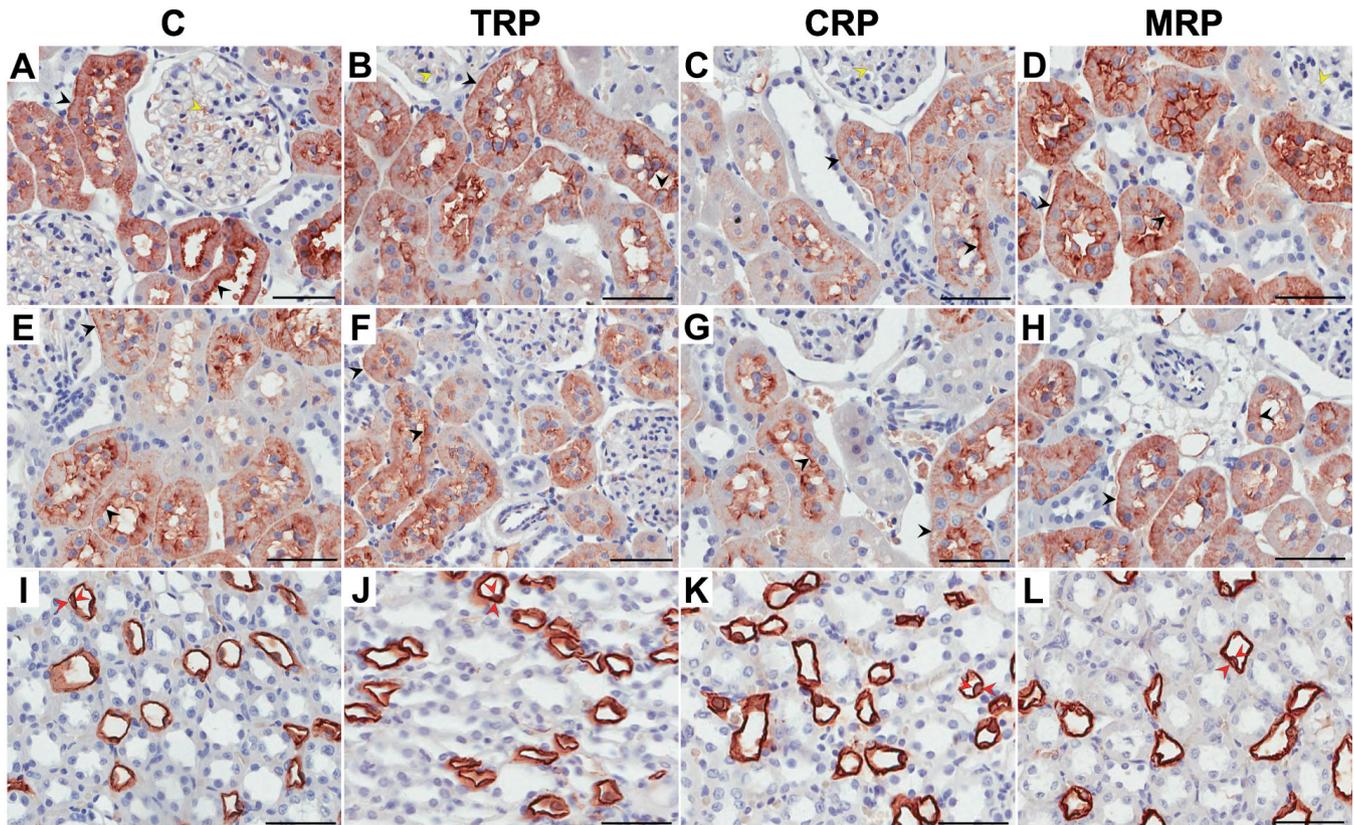
Fig. 1. Representative light micrographs of immunolocalization of aquaporin 1 (AQP1) **(A-L)** in the rat kidney. Immunoexpression of AQP1 (brown colour) in the apical (brush border) and basolateral plasma membranes of the epithelial cells of proximal tubules **(A-H;** black arrowheads) and descending thin limb of Henle's loop **(I-L;** red arrowheads) and also in the endothelium of glomeruli capillaries **(A-D;** yellow arrowheads) in the control group **(A, E, I)**, the TRP group **(B, F, J)**, the CRP group **(C, G, K)**, and the MRP group **(D, H, L)**. In proximal tubules different intensity of labeling of AQP1 was found (details in the text). The epithelial cells of distal tubules and collecting ducts were unlabeled. C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone. Scale bars: 50 μm.

Immunoexpression of AQP2 (Fig. 2) was found in the apical and basolateral membranes, and also intracellular vesicles of the cortical and medullary collecting duct (CD) principal cells, and in the connecting tubules in the rat kidney in the control (Fig. 2A,E) and all experimental groups - TRP (Fig. 2B,F), CRP (Fig. 2C,G) and MRP (Fig. 2D,H) groups. Apical membrane of CD principal cells mostly exhibited strong intensity of AQP2 labeling, while basolateral membranes were characterized by weak intensity.

Immunoexpression of AQP3 (Fig. 3) was revealed in the basolateral plasma membrane of the cortical and medullary collecting duct principal cells in rat kidney in the control (Fig. 3A,E) and all experimental groups - TRP (Fig. 3B,F), CRP (Fig. 3C,G) and MRP (Fig. 3D,H) groups. In all groups, labeling of AQP3 in CD principal cells in medulla was weaker than AQP4.

Immunoexpression of AQP4 (Fig. 4), similarly to AQP3, was noted in the basolateral plasma membrane of the cortical and medullary collecting duct principal cells in rat kidney in the control (Fig. 4A,E) and all experimental groups - TRP (Fig. 4B,F), CRP (Fig. 4C,G) and MRP (Fig. 4D,H) groups. Both outer and inner medullary CD exhibited mostly moderate intensity of AQP4 labeling, while cortical collecting ducts were characterized by weaker intensity. In all groups, labeling of AQP4 in CD principal cells in medulla was stronger than AQP3.

Quantitative evaluation of AQP1, 2, 3 and 4 immuno-expression in rat kidney

The percentage of proximal tubular epithelial cells characterized by weak, moderate, and strong expression of AQP1 in the apical membrane (Fig. 5) in the control group was statistically significant compared to TRP (p=0.006 for weak expression) and CRP (p=0.001 and p=0.003 for moderate and strong expression, respectively) groups. The highest percentage of proximal tubule cells characterized by weak expression of AQP1 in the apical membrane was found in the TRP group (28.9±3.4%), while the lowest percentage was found in the control group (21.2±9.3%). The highest percentage of proximal tubule cells characterized by moderate expression of AQP1 in the apical membrane was noted in the CRP group (48.2±8.1%), while the lowest percentage was noted in the TRP group (29.0±15.6%). The highest percentage of proximal tubule cells characterized by strong expression of AQP1 in apical membrane was found in the control group (44.7±17.9%), while the lowest percentage was found in the CRP group (26.9±10.5%). The highest decrease of AQP1 expression in the apical membrane of proximal tubular epithelial cells was revealed in the CRP group.

The percentage of proximal tubular epithelial cells with weak and moderate expression of AQP1 in the basolateral membrane (Fig. 5) in the control group
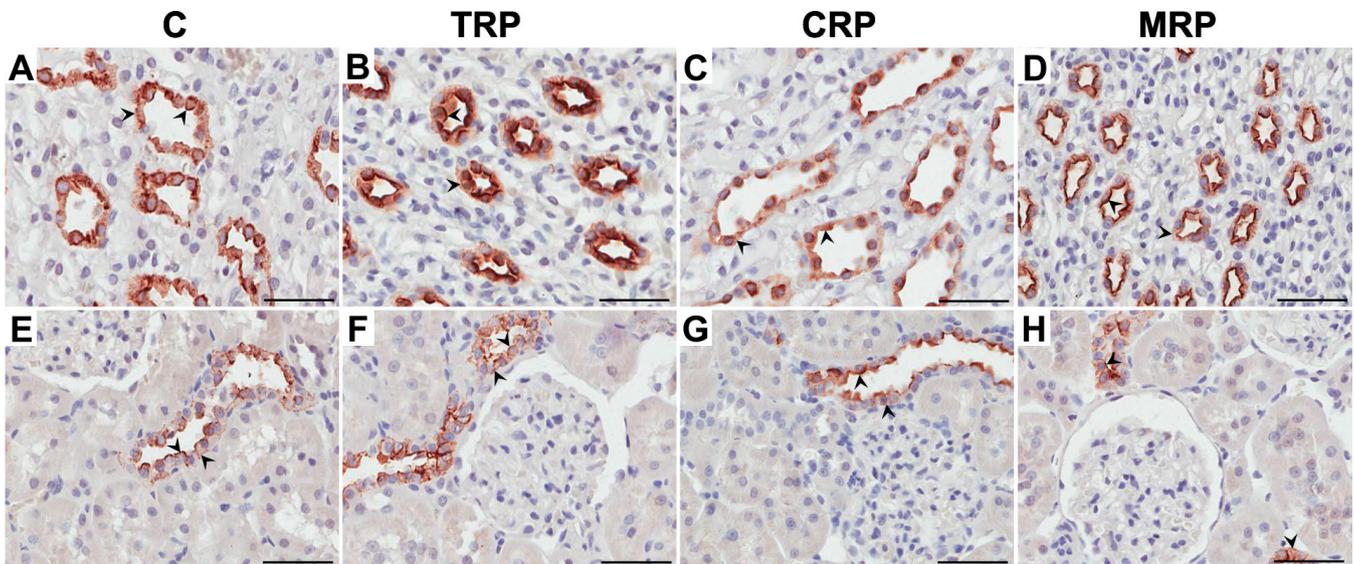
Fig. 2. Representative light micrographs of immunolocalization of aquaporin 2 (AQP2) **(A-H)** in the rat kidney. Immunoexpression of AQP2 (brown colour) in the apical and basolateral plasma membranes (black arrowheads) and intracellular vesicles in the medullary **(A-D)** and cortical **(E-H)** collecting duct principal cells in the control group **(A, E)**, the TRP group **(B, F)**, the CRP group **(C, G)**, and the MRP group **(D, H)**. In collecting duct principal cells different intensity of labeling of AQP2 was found (details in the text). The epithelial cells of proximal and distal tubules and also descending thin limb of Henle's loop were unlabeled. C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone. Scale bars: 50 μm.

differed statistically from the CRP group (p=0.027 and p=0.003, respectively). The highest percentage of proximal tubule cells with weak expression of AQP1 in basal membrane was found in the CRP group (66.3±7.1%), while the lowest percentage was found in the TRP group (54.3±8.2%). The highest percentage of proximal tubule cells with moderate expression of AQP1 in basal membrane was noted in the control group
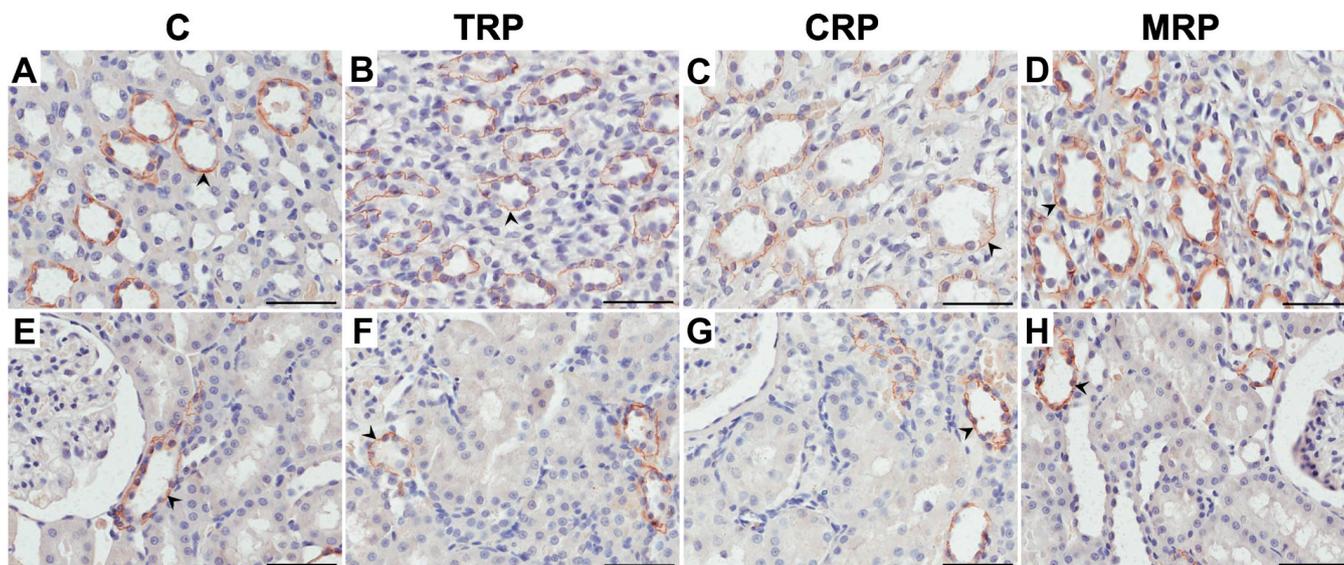
Fig. 3. Representative light micrographs of immunolocalization of aquaporin 3 (AQP3) **(A-H)** in the rat kidney. Immunoexpression of AQP3 (brown colour) in the basolateral plasma membranes (black arrowheads) in the medullary **(A-D)** and cortical **(E-H)** collecting duct principal cells in the control group **(A, E)**, the TRP group **(B, F)**, the CRP group **(C, G)**, and the MRP group **(D, H)**. In collecting duct principal cells different intensity of labeling of AQP3 was found (details in the text). The epithelial cells of proximal and distal tubules and also descending thin limb of Henle's loop were unlabeled. C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone. Scale bars: 50 μm.
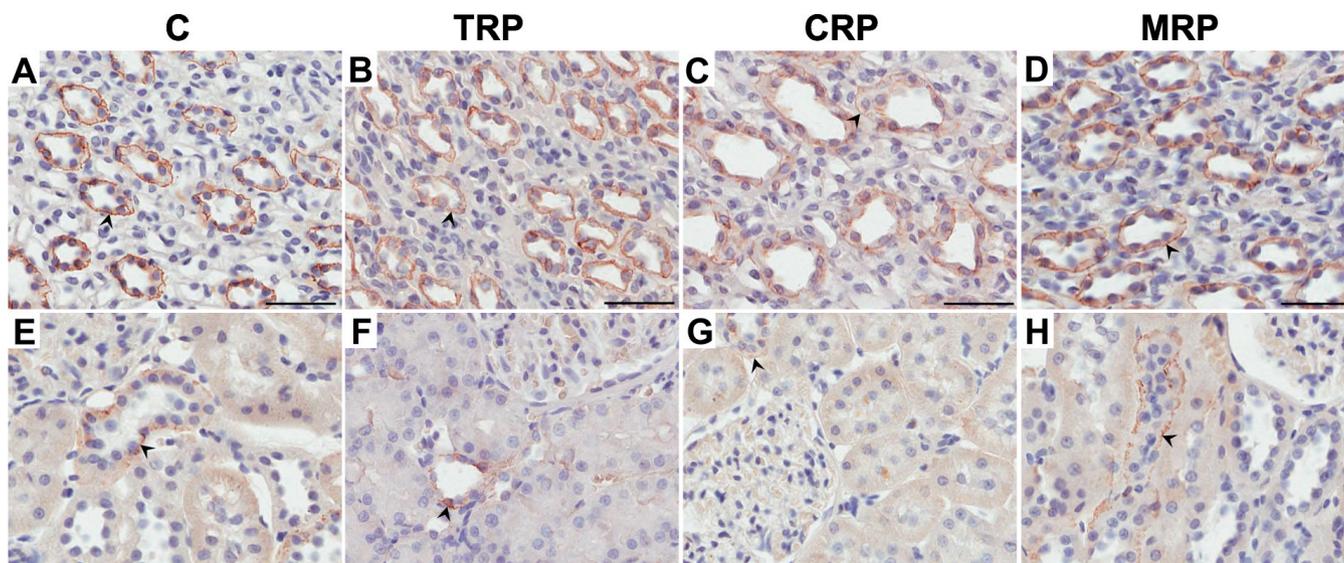
Fig. 4. Representative light micrographs of immunolocalization of aquaporin 4 (AQP4) **(A-H)** in the rat kidney. Immunoexpression of AQP4 (brown colour) in the basolateral plasma membranes (black arrowheads) in the medullary **(A-D)** and cortical **(E-H)** collecting duct principal cells in the control group **(A, E)**, the TRP group **(B, F)**, the CRP group **(C, G)**, and the MRP group **(D, H)**. In collecting duct principal cells different intensity of labeling of AQP4 was found (details in the text). The epithelial cells of proximal and distal tubules and also descending thin limb of Henle's loop were unlabeled. C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone. Scale bars: 50 μm.

(37.5±11.2%), while the lowest percentage was noted in the CRP group (23.1±11.6%). The highest percentage of proximal tubule cells with strong expression of AQP1 in basal membrane was found in the control group (1.9±2.5%), while the lowest percentage was found in the CRP group (1.0±2.0%). Similarly to apical membrane, the highest decrease of AQP1 expression in basolateral membrane of proximal tubular epithelial cells was noted in the CRP group.

The percentage of descending thin limb of Henle's loop cells with moderate, and strong expression of AQP1 in the apical membrane (Fig. 6) in the control group differed statistically from the CRP group (p=0.002 and p=0.001, respectively). The highest percentage of Henle's loop cells with weak expression of AQP1 in apical membrane was found in the MRP group

(2.6±1.1%), while the lowest percentage was found in the control (1.2±0.8%) and TRP (1.2±1.2%) groups. The highest percentage of Henle's loop cells with moderate expression of AQP1 in apical membrane was noted in the CRP group (35.2±8.9%), while the lowest percentage was noted in the control group (24.5±9.7%). The highest percentage of Henle's loop cells with strong expression of AQP1 in apical membrane was found in the control (73.2±10,8%) and TRP (73.2±4.2%) groups, while the lowest percentage was found in the CRP group (63.1±9.2%). The highest decrease of AQP1 expression in apical membrane of descending thin limb of Henle's loop cells was observed in the CRP group.

The percentage of descending thin limb of Henle's loop cells with weak and moderate expression of AQP1 in the basolateral membrane (Fig. 6) in the control group
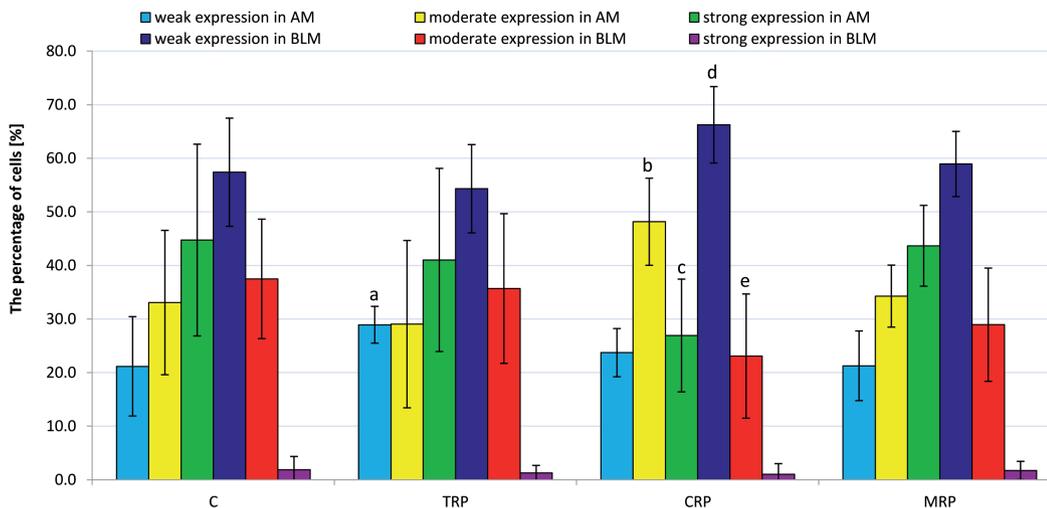
Fig. 5. The percentage of epithelial cells of proximal tubules with aquaporin 1 (AQP1) expression in the apical (brush border) and basolateral plasma membranes in the rat kidney. AM - apical membrane; BLM - basolateral membrane; C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone; p<0.05 vs. control for weak (a), moderate (b), and strong (c) expression of AQP1 in the apical membrane; p<0.05 vs. control for weak (d) and moderate (e) expression of AQP1 in the basolateral membrane (Kruskal-Wallis test); error bars show standard deviations.
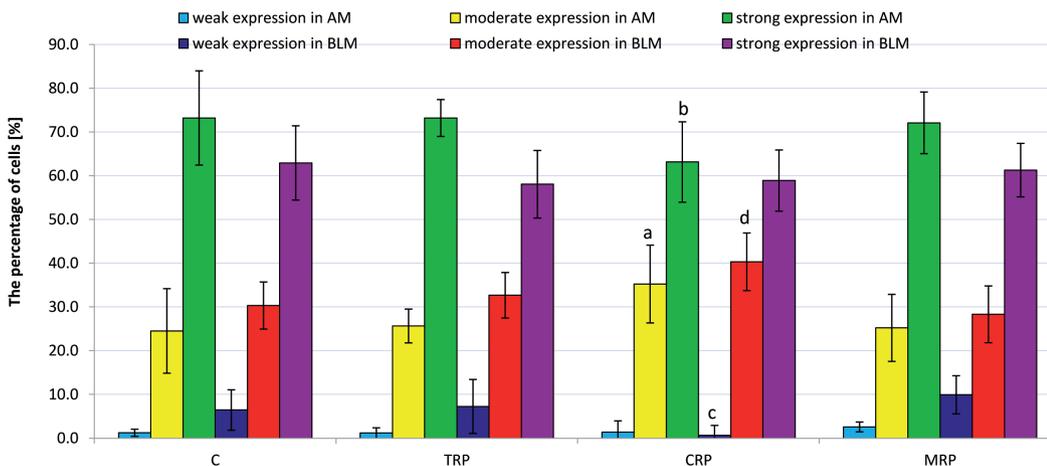
Fig. 6. The percentage of descending thin limb of Henle's loop cells with aquaporin 1 (AQP1) expression in the apical and basolateral plasma membranes in the rat kidney. AM - apical membrane; BLM - basolateral membrane; C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone; p<0.05 vs. control for moderate (a) and strong (b) expression of AQP1 in the apical membrane; p<0.05 vs. control for weak (c) and moderate (d) expression of AQP1 in the basolateral membrane (Kruskal-Wallis test); error bars show standard deviations.

differed statistically from the CRP group (p=0.001 and p<0.001, respectively). The highest percentage of Henle's loop cells with weak expression of AQP1 in basal membrane was found in the MRP group (9.9±4.4%), while the lowest percentage was found in the CRP group (0.7±2.3%). The highest percentage of Henle's loop cells with moderate expression of AQP1 in basal membrane was noted in the CRP group (40,3±6,6%), while the lowest percentage was noted in the MRP group (28.3±6.5%). The highest percentage of Henle's loop cells with strong expression of AQP1 in basal membrane was found in the control group (62.9±8.5%), while the lowest percentage was found in the TRP group (58.0±7.7%).

The percentage of collecting duct cells with weak and strong expression of AQP2 in the apical membrane (Fig. 7) in the control group differed statistically from the CRP group (p<0.001). The highest percentage of collecting duct cells with weak and moderate expression of AQP2 in apical membrane was found in the CRP group (20.3±4.5% and 36.4±10.1%, respectively), while the lowest percentage was found in the control (2.0±3.2% and 32.2±12.6%, respectively) and TRP (32.2±8.5% for moderate expression) groups. The highest percentage of collecting duct cells with strong expression of AQP2 in apical membrane was found in the control group (65.8±10.5%), while the lowest percentage was found in the CRP group (42.5±10.9%). The highest decrease of AQP2 expression in apical membrane of collecting duct cells was noted in the CRP
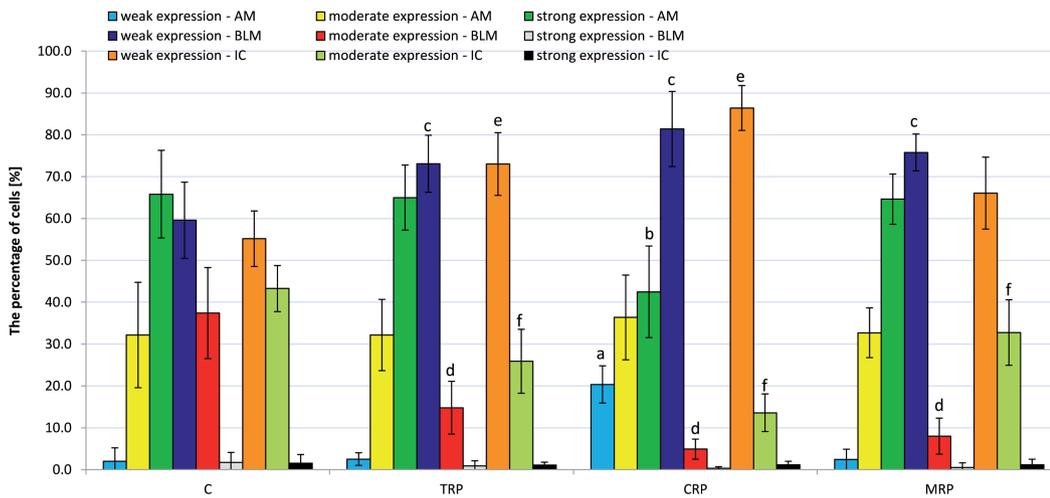
Fig. 7. The percentage of collecting duct cells with aquaporin 2 (AQP2) expression in the apical and basolateral plasma membranes, and also its intracellular expression in the rat kidney. AM - apical membrane; BLM - basolateral membrane; C - control group without any medication; IC - intracellular; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone; p<0.05 vs. control for weak (a) and strong (b) expression of AQP2 in apical membrane; p<0.05 vs. control for weak (c) and moderate (d) expression of AQP2 in basolateral membrane; p<0.05 vs. control for weak (e) and moderate (f) intracellular expression of AQP2 (Kruskal-Wallis test); error bars show standard deviations.
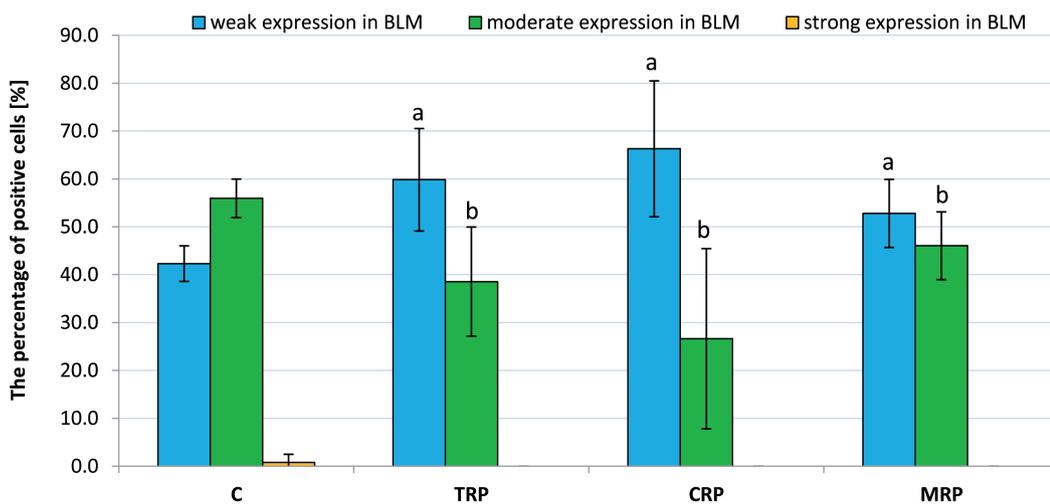
Fig. 8. The percentage of collecting duct cells with aquaporin 3 (AQP3) expression in the basolateral plasma membrane in the rat kidney. BLM - basolateral membrane; C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone; p<0.05 vs. control for weak (a) and moderate (b) expression of AQP3 in the basolateral membrane (Kruskal-Wallis test); error bars show standard deviations.

group.

The percentage of collecting duct cells with weak and moderate expression of AQP2 in the basolateral membrane (Fig. 7) in the control group differed statistically from the TRP (p=0.003 and p=0.006, respectively), CRP (p<0.001), and MRP groups (p<0.001). The highest percentage of collecting duct cells with weak expression of AQP2 in basal membrane was found in the CRP group (81.4±9.0%), while the lowest percentage was found in the control group (59.6±9.1%). The highest percentage of collecting duct cells with moderate and strong expression of AQP2 in basal membrane was noted in the control group (37.4±10.9% and 1.7±2.4%, respectively), while the lowest percentage was noted in the CRP group (4.9±2.4% and 0.3±0.4%, respectively). Similarly to apical membrane, the highest decrease of AQP2 expression in basolateral membrane of collecting duct cells was noted in the CRP group.

The percentage of collecting duct cells with weak and moderate intracellular expression of AQP2 (Fig. 7) in the control group differed statistically from the RTP (p<0.001), the CRP (p<0.001), and the MRP (p=0.033 for moderate expression) groups. The highest percentage of collecting duct cells with weak intracellular expression of AQP2 was found in the CRP group (86.4±5.4%), while the lowest percentage was found in the control group (55.2±6.6%). The highest percentage of collecting duct cells with moderate and strong intracellular expression of AQP2 was noted in the control group (43.3±5,5% and 1.6±2.1%, respectively), while the lowest percentage was noted in the CRP (13.6±4.5% and 1.1±0.9%, respectively) and the TRP (1,1±0,7% for strong expression) groups. Similarly to apical and basolateral membranes, the highest decrease of AQP2 intracellular expression in collecting duct cells was noted in the CRP group.

The percentage of collecting duct cells with weak and moderate expression of AQP3 in the basolateral membrane (Fig. 8) in the control group differed statistically from the TRP (p<0.001), CRP (p<0.001), and MRP (p=0.005 and p=0.010, respectively) groups. The highest percentage of collecting duct cells with weak expression of AQP3 in the basal membrane was found in the CRP group (66.3±14.2%), while the lowest percentage was found in the control group (42.3±3.7%). The highest percentage of collecting duct cells with moderate expression of AQP3 in basal membrane was noted in the control group (56.0±4.0%), while the lowest percentage was noted in the CRP group (26.6±18.8%). The collecting duct cells with strong expression of AQP3 in basal membrane was found only in the control group (0.8±1.7%). The highest decrease of AQP3 expression in the basolateral membrane of collecting duct cells was found in the CRP group.

The percentage of collecting duct cells with weak, moderate, and strong expression of AQP4 in the basolateral membrane (Fig. 9) in the control group differed statistically from the TRP (p<0.001 for weak and strong expression), the CRP (p<0.001 for moderate and strong expression), and the MRP (p=0.006 for weak expression) groups. The highest percentage of collecting duct cells with weak expression of AQP4 in basal membrane was found in the TRP group (45.2±5.2%), while the lowest percentage was found in the control group (36.7±4.1%). The highest percentage of collecting duct cells with moderate expression of AQP4 in basal membrane was noted in the CRP group (57.8±4.6%), while the lowest percentage was noted in the MRP group (43.2±7.3%). The highest percentage of collecting duct cells with strong expression of AQP4 in basal membrane was found in the control group (18.7±11.2%), while the lowest percentage was found in the CRP group (0.2±0.5%). The highest decrease of AQP4 expression in the basolateral membrane of collecting duct cells was found in the CRP group.
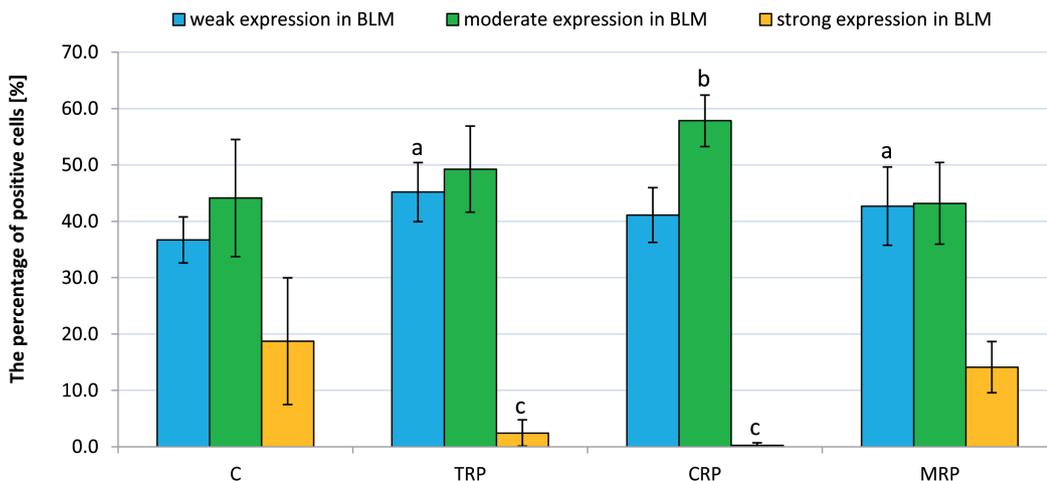
Fig. 9. The percentage of collecting duct cells with aquaporin 4 (AQP4) expression in the basolateral plasma membrane in the rat kidney. BLM - basolateral membrane; C - control group without any medication; CRP - rats received cyclosporine A, rapamycin, and prednisone; MRP - rats received mycophenolate mofetil, rapamycin, and prednisone; TRP - rats received tacrolimus, rapamycin, and prednisone; p<0.05 vs. control for weak (a), moderate (b), and strong (c) expression of AQP4 in the basolateral membrane (Kruskal-Wallis test); error bars show standard deviations.

Discussion

Although many studies have been conducted on the impact of immunosuppressants on the urinary tract (Kędzierska et al., 2015, 2016; Grabowska et al., 2016, 2020), still little is known about the effects of these drugs on AQP expression. This is the first report concerning the influence of the multi-drug rapamycin-based immunosuppressive regimens on the immunoexpression of the AQP1-4 water channels in the rat kidney. The presented study has demonstrated that a long-term immunosuppressive treatment significantly decreased expression of the AQP1-4 in the renal tubules in rat.

We have developed an experimental model of the immunosuppressive treatment which might be comparable to the multi-drug long-term therapy commonly used in renal transplant recipients. The time of experiment was 6 months, which reflects about 15 years of human life (the average rat lifetime is 2-3 years) (Quinn, 2005). In many studies on animal models, concerning the effects of immunosuppressive treatment, a relatively short-term treatment was applied (Van Westrhenen et al., 2007). It is worth noting that our research was performed on animals in which kidney transplantation was not done. This allowed for the analysis of the direct effect of immunosuppressive drugs, excluding factors which interfere with their picture of action (e.g. ischemia/reperfusion-associated tissue damage, or humoral and cellular immunological factors associated with the response mechanisms against transplanted tissues). Moreover, we have decided on oral administration of immunosuppressants rather than intro-peritoneal or subcutaneous usually used in other studies (Ysebaert et al., 2003). This route of drug administration was physiological and safest for animals.

In our study, a mild increase of MCHC and HCT was observed in groups in which FK-506, rapamycin, and prednisone as well as CsA, rapamycin, and prednisone were administered (statistically insignificant). This may indicate mild cellular RBC dehydration. Water loss causes both reduction in cell volume and an increase of haemoglobin concentration (Berda-Haddad et al., 2017). Moreover, it has been confirmed that selected immunosuppressive agents (mostly steroids) may slightly increase red cell indices (King et al., 1988). Interestingly, a mild decrease of MCHC in the MRP group was observed. Literature data indicates that values of MCHC were lower in patients receiving mycophenolate mofetil at 1 week, 1 month and 6 months after transplantation (Khosroshahi et al., 2006). It confirms the results obtained in our experiment in the group in which MMF, rapamycin, and prednisone were administered. Rapamycin included in all experimental protocols also could influence hematological parameters. It is worth mentioning that the mammalian target of rapamycin complex 1 (mTORC1) pathway is a key regulator of RBC proliferation and growth. Therefore disorders in this pathway may result in anemia (Knight et al., 2014).

In the present study, an increase of total calcium in the blood of rats was found in all experimental groups, with the highest concentration in the CsA, rapamycin, and prenisone-treated rats. Einollahi et al. (2012) have reported that hypercalcemia was observed in 66% of the examined kidney transplant patients. Under normal conditions, CsA and FK-506 in renal transplant patients increase the excretion of magnesium and calcium in the urine. Supplementation is sometimes ineffective due to the reduced kidney threshold for these ions. However, in our experiment, in rats, kidney transplantation was not performed, therefore we suspected that animals did not excrete calcium in excess. The results of other researchers have confirmed our suspicions. Tsuruoka et al. (2007) have revealed that in rats which received CsA serum Ca concentrations were increased, but urinary reabsorption ratios of Ca were not affected by the drug. Moreover, the increase of urine deoxypyridinoline, an index of bone resorption, was observed. Therefore, it can be stated that the increase of total Ca in blood of rats in the CsA, rapamycin, and prednisone-treated rats is mainly due to bone resorption.

In our research, in all experimental groups a decreased expression of AQP1, AQP2, AQP3 and AQP4 was found, with the highest decrease in the CsA, rapamycin, and prenisone-treated rats. The presented results suggest that different multi-drug rapamycin-based immunosuppressive regimens to varying degrees inhibit water reabsorption by downregulating AQPs. Unfortunately, in the available literature, we could not find any research concerning the influence of multi-drug protocols on AQP expression that can be referred to. However, in a very few studies with use single immunosuppressive drugs it has been shown that CsA treatment decreases AQP expression in different experimental models (Lim et al., 2004; Rinschen et al., 2011). Lim et al. (2004) have reported that CsA treatment for 4 weeks significantly decreases the expression of AQP1-4 and urea transporters in rats.

One of the reasons for the downregulation of the AQPs may be renal tubular damage caused by the immunosuppressive drugs. It has been proved that long-term use of CNI leads to irreversible renal functional deterioration as a result of irreversible and progressive tubulo-interstitial injury and glomerulosclerosis (Naesens et al., 2009). However, in our previous studies we did not find any histopathological changes (eg. fibrosis, inflammatory infiltration, tubular atrophy, vacuolation of the tubular cells) in any experimental groups (Kędzierska et al., 2015). Our previous studies have shown that the applied drug combinations did not cause significant damage of the renal tubules. The blood plasma creatinine in the tested animals was relatively stable, there was no significant difference between the study groups and the control group (Kędzierska et al., 2015). In order to determine potential nephrotoxic changes, an analysis of concentrations of kidney damage markers in rat blood plasma, kidney injury molecule-1

(KIM-1) and monocyte chemoattractant protein-1 (MCP-1) was additionally carried out (Kędzierska et al. 2016). In all experimental groups no changes in the KIM-1 concentration were found. Interestingly, in the group in which mycophenolate mofetil, rapamycin and prednisone were administered, a significant reduction in MCP-1 concentration was found. MCP-1 influences the activation and migration of leucocytes into the tubule-interstitial space of the kidneys and plays an important role in renal fibrosis. Therefore, it may be that downregulation of AQPs in our experimental model could be the effect of specific action of immunosuppressive drugs applied and not the result of damage to the renal tubules.

Most data that allow for a broader explanation of the mechanisms of water reabsorption, in the renal tubules in response to body needs, concern AQP2. In our study, the observed downregulation of AQP2 is undoubtedly associated with long-term administration of CsA, rapamycin, and prednisone. Under normal physiological conditions the main factor regulating expression and distribution of AQP2 is the AVP. In response to hypernatremia or hypovolemia, AVP is secreted from the posteriori pituitary gland into the blood. Then via the bloodstream, AVP reaches the kidneys and binds to the vasopressin type-2 receptor (V2R), which is localized in the basolateral membrane of collecting duct principal cells (Ando and Uchida, 2018). In the short-term regulation, binding AVP to V2R activates a cascade signaling pathway leading to an increase of cyclic adenosine monophosphate (cAMP), activation of protein kinase A and an increased intracellular level of Ca^{2+}. This leads to AQP2 phosphorylation and its trafficking to the apical plasma membrane increasing the osmotic water permeability. After fusion with the apical plasma membrane of collecting duct principal cells, AQP2 is excreted into the urine or undergoes endocytosis. In the long-term regulation of AQP2, a prolonged stimulation of vasopressin markedly increases AQP2 abundance in the renal collecting duct (Kwon et al., 2013).

Besides AVP, several other factors modulate AQP2 expression and distribution, including extracellular tonicity and calmoduline-dependent serine/threonine phosphatase calcineurin (CaN) (Jo et al., 2001; Vukićević et al., 2016; Ando and Uchida, 2018). Calcineurin is an important signaling molecule in many cells. It is a heterodimer made up of two subunits, catalic A (CnA) and regulatory B (CnB) (Gooch et al., 2004). The involvement of the cell receptor with a related ligand induces an increase in intracellular calcium concentration, followed by the activation of calmodulin, which interacts with CaN, leading to its activation. Calcineurin dephosphorylates nuclear regulatory proteins such as nuclear factor of activated T-cells (NFATc), thereby facilitating their translocation from the cytoplasm to the nucleus, where they regulate gene expression for numerous proteins (Knoop et al., 2004). In the kidney, the expression of CaN was observed in the glomerulus, proximal tubule, medullary thick ascending

limbs, cortical and medullary collecting duct and connecting tubule (Tumlin et al., 1995; Gooch et al., 2004). In the CD, calcineurin is co-localized with AQP2 in intracellular vesicles (Gooch et al., 2004). The role of CaN in regulation of expression and trafficking of AQP2 is not fully explained. However, it is known that genetic loss of CaN Aα causes impaired urine-concentrating response to vasopressin with no accumulation of AQP2 in the apical plasma membrane and loss of this protein in the vesicle fraction (Gooch et al., 2006). Moreover, it is revealed that long-term CaN inhibition by CsA results in polyuria, and also decreased expression of renal AQPs and urinary osmolality (Batlle et al., 1986; Lim et al., 2004).

Under normal physiological conditions, an increase of hipertonicity of interstitium, and associated increased release of Ca^{2+} from endoplasmic reticulum and binding them to CnB causes dephosphorylation of NFATc in the cytosol and its subsequent translocation to the nucleus, where it binds to the promoter region of AQP2 gene (Gooch et al., 2006; Lim et al., 2007) (Fig. 10). Binding of calcium ions and CnB activation is also accompanied by an increase in AQP2 phosphorylation and increased expression in apical plasma membrane (Ando et al., 2016). Ambient hypertonicity also stimulates the activity of transcriptomal activator, tonicity-responsive enhancer binding protein (TonEBP). This protein protects renal cells from hypertonic stress and a high concentration of urea by stimulating the transcription of specific genes (Hasler et al., 2006; Lim et al., 2007). An increase of TonEBP also induces nuclear localization of NFATc, and contributes to upregulation of AQP2 expression (Li et al., 2007). The observed decrease in AQP2 expression in the experimental rats is probably associated with long-term inhibition of CaN.

As mentioned earlier, the long-term inhibition of calcineurin with CsA results in polyuria, decreased expression of renal AQPs and urinary osmolality (Batlle et al., 1986; Lim et al., 2004; Gooch et al., 2006). It is still unknown whether CsA affects AQPs, including AQP2, directly, or alters it through other mechanisms. In the course of the long-term treatment of CsA a reduction of medullary osmotic gradient was observed by decreased expression of active sodium transporters (sodium/potassium/chloride transporter type 2 (NKCC2), sodium/chloride transporter, sodium-potassium pump Na^+/K^+-ATPase), increased sodium excretion, and also reduced urinary concentration (Lim et al., 2007). In addition, a decrease in urea transporters that are responsible for accumulation of urea in the inner medullary collecting duct was also found (Lim et al., 2004). The decrease of hypertonicity of the renal medullary interstitium causes a decrease in the release of calcium ions into the cytoplasm, a decrease in CnB activity, and a decrease in TonEBP expression, which consequently leads to a reduction in NFATc nuclear translocation and downregulation of AQP2.

Decreased AQP2 expression under the influence of CsA was observed both in the studies conducted on

laboratory animals and in vitro in cultured inner medullary collecting duct (IMCD) cells. Despite the fact that this mechanism is still not fully explained, to date, it is known that apart from decreasing the molality of medullary interstitium, CsA decreases AQP2 protein level and mRNA expression, although it is suggested that this process probably is not associated with inhibition of calcineurin A (Rinschen et al., 2011). The incubation of IMCD with CsA causes an increased expression of glycogen synthase kinase type 3b (Gsk3b) that is involved in the regulation of AQP2 abundance by modulating adenylate cyclase activity (Rao et al., 2010; Rinschen et al., 2011). Under the influence of CsA, a decrease in expression of β-catenin, which is a downstream effector of Gsk3b activity, was also observed. Still, there is little known about β-catenin and Gsk3b. However, the proteomics study conducted by Nielsen and co-workers (Nielsen et al., 2008) shows that an increase in the expression of the mentioned proteins is observed in lithium-induced diabetes insipidus in rat.

Under the influence of CsA, a decrease in phosphorylation of cAMP-responsive element binding protein (CREB), which stimulates the transcription of AQP2, was also observed. In comparison to Rinschen and co-workers (2011), who suggest a lack of association between inhibition of CnA and a decrease in AQP2 expression, Gooch et al. (2006) have shown that loss of CnA causes disturbances in intracellular trafficking of the AQP2 in the apical plasma membrane.

Interestingly, the incubation at 48 h of IMCD in FK-506 does not cause a reduction in the expression of AQP2 protein and mRNA, which were observed under the influence of CsA. Under the influence of FK-506, a reduction in β-catenin expression was not observed, although there was an increase in Gsk3b expression and no changes in the CREB expression (Rinschen et al., 2011). However, it should be emphasized that there is no literature data concerning the long-term effects of FK-506 on AQPs, which does not preclude a reduction in their expression under long-term treatment.
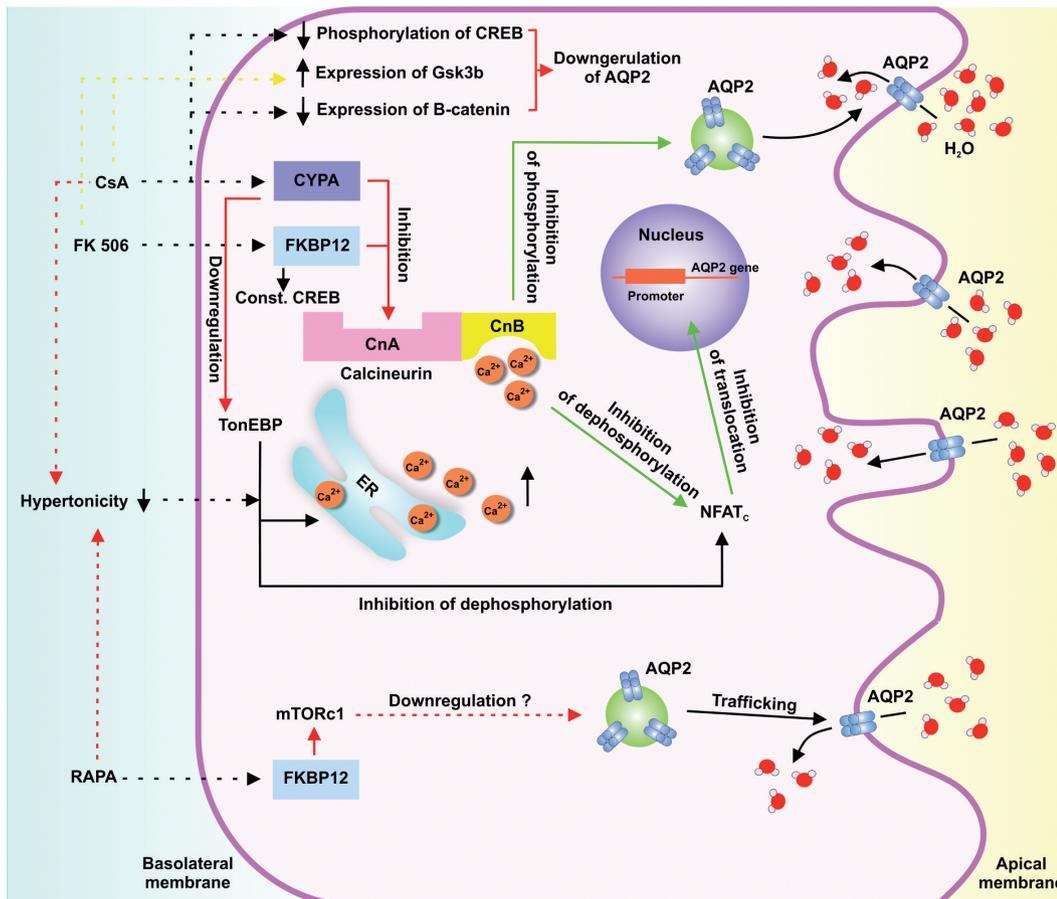
Fig. 10. Scheme showing the possible pathomechanism of cyclosporine A (CsA), tacrolimus (FK506) and rapamycin (RAPA) on aquaporin 2 (AQP2) expression in the collecting duct principal cells. CsA binds with the cyclophilin A (CYPA) and inhibits calcineurin A subunit (CnA). Deactivation of CnA results in impaired urine-concentrating response to vasopressin with no accumulation of AQP2 in the apical plasma membrane and loss of this protein in the vesicle fraction (not shown on the diagram). A long-term treatment of CsA decreased the hypertonicity of medullary interstitium. As a result, there is a decreased expression of tonicity-responsive enhancer binding protein (TonEBP) and translocation to the nucleus of nuclear factor of activated T cells (NFATc) that reduces the AQP2 transcription. The reduction of medullary osmotic gradient caused a decrease in the intracellular Ca^{2+} level that is necessary to activate the calcineurin B subunit (CnB), which 1) specifically dephosphorylates NFATc and 2) phosphorylates AQP2 allowing its trafficking to apical plasma membrane. A long-term treatment of CsA decreased phosphorylation of cAMP-responsive element binding protein (CREB), expression of β-catenin and increased glycogen synthase kinase type 3b (Gsk3b) expression. Changing the activity of these factors downregulates AQP2. FK506 binds with FK506-binding protein of 12 kDa (FKBP12) and inhibits CnA. A short-term treatment of FK506 does not downregulate AQP2, but increases the expression of Gsk3b. Moreover, FK506 does not change the expression of CREB. RAPA binds to FKBP12, which directly interacts with the FKBP-binding domain (FRB) of mammalian target of rapamycin complex 1 (mTORC1). A prolonged exposure of RAPA reduces the hypertonicity of medullary interstitium and probably downregulates the AQP2.

The mechanism of action of rapamycin is still not fully understood, however, it is known that rapamycin binds to FK-506-binding protein of 12 kDa (FKBP12), which directly interacts with the FKBP-binding domain (FRB) of mTORC1 (Brown et al., 2018). It has also been revealed that chronic treatment with rapamycin may result in some loss of mammalian target of rapamycin complex 2 (mTORC2) activity (Gaubitz et al., 2015). It is worth mentioning that in selected experimental models beneficial effects of rapamycin on the kidneys were noted. Yang et al. (2018) have found that in a unilateral ureteral obstruction model, rapamycin protects against renal fibrosis, tubular dilation and atrophy and also moderates the tubular proliferation and apoptosis processes. However, despite numerous studies on the effects of immunosuppressants on the kidneys, little is known about the impact of rapamycin on renal AQP expression. To date, only three papers have been published, including AQP2 expression under activation or inhibition of mTORC1 (da Silva et al., 2009; Gao et al., 2013; Chen et al., 2014). Unfortunately, these results do not explain how mTORC1 affects the expression of AQP2. Chen et al. (2014) have reported that the activation of mTORC1 causes many undesirable renal changes, including columnar cell lesions and dedifferentiation of collecting duct principal cells with the loss of AQP2. Da Silva et al. (2009) have found that a long-term treatment of rapamycin decreased the expression of NKCC2 in the thick ascending limb of Henle loop, increased the urinary exertion of sodium, potassium and magnesium and reduced by 54% the expression of AQP2. One of the causes of AQP2 downregulation may be the decrease in molality of renal medulla interstitium associated with the reduction in NKCC2 expression and increased Na^+ and K^+ excretion. In turn, Gao et al. (2013) have reported that changes in AQP2 expression have not been observed in the kidney of healthy mice treated with rapamycin. In contrast, in mice treated with lithium and induced nephrogenic diabetes isipidus, which is characterized by a reduction in AQP2 expression, co-administration of rapamycin resulted in additional downregulation of this protein. Decreased aquaporin expression was observed in all experimental groups, indicating impairment of water reabsorption in renal tubules. In addition, elevated MCHC and HCT values indicate mild cell dehydration. We suspect that high levels of total calcium in the blood of rats also contributed to their dehydration, which, with reduced expression of aquaporins, further contributed to their blood concentration. The mechanism of extracellular calcium activity in the kidneys is complex and still not fully understood (Michałek et al., 2014). To date, many authors have observed a correlation between the calcium plasma concentration and expression of total AQP2 in the kidney and urine (Sands et al., 1998; Valenti et al., 2000; Németh-Cahalan et al., 2004). Conducted studies in rats have shown that an increase in total concentration of calcium serum from 2.01 mmol/L to 2.35 mmol/L causes a reduction of 50% in total AQP2

expression in renal collecting ducts, and also a decrease of AQP2 expression in the apical membrane and the urine (Earm et al., 1998). Interestingly, in calves the decrease in plasma calcium concentration was accompanied by an increase of renal excretion of AQP2 in the urine (Michałek et al., 2014). Therefore, we suspect that the reduction of aquaporin expression under the influence of immunosuppressive drugs made rats particularly susceptible to dehydration.

Our study has limitations. During the experiment rats were not kept in metabolic cages and therefore urine was not collected from them. Data concerning urine parameters would undoubtedly complete the presented results.

Conclusions

In conclusion, long-term immunosuppressive treatment using multi-drug protocols can contribute to impair urine-concentrating ability by decreasing expression of the AQP1-4 in renal tubules in rat. Moreover, a mild increase of red cell indices and total calcium in the blood of rats may indicate cell dehydration tendency. Therefore, we suspect that the downregulation of AQPs by immunosuppressive drugs made rats susceptible to dehydration.

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