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Influence of Intrauterine Exposure to Safe and Contraindicated Immunosuppressive Drugs In Combinations during Pregnancy on Morphology and Function of Kidneys in Juvenile Wistar Rats

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Abstract

Objective: In the study we focused on the impact of "safe" and "contraindicated" immunosuppressive drugs in combinations during pregnancy on changes in native kidneys in juvenile Wistar rats after exposure of pregnant female rats to these drugs.

Method: The study was conducted on 32 female (full dose of drugs) and 8 female Wistar rats (half dose of drugs), subjected to immunosuppressive regimens commonly used in therapy of human kidney transplant recipients. The animals received drugs by oral gavage 2 weeks before pregnancy and during 3 weeks of pregnancy.

Results: Basing on serum creatinine concentrations, combination of tacrolimus, mycophenolate mofetil and prednisone turned out to be less harmful to the kidney than combination of cyclosporine A, mycophenolate mofetil and prednisone or cyclosporine A, everolimus and prednisone. Neutrophil-gelatinase associated lipocalin (NGAL) concentration in kidney seemed to be dose-dependent in rats treated with cyclosporine A, mycophenolate mofetil and prednisone. Morphological changes in kidneys in juvenile rats exposed to immunosuppressive treatment *in utero* were more pronounced in the first 3 weeks of life and diminished with age. In rats exposed to cyclosporine A, everolimus and prednisone we have observed a decrease in thickness of renal cortex and reduced diameter of glomeruli. These changes were still evident in 8-week-old animals.

Conclusion: Combination of tacrolimus, mycophenolate mofetil and prednisone turned out to be the least harmful to the kidney (the lowest creatinine concentrations); combination of cyclosporine A, everolimus and prednisone appeared to be the most harmful one – we observed not only an increase in serum creatinine concentrations but a decrease in thickness of renal cortex and reduced diameter of glomeruli as well.

Keywords: Combination of drugs; Immunosuppressive drugs; Kidney; Pregnancy; Therapy; Transplantation; Wistar rats

Objective

Successful renal transplantation improves the functions of many organs and systems previously affected by the uremic state. Female kidney graft recipients in reproductive age recover their menstrual cycles and fertility in 6 months following renal transplantation. While planning pregnancy in female recipient it appears advisable to wait one to two years after transplant to achieve stable graft function as well as stabilization of immunosuppressive medications. Such pregnancies are high-risk ones, immunosuppressive drugs and their active metabolites can cross the placental barrier and enter fetal circulation. During pregnancy, stable medication regimens should be changed as little as possible, and close maternal and fetal surveillance are required. Some immunosuppressive drugs are considered to be relatively safe during pregnancy (cyclosporine A, CsA; tacrolimus, Tc; azathioprine, steroids), others, relatively new, are contraindicated (mycophenolate mofetil, MMF; mammalian target of rapamycin inhibitors, mTOR inhibitors). Recently the use of azathioprine is decreasing concomitantly with an increase in the use of Tc or MMF. However, experience regarding use of many immunosuppressive drugs in human pregnancy is limited. The pregnancy issues that face recipients and caretakers with the current adjunctive therapies and differing combinations of immunosuppressive regimens continue to require further study. The most pressing issue is the question of whether fetal exposure to new drugs like MMF or mTOR inhibitors confers additional risk, relative to the potential improvement in maternal survival and maternal graft function/survival conferred by these drugs [1]. In rats nephrogenesis begins on embryonic day 12 and is completed between 10 and 15 days postnatally [2]. In previous studies in pregnant rats and humans some adverse effects of immunosuppressive drugs on the development of kidneys were confirmed. Prolonged exposure to steroids resulted in reduced number of nephrons, glomerulosclerosis and hypertension [3], but rats generally were not so sensitive to teratogenic effects of corticosteroids like mice and rabbits [4]. CsA in rats induced morphological alterations in renal parenchyma (reduced size of glomeruli, tubulopathy) of neonates and an increase in expression of proteins responsible for nephrotoxicity (iNOS, MMP2) [5]. Tacrolimus

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use in humans led to an incomplete maturation of renal potassium transport resulting in transient hyperkalemia and an increase in plasma creatinine concentration [6]. Other drugs were not harmful - no renal morphologic lesions were found in sirolimus-treated rats [7]. MMF was nephrotoxic neither in rats [8] nor in humans [9].

The aim of our study was to investigate the impact of "safe" and "contraindicated" immunosuppressive drug regimens (combinations of three drugs) on changes and development of kidneys in juvenile Wistar rats after exposure of female rats to immunosuppressive drugs during pregnancy. The medication doses used during the experiment reached levels in the therapeutic range.

Materials and Method

Animals and treatment

The study was conducted on 32 female and 8 male Wistar rats (the Centre of Experimental Medicine, Medical University in Bialystok, Poland). At the start of the experiment, the rats were 12 weeks old and their mean body mass was 230 g. The animals had genetic and health certificates issued by a veterinarian. This study was approved by the Local Ethical Committee for Experiments on Animals in Szczecin (No. 12/2013, dated 24 Oct 2013). The animals were housed singly, kept on a 12-hour-light-dark cycle and were given feed Labofeed H (Morawski, Kcynia, Poland) and water ad libitum.

We have chosen the combinations of three immunosuppressive drugs which are most frequently used in clinical practice in humans during maintenance immunosuppressive therapy. The experiments were performed using the pharmaceutical form of each drug. The animals received drugs by oral gavage (at a dose volume of 5 ml/kg daily). The doses used in the study were as follows: tacrolimus (Prograf, Astellas): 4 mg/kg/day; mycophenolate mofetil (CellCept, Roche): 20 mg/kg/day; cyclosporin A (Sandimmun Neoral, Novartis): 5 mg/kg/day; everolimus (Certican, Novartis): 0.5 mg/kg/day and prednisone (Encorton, Polfa): 4 mg/kg/day. The drug doses were based on data available in the literature [10-18]. The rats (n=32) were divided into four groups:

- control group (n=8) – control group did not receive the drugs, but rats were given the gavage base and olive oil under otherwise identical conditions as the other rats used in the experiment;
- CMG group (n=8) – received cyclosporine, mycophenolate mofetil and prednisone;
- TMG group (n=8) - received tacrolimus, mycophenolate mofetil and prednisone;
- CEG group (n=8) - received cyclosporine, everolimus and prednisone.

The animals received medication every 24 hours for approximately 5 weeks (2 weeks after the acclimatization period prior to mating-when placed with males 1:1 in separate cages - and later after mating during 3 weeks of pregnancy). Day 0 of pregnancy was confirmed the next morning by the presence of a vaginal plug. After mating each pregnant female rat was housed in a separate cage. Once a week the animals were weighed again, and medication dose was adequately adjusted based on the changed body mass. After delivery the treatment was stopped (no drugs administration during lactation period- women generally are advised not to breastfeed while taking immunosuppressive treatment as secretion of drugs into breast milk may attain high levels of drugs concentration in the suckling infant). Thirty one female rats completed the study.

Eighty three pups from 9 litters were born numbers of dams, litters and new-born rats in control and treatment groups at the time of birth are presented in Table 1. One pup from CMG group died in the age of 3 days.

Six 19-day-old rats from CMG group were euthanized as they seemed unable to live longer and reach the age of 8 weeks because of visible abnormalities (hydrocephaly, anophthalmia, apathy). They were euthanized together with 6 rats from a control group in the age of the corresponding day. The rest of juvenile rats from CMG group (5, 1 more died later in age of 28 days) was sacrificed after 8 weeks from birth (with corresponding group of 12 rats born from mothers from a control group).

As small numbers of rats from treatment groups were born, the experiment was repeated with half dose of study drugs (except of the dose of steroid which remained the same). The study included as few animals as possible. We used additional group of eight 12-week-old female rats divided in 3 separate groups (2 rats received the same drug combination as CMG group –named CMG group 0.5; 3 rats the same drug combination as TMG group – named TMG group 0.5 and 3 rats the same drug combination as CEG group- named CEG group 0.5). This additional study was approved by the Local Ethical Committee for Experiments on Animals in Szczecin (No.10/2014 and No.11/2014, both dated 06 Jun 2014). The doses used in the study were as follows: tacrolimus (Prograf, Astellas): 2 mg/kg/day; mycophenolate mofetil (CellCept, Roche): 10 mg/kg/day; cyclosporin A (Sandimmun Neoral, Novartis): 2.5 mg/kg/day; everolimus (Certican, Novartis): 0.25 mg/kg/day and prednisone (Encorton, Polfa): 4 mg/kg/day. The rest of procedures were identical as in the first part of experiment.

All 8 female rats completed the second part of the study. 63 pups from 7 litters were born-numbers of dams, litters and new-born rats in control and treatment groups at the time of birth are presented in Table 1.

The juvenile rats from these groups were sacrificed after 8 weeks from birth (we have used for analysis 12 rats from CMG group 0.5, 12 rats from TMG group 0.5 and 7 rats from CEG group 0.5).

All juvenile rats were euthanized by penthobarbitalum sodium

Number		Group			
		Control	CMG	TMG	CEG
Full dose of drugs	Dams	7	8	8	8
	Litters	6	2	-	1
	Rats	69	13	-	1
Half dose of drugs	Dams	-	2	3	3
	Litters	-	2	3	2
	Rats	-	24	32	7

CMG - CsA+MMF+Prednisone; TMG - Tc+MMF+Prednisone; CEG - CsA+Everolimus+Prednisone

Table 1: Number of dams, litters and newborn rats in control and treatment groups (full dose and half dose groups) at the time of birth.

(Polpharma) injection administered intraperitoneally at 40 mg/kg body weight. Their body weight was measured. Blood samples of rats in age of 8 weeks were obtained to determine lab tests. Blood tests included sodium, potassium and chloride level, urea, creatinine and uric acid concentration, total protein and albumin. Subsequently, necropsies of all rats were performed, and the collected kidneys were weighted. The right kidney was placed in a vat of liquid nitrogen (and later all samples were stored in -80°C for enzyme activity analysis). The left kidney was fixed in 4% buffered formalin solution for histological examination.

Markers of kidney injury

Kidney injury molecule KIM-1 (TIM-1), monocyte chemoattractant protein 1 (MCP-1) and neutrophil-gelatinase associated lipocalin (NGAL) were assessed in homogenized renal tissue of 8-week-old rats as the markers of kidney injury.

Homogenization protocol: Frozen whole kidneys were taken from liquid nitrogen and placed in a thermobox (-21°C). A small fragment of the tissues was placed in a metal homogenizator (previously cooled in a container with liquid nitrogen) and poured on 2-3 times with liquid nitrogen; then it was fragmented with a few hammer blows (4-5 times) against a metal mandrel (also previously cooled in a container with liquid nitrogen). Pulverized and frozen sample (volume equal to an approximately 1 mg of protein) was placed with a cooled spoon in an Eppendorf tube containing 500 µL of appropriate buffer (according to commercial enzyme assay kit procedure) previously cooled to the temperature of 4°C. After a short vortexation, homogenization was carried out with a knife homogenizator for about 15 s. Extract mixtures were centrifuged (3000 g for 10 min, at 4°C) and the supernatants stored at -80°C and used for enzyme assays.

Kidney injury molecule: *KIM-1 (TIM-1)* was assessed using the Quantikine Rat TIM-1/KIM-1/HAVCR Immunoassay (RnD System, USA). This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rat TIM-1 has been pre-coated onto a microplate. Standards control, and samples were pipetted into the wells and any rat TIM-1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rat TIM-1 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The intensity of the color measured was in proportion to the amount of at TIM-1 bound in the initial step. The sample values were then read off the standard curve.

Monocyte chemoattractant protein 1: MCP-1 was assessed using the rat MCP-1 Instant ELISA (an enzyme-linked immunosorbent assai for the quantitative detection of rat MCP-1, eBioscience, An Affymetrix Company, Viena, Austria). The tissue was homogenized as described above and diluted 1:25 with Sample Diluent. An anti-rat MCP-1 monoclonal coating antibody was adsorbed onto microwells. Rat MCP-1 present in the sample or standard binded to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-rat MCP-1 antibody binded to rat MCP-1 captured by the first antibody. Streptavidin-HRP binded to the biotin conjugated anti-rat MCP-1. Following incubation unbound biotin conjugated anti rat MCP-1 and Streptavidin- HRP was removed during a wash step, and substrate solution reactive with HRP was added to the wells. A coloured product was formed in proportion to the amount of soluble rat MCP-1 present in the sample. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. A standard curve was prepared from seven rat MCP-1 standard dilutions and rat MCP-1 sample concentration determined.

Neutrophil-gelatinase associated lipocalin: *NGAL* was assessed using the rat NGAL/lipocalin2/oncogene24p3 ELISA (Wuhan EIAab Science Company, Wuhan, China). The microtiter plate has been pre-coated with an antibody specific to NGAL. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for NGAL and Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB substrate solution was added to each well. Only those wells that contain NGAL, biotin-conjugated antibody and enzyme-conjugated Avidin would exhibit a change in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of NGAL in the samples was then determined by comparing the optical density of the samples to the standard curve.

Protein concentration in samples measurement: All results were expressed as a concentration in 1 mg of protein. To determine the protein content in the sample The Micro BCA Protein Assay Kit (Thermo Scientific, Pierce Biotechnology, USA) was used and the measurement was made according to the manufacturer's protocol. This Kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. The method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{1+}, which is formed when Cu^{2+} is reduced by protein in an alkaline environment [19].

Histological evaluation and its criteria

Paraffin slides (3 µm) were stained with hematoxylin-eosin (HE) and underwent general histological examination. Additionally, the thickness of renal cortex and diameter of glomeruli in kidneys were measured. The samples were independently examined by two experienced pathologists. All discrepancies were discussed right after the examination, problematic issues were resolved and representative images were chosen.

Drug concentration in blood: For the evaluation of drug concentrations in rats' blood we used two separate groups of female rats (n=14 x 2) in the corresponding age which were not pregnant. These rats were given identical doses of the drugs by oral gavage (full dose group and half dose group; every medication dose was adjusted based on weight). The drug concentration was determined in accordance with the literature [12,20] after 4 hours of oral administration. The concentration of drugs in blood was determined after 1 week of taking drugs once daily from the time of first administration. The concentrations of drugs were determined in all of the rats' blood. The concentration of CsA was determined with Abbott AxSYM assay, which is based on fluorescence (fluorescence polarisation immunoassay - FPIA). To determine Tc level we used IMx assay based on microparticle enzyme immunoassay (Microparticle Enzyme Immunoassay - MEIA). The test was performed using an Abbott analyser (Abbott Laboratories, Park, USA). The study was carried out at the Clinical Central Laboratory in Szczecin. The concentration of everolimus was determined at the Laboratory of Mass Spectometry IBB PAN in Warsaw using original author's method (ultra performance liquid chromatography/tandem mass spectrometry UPLC/MS/MS) [21].

Statistical analysis: The values of quantitative variables were compared between groups using non-parametric tests (Kruskal-Wallis and Mann-Whitney U test), due to most of the data being not normally

distributed (as assessed by Shapiro-Wilk's test). Kruskal-Wallis test is used for comparing two or more independent samples of equal or different sample sizes and extends the Mann-Whitney test when there are more than two groups. A significant Kruskal-Wallis test indicates that at least one sample stochastically dominates one other sample. The mean, standard deviation, median, minimum and maximum values were calculated for each group. The cut-off level of statistical significance was set at $p<0.05$. Calculations were performed using Statistica 10 software.

Results

The concentrations of drugs in blood are shown in Table 2. The results of the research and statistical analysis are presented in Tables 3-6 and Figures 1 and 2.

Body and kidneys mass

Analyzing obtained results we have found enlargement of kidneys in new-born rats from CMG group in the age of 19 days; however, rats from this group reached higher body weight comparing to control rats. Looking at kidney/body weight ratio we have obtained 0.006 for control rats and 0.007 for rats from CMG group – the difference seemed to be not significant. Statistically significant differences were not observed in 8-week-old rats in full and half dose regimen. Although 8-week-old rats from TMG group 0.5 reached lower body weight, their kidneys were not significantly reduced in size comparing to control rats (Table 3).

Laboratory blood test results

Blood biochemical parameters were analyzed only in 8-week-old rats. We have found lower value of serum chloride concentration in rats from CMG group when full dose of immunosuppressive drugs was used (Table 4). These differences were not observed in 8-week old rats when half dose of immunosuppressive drugs was used (Table 5). However, in this group of rats we noticed other changes – elevated total protein and albumin concentration in rats from TMG group 0.5. Increase in albumin concentration was also observed in rats from CMG group 0.5. Creatinine concentration was increased in rats from two groups – CMG group 0.5 and CEG group 0.5, but urea concentration was decreased in rats from CEG group 0.5 (Table 5).

Markers of kidney injury

Analyzing results of renal injury markers in kidney we have found higher levels of NGAL in 8-week-old rats from CMG group (full dose regimen) in comparison to rats from control group. There were no statistically significant differences in concentration of KIM-1 (TIM-1) and MCP-1 between these groups. In half dose regimen there were not statistically significant changes in concentrations of all analyzed renal injury markers between control and treatment groups (Table 6). Therefore level of NGAL concentration seemed to be dose-dependent-increased after full dose of drugs (CMG group) and comparable to control after reduction of dose (CMG group 0.5).

Histopathological evaluation

In histology the kidneys of 19-day-old rats from CMG group were changed as compared to control. Organs showed hypertrophy (in most cases) or hypotrophy. The morphometric measurements comparing the kidneys of control rats with kidneys of rats from CMG group showed statistical changes in the diameter of glomeruli. Diameter of glomeruli in rats from CMG group was higher - on average 50.281 μm versus 37.299 μm in control group ($p=0.002$). Thickness of renal cortex in rats from CMG group was on average 953.85 μm versus 1104.704 μm in control group (difference not statistically significant). In 19-day-old control kidney, at the periphery of cortex there was well visible, darker-staining zone with regularly arranged glomeruli. This zone was invisible in rats from CMG group. The border between cortex and medulla was clearly visible in control animals, whereas it was more difficult to distinguish it in rats from CMG group (Figure 1). In rats from CMG group glomeruli were located deeper, almost within medulla. There were also observed a cluster of darker-staining cells and the glomeruli were more frequently arranged in double (some of them were residual/underdeveloped, or two glomeruli were in common Bowman's capsule). In rats from CMG group there were also many single residual/underdeveloped glomeruli (Figure 2).

The renal morphology of control 8-week-old rats and rats from CMG group (full dose regimen) in the same age was similar. The arrangement of individual elements in the kidney parenchyma was unchanged. Thickness of renal cortex in rats from CMG group was on average 840.918 versus 933.503 in the control group; diameter of glomeruli in rats from CMG group was on average 65.679 μm versus 63.745 μm in the control group (differences not statistically significant).

In 8-week-old rats from half dose groups thickness of renal cortex in rats from CMG group 0.5 was on average 842.014 μm, from TMG group 0.5–852.701 μm versus 933.503 μm in the control group (differences not statistically significant). Diameter of glomeruli in rats from CMG group 0.5 was on average 59.859 μm, from TMG group 0.5–61.461 μm versus 63.745 μm in the control group (differences not

	Dose	Group			
		CMG (n=3 × 2)	TMG (n=4 × 2)	CEG (n=4 × 2)	Control (n=3 × 2)
CyclosporinA (ng/mL)	5 mg/kg	69.37 ± 45.61	-	50.35 ± 8.80	-
	2.5 mg/kg	33.57 ± 41.04	-	33.53 ± 12.84	-
Tacrolimus (ng/mL)	4 mg/kg	-	7 ± 6,61	-	-
	2 mg/kg	-	0.77 ± 0.57	-	-
Everolimus (ng/mL)	0.5 mg/kg	-	-	1.43 ± 0.17	-
	0.25 mg/kg	-	-	0.83 ± 0.22	-
Body mass (g)	Full dose group	240 ± 21	255 ±12.5	245 ± 22.5	260 ±16
	Half dose group	250 ± 50.1	235 ± 20	225± 11.5	255 ±15

CMG - CsA+MMF+Prednisone; TMG - Tc+MMF+Prednisone; CEG - CsA+Everolimus+Prednisone

Table 2: The medication concentrations (full dose and half dose) and weight of rats in the study groups (additional study groups). Results are presented as arithmetic mean ± standard deviation.

parameter		group				p (Mann-Whitney test)
		control	CMG	TMG	CEG	
19-day-old rats						
Body mass (g)	n	6	6			
	AM±SD	23.42 ± 0.78	30.74 ± 4.81	-	-	
	Med	23.25	31.49	-	-	
	range	22.42-24.72	25.00-36.88	-	-	0.002
Kidney mass (g)	n	6	6			
	AM±SD	0.14 ± 0.09	0.2 ± 0.07	-	-	
	Med	0.14	0.2	-	-	
	range	0.12-0.18	0.14-0.24	-	-	0.01
8-week-old rats (full dose of drugs)						
Body mass (g)	n	12	5			
	AM ± SD	210.65 ± 42.52	188.03 ± 45.97	-	-	
	Med	213.63	212.64	-	-	
	range	149.56-77.70	126.6-228.88	-	-	NS
Kidney mass (g)	n	12	5			
	AM ± SD	0.76 ± 0.16	0.65 ± 0.14	-	-	
	Med	0.76	0.73	-	-	
	range	0.54-0.98	0.42-0.80	-	-	NS
8-week-old rats (half dose of drugs)						
Body mass (g)	n	12	12	12	7	p (Kruskal-Wallis test)
	AM±SD	210.65 ± 42.52	185.895 ± 24.06	171.62 ± 34.84	207.17 ± 42.38	
	Med	213.63	181.2	164.83*	181.22	
	range	149.56-277.7	150.56-214.64	129.64-226.66	164.34-275.3	0.077
Kidney mass (g)	n	12	12	12	7	
	AM ± SD	0.76 ± 0.16	0.68 ± 0.075	0.63 ± 0.12	0.76 ± 0.15	
	Med	0.76	0.66	0.64	0.72	
	range	0.54-0.98	0.60-0.86	0.44-0.78	0.58-1.02	NS

CMG - CsA+MMF+Prednisone; TMG - Tc+MMF+Prednisone; CEG - CsA+Everolimus+Prednisone
AM – Arithmetic Mean; SD – Standard Deviation; Med – Median; p – Level of Significance; NS - Difference Non-Significant, * p<0,05 vs. Control group (Mann-Whitney test)

Table 3: Body and kidney mass results of rats.

Parameter		**Group**		**p (Mann-Whitney test)**
		Control n=12	CMG n=5	
Sodium (mmol/L)	AM ± SD	147.00 ± 2.26	146.20 ± 2.17	NS
	Med	147	145	
	range	143-151	144-149	
Potassium (mmol/L)	AM ± SD	5.24 ± 0.98	5.92 ± 1.00	NS
	Med	4.95	6.1	
	range	4.2-7.6	4.9-7.3	
Chloride (mmol/L)	AM ± SD	102.08 ± 1.51	98.80 ± 2.59	0.02
	Med	102	98	
	range	100-105	96-102	
Total protein (g/L)	AM ± SD	61.00 ± 2.17	61.80 ± 1.48	NS
	Med	60.5	62	
	range	58-64	60-64	
Albumin (g/L)	AM ± SD	32.50 ± 1.73	32.00 ± 1.58	NS
	Med	32	32	
	range	30-36	30-34	
Creatinine (mg/dL)	AM ± SD	0.50 ± 0.04	0.49 ± 0.05	NS
	Med	0.49	0.48	
	range	0.42-0.57	0.45- 0.56	
Urea (mg/dL)	AM ± SD	53.42 ± 8.84	54.6 ± 6.5	NS
	Med	52	55	
	range	42-74	48-61	
Uric acid (mg/dL)	AM ± SD	4.11 ± 2.29	3.94 ± 2.85	NS
	Med	4.5	4.1	
	range	1.3-7.7	1.0-7.4	

CMG - CsA+MMF+Prednisone, p – Level of Significance; NS - Difference Non-Significant,
AM – Arithmetic Mean; SD – Standard Deviation; Med – Median

Table 4: Laboratory blood test results of 8-week-old rats (full dose of drugs).

Parameter		Group				p(Kruskal- Wallis test)
		Control (n=12)	CMG 0.5 (n=12)	TMG 0.5 (n=12)	CEG 0.5 (n=7)	
Sodium (mmol/L)	AM ± SD	147.00 ± 2.26	147.58 ± 1.56	146.92 ± 0.07	146.00 ± 1.83	NS
	Med	147	147.5	146.5	146	
	range	143-151	145-150	144-150	143-148	
Potassium (mmol/L)	AM ± SD	5.24 ± 0.98	5.52 ± 1.11	5.00 ± 0.71	5.93±1.02	NS
	Med	4.95	5.40	5.00	5.90	
	range	4.2-7.6	3.7-7	3.9-6.1	4.7–7.5	
Chloride (mmol/L)	AM ± SD	102.08 ± 1.51	103.17 ± 2.44	102.25± 1.76	102.86 ± 3.24	NS
	Med	102	103	103	103	
	range	100-105	100-107	99-105	99-107	
Total protein (g/L)	AM ± SD	61.00 ± 2.17	58.51± 2.52	63.92 ± 3.50*	62.29 ± 4.89	**0.15**
	Med	60.5	64	63.5	63	
	range	58-64	61-68	59-72	55-69	
Albumin (g/L)	AM ± SD	32.50 ± 1.73	35.33±1.50***	35.42 ± 2.02**	34.71 ± 2.69	**0.0042**
	Med	32	35	35.5	35	
	range	30-36	33-38	33-40	31-38	
Creatinine (mg/dL)	AM ± SD	0.50 ± 0.04	0.56 ± 0.04**	0.53 ± 0.05	0.55 ± 0.03*	**0.010**
	Med	0.49	0.565	0.53	0.55	
	range	0.42-0.57	0.50-0.62	0.48-0.65	0.50–0.59	
Urea (mg/dL)	AM ± SD	53.42 ± 8.84	48.50 ± 9.97	49.50 ± 4.96	45.86 ± 4.06*	**0.043**
	Med	52	46	47	45	
	range	42-74	39-69	45-62	41-54	
Uric acid (mg/dL)	AM ± SD	4.11 ± 2.29	5.60 ± 2.84	5.36 ± 2.94	5.97 ± 2.58	NS
	Med	4.5	5.75	6.25	7	
	range	1.3-7.7	2.0-10.1	1.6-9.4	2.2-8.4	

CMG - CsA+MMF+Prednisone; TMG - Tc+MMF+Prednisone; CEG - CsA+everolimus+Prednisone; p – Level of Significance; NS - Difference Non-Significant, * p<0,05 vs. Control Group, ** p<0,01 vs. Control Group, *** p<0,001 vs. Control Group, AM – Arithmetic Mean; SD – Standard Deviation; Med – Median

Table 5: Laboratory blood test results of 8-week-old rats (half dose of drugs).

Parameter		Group				p (Mann-Whitney test)
		Control	CMG	TMG	CEG	
Full dose of drugs						
TIM-1 (pg/mg protein)	n	12	5	-	-	NS
	AM ± SD	94.70 ± 19.42	103.06 ± 47.73	-	-	
	Med	98.86	112.09	-	-	
	range	57.81-131.96	23.02-142.77	-	-	
MCP-1 (pg/mg protein)	n	12	5	-	-	NS
	AM ± SD	25.83 ± 20.12	32.65±19.73	-	-	
	Med	22.12	33.53	-	-	
	range	3.53-58.89	10.6-60.47	-	-	
NGAL (pg/mg protein)	n	12	5	-	-	0.026
	AM ± SD	129.86±15.49	169.9 ± 32.03	-	-	
	Med	122.85	155.46	-	-	
	range	110.29-157.17	144.44-218.90	-	-	
Half dose of drugs						p (Kruskal-Wallis test)
TIM-1 (pg/mg protein)	n	12	12	12	7	NS
	AM ± SD	94.70 ± 19.42	89.25 ± 18.97	70.98±25.54	97.53±36.09	
	Med	98.86	95.58	74.82	106.20	
	range	57.81-131.96	61.39-114.64	35.34-104.79	25.84-126.95	
MCP-1 (pg/mg protein)	n	12	12	12	7	NS
	AM ± SD	25.83 ± 20.12	18.33±12.06	16.85±17.96	34.96 ±23.60	
	Med	22.12	17.61	16.68	35.83	
	range	3.53-58.89	3.06-44.63	0.59-53.41	4.27-71.16	

NGAL (pg/mg protein)	n	12	12	12	7	NS
	AM ± SD	129.86 ±15.49	118.34 ± 23.33	122.78 ± 23.57	142.91 ± 26.26	
	Med	122.85	95.58	74.82	106.20	
	range	110.29-157.17	86.12-146.34	98.71-166.21	105.82-166.21	

CMG - CsA+MMF+Prednisone; TMG - Tc+MMF+Prednisone; CEG - CsA+Everolimus+Prednisone; TIM-1 - Kidney Injury Molecule KIM-1 (TIM-1); MCP-1 - Monocyte Chemoattractant Protein; NGAL - Neutrophil-Gelatinase Associated Lipocalin; AM – Arithmetic Mean; SD – Standard Deviation; Med – Median; p – Level of Significance; NS - Difference Non-Significant

Table 6: Concentrations of renal injury markers in 8-week-old rat kidney in the study groups.

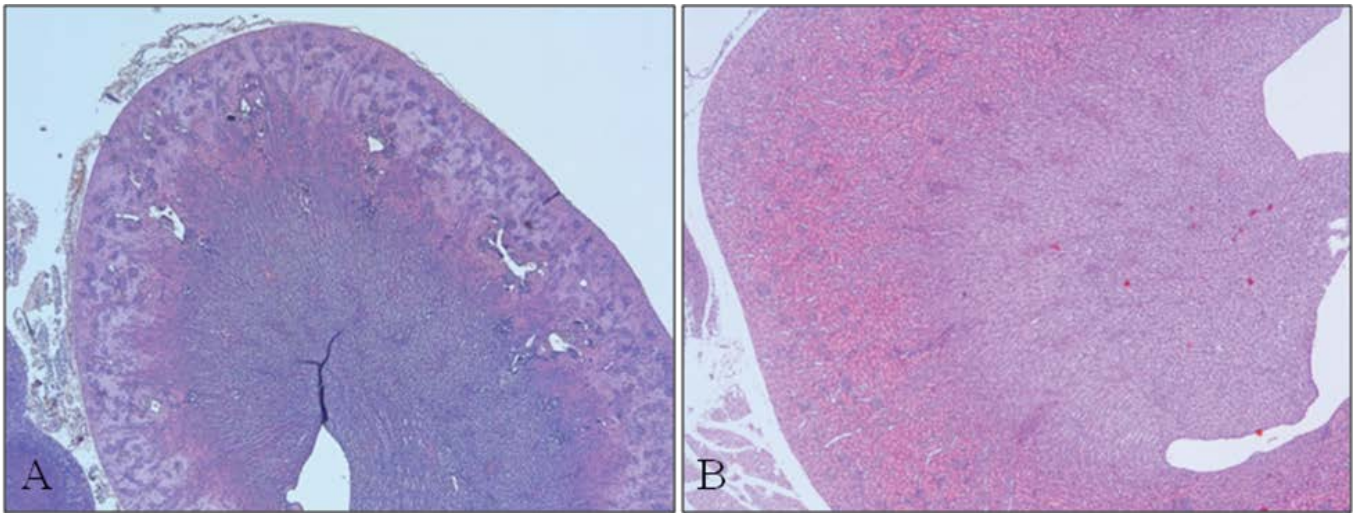

Figure 1: The image of kidney of 19-day-old rat from control (A) and CMG group (B). Objective magnification: x5. Staining: HE.

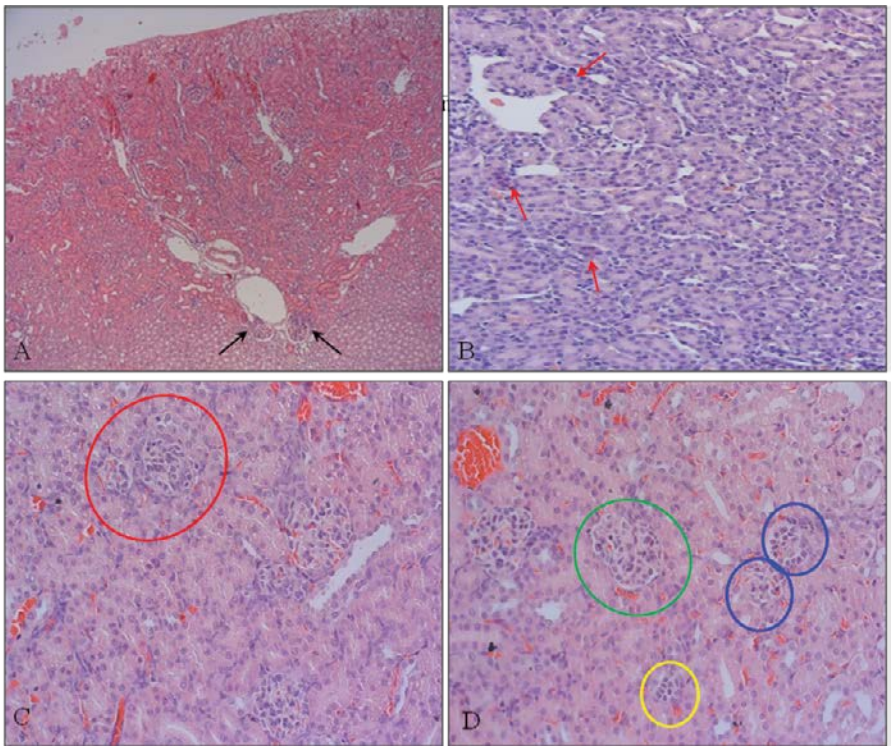

Figure 2: The renal morphology of 19-day-old rat from CMG group. The glomeruli located deeper (A; black arrows). The cluster of darker staining cells (B; red arrows). Single residual/underdeveloped glomerulus (C; red sphere and D; yellow sphere). The glomeruli in double (C, D); both glomeruli underdeveloped/very small (D; blue sphere); two glomeruli in one common Bowman's capsule (D; green sphere). Objective magnification: A x10, B x20, C, D x40. Staining: HE

statistically significant). Thickness of renal cortex in rats from CEG group 0.5 was 757.992 μm – lower than in the control group (p=0.018). Diameter of glomeruli in rats from CEG group 0.5 was 54.018 μm – lower than in the control group (p=0.006), as well (image not shown). Comparing results of morphometric measurements in rats from CMG groups (full dose regimen – CMG group and half dose regimen - CMG group 0.5) we have found no differences in the thickness of cortex and the diameter of the glomeruli.

Discussion

Effect of MMF and mTOR inhibitors on the development of fetal organs *in utero* in prospective study can be examined only in animal model. In most animal studies on the toxicity of immunosuppressive drugs during pregnancy the effect of only single drug was examined – in most studies it was CsA [4,5]. In our experiment the creation of a model of immunosuppressive drugs action, which was comparative to chronic immunosuppressive therapy commonly used in clinical practice in humans (combination of three drugs), was attempted. The applied doses of immunosuppressive drugs warranted that their blood concentrations could reach the level within a therapeutic range (but non-toxic one), as in the work of other investigators [22-24]. Drug trough levels were measured 4 hours after oral supply, based on the literature data [12,20]. This was considered an optimal time for determining the concentration of drugs in blood, due to different drug metabolism in rats compared to humans.

Analyzing the mass of kidney we have found enlarged kidneys only in very young rats from CMG group comparing to controls (in the age of 19 days), however, rats from CMG group reached higher body weight comparing to control rats. These rats had many abnormalities. During necropsies we have observed hydrocephaly, anophthalmia, organomegaly (photo not shown); higher heart and liver masses were also found. In the study of Kędzierska et al. [25] the rats receiving CsA were seen to have significantly higher body weight than rats receiving CsA-free regimen. This effect could be probably dose-dependent. In rats from TMG group 0.5 we noticed even reduction in body weight compared to control rats, like in the above mentioned study [25] where Tc had the strongest negative influence on the rat body weight. The hyper- or hypotrophy of kidneys were not observed in 8-week old rats independently on the dose. Decrease in body mass and the mass of kidney were not observed in CEG group 0.5 (where mTOR inhibitor, everolimus was used). In observations of other authors treatment with mTOR inhibitor, sirolimus led to lower body mass in adult rats [22]; fetal hypothrophy was associated with *in utero* exposure to this drug in animals [6]. Impact of CsA (rats were treated with combination of everolimus and CsA) could partially explain this phenomenon.

Analysing biochemical parameters from blood we have found decreased concentration of chloride in rats from CMG group. It is possible that exposure to immunosuppressive drugs like CsA *in utero* could influence the transport of ions in nephrons. Cui et al. [26] identified two genes, Slc12a3 and kidney-specific Wnk1 (KS-Wnk1), that are known to be involved in sodium transport in the distal nephrons and could potentially be involved in the mechanism of calcineurin inhibitors induced nephrotoxicity. They have found down-regulation of these genes in animals treated with CsA or Tc and hypothesized that decreased expression of Slc12a3 and KS-Wnk1 could have altered the sodium chloride reabsorption in the distal tubules. Esteva-Font et al. [27] confirmed an increase in the Na-K-2Cl co-transporter of the loop of Henle (NKCC2) in CsA-treated rats. Therefore the lower level of chloride in our experiment could be explained by influence of CsA on the function of ions transporters. This effect disappeared after reduction of dose - in rats from CMG group 0.5 level of chloride in serum was comparable to control rats.

We have not found differences in concentrations of creatinine, urea, total protein and albumin levels between control rats and rats from CMG group (full dose group, 5 rats). After reduction of doses of immunosuppressive drugs some differences in these parameters occurred. One should remember that we were able to analyze more rats per each group – CMG group 0.5 – 12 animals, TMG group 0.5 – 12 animals; CEG group 0.5–7 animals, so some changes could become more noticeable. Creatinine concentration was increased in rats from two groups – CMG group 0.5 and CEG group 0.5. Urea concentration was decreased in rats from CEG group 0.5. In both combinations one drug was potentially nephrotoxic (CsA), the other one not (MMF, everolimus). The antenatal exposure to CsA could cause oligonephronia; this drug in the dose of 25 mg/kg exerted the most striking fetotoxic effect accompanied by maternal and fetal renal tubulotoxicity (focal proximal straight tubular cell vacuolation). In electron microscopy cytoplasmic vacuolisation was due to dilatation of the endoplasmic reticulum. Kidneys of fetuses showed focal, proximal straight tubular cell necrosis-the damage was more severe than the sublethal tubular cell injury observed in the mothers [4]. In contrast, rats treated with MMF had lower creatinine serum concentrations compared to rats not treated with this drug [25]. This relationship was found in another studies, too, MMF was not nephrotoxic [8,9]. Equidosed sirolimus (rapamycin) did not alter the glomerular filtration rate. No renal morphologic lesions were found in rapamycin-treated animals [7]. However, when combination of sirolimus and CsA was used [28], sirolimus has been shown to exacerbate CsA-induced nephrotoxicity (a significant decrease in creatinine clearance). In study of Piao et al. [18], both sirolimus and everolimus aggravated CsA-induced renal injury, but the pharmacologic interaction between everolimus and CsA at the tissue level was less pronounced than between CsA and sirolimus. In our study, combination of Tc with MMF and prednisone (TMG group) turned out to be less nephrotoxic than combination of CsA with MMF and prednisone (CMG group) or CsA with everolimus and prednisone (CEG group).

We have also noticed increased total protein and albumin concentration in rats from TMG group 0.5 and increase in albumin concentration in rats from CMG group 0.5. Due to technical difficulties we have not collected rats' urine for daily proteinuria during the experiment. Nevertheless, calcineurin inhibitors, like CsA and Tc, exert well-known effect of proteinuria reduction via constriction of afferent arteriole in kidney [29] what can be responsible for these results. Additionally, in study of Wu et al. treatment with MMF prevented increased urinary albumin excretion in diabetic rats [30].

Recently, tree novel biomarkers turned out to be eligible in detecting kidney injury, including NGAL, KIM-1 and MCP-1. NGAL protein is expressed in several tissues, including neutrophils, kidney (proximal tubule), liver, adipocytes and macrophages. Induction and expression of NGAL was observed in acute, ischemic and nephrotoxic kidney injury [31-33]. In our study we have found higher levels of NGAL in 8-week-old rats from CMG group (full dose) in comparison to rats from control group. In half dose regimen there were not statistically significant changes in concentrations of NGAL between control and treatment groups. This last observation was consistent with results obtained in another study [34]. Therefore level of NGAL concentration seemed to be dose-dependent- higher after full dose of drugs (CMG group) and lower after reduction of dose (CMG group 0.5). T cell Immunoglobulin Mucin (TIM-1, also known as KIM-1) is expressed

at low levels in the normal kidney but it is markedly upregulated in the proximal tubules of the postischemic kidney, regenerating proximal tubular cells or tubules surrounded by a highly proliferative and fibrogenic interstitial response [35]. It is also involved in the regulation of Th1- and Th2-cell-mediated immunity [36,37]. KIM-1 is one of sensitive indicators of acute renal failure due to toxins. MCP-1 is a potent chemoattractant for macrophages and monocytes and was later shown to attract also CD4+ and CD8+ T lymphocytes and NK cells. Numerous cell types, including tubular epithelial cells and mesangial cells, are known to be capable of expressing this molecule. MCP-1 was postulated to play a pathogenic role in a variety of diseases characterized by mononuclear cell infiltration, including chronic inflammatory states and allergic responses. In kidney presence of MCP-1 triggered inflammation and fibrosis in renal parenchyma [38]. There were no statistically significant differences in concentration of KIM-1 (TIM-1) and MCP-1 in 8-week-old rats from treatment groups independently on dose in comparison to rats from control group. In previous studies it looked different - expression of KIM-1 was increased in rat kidney in a model of cyclosporine-induced nephrotoxicity and in rats treated with combination of cyclosporine and mTOR inhibitor sirolimus [23,28]. Tacrolimus up-regulated renal cortical gene for MCP-1 [39]. On the other hand, in the study of Wu et al [30] MMF might have suppressed up-regulation of MCP-1 expression in diabetic kidneys in rats mainly via suppression of macrophage infiltration. These data suggest that there is a possibility that use of calcineurin inhibitors like CsA and Tc together with MMF or everolimus in one combination could prevent an increase in concentration of KIM-1 (TIM-1) and MCP-1 in renal tissue.

In everyday practice histological assessment is used in kidney quality evaluation so preparations made from rat kidney, stained with HE method were evaluated in search of potential acute or chronic lesions within the kidney tubules, glomeruli and vessels. In histology, the kidneys of 19-day-old rats from CMG group were changed as compared to control - in most cases hypertrophic with decrease in thickness of renal cortex and increase in diameter of glomeruli – as described in results. The renal morphology of older 8-week old rats from control and treatment groups was similar. The impact of immunosuppressive drugs on renal development can be more clearly visible in very young animals as in rats nephrogenesis begins on embryonic day 12 and is continued during early postnatal life (is completed between 10 and 15 days postnatally) [2]. Thus the differences between control and treated group may be less distinct in older rats. Some injuries can be even reversible after delivery when organism is not exposed to immunosuppression any longer. Thickness of renal cortex and diameter of glomeruli in rats from CEG group 0.5 was decreased than in the control group. It could be explained with effect of mTOR inhibitors. In study of Viklický et al. [15] rapamycin effectively reduced compensatory renal allograft hypertrophy. Kurdián et al. [17] proved that treatment with everolimus diminished glomerular hypertrophy and blunted the increased expression of TGF β (what could partially explain its anti-fibrotic effect).

In summary, changes in kidneys in juvenile Wistar rats after exposure to immunosuppressive treatment *in utero* (CsA, MMF, prednisone) were more pronounced in first 3 weeks of life and diminished with age. NGAL concentration in kidney seemed to be dose-dependent- increased after full dose of drugs (CsA, MMF, prednisone) and comparable to control group after reduction of dose. There were no statistically significant differences in concentration of KIM-1 (TIM-1) and MCP-1 in rats from treatment groups independently of the dose in comparison to rats from control group. In our study combination of Tc with MMF and prednisone turned out to be less harmful to the kidney than combination of CsA with MMF and prednisone or CsA with everolimus and prednisone. In rats exposed prenatally to CsA, everolimus and prednisone we have observed a decrease in thickness of renal cortex and reduced diameter of glomeruli, the effects were still present in 8-week-old animals.

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