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From Cell Biology to Tissue Engineering

Birth defects in juvenile Wistar rats after exposure to immunosuppressive drugs during pregnancy

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Summary. Introduction: Immunosuppressive drugs and their active metabolites can cross the placental barrier and enter fetal circulation. The adverse effects on the fetus include chromosomal aberrations, structural malformations, organ-specific toxicity and intrauterine growth retardation. The aim of our study was to investigate the impact of "safe" and "contraindicated" immunosuppressive drugs on birth defects in juvenile Wistar rats after exposure of pregnant female rats to these drugs.

Material and methods: The study was conducted on 32 female Wistar rats, subjected to immunosuppressive regimens most 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: Treatment with mycophenolate mofetil and everolimus turned out to be toxic. We have noticed a significantly reduced number of live births in all pregnant rats exposed to these drugs in combination with calcineurin inhibitors and prednisone. Malformations and histological changes of fetal organs were confirmed after mycophenolate mofetil exposure during pregnancy.

Conclusions: Mycophenolate mofetil turned out to be more toxic when used with tacrolimus than with cyclosporin (delivery of live offspring was possible only in the latter group). Everolimus in combination with cyclosporin effectively suppressed the fetal maturation in utero, but did not contribute to the development of malformations.

Key words: Congenital malformation, Rats, Pregnancy, Immunosuppressive Drugs, Transplantation

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Introduction

As the first reported pregnancy after transplantation occurred in a kidney recipient in 1958, the National Transplantation Pregnancy Registry (NTPR) was established in 1991 to study the outcomes of pregnancies in female transplant recipients and pregnancies fathered by male transplant recipients. Such pregnancies are high-risk and require coordinated care among maternal fetal medicine and transplant specialists as immunosuppressive treatment must be continued to preserve all functions of transplanted kidney. According to data from NTPR there are significantly more stillbirths, preterm deliveries and increased incidence of low birth weight in

Abbreviations. ALT, alanine transaminase; Ang II, angiotensin II; AST, aspartate transaminase; CEG, cyclosporine A + everolimus + prednisone; CMG, cyclosporine A + mycophenolate mofetil + prednisone; CsA, cyclosporine A; FPIA, fluorescence polarisation immunoassay; HE, hematoxylin-eosin; MEIA, Microparticle Enzyme Immunoassay; MMF, mycophenolate mofetil; mTOR, mammalian target of rapamycin; NTPR, National Transplantation Pregnancy Registry; ROS, reactive oxygen species; Tc, tacrolimus; TMG, tacrolimus + mycophenolate mofetil + prednisone; UPLC/MS/MS, ultra performance liquid chromatography/tandem mass spectrometry

the transplant group (Armenti et al., 2008). However, most pregnancies go well and their offspring have normal postnatal growth and development. Immunosuppressive drugs and their active metabolites can cross the placental barrier and enter fetal circulation. The adverse effects on the fetus could include chromosomal aberrations, structural malformations, organ-specific toxicity and intrauterine growth retardation (Armenti et al., 2008). Some immunosuppressive drugs are considered to be relatively safe during pregnancy (cyclosporine A, CsA; tacrolimus, Tc; azathioprine and steroids), others, relatively new, are contraindicated because of possible and suspected toxicity (mycophenolate mofetil, MMF and mammalian target of rapamycin, mTOR inhibitors). In the United States of America MMF is now used by nearly 80% of kidney transplant patients (Anderka et al., 2009). We have limited data from case reports regarding fetotoxicity of drugs contraindicated during pregnancy. In human pregnancies exposed to MMF there is a high incidence of birth defects and spontaneous miscarriage (Armenti et al., 2008; Hirachan et al., 2012). In the general population malformations occur in approximately 3-5% of live births (Armenti et al., 1997) and the incidence of birth defects in the live-born of female kidney recipients appears similar to the general population. Pregnancies with MMF exposure have a 23% incidence of birth defects. Structural malformations include: hypoplastic nails and shortened fifth fingers, microtia (ear deformity) with cleft lip and palate, microtia alone, and neonatal death with multiple malformations (Sifontis et al., 2006). In another observation the fetus had facial dysmorphology and midline anomalies, including agenesis of the corpus callosum (Le Ray et al., 2004). In a study of Perez-Aytes et al. (2008) the infant born to a recipient of renal transplantation, who became pregnant while taking MMF, exhibited cleft lip and palate, bilateral microtia and atretic external auditory canals, chorioretinal coloboma, hypertelorism, and micrognathia. In a study of Schoner et al. (2008) MMF may have contributed to or even caused acrofacial dysostosis phenotype and extensive clefting in the newborn, together with internal cardiovascular, gastrointestinal and urogenital malformations. The above-described pattern establishes a link between exposure to MMF and development of the structures derived from the frontonasal prominence and first pharyngeal arch (MMF embryofetopathy?). However, live-born outcomes without structural malformations have also been noted in the MMF cohort (Armenti et al., 2008). No structural defects have yet been reported after exposure to mTOR inhibitor, sirolimus during early pregnancy in a limited number of recipients evaluated (Sifontis et al., 2006; Armenti et al., 2008), but 3 pregnancies of 7 ended up with spontaneous abortion (all occurred in the first trimester). Differing combinations of immunosuppressive regimens continue to require further study and it is critical to determine the safety of newer adjunctive agents during pregnancy, relative to the potential improvement in maternal survival and maternal graft function/survival conferred by these drugs (Armenti et al., 2008). Although experience has shown that animal studies are not entirely accurate in the evaluation of teratogenicity, they continue to provide a necessary assessment of potential toxicities that may exist in humans (Farley et al., 1991). We are unable to carry out a prospective and controlled study on human pregnancy so we used Wistar rats as suitable for toxicity studies (small body size, long survival rate, ease of handling and fact that immunosuppressive drugs are likely to be teratogenic in rats - Yamatoya et al., 2012). The aim of our study was to investigate the impact of recommended and not recommended immunosuppressive drugs on birth defects in juvenile Wistar rats after exposure of pregnant female rats to these drugs. The immunosuppressive regimens most commonly used in therapy of human kidney recipients were examined. The medication doses used during the experiment reached therapeutic ranges.

Materials and methods

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 weight was 230g. 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*.

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 (Katz et al., 1991; Viklicky et al., 2001; Jolicoeur et al., 2003; Ma et al., 2006; Westrhenen et al., 2007; Martinez-Palli et al., 2011; Kuridan et al., 2012; Piao et al., 2012; Sagiroglu et al., 2014). 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 identical conditions as the other rats used in the experiment;
- CMG group (n=8) - received cyclosporine A, mycophenolate mofetil and prednisone;
- TMG group (n=8) - received tacrolimus,

mycophenolate mofetil and prednisone;

•CEG group (n=8) - received cyclosporine A, 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 weight. After delivery the treatment was stopped (no drugs administration during lactation period, as in humans breastfeeding is not advised while taking immunosuppressive drugs). 31 female rats completed the study.

Only pups from control group and CMG group were born. The rats at age of 19 days and 8 weeks were euthanized by intraperitoneal penthobarbitalum sodium (Polpharma) injection administered intraperitoneally at 40 mg/kg body weight. Their body and organ weights were measured. Blood samples of rats at age of 8 weeks were obtained to determine lab tests. Blood tests included: basic haematology, sodium, potassium and chloride level, urea, creatinine and uric acid concentration, total protein and albumin, AST, ALAT. Subsequently, necropsies of all rats were performed, the collected organs (brain, liver, heart, kidneys) were weighted and fixed in 4% buffered formalin solution for histological examination.

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.

Drug concentration in blood

For the evaluation of drug concentrations in rats' blood we used a separate group of female rats (n=14) at the corresponding age which were pregnant. These rats were given identical doses of the drugs by oral gavage (every medication dose was adjusted based on weight). The drug concentration was determined in accordance with the literature (Ma et al., 2006; Schmitz et al., 2009) 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 - Tszyrsznic et al., 2013).

Statistical analysis

Quantitative variables were compared between groups with nonparametric Mann-Whitney U test, which was used because the number of rats was too small to assess reliably the normality of distribution. The mean (AM), standard deviation (SD), median (Med), 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

Offspring

The results of drug concentrations in blood are shown in Table 1. The results of the research and statistical analysis are presented in tables 2-4 and figures 1-10. Eighty three pups from 9 litters were born- 69 pups from a control group (6 litters), 13 pups from CMG group (2 litters; 1 pup died at the age of 3 days) and only 1 pup from CEG group. No pups from TMG group were

Table 1. The medication concentration in blood and weight of female rats (additional study groups). Results are presented as arithmetic mean ± standard deviation.

	Control Group (n=3)	CMG Group (n=3)	TMG Group (n=4)	CEG Group (n=4)
Cyclosporin A (ng/mL)	-	69.37±45.61	-	50.35±8.80
Tacrolimus (ng/mL)	-	-	7.00±6.61	-
Everolimus (ng/mL)	-	-	-	1.43±0.17
Body mass (g)	260.00±16.00	240.00±21.00	255.00±12.50	245.00±22.50

CMG, CsA+MMF+prednisone; TMG, Tc+MMF+prednisone; CEG, CsA+everolimus+prednisone.

born. We have noticed a reduced number of litters and rats per litter in all rats treated with immunosuppressive drugs in comparison to control group. Treatment in TMG and CEG groups turned out to be very toxic – no live births in TMG group and only 1 rat in CEG group (without malformations). In CMG group there were 2 litters - half of the rats were malformed and half looked quite normal.

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 (Fig. 1). They were euthanized together with 6 rats from a control group at the same age. The rest of the juvenile rats from CMG group (5, 1 more died later at age of 28 days) was sacrificed after 8 weeks from birth (with corresponding group of 12 rats born from mothers from a control group).

Body and collected organs weight

Analyzing the obtained results we found

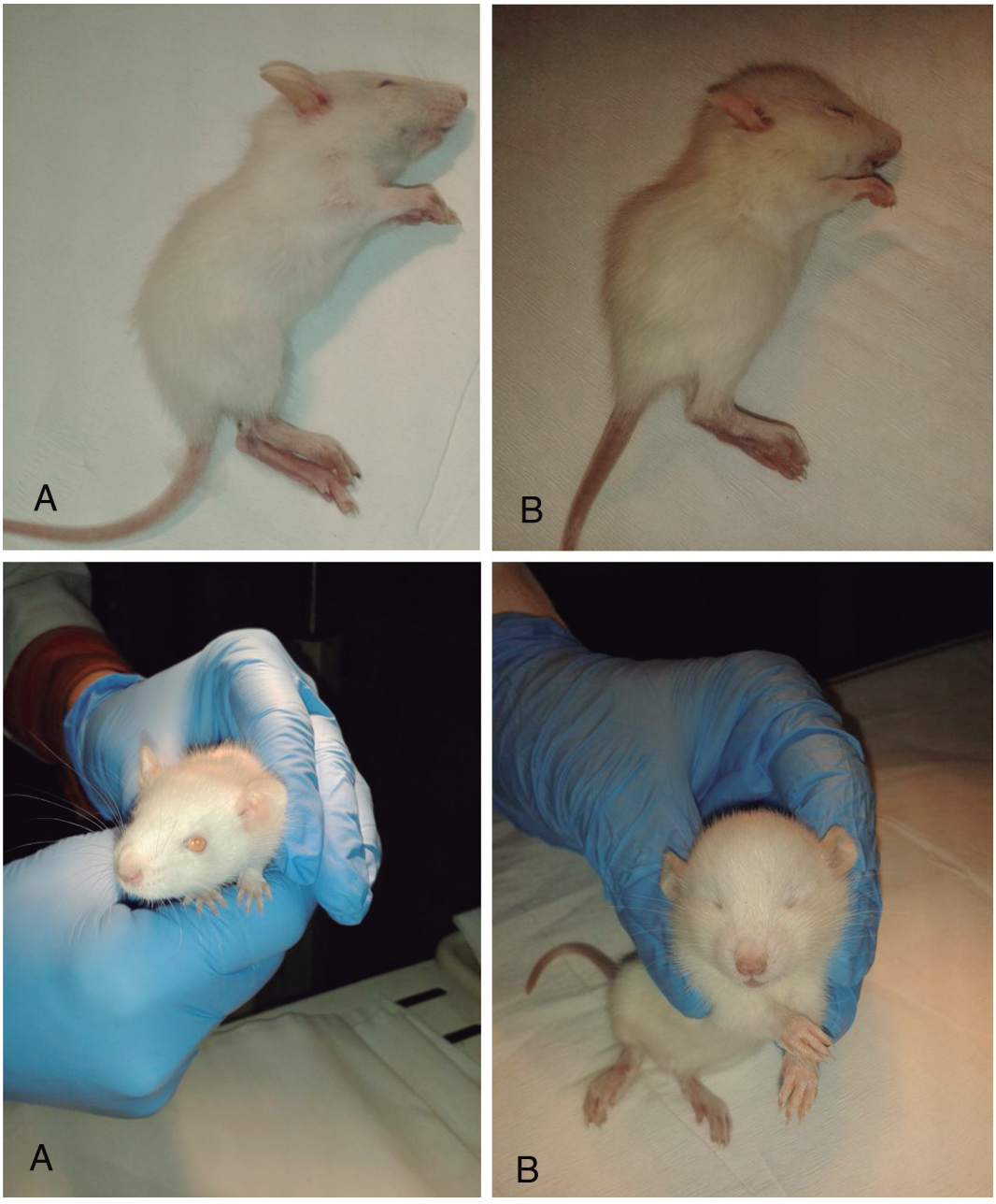

Fig.1. Morphology of 19-day-old rat from control **(A)** and CMG group **(B)**. Hydrocephaly and anophthalmia in rat from CMG group.

enlargement of organs in newborn rats from CMG group at age of 19 days; rats from this group reached higher body weight compared to control rats (Table 2). Due to hydrocephaly we were unable to assess precisely the mass of the brain. The heart and kidneys were statistically heavier, as well as the livers ("borderline" statistical significance). These differences were not observed in 8-week old rats (Table 3).

Laboratory blood test results

Blood haematological and biochemical parameters were analyzed only in 8-week-old rats. We found higher values of white cell count and lymphocytes cell count, as well as AST activity in rats from CMG group; and lower value of chloride concentration (Tab. 4).

Histopathological evaluation

Central nerve system

Brains collected from 19-day-old rats from CMG group were highly deformed (Fig. 2B) in comparison to control rats (Fig. 2A). Brains were extended in anterior-posterior axis and their topography was robustly altered. Forebrains were extremely elongated and their thickness was slender as a result of enormous volume of ventricles. The cerebellum of 19-day-old rats from CMG group was elongated as compared to control rats (Fig. 4). The hippocampus of rats from CMG group was

Table 2. Body and organ weight results of 19-day-old rats in control and CMG group.

parameter/group	Control group (n=6)	CMG group (n=6)	p (Mann-Whitney test)
Body mass (g)			
AM±SD	23.42±0.78	30.74±4.81	0.002
Med	23.25	31.49	
range	22.42-24.72	25.00-36.88	
Heart mass (g)			
AM±SD	0.11±0.01	0.22±0.05	0.004
Med	0.12	0.20	
range	0.10-0.12	0.18-0.30	
Liver mass (g)			
AM±SD	0.80±0.14	0.98±0.14	0.051
Med	0.84	0.98	
range	0.56-0.90	0.76-1.10	
Spleen mass (g)			
AM±SD	0.07±0.02	0.10±0.03	NS
Med	0.07	0.10	
range	0.04-0.08	0.06-0.12	
Thymus mass (g)			
AM±SD	0.10±0.04	0.12±0.05	NS
Med	0.10	0.14	
range	0.06-0.18	0.04-0.02	
Kidney mass (g)			
AM±SD	0.14±0.09	0.20±0.07	0.019
Med	0.14	0.20	
range	0.12-0.18	0.14-0.24	

AM, arithmetic mean; SD, standard deviation; Med, median; p, level of significance; NS, difference non-significant; CMG, CsA+MMF+prednisone.

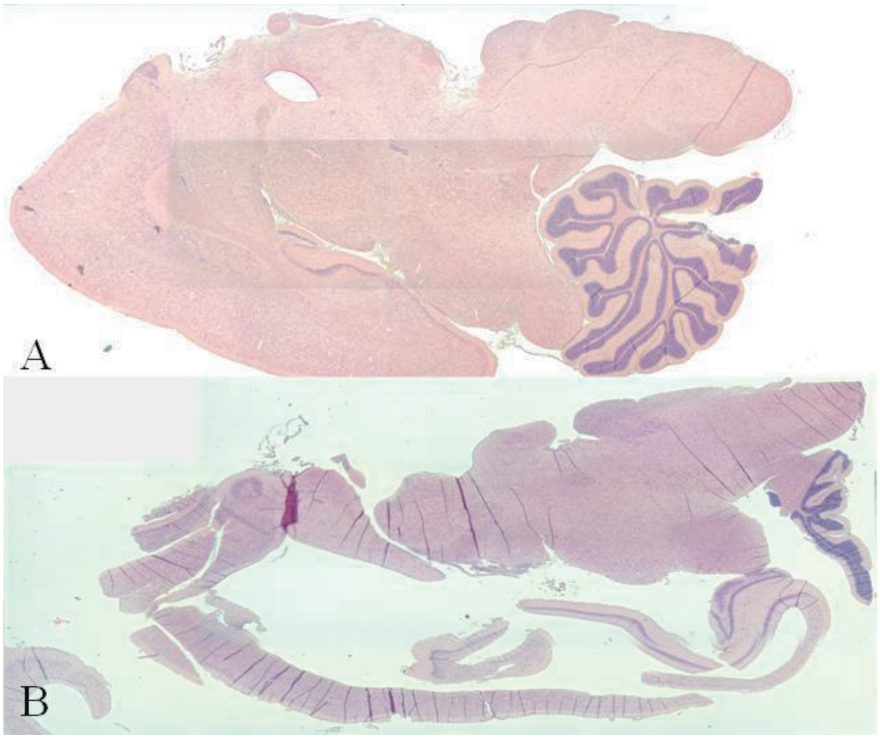

Fig. 2. Image of sagittal section of brain collected from 19-day-old control rat **(A)** and rat from CMG group **(B).** Staining: HE. x 5.

Table 3. Body and organ weight results of 8-week-old rats in control and CMG group.

parameter/group	control group (n=12)	CMG group (n=5)	p (Mann-Whitney test)
Body mass (g)			
AM±SD	210.65±42.52	188.03±45.97	NS
Med	213.63	212.64	
range	149.56-277.70	126.60-228.88	
Brain mass (g)			
AM±SD	1.77±0.09	1.68±0.08	NS
Med	1.74	1.70	
range	1.68-1.96	1.54-1.76	
Heart mass (g)			
AM±SD	0.84±0.25	0.67±0.16	NS
Med	0.77	0.72	
range	0.62-1.52	0.42-0.84	
Liver mass (g)			
AM±SD	10.07±2.60	8.78±2.10	NS
Med	10.01	9.94	
range	6.38-14.78	5.82-10.44	
Spleen mass (g)			
AM±SD	0.54±0.10	0.50±0.18	NS
Med	0.52	0.52	
range	0.40-0.74	0.30-0.70	
Thymus mass (g)			
AM±SD	0.46±0.09	0.55±0.13	NS
Med	0.44	0.62	
range	0.34-0.64	0.38-0.66	
Kidney mass (g)			
AM±SD	0.76±0.16	0.65±0.14	NS
Med	0.76	0.73	
range	0.54-0.98	0.42-0.80	

AM, arithmetic mean; SD, standard deviation; Med, median; p, level of significance; NS, difference non-significant, CMG, CsA+MMF+prednisone.

incorrectly shaped with many neurons in gyrus dentatus and cornu ammonis that showed chromatolysis (which was also observed in many nerve cells of cerebral

Table 4. Basic laboratory blood test results of 8-week-old rats in control and CMG group –results are presented as arithmetic mean ± standard deviation.

parameter/group	control group (n=12)	CMG group (n=5)	p (Mann-Whitney test)
HGB (mmol/L)	9.02±0.65	9.57±0.57	NS
RBC count (T/L)	7.90±0.30	8.46±0.58	NS
HCT (L/L)	0.46±0.30	0.48±0.58	NS
PLT (G/L)	881.45±0.30	958.25 ± 0.58	NS
WBC count (G/L)	6.00±1.56	8.53±1.53	0.039
LYM (G/L)	4.59±0.30	6.64±0.58	0.013
NEU (G/L)	0.78±0.30	1.12±0.58	0.077
MONO (G/L)	0.41±0.30	0.67±0.58	0.077
EOS (G/L)	0.16±0.24	0.07±0.02	NS
BASO (G/L)	0.02±0.03	0.02±0.01	NS
IG (G/L)	0.02±0.02	0.02±0.01	NS
Sodium (mmol/L)	147.00±2.26	146.20±2.17	NS
Potassium (mmol/L)	5.24±0.98	5.92±1.00	NS
Chloride (mmol/L)	102.08±1.51	98.80±2.59	0.026
Total protein (g/L)	61.00±2.17	61.80±1.48	NS
Albumin (g/L)	32.50±1.73	32.00±1.58	NS
Creatinine (mg/dL)	0.50±0.04	0.49±0.05	NS
Urea (mg/dL)	53.42±8.84	54.60±6.50	NS
Uric acid (mg/dL)	4.11±2.29	3.94±2.85	NS
AST (U/L)	116.83±26.23	174.00±51.66	0.006
ALT (U/L)	55.58±13.69	62.60±4.88	NS

HGB, haemoglobin; RBC, red blood cell; HCT, packet cell volume; PLT, platelets; WBC, white blood cells; LYM, lymphocytes; NEU, neutrophils; MONO, monocytes; EOS, eosinophils; BASO, basophils; p, level of significance; NS, difference non-significant; CMG, CsA+MMF+prednisone.

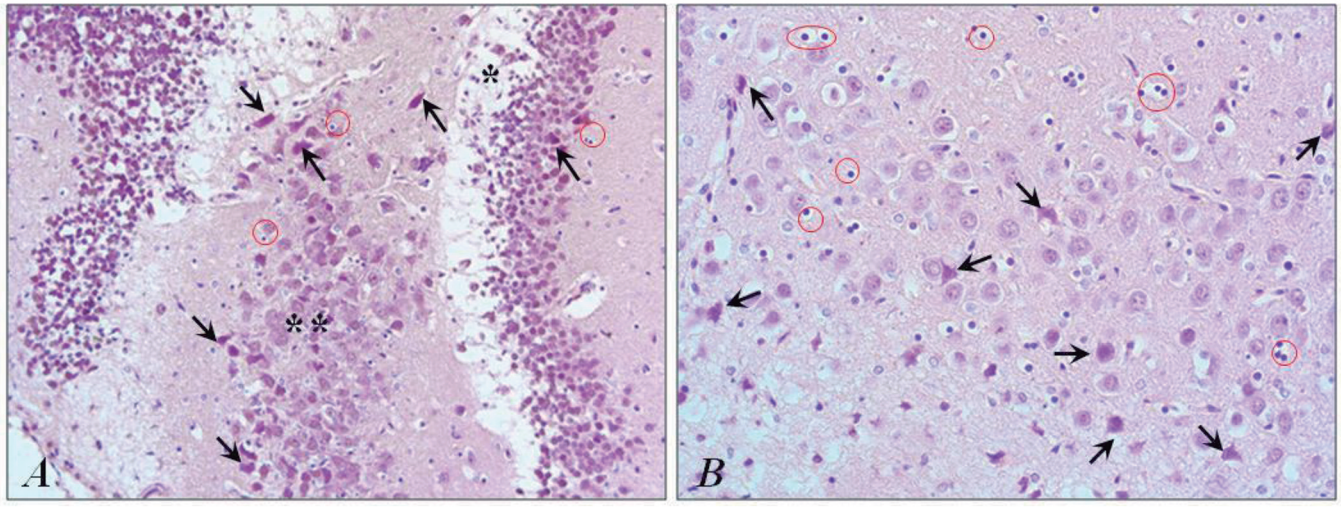

Fig. 3. Intensification of chromatolysis in neurons (black arrows) of gyrus dentatus (*) and cornu ammonis (**) of hippocampus **(A)** and cerebral cortex **(B)** of 19-day-old rat from CMG group. Brain tissue infiltration by mononuclear small, dark staining cells resembling lymphocytes (cells with dark staining nucleus and light halo envelope) in red circles. Staining: HE. x 40.

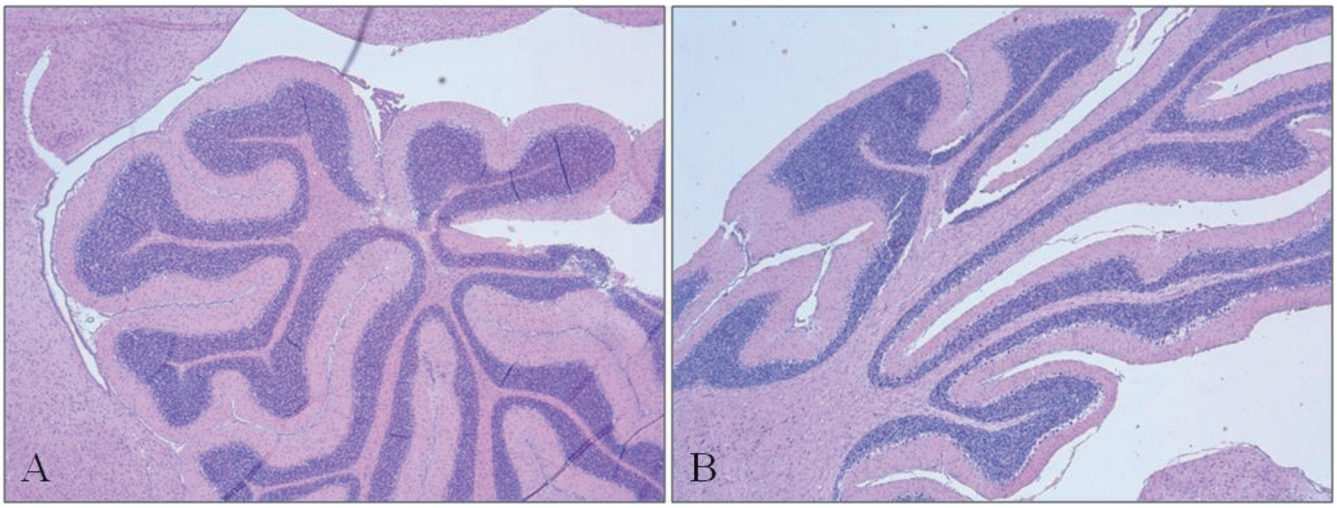

Fig. 4. Image of cerebellum of 19-day-old rat from control **(A)** and CMG group **(B)**. Enlarged cerebellum in rat from CMG group. Staining: HE. x 5.

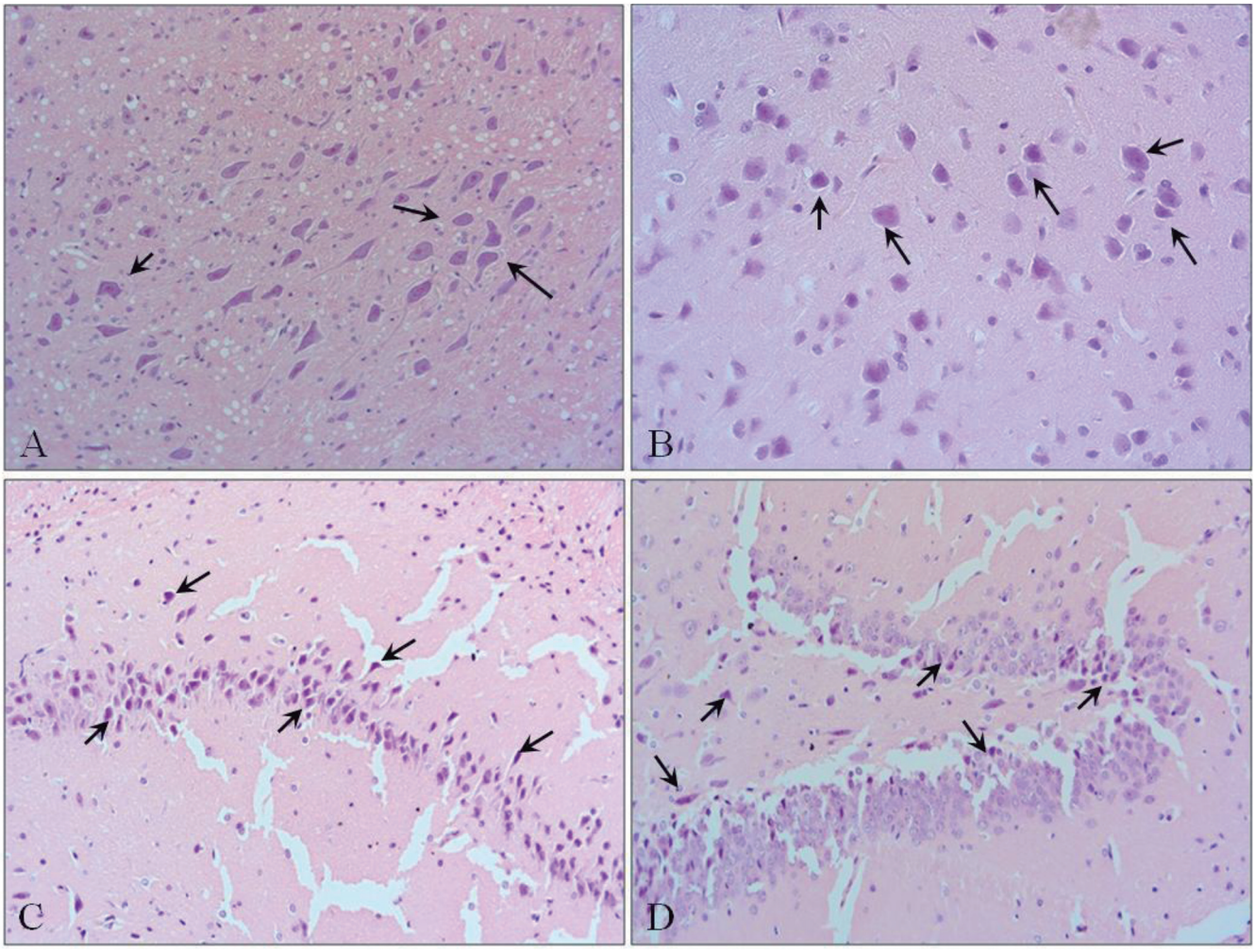

Fig. 5. Image shows the great intensification of chromatolysis in neurons (black arrows) in cerebrum **(A)**, its cortex **(B)** and hippocampus (**C**, gyrus dentatus; **D**, cornu ammonis) of 8-week-old rat from CMG group. Staining: HE. x 40.

cortex). Brain tissue infiltration by mononuclear small, dark staining cells resembling lymphocytes was also observed (Fig. 3).

The hippocampus of 8-week-old control rats and rats from CMG group in the same age was correctly shaped, gyrus dentatus as well as C1-C4 zona were correctly arranged. In many different areas of brain of 8-week-old rats from CMG group, like neocortex, gyrus dentatus and cornu ammonis of hippocampus there were visible numerous neurons that showed chromatolysis (Fig. 5A-D). In the histological image of cerebellum of rats from CMG group within the third layer of cortex there was the relaxation between granular cells (Fig. 6B). The thickness of molecular layer and granule cell layer in both groups of rats was similar.

Heart

The general image of heart from control 19-day-old rats (Fig. 7A) was similar to those from rats of CMG group (Fig. 7B). In both groups of rats all elements of heart (the ventricular and atrial walls, cardiac septum, heart valve) were correctly developed, but under magnification 40x, the structure of cardiomyocytes of rats from CMG group was more relaxed/loose (higher content of connective tissue of endomysium) in comparison to control rats.

In the myocardium of 8-week-old rats from CMG group there were many randomly located cells with light staining cytoplasm (vacuolated cardiac muscle cells) (Fig. 8A) and the area extended through the entire of

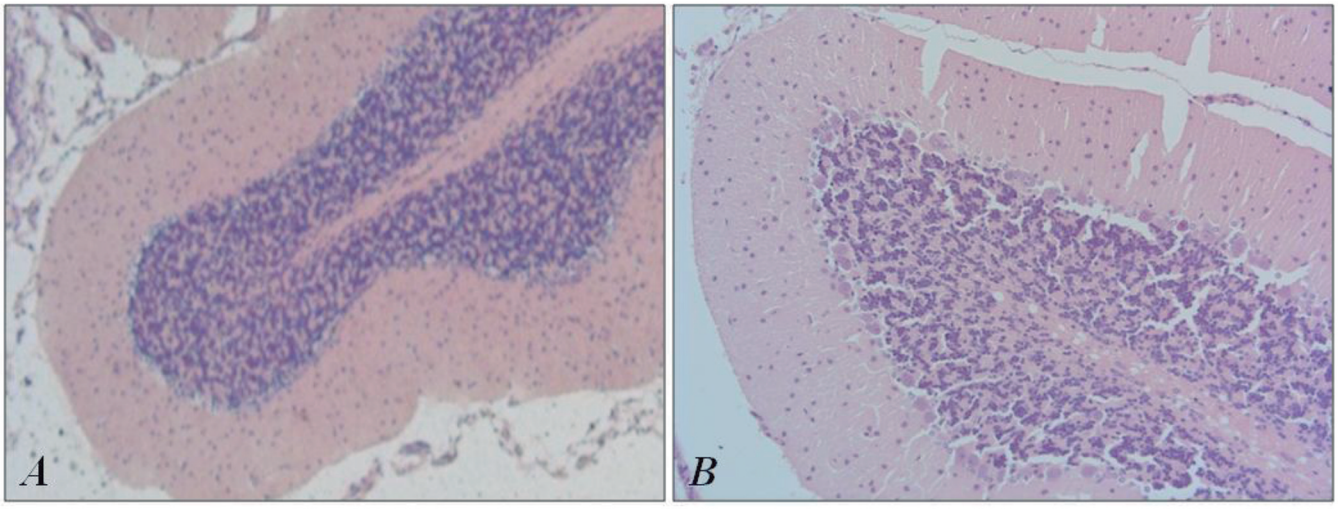

Fig. 6. The cerebellum of 8-week-old rat from control **(A)** and CMG group **(B)**. The relaxation between granular cells in rat from CMG group. Staining: HE. x 40.

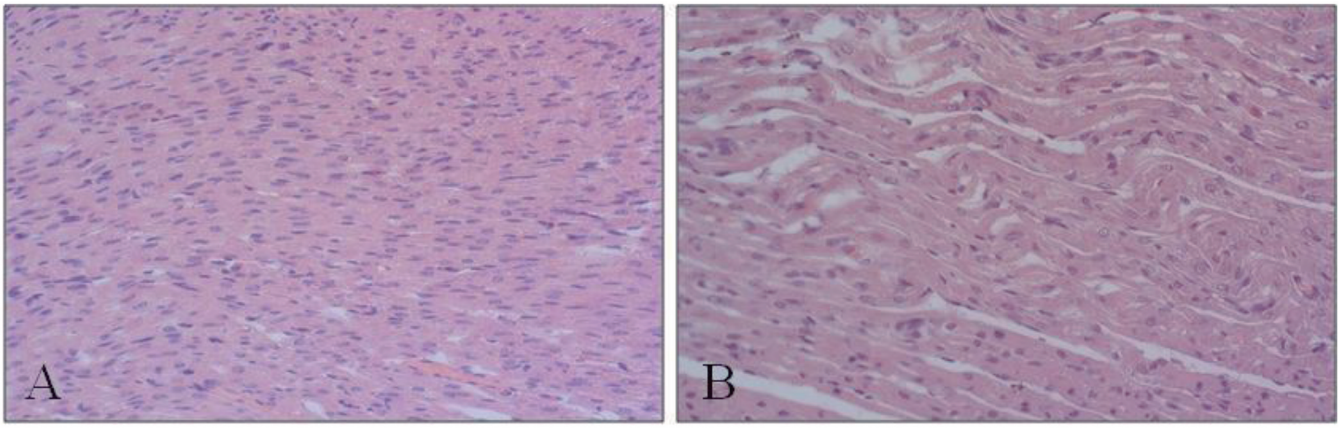

Fig. 7. Arrangement of cardiomyocytes within myocardium of control rat **(A)** and rat from CMG group **(B)** (19-day-old rats). The structure of cardiomyocytes of rat from CMG group more relaxed/loose. Staining: HE. x 40.

myocardium, containing darker (more acidophilic) cells (like in coagulative necrosis) (Fig. 8B). Generally, endomysial connective tissue with many adipose cells was much more abundant than in control rats (data not shown).

Liver

The liver morphology of 19-day-old control rats (Fig. 9A) and rats from CMG group (Fig. 9B) was comparable: the interconnected plates of hepatocytes showed typical arrangement, the lobules with centrally located central vein and peripherally located portal space. Between hepatocytes of rats from CMG group, there were observed many cells with small, dark staining nucleus resembling lymphocytes (Fig. 9B; black arrows). In the morphometric measurement comparing the sinusoids' diameter of control rats with rats from CMG group liver sinusoids seemed to be wider, more extended in rats from CMG group but these results were not statistically significant.

The liver morphology of 8-week-old control rats and rats from CMG group was comparable: the lobules had normal/typical arrangement, "spongy" image of

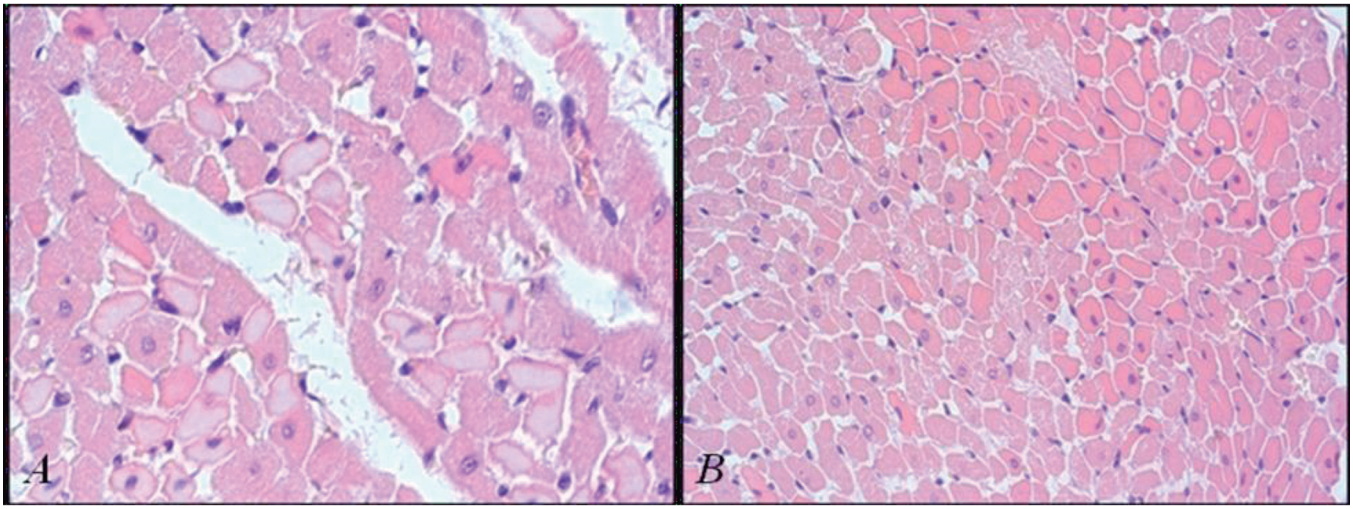

Fig. 8. Structure of myocardium of 8-week-old rat from CMG group. Many cardiomyocytes manifested light staining cytoplasm (vacuolated cardiac muscle cells) **(A)** and more acidophilic, dark staining cytoplasm without cross-striations (coagulative necrosis) **(B).** Staining: HE. x 40.

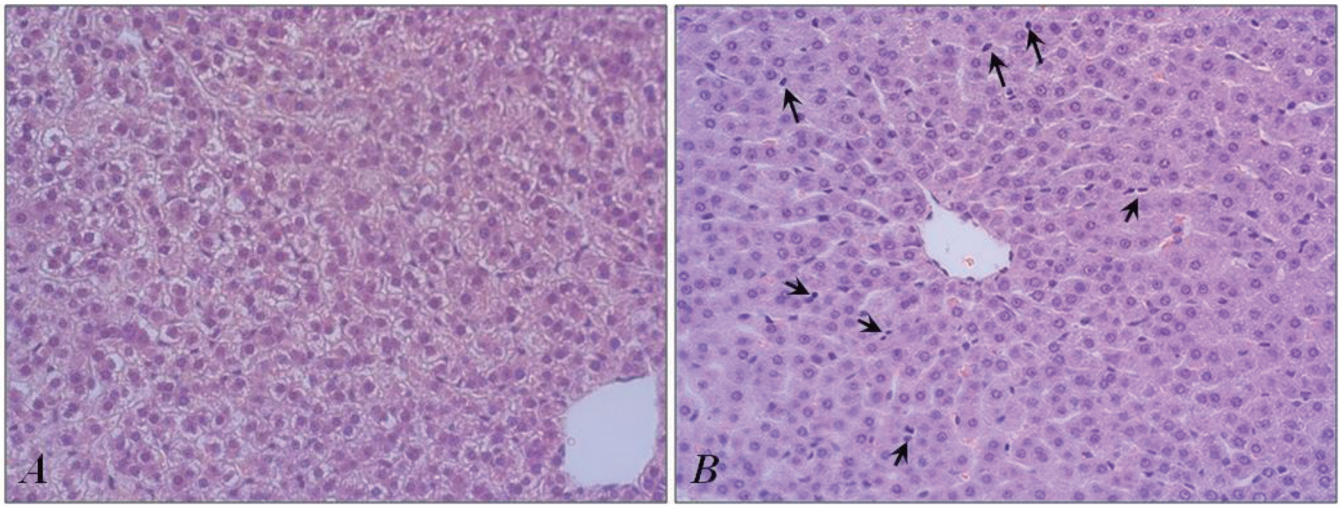

Fig. 9. The histological structure of liver of 19-day-old control **(A)** and CMG **(B)** rat. Between hepatocytes of rat from CMG group there were observed numerous small, dark staining cells (lymphocytes) (B; black arrows). Staining: HE. x 40.

hepatocytes looked similar. The morphometric measurements comparing the sinusoids' diameter of control rats with CMG rats did not show statistically significant changes.

Kidneys

The kidneys of 19-day-old rats from CMG group (Fig.10 B,b) were changed as compared to control (Fig. 10A) - in most cases hypertrophic (organ underwent hypertrophy Fig. 10B) or hypotrophy (Fig. 10b) with renal calyxes and pelvis enlargement. 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. Morphometric analysis showed an increase in diameter of glomeruli (diameter of glomeruli in control rats-mean 37.299 μm and in rats from CMG group-mean 50.281 μm). The differences were statistically significant (p=0.002).

The renal morphology of 8-week old rats from control and CMG group was similar.

Discussion

The most recent European Best Practice Guidelines recommend that immunosuppressive therapy based on CsA or Tc with or without corticosteroids and azathioprine may be continued in renal transplant recipients during pregnancy (Armenti et al., 2008). Other drugs, such as MMF or sirolimus are not recommended based on the current information available (Sifontis et al., 2006). During pregnancy, stable medication regimens should be changed as little as possible and changes in drug pharmacokinetics can be observed (Sgro et al., 2002).

The effect of MMF and mTOR inhibitors on the development of fetus in utero in a prospective study can be examined only in an animal model. In most animal studies focused on toxicity of immunosuppressive drugs the effect of a single drug was examined. 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, was attempted. We used immunosuppressive drugs considered to be acceptable during pregnancy (CsA, Tc, steroids), in combinations together with not recommended drugs during pregnancy (MMF and everolimus, new mTOR inhibitor). In each combination of three drugs only one of them was contraindicated during pregnancy (in CMG and TMG group-MMF; in CEG group-everolimus). We enquired which combination of drugs regarding MMF would be more harmful-with CsA or with Tc. In CEG group we added mTOR inhibitor-everolimus to therapy, because data on its effects during pregnancy are scarce as well as both in animals and humans.

The applied doses of immunosuppressive drugs meant that blood concentrations could be reached within a therapeutic range. Drug concentrations were measured 4 hours after oral supply - this was considered an optimal time for determining the concentration of drugs in blood, due to different drug metabolism in rats compared to humans (Ma et al., 2006; Schmitz et al., 2009). We did not monitor therapeutic doses of MMF as monitoring of this drug has not been the standard approach in adjusting doses to maintain efficacy and prevent its toxicity in daily practice.

In our study we observed a reduced number of litters

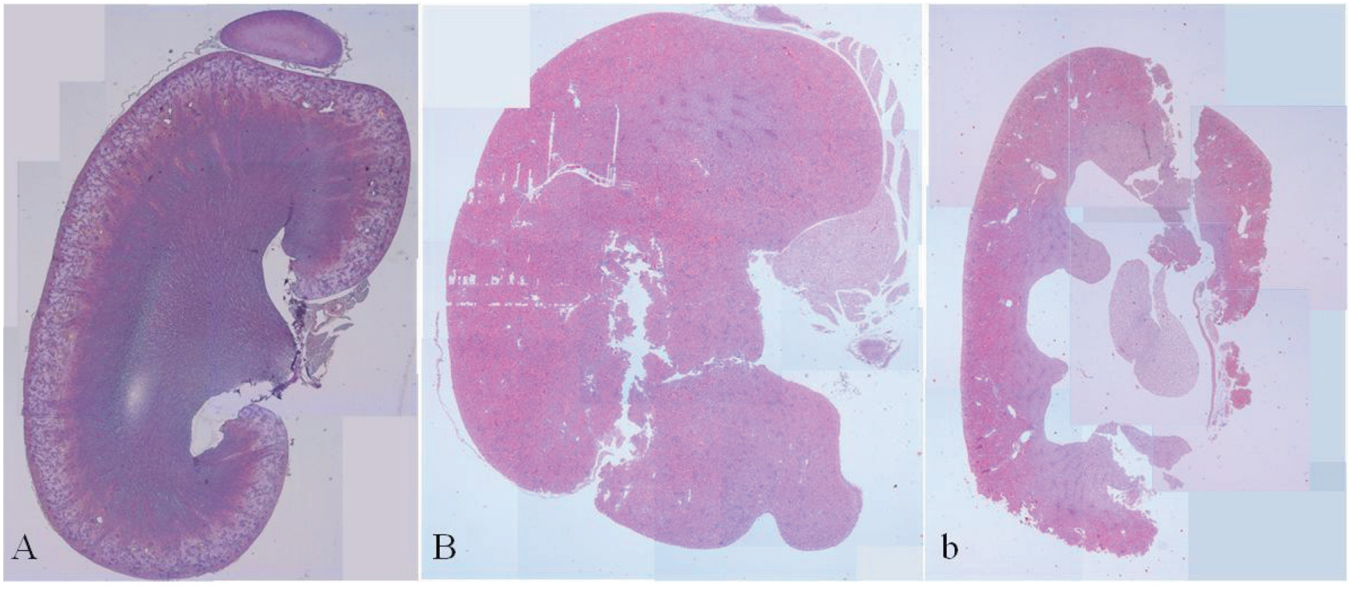

Fig. 10. The sagittal section of kidney of 19-day-old rat from control **(A)** and CMG group (B-hypertrophy, b-hypotrophy). Staining: HE. x 5.

and rats per litter in all rats exposed to immunosuppressive treatment. In CMG group we observed approximately 50% malformed juvenile rats with hydrocephaly, anophthalmia and apathy. In TMG and CEG group a great increase in fetal loss was observed (delivery of living offspring almost impossible) at term but no malformed fetuses could be found. MMF turned out to be more toxic when used with Tc than with CsA (delivery of live offspring was possible only in CMG group). Everolimus in combination with CsA was also very toxic because it effectively suppressed the fetal development in utero, although the only one live pup has not got malformations.

In the animal model the immunosuppressive drugs considered to be relatively safe during pregnancy (CsA, Tc and steroids) caused an impairment only when administered in high doses. CsA had fetotoxic effects in at a dose of 25 mg/kg/d during different phases of gestation. CsA given from days 1 to 7 caused a small reduction in litter size; from day 8 to 14 an increase in the incidence of resorptions and a reduction in weight (Brown et al., 1985). In another study high-dose CsA resulted in a high incidence of fetal mortality or in runting and fetal kidney impairment (Mason et al., 1985). The administration of Tc to pregnant rats during the preimplantation phase failed to generate any toxic effect to on mother or embryo (Ramos et al., 2008). In this study on mice the administration of low-dose Tc resulted in a higher number of resorptions, but pups that survived did not appear different from controls. High-dose Tc had no obvious detrimental effects on maternal health but caused resorption of all fetuses. Thus, Tc and CsA can have adverse effects on pregnancy, but the fetal toxicity was dose-dependent (Farley et al., 1991). No consistent malformative pattern was observed (Perez-Aytes et al., 2008). Data remain limited. In animal studies after exposure to mTOR inhibitor sirolimus decreased weight of pups and delayed ossification of skeletal structures have been reported, although, no evidence of teratogenicity was noted (Tendron et al., 2002; Sifonitis et al., 2006; Armenti et al., 2008).

Analysing laboratory results 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. (2011) identified two genes, Slc12a3 and kidney-specific Wnk1 (KS-Wnk1), which 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. (2007) 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 the influence of CsA on the function of ions transporters.

We also observed elevated activity of AST in rats from CMG group. CsA-induced toxicity is associated with increased production of reactive oxygen species (ROS) and lipid peroxidation in the kidney and liver. Korolczuk et al. (2013) treated rats with CsA at a high dose of 25 mg/kg/day. Animals developed failure of kidney and the liver functions, manifested by an increase in serum levels of creatinine, urea, uric acid, bilirubin, AST and ALT and a decrease in total proteins. Ultrastructural examination of tubular epithelial cells and hepatocytes revealed dilatation of endoplasmic reticulum and injury to mitochondria, formation of autolysosomes, and the presence of single apoptotic cells. Kaya et al. (2008) also observed a liver injury and significant elevation in serum AST and ALT activities in rats treated with CsA. In our observation ALT activity was comparable in control rats and rats from CMG group. One should remember that AST can derive from liver as well as from other organs like brain and heart, which organs were altered (the structure of liver mostly unchanged) in 8-week-old rats from CMG group compared to control rats.

In hematology we have found elevated level of WBC and lymphocyte count in rats from CMG group. Immunosuppressive drugs potentially influence maternal and fetal immune system during pregnancy and can cause changes in peripheral blood cells after birth. Tamer et al. (2007) confirmed that CsA administration increased lipid peroxidation in the peripheral lymphocytes of rats; this effect was accompanied by a decrease in lymphocytes deformability. In a model of chronic experimental pyelonephritis (Findon and Miller, 1989), in animals treated with CsA, bacterial numbers increased markedly, although circulating neutrophil numbers were relatively unaffected. Other studies had different observations - Roman et al. (2004) revealed a decrease in the absolute number of T lymphocytes in peripheral white blood cells in rats treated with CsA and Tc. More detailed observation requires immunophenotyping assessment (evaluation of subsets of lymphocytes).

Increased body weight of rats from CMG group could be related to enlargement of heart, kidney, liver mass (borderline) and hydrocephaly. Brains collected from 19-day-old rats from CMG group were highly deformed and their topography was robustly altered by the presence of hydrocephaly. The neurons of hippocampus showed chromatolysis in younger and older rat; and infiltration by mononuclear small, dark staining cells resembling lymphocytes in younger animals. In previous studies rats' offspring whose mothers had been exposed to MMF exhibited similar changes in central nervous system such as anophthalmia, hydrocephaly; agnathia and fetal resorptions were also observed (Vento et al., 2008; Kędzierska et al., 2015). The most common defect observed in humans, microtia, was not seen in the animal model.

Other malformations observed in previous animal

studies were ectopic kidney and diaphragmatic hernia (Perez-Aytes et al., 2008). In our study kidneys of 19-day-old rats from CMG group were in most cases hypertrophic with an increase in diameter of glomeruli. In older, 8-week-old rats these differences were not present any more. Data from animal studies suggest that antenatal exposure to CsA can cause oligonephronia; CsA at a high dose (25 mg/kg) caused fetal renal tubulotoxicity (focal proximal straight tubular cell vacuolisation and necrosis - Mason et al., 1985). On the other hand rats treated with MMF had lower creatinine serum concentrations compared to rats not treated with this drug (Kędzierska et al., 2015). This dependence was found as well in other studies, MMF was not nephrotoxic (Vanrenterghem, 1997; Tian et al., 2007; Heemann et al., 2012).

In the heart the structure of cardiomyocytes of rats from CMG group was more loosely arranged in comparison to control rats. Generally, the endomysial connective tissue with adipose cells was much more abundant than in control rats, independently of age. It is possible that calcineurin inhibitors can influence the development of heart. In the study of Fu et al. (1999) calcineurin was involved in the signal transduction of Ang II-induced cardiomyocyte hypertrophy and fibroblast hyperplasia. In another study (Han et al., 2005) treatment with CsA suppressed the Ang II-induced c-fos protein expression and $[Ca^{2+}]$ elevation in a single cardiomyocyte, which might play a role in the prevention of Ang II-induced cardiomyocyte hypertrophy.

Conclusions

In summary, we have noticed a significantly reduced number of live births in all pregnant rats exposed to contraindicated immunosuppressive drugs in combination with calcineurin inhibitors and prednisone. Treatment with MMF and everolimus turned out to be very toxic. Malformations and histological changes of fetal organs were confirmed after MMF exposure during pregnancy. MMF turned out to be more toxic when used with Tc than with CsA (delivery of live offspring was possible only in the latter group). Everolimus in combination with CsA is also very toxic because it effectively suppressed the fetal development in utero, although the only one live pup has not got malformations.

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