

# The effect of immunosuppressive therapy on renal cell apoptosis in native rat kidneys

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**Summary.** Aim: To analyse the impact of the most commonly used immunosuppressive drugs on the occurrence of apoptosis in the native kidneys of Wistar rats. Method: The study involved 36 rats. The animals grouped according to the immunosuppressive regimen used (tacrolimus, mycophenolate mofetil, cyclosporine A, rapamycin and prednisone). The rats in all study groups were treated with a 3-drug protocol for 6 months. The medication dose was adjusted based on available literature data. No drugs were administered to the control group. The rats were then killed. Autopsies of all animals were performed and the kidneys were isolated for histopathology (HE + PAS). To assess cell apoptosis the TUNEL reaction was performed. Blood trough levels of immunosuppressive drugs as well as the parameters of peripheral blood were determined. Results: 1. In rats treated with cyclosporine A distal nephron tubules were characterised by more pronounced apoptosis. 2. In tacrolimus-treated rats a lower intensity of apoptosis was found in the distal tubules. 3. In rapamycin-treated rats the apoptosis was inhibited both in the distal and proximal nephron tubules. 4. In MMF treated rats intense apoptosis was observed in the proximal nephron tubules. 5. There were no significant changes in renal

histopathology (HE + PAS). Conclusions: The apoptosis in nephron tubules caused by immunosuppressive therapy is not accompanied by any histopathological changes (eg fibrosis, inflammation, tubular atrophy, vacuolation of the tubular cells) in light microscopy.

**Key words:** Apoptosis, Immunosuppression, Drug effects, Transplantation, Kidney

## Introduction

Modern immunosuppressive regimens have reduced the incidence of acute rejection episodes with the early results of organ survival being satisfactory-one-year survival in renal transplantation is currently at around 90-95% (Moudgil et al., 2013). According to data published by the Collaborative Transplant Study the average renal allograft half life in 1988 was 7.5 years, while currently it is approximately 8.8 years in the USA (Meier-Kriesche et al., 2004; Brennan, 2006) and 15.1 years in Europe. Approximately 5% of transplanted kidneys cease to function annually due to chronic damage of different aetiology. Chronic transplant dysfunction is responsible for chronic renal allograft disease (CRAD) in up to 60-80% of patients this may have an immunological or non-immunological background. A typical histopathological finding in such cases is chronic allograft nephropathy (CAN). In the

new Banff classification (2007) the CAN concept was withdrawn and replaced by an IF/TA (interstitial fibrosis/tubular atrophy) which is a non-specific tubular atrophy and interstitial fibrosis. It is believed that the imbalance between the formation of the extracellular matrix (ECM) and its degradation is the main cause of IF/TA (Esposito et al., 1996).

Apoptosis plays an interesting role in the development of chronic graft dysfunction and it is the process of cell death which occurs in the body under physiological conditions which leads to the elimination of unwanted cells. The decisive moment in apoptosis is the activation of caspases, proteolytic enzymes, leading to the degradation of DNA, the damage of protein structures and ultimately to the disintegration of the cell. Abnormalities in the process of apoptosis can lead to the survival of pathological cells and their products with further adverse consequences such as the development of fibrosis.

Evidence on the impact of calcineurin inhibitors on renal tubular cell apoptosis comes from studies *in vivo* (Xiao et al., 2013) and *in vitro* (Xiao et al., 2011). Cyclosporine A may affect apoptosis through various mechanisms the most important are; oxidative stress within the mitochondria and the endoplasmic reticulum, the impact on nitrous oxide synthase (NOS) and an increase in Fas/Fas-L or reduction in Bcl-2/Bcl-XL expression and as a result increased caspase 1 and 3 activity. Intramitochondrial oxidative stress results in a decrease in catalase and NADPH activity as well as the release of cytochrome c into the cytoplasm.

The aim of this study was to:

1. Prove what if any histopathological changes develop in the kidneys of rats under the influence of immunosuppressive therapy.
2. Determine whether medications affect the activity of apoptosis in the cells of nephron tubule.

## Materials and methods

### Material

The study was conducted on 36 male Wistar rats from a licensed breeder. At the start of the experiment the rats were 14 weeks old. Prior to testing all of the animals were weighed their mean weight being 305g. Experiments were carried out using a pharmaceutical form of the drug. The test animals received the drug formed in a ball of bread orally. The drug doses were based on available literature data (Katz et al., 1991; Joffe et al., 1993; Jolicœur et al., 2003; Ma et al., 2006; Westrhenen et al., 2007). The doses used in the experiment were as follows: tacrolimus (Prograf)-4 mg/kg/day, mycophenolate mofetil (Cellcept)-20 mg/kg/day, cyclosporin A (Sandimmun-Neoral)-5 mg/kg/day, rapamycin (Rapamune)-0.5 mg/kg/day, prednisone (Encorton)-4 mg/kg/day. The animals received medication every 24 hours for six months. After 3 months of the experiment the animals were

weighed again and the medication dose was adjusted adequately to the altered weight. Six rats did not receive treatment as they made up the control group. A Diagram of the experiment is presented in Table 1.

### Methods

34 rats completed the study. Two rats from the CRG group died before the end of the study. At 6 months all of the animals were killed. Their body weight was measured, their blood taken for laboratory evaluation and during the autopsy their kidneys were harvested for histopathological examination. Blood tests included: urea and creatinine concentration, total protein, total calcium and others (Table 2).

### Histopathological evaluation and its criteria

An assessment of the histological preparations stained with hematoxylin/eosin and PAS was performed. The samples were independently examined by two experienced pathologists. The material was evaluated in terms of potential acute or chronic inflammatory changes within the renal tubules, glomeruli and vessels in accordance with the modified criteria of Banff 2009 (the criteria for graft rejection were omitted) (Perkowska-Ptasińska, 2011).

### Apoptosis assessment

In order to evaluate tubular cell apoptosis the TUNEL reaction was performed (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-labeling). An important feature of apoptotic cells used for apoptosis detection is the fragmentation of DNA into pieces whose length is equal to a multiple nucleosome length (180-200 bp) reflecting the structure of the histone octamers. The reaction product was assessed by light microscopy. This test was considered positive in the presence of colour reaction in at least one nucleus in the tubule. Positive results are expressed as a percentage of proximal or distal tubules with the stained nucleus. The number of apoptotic nuclei were analysed at each proximal and distal tubule in the samples separately. The results are presented as a

**Table 1.** The study design. Abbreviations of the drugs that are used to name the study groups in brackets.

| Group         | Glucocortico-steroids (G) | Tacrolimus (T) | Cyclosporin A (C) | Rapamycin (R) | Mycophenolate mofetil (M) |
|---------------|---------------------------|----------------|-------------------|---------------|---------------------------|
| Control (n=6) | -                         | -              | -                 | -             | -                         |
| TRG (n=6)     | +                         | +              | -                 | +             | -                         |
| CRG (n=6/4)   | +                         | -              | +                 | +             | -                         |
| MRG (n=6)     | +                         | -              | -                 | +             | +                         |
| CMG (n=6)     | +                         | -              | +                 | -             | +                         |
| TMG (n=6)     | +                         | +              | -                 | -             | +                         |

percentage of nuclei with features of apoptosis among all the nuclei of the tubule (Fig. 1).

#### Drug concentration

The drug concentration was determined in accordance with the literature (Ma et al., 2006; Schmitz et al., 2009) after 4 hours of enteral administration.

The concentration of rapamycin was determined at the Department of Biochemistry of Pomeranian Medical University with a multistep liquid-liquid extraction of whole blood samples followed by reversed-phase HPLC with ultraviolet detection (1-chlorobutane and 32-desmethoxy rapamycin extraction as internal standard) using Hewlett-Packard 1050 Alltech Alltima C18 5µm 150×2.1mm column at 50°C. The flow rate of the mobile phase containing 60% acetonitrile and 40% water was 0.5 mL/min. The detection wavelength was set at 278 nm Rapamycin detection limit was 1 ng/mL.

The concentration of tacrolimus and cyclosporin A were determined in all of the rats' blood. The study was carried out at the Clinical Central Laboratory in Szczecin. IMx assay based on microparticle enzyme immunoassay (Microparticle Enzyme Immunoassay-MEIA) was used to determine the tacrolimus level. The test was performed using an Abbott analyser (Abbott Laboratories Park USA).

The concentration of CsA was determined with Abbott AxSYM assay which is based on fluorescence (fluorescence polarisation immunoassay-FPIA).

The results are shown in Table 3.

#### Statistical analysis

The values of measurable variables were compared between groups using nonparametric tests (Kruskal-Wallis and U Mann-Whitney test) due to most of the data being non-normal distributed (as assessed in Shapiro-Wilk test).

The mean, standard deviation, median, minimum and maximum values were calculated for each group. Multivariate analysis was performed based on a general linear model. The standardised regression coefficient  $\beta$ , 95% confidence interval (95% CI) and p-value for each independent variable were presented.

If the distribution of the dependent variable differed from the norm it was subjected to a logarithmic transformation. The cut-off level of statistical significance was set at  $p < 0.05$  for bilateral statistical hypothesis tests. Calculations were performed using Statistica 10 software.

#### Results

The results of the research and statistical analysis are presented in tables 2-4 and figures 2-8. Any differences between treatment groups were calculated using ANOVA while variances between the study groups and the control group were calculated using U Mann-Whitney test and p-value was presented as a result.

**Table 2.** Body weight and basic laboratory blood test results of rats in the treatment groups.

| parameter/group      |         | control group | TRG       | CRG       | MRG        | CMG       | TMG        | p (Anova) |
|----------------------|---------|---------------|-----------|-----------|------------|-----------|------------|-----------|
| Total protein (g/dl) | av± SD  | 6.12±0.30     | 6.06±0.58 | 5.29±1.19 | 5.83±0.20  | 5.84±0.23 | 6.07±0.17  | NS        |
|                      | median  | 6.12          | 6.16      | 5.81      | 5.86       | 5.75      | 6.04       |           |
|                      | min-max | 5.78-6.45     | 5.23-6.89 | 3.52-6.02 | 5.46-6.05  | 5.63-6.12 | 5.88-6.37  |           |
| Total calcium (g/dl) | av± SD  | 2.13±0.35     | 2.11±0.36 | 2.45±0.08 | 2.15±0.09  | 2.13±0.27 | 2.06±0.19  | NS        |
|                      | median  | 2.06          | 2.16      | 2.44      | 2.17       | 2.17      | 2.11       |           |
|                      | min-max | 1.83-2.8      | 1.50-2.52 | 2.36-2.54 | 2.02-2.27  | 1.75-2.49 | 1.72-2.23  |           |
| GFR (ml/min)         | av± SD  | 2.99±1.52     | 2.76±0.68 | 3.09±2.31 | 3.43±0.39  | 3.19±0.64 | 3.66±0.79  | NS        |
|                      | median  | 2.68          | 2.57      | 2         | 3.4        | 3.2       | 3.47       |           |
|                      | min-max | 1.59-5.5      | 1.96-3.76 | 1.82-6.55 | 2.97-3.92  | 2.44-4.09 | 2.71-4.89  |           |
| Creatinine (mg/dl)   | av± SD  | 0.78±0.16     | 0.77±0.08 | 0.79±0.18 | 0.7±0.03   | 0.72±0.06 | 0.68±0.06  | NS        |
|                      | median  | 0.78          | 0.78      | 0.87      | 0.7        | 0.71      | 0.69       |           |
|                      | min-max | 0.57-0.95     | 0.67-0.87 | 0.53-0.9  | 0.66-0.74  | 0.65-0.8  | 0.6-0.77   |           |
| Urea (mg/dl)         | av± SD  | 71.9±24.6     | 51.06±9.3 | 62.5±38.5 | 91±23.9    | 24.9±11.8 | 73.4±17.8  | 0.02      |
|                      | median  | 71.88         | 48.51     | 50        | 83.05      | 22.67*    | 74.41      |           |
|                      | min-max | 37.5-106.3    | 42.6-66.7 | 31.2-119  | 75-137.5   | 12.5-43.8 | 50-100     |           |
| Body mass (g)        | av± SD  | 573.2±856     | 403±54.3  | 467±54.9  | 435.6±58.3 | 544±50.2  | 482.5±66.7 | 0.01      |
|                      | median  | 562.5         | 417*      | 470       | 414        | 554       | 482.5      |           |
|                      | min-max | 474-700       | 331-480   | 398-530   | 385-546    | 481-600   | 405-580    |           |
| Kidney mass (g)      | av± SD  | 2.03±0.39     | 1.6±0.25  | 1.68±0.25 | 2±0.18     | 1.82±0.27 | 1.72±0.29  | NS        |
|                      | median  | 2.1           | 1.6       | 1.65      | 1.95       | 1.75      | 1.7        |           |
|                      | min-max | 1.4-2.5       | 1.4-2.1   | 1.4-2     | 1.8-2.3    | 1.5-2.3   | 1.4-2.2    |           |

\*  $p < 0.01$  vs K. \*\* $p < 0.05$  vs K

The results of kidney preparation analysis by light microscopy

The analysis of the HE and PAS stained slides obtained from each group showed (Figs. 2, 3).

Control group

Normal glomeruli tubules and vessels structure. According to Banff '09 criteria it was assessed as: ct0, Ci0, cg0, ah0, t0, i0.

Table 3. The medication concentration and weight of rats in study groups. Results are presented as mean±SD.

|                       | CMG Group (n=6) | TMG Group (n=6) | TRG Group (n=6) | CRG Group (n=6) | MRG Group (n=6) | Control (n=6) |
|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|---------------|
| Cyclosporin A (ng/ml) | 785.2 ±83.3     | -               | -               | 1272±556.7      | -               | -             |
| Tacrolimus (ng/ml)    | -               | 14.1±13.1       | 15.3±9.2        | -               | -               | -             |
| Rapamycin (ng/ml)     | -               | -               | 6.5 ±2.4        | 6.3±4.4         | 2.3±2.1         | -             |

Table 4. Immunohistochemical reactions and apoptosis intensity in the study groups.

| IHC reaction/group                        |         | control   | TRG       | CRG       | MRG       | CMG       | TMG       | p (Anova) |
|-------------------------------------------|---------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| apoptosis in distal tubules               | mean±SD | 0.81±0.21 | 0.89±0.16 | 1±0.00    | 1±0.00    | 0.99±0.02 | 0.96±0.05 | 0.01      |
|                                           | median  | 0.91      | 0.96      | 1.00*     | 1.00*     | 1.00*     | 0.98      |           |
|                                           | min-max | 0.42-0.97 | 0.58-1    | 1-1       | 1-1       | 0.94-1    | 0.86-1    |           |
| apoptosis in proximal tubules             | mean±SD | 0.37±0.29 | 0.44±0.22 | 0.24±0.06 | 0.32±0.32 | 0.69±0.25 | 0.67±0.17 | 0.03      |
|                                           | median  | 0.27      | 0.45      | 0.23      | 0.21      | 0.73      | 0.61      |           |
|                                           | min-max | 0.08-0.77 | 0.12-0.65 | 0.18-0.32 | 0.13-0.98 | 0.23-1    | 0.49-0.96 |           |
| apoptotic nuclei of distal tubule cells   | mean±SD | 0.5±0.26  | 0.41±0.21 | 0.52±0.04 | 0.56±0.22 | 0.83±0.23 | 0.52±0.10 | 0.05      |
|                                           | median  | 0.52      | 0.44      | 0.53      | 0.52      | 0.97      | 0.5       |           |
|                                           | min-max | 0.16-0.85 | 0.15-0.75 | 0.46-0.56 | 0.38-0.99 | 0.53-1    | 0.41-0.7  |           |
| apoptotic nuclei of proximal tubule cells | mean±SD | 0.16±0.17 | 0.16±0.10 | 0.06±0.03 | 0.19±0.31 | 0.5±0.31  | 0.27±0.13 | 0.01      |
|                                           | median  | 0.09      | 0.14      | 0.06      | 0.08      | 0.47**    | 0.23      |           |
|                                           | min-max | 0.02-0.43 | 0.05-0.32 | 0.04-0.1  | 0.04-0.83 | 0.08-1    | 0.17-0.51 |           |

\*p<0.01 vs control. \*\* p<0.05 vs control.

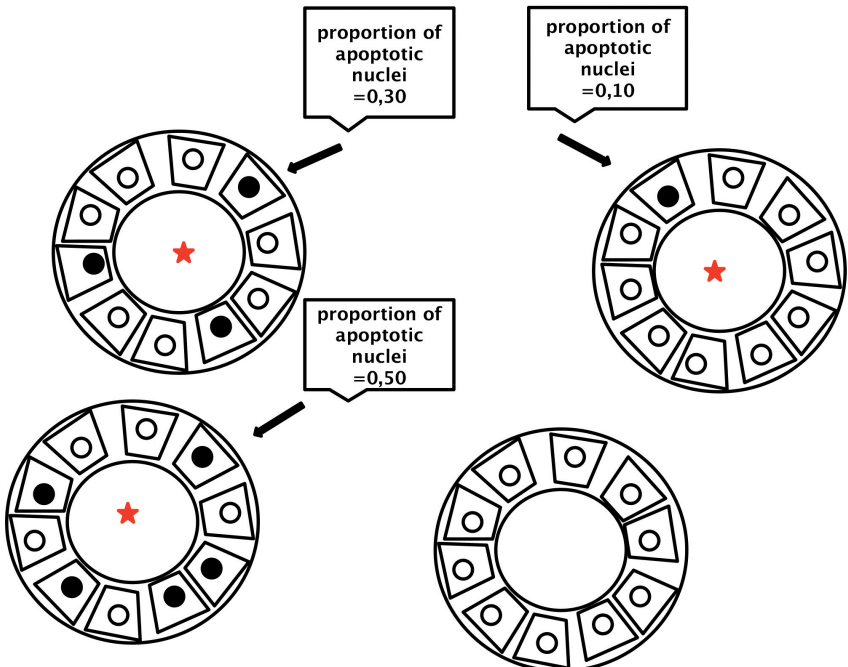


Fig. 1. Renal tubules cross sectional structures with a positive staining to detect apoptosis (marked with an star). Calculation method for percentage of apoptotic nuclei in each tubule.



**TRG Group**

Slightly more lymphocytes (<10%) in the interstitium between the tubules. The normal kidney glomeruli, proximal and distal tubules were observed. According to Banff '09 criteria: ct0, Ci0, cg0, ah0, t0, i0.

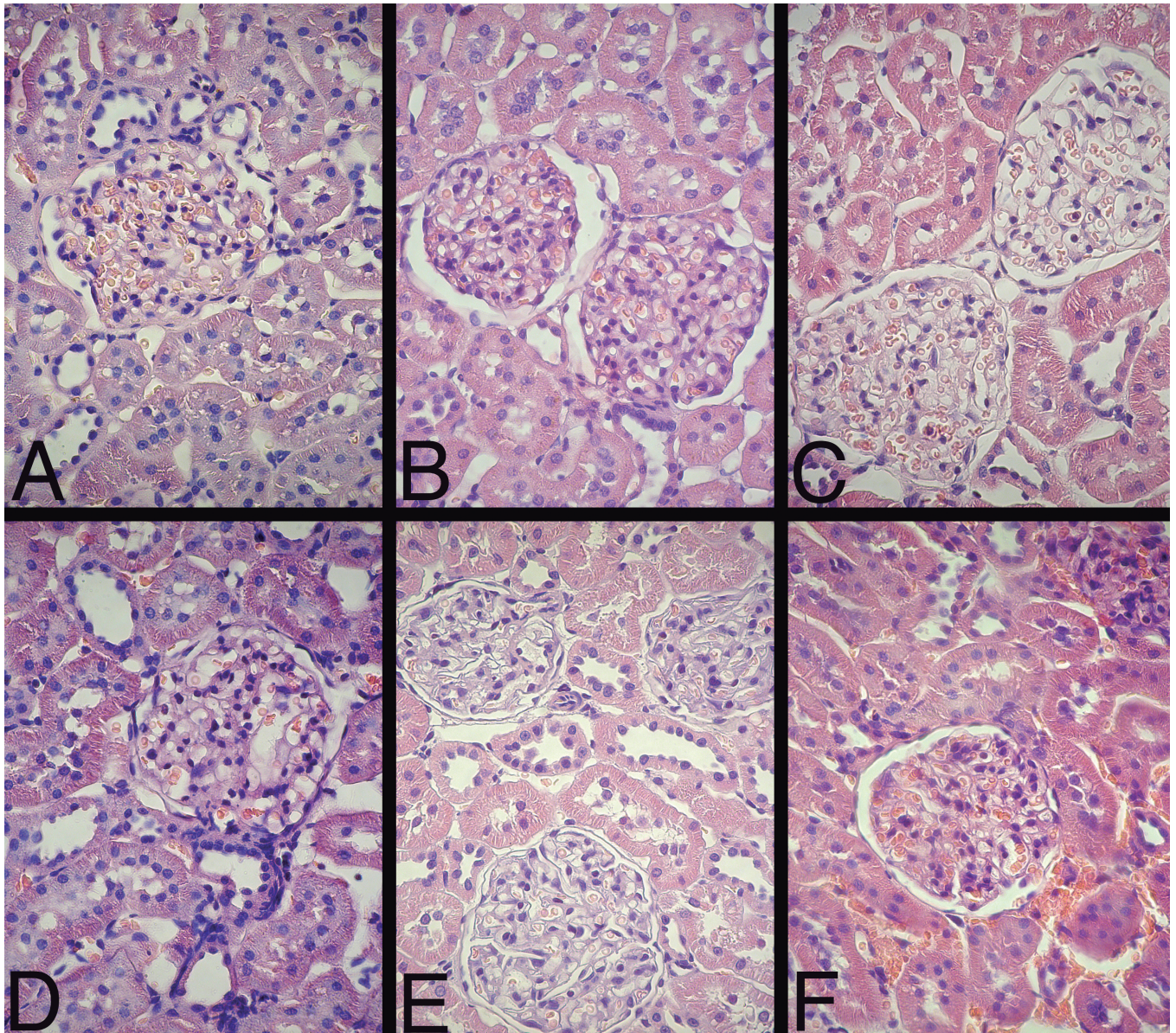
**CRG group**

A slight proximal tubular cell cytoplasm decomposition. mainly in the apical cells. In some regions the individual cells were detached from the

basement membrane. but there were no signs of renal tubular necrosis. Banff '09 criteria: ct0, Ci0, cg0, ah0, t0, i0.

**MRG Group**

No significant changes to the compared control group were found except for an increased cellularity of the interstitium (<10%). Besides cytoplasm degradation of proximal tubular cells is well pronounced which was similar to the CRG group. Banff '09: ct0, Ci0, cg0, ah0, t0, i0.



**Fig. 2.** HE staining of rat kidney. CMG group (A). TRG group (B). TMG group (C). MRG group (D). CRG group (E). The control group (F). x 40



**CMG Group**

No differences were noted when compared to the control group. Banff '09: ct0, Ci0, cg0, ah0, t0, i0.

**TMG Group**

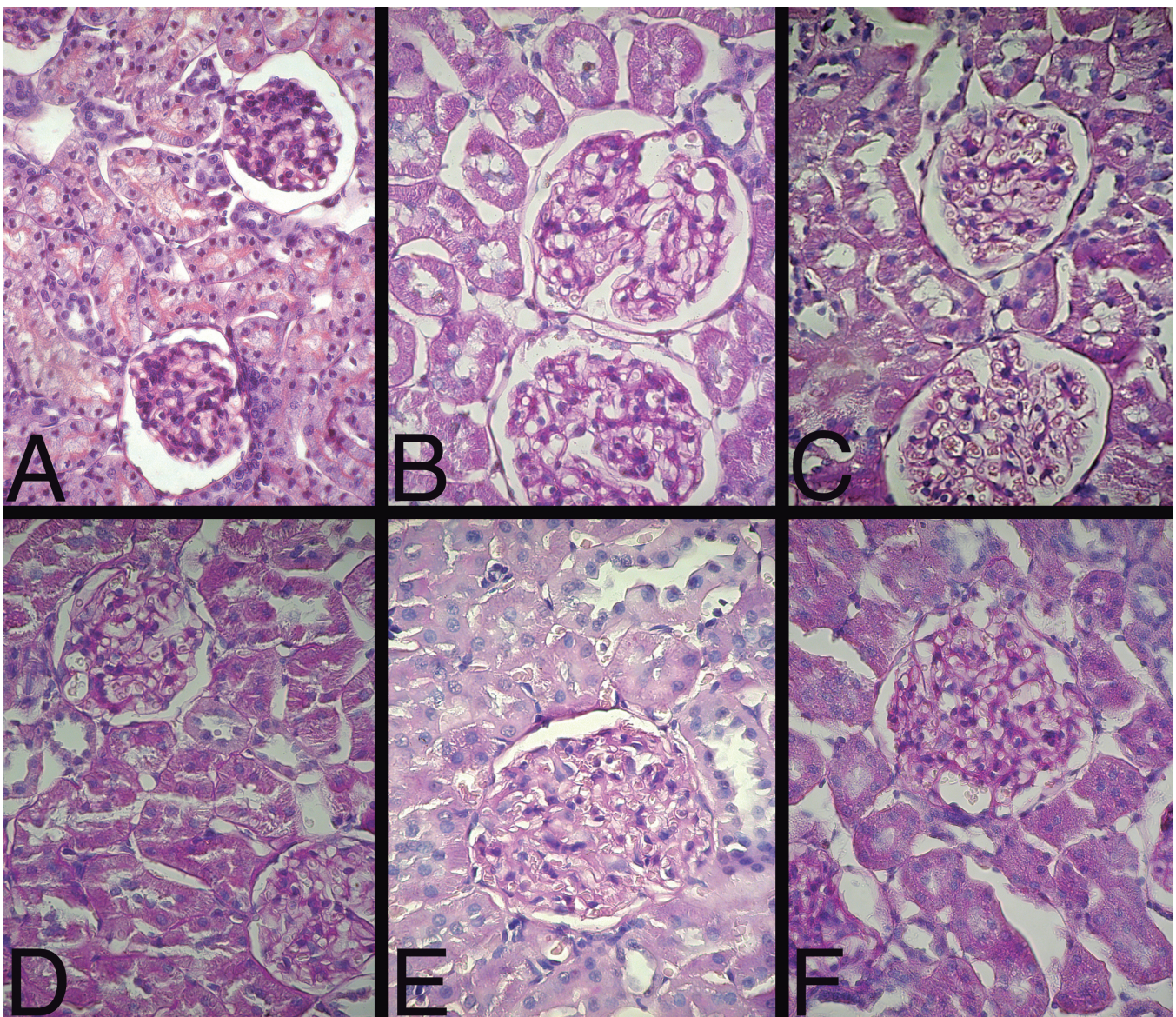
Well preserved proximal tubules with homogeneous cytoplasm and single cells in the interstitium (<10%) likewise noted in the control group. Banff '09: ct0, Ci0, cg0, ah0, t0, i0.

**Apoptosis assessment**

Apoptosis was also evaluated in proximal and distal

tubules with significant differences between the two groups being noted ( $p=0.03$  and  $p=0.01$  respectively for proximal and distal tubules). The percentage of apoptotic nuclei in the cells of the proximal and distal tubules was analysed (Fig. 4) and also significant differences between all treatment groups were found ( $p=0.01$  and  $p=0.05$  respectively) (Table 4). Statistical analysis between treatment groups proved that CRG ( $p<0.01$ ), MRG ( $p<0.01$ ) and CMG ( $p<0.01$ ) groups exhibited significantly more pronounced apoptosis in the distal nephron tubules compared to the control group. In the CMG group the percentage of apoptotic nuclei in the proximal tubule cells was also higher than the control group ( $p<0.05$ ).

Immunosuppressive regimens and their impact on



**Fig. 3.** PAS staining of rat kidney. The control group (A). TRG group (B). TMG group (C). MRG (D). CRG (E). CMG (F). x 40



blood parameters together with the expression of selected markers of apoptosis were compared in the rat kidneys. The control group which did not receive treatment was excluded from the assessment.

#### *Cyclosporine A based protocols*

Cyclosporine A based protocols (CMG, CRG) were compared to other drug combinations (TMG, TRG, MRG). It was observed that the rats treated with CsA containing regimen had a higher body weight (median 509 vs 422 g  $p<0.01$ ) and lower serum urea (median 31.3 vs 71.3 mg/dl  $p<0.003$ ) than those that did not receive any medication ( $p<0.01$   $p<0.003$ ). In rats treated with CsA there were significantly more apoptotic nuclei in the distal tubule cells (median 0.56 vs 0.48  $p<0.05$ )

compared to rats not treated with CsA (Fig. 5).

#### *Tacrolimus based protocols*

Tacrolimus based treatment (TMG, TRG) was compared to tacrolimus-free combinations (CMG, CRG, MRG). More pronounced apoptosis in the distal tubule and a higher percentage of apoptotic nuclei in the distal tubular cells (median 1 vs 0.97 and 0.56 vs 0.47  $p<0.01$  and 0.05 respectively) were found in rats treated with the tacrolimus-free regimen (Fig. 6).

#### *Mycophenolate mofetil based regimen*

MMF based treatment had the lowest effect on assessed parameters in comparison to other protocols

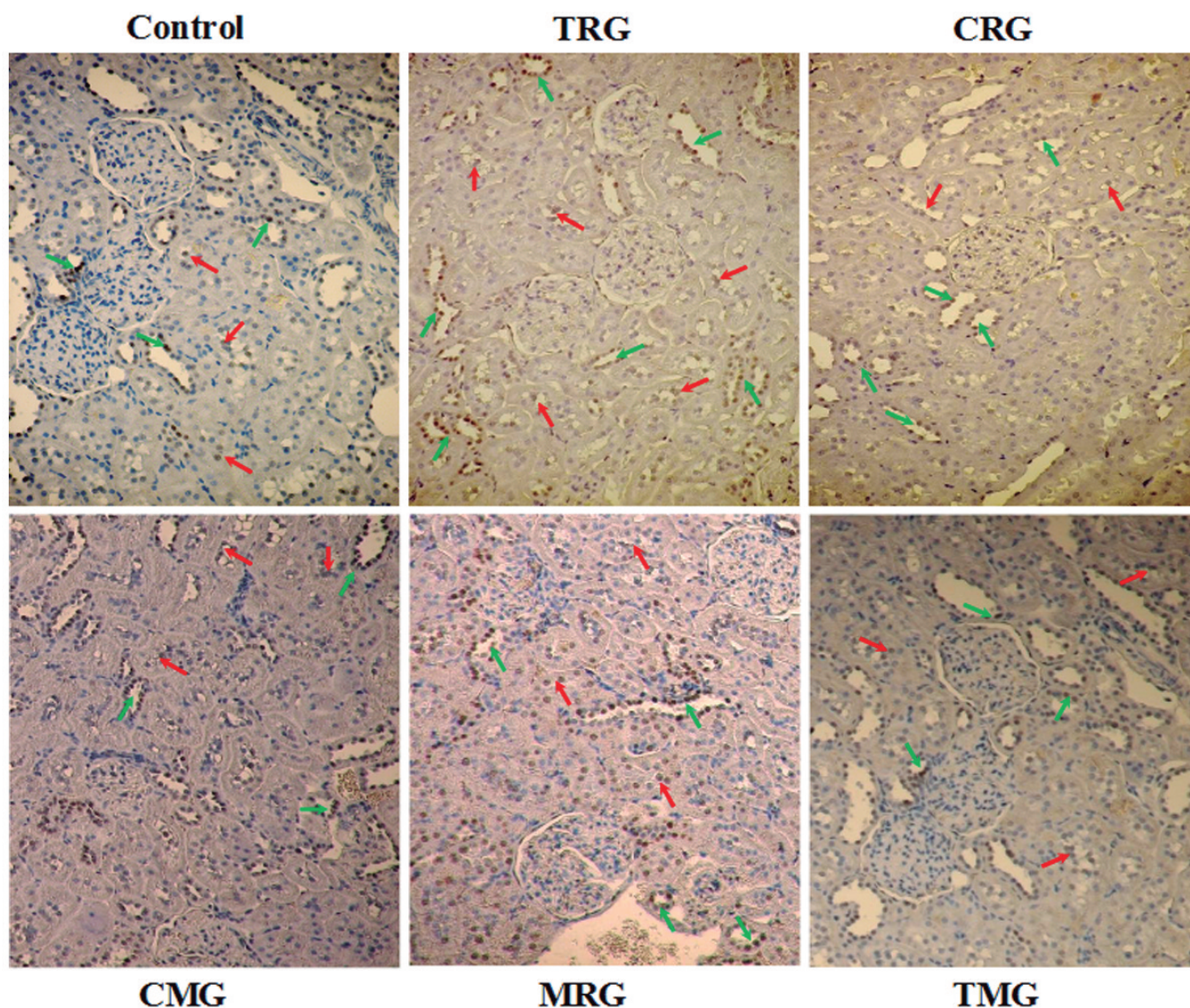


Fig. 4. TUNEL reaction-apoptosis. Control vs. all treatment groups. Red arrows-proximal convoluted tubule. Green arrows-distal convoluted tubule. x 20

(CRG, TRG). Rats treated with the MMF-based combination had lower serum creatinine (median 0.7 vs 0.8 mg/dl  $p<0.02$ ) and higher GFR (median 3.4 vs 2.4 ml/min  $p<0.02$ ) a higher percentage of apoptotic nuclei in the proximal tubule (median 0.23 vs 0.09  $p<0.05$ ) and a higher weight (median 484 vs 422 g borderline statistical significance- $p<0.06$ ) (Fig. 7).

#### Rapamycin-containing protocols

Rapamycin-based treatment (CRG, TRG, MRG) had the highest impact on assessed parameters. Rats treated with rapamycin had significantly lower body weight (median 418 vs 518 g  $p<0.001$ ) than the rats treated with the rapamycin-free regimen. Furthermore apoptosis in proximal tubules and the percentage of apoptotic nuclei in proximal tubular cells was significantly less pronounced than in the groups treated with rapamycin-free protocols (median 0.26 vs 0.7  $p<0.01$  and 0.08 vs 0.35  $p<0.001$ ). The distal tubules also had less apoptotic nuclei (median 0.48 vs 0.57  $p<0.05$ ) (Fig. 8).

#### Multivariate analysis

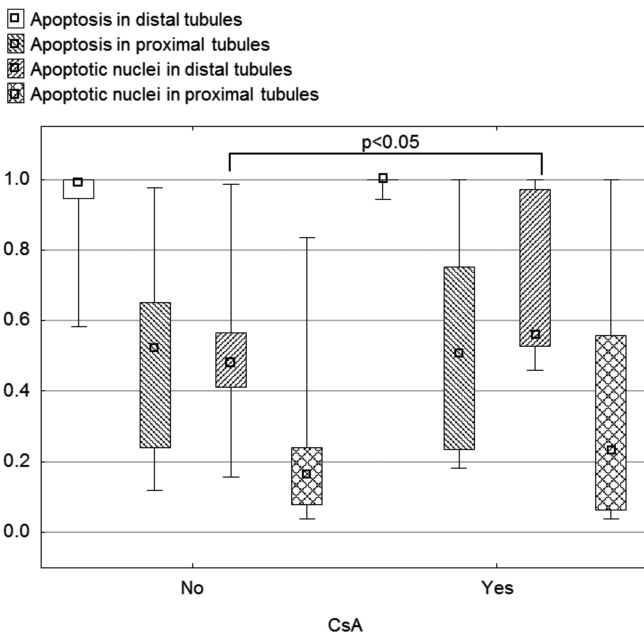
A logistic regression model was made for apoptosis in the distal tubules as most of them showed an apoptotic rate percentage of 100%. It has been shown that tacrolimus is the only significant independent predictor of a lower value ( $<100\%$  vs.  $100\%$ ) of distal tubular apoptosis (the level of statistical significance  $p=0.002$  odds ratio 0.022 confidence intervals  $-95\%=0.002$  to

$+95\%=0.278$ ). After analysing the effects of drugs on the rate of apoptosis in the distal tubule cell nuclei multiple regression models were built. The immunosuppressive medication groups were used as independent variables. It was observed that cyclosporin A ( $p=0.005$ , CI  $-95\%=0.154$ , CI  $+95\%=0.801$ ,  $\beta=0.477$ ) and mycophenolate mofetil ( $p=0.011$ , CI  $-95\%=0.108$ , CI  $+95\%=0.755$ ,  $\beta=0.432$ ) were significant independent predictors of greater apoptosis value in distal tubule cells. The addition of rapamycin to the model resulted in the loss of mycophenolate influence significance in the absence of rapamycin effect ( $p=0.095$ ).

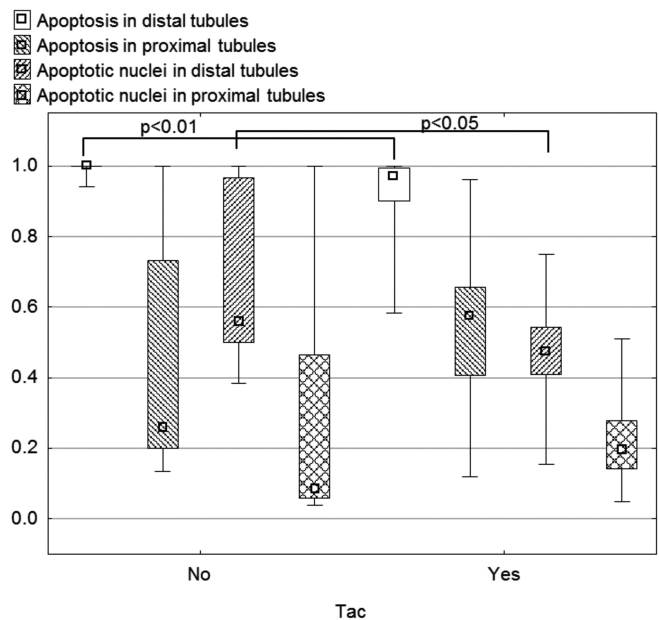
Another multiple regression model was constructed using tacrolimus and MMF as independent variables. There was no effect of such a model on the apoptotic nuclei percentage in distal tubule cells (MMF  $p=0.09$ , CI  $-95\%=-0.052$ , CI  $+95\%=0.681$ ,  $\beta=0.314$ , tacrolimus  $p=0.07$ , CI  $-95\%=-0.697$ , CI  $+95\%=0.035$ ,  $\beta=-0.031$ ).

#### The effect of medications on apoptosis phenomenon and the percentage of apoptotic nuclei in proximal tubules

A model of multiple regression was constructed to estimate the effect of cyclosporine A rapamycin and mycophenolate mofetil on apoptosis processes. It was observed that rapamycin is the only significant independent predictor of the lower apoptosis intensity in the proximal tubule (rapamycin  $p=0.005$ , CI  $-95\%=-1.182$ , CI  $+95\%=-0.239$ ,  $\beta=-0.711$ ; cyclosporine A  $p=0.467$ , CI  $-95\%=-0.490$ , CI  $+95\%=0.232$ ,  $\beta=-0.129$ ; MMF  $p=0.624$ , CI  $-95\%=-0.566$ , CI



**Fig. 5.** Comparison of apoptosis intensity and number of apoptotic nuclei in groups treated with CsA-based (CMG, CRG) or CsA-free (TMG, TRG, MRG) regimen.



**Fig. 6.** Comparison of apoptosis intensity and number of apoptotic nuclei in groups treated with tacrolimus-based (TRG, TMG) or tacrolimus-free regimen (CRG, CMG, MRG).



## Effects of immunosuppression on apoptosis

+95%=0.347, beta=-0.110).

To assess the impact of immunosuppressive therapy on the phenomenon of apoptosis in proximal tubules another model of multiple regression with a different set of drugs was constructed: tacrolimus, rapamycin and mycophenolate mofetil. Again it was observed that only rapamycin proved to be a significant independent predictor of lower apoptosis intensity in the proximal tubule (rapamycin  $p=0.019$ , CI -95%=-1.049, CI +95%=-0.106, beta=-0.577; tacrolimus  $p=0.467$ , CI -95%=-0.239, CI +95%=0.506, beta=0.133; MMF  $p=0.935$ , CI -95%=-0.465, CI +95%=0.504, beta=0.019).

The effects of rapamycin on the reduction of apoptosis intensity in the proximal tubules was maintained independently of the immunosuppressive regimen used. A similar situation was observed assessing the therapy effects on the rate of apoptosis in proximal tubular cell nuclei. A model with cyclosporine A rapamycin and mycophenolate mofetil was built. Rapamycin proved to be the only significant independent predictor of a lower percentage of apoptosis in proximal tubular cell nuclei (rapamycin  $p=0.009$ , CI -95%=-1.119, CI +95%=-0.176, beta=-0.647; cyclosporine A  $p=0.781$ , CI -95%=-0.410, CI +95%=0.312, beta=-0.049; MMF  $p=0.904$ , CI -95%=-0.484, CI +95%=0.430, beta=-0.027).

Another multiple regression model was constructed using a different set of medications: tacrolimus, rapamycin and mycophenolate mofetil as independent variables. Again it was found that rapamycin is the only

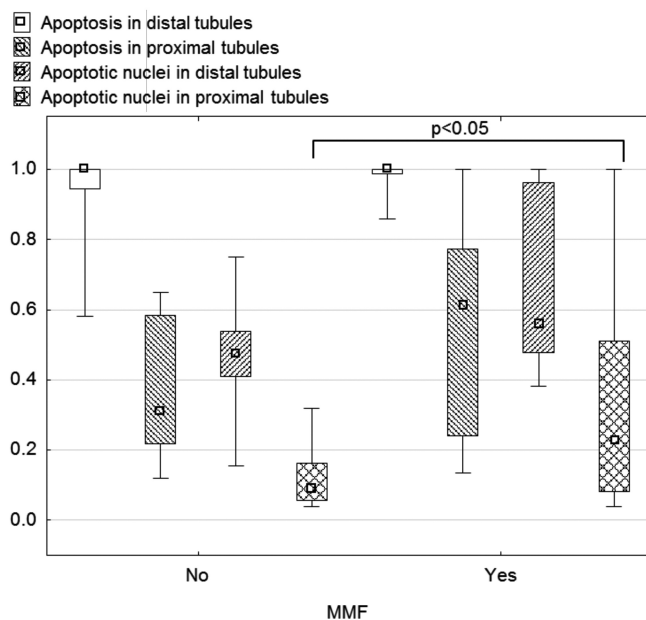
significant independent predictor of a lower percentage of apoptosis in proximal tubular cell nuclei (rapamycin  $p=0.015$ , CI -95%=-0.229, CI +95%=-1.068, beta=-0.597; tacrolimus  $p=0.781$ , CI -95%=-0.181, CI +95%=-0.322, beta=0.051, MMF  $p=0.925$ , CI -95%=-0.235, CI +95%=-0.462, beta=0.022).

The effects of rapamycin on the reduction in apoptosis rate in proximal tubular cell nuclei was maintained regardless of the immunosuppressive model used.

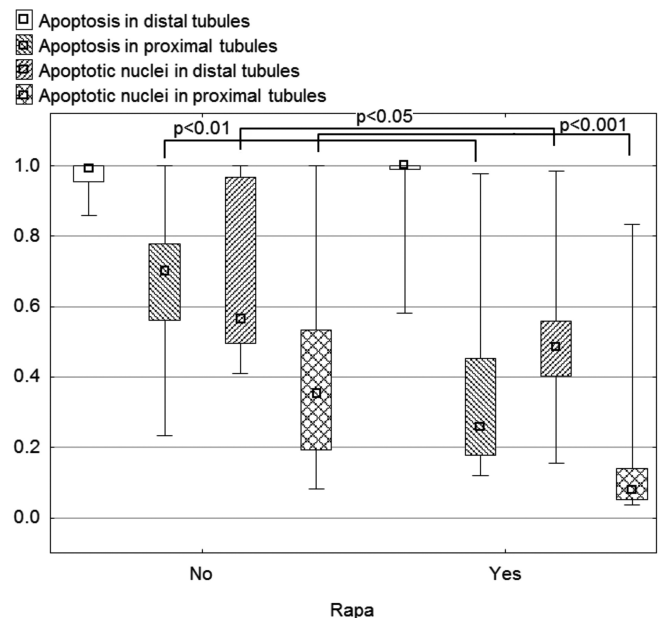
### The effect of treatment on rat body weight

The influence of immunosuppressive treatment on the body weight of the animals in the study groups was constructed. Various regression models were constructed using multiple drugs as independent variables. Rapamycin and tacrolimus had the strongest negative influence on the rat body weight (for rapamycin  $p=0.002$ , CI -95%=-1.191, CI +95%=-0.318, beta=-0.754 and for tacrolimus  $p=0.016$ , CI -95%=-0.779, CI +95%=-0.089, beta=-0.434). Mycophenolate mofetil had no significant effect on animal body weight ( $p=0.355$ , CI -95%=-0.653, CI +95%=0.243, beta=-0.205).

In a separate model the effect of cyclosporin A and rapamycin on rat body weight was evaluated. The positive effect of cyclosporin A was observed ( $p=0.025$ , CI -95%=0.049, CI +95%=0.683, beta=0.366). As in the previous model. the effect of rapamycin on rat body weight was negative ( $p=0.005$ , CI -95%=-0.790, CI



**Fig. 7.** Comparison of selected parameters in groups treated with MMF-based (CMG, TMG, MRG) and MMF-free (CRG, TRG) regimen.



**Fig. 8.** Comparison of selected parameters in groups treated with rapamycin-based (CRG, TRG, MRG) or rapamycin-free (CMG, TMG) protocols.



+95%=-0.156, beta=-0.473).

#### *The effects of immunosuppressive therapy on creatinine clearance in rats*

The effects of drugs on creatinine clearance in rats was analysed. A model with tacrolimus and MMF was constructed. The positive effect of MMF on creatinine clearance ( $p=0.037$ , CI -95%=0.029, CI +95%=0.820, beta=0.424) was noted. Tacrolimus had no significant effect on creatinine clearance ( $p=0.575$ , CI -95%=-0.287, CI +95%=0.504, beta=0.109). In a model in which cyclosporin A rapamycin and MMF were used as independent variables there was no effect by the medications on creatinine clearance. Therefore it was concluded that only MMF had a significant influence on creatinine clearance in the study groups.

#### **Discussion**

Nephrotoxic effects of immunosuppressants particularly calcineurin inhibitors (CNI) are one of the major causes of non-immune renal graft function loss. In their study, Nankivell et al. (2003) demonstrated that 10 years after kidney transplantation histopathological changes typical for calcineurin inhibitor nephrotoxicity occurred in all patients treated with those drugs. There is still a problem with interpretation of such histopathological findings because arterioles hyalinisation, glomerular sclerosis and tubulointerstitial damage are not specific to drug-induced nephrotoxicity but can be influenced by many other factors, such as the quality of the harvested organ, recipient age, ischemia-reperfusion damage, humoral and cellular rejection. El-Zoghby et al. (2009) showed isolated calcineurin inhibitor nephrotoxicity in only 0.7% of renal transplant recipients. Interestingly, Snanoudj et al. (2011) demonstrated the presence of typical features of CNI nephrotoxicity in up to 64% of kidney transplant recipients who had never received a calcineurin inhibitor based immunosuppressive regimen.

In our experiment the creation of a model of immunosuppressive drugs action which was comparative to chronic immunosuppressive therapy in humans was attempted. A "physiological" route of medication administration was chosen, and the time of the experiment was extended to six months for rats (life duration average of 2-3 years) which may be compared to approximately 12-15 years of human life. The effects of immunosuppression regimen commonly used in clinical practice were evaluated. The applied doses of immunosuppressive drugs meant that blood concentrations could be reached that are within a therapeutic range, but non-toxic, as in the work of other investigators (Rovira et al., 2008; Sánchez-Pozos et al., 2010; Shing et al., 2012). Drug trough levels were measured 4 hours after enteral supply, based on the literature data (Ma et al., 2006; Schmitz et al., 2009). This was considered an optimal time for determining the

concentration of drugs in blood due to different drug metabolism in rats compared to humans.

There was a significant difference in body weight in the study groups, depending on immunosuppressive therapy. The rats in the control group reached the highest weight, whereas the TRG group were the lowest-the difference was statistically significant. In the remaining 4 groups a lower body weight was observed in rats though the differences did not reach statistical significance compared to the control group. Other researchers have also observed a decrease in body weight in rats treated with immunosuppressants. Lloberas et al. (2008) treated rats with cyclosporine A (15 mg/kg) and rapamycin (3 mg/kg) in a 30-day-long experiment and rat body mass was lower after that period. Unfortunately the medication trough level was not specified. Other researchers have also confirmed this observation in a group of rats in which rapamycin was administered at a dose of 1 mg/kg body weight 3 times a week for 12 weeks (Rovira et al., 2008). Trough levels in the cited experiment were 38 ng/ml-12 hours after intraperitoneal administration. In our own research a dose of 0.5 mg per kg of rapamycin was administered. In the MRG group rapamycin trough level averaged 2.3 ng/ml 4 hours after oral supply while in the CRG group it reached an average of 6.3 ng/ml. Therefore the drug concentrations were considerably lower than the other experiments cited earlier in this work.

The effect of particular medications used in the protocols on rat body weight was also analysed. The rats receiving cyclosporine were seen to have significantly higher body weight than rats receiving cyclosporin-free regimen. This relationship was confirmed in the multiple regression model where the effect of cyclosporin A and rapamycin on body weight was assessed. A positive effect of cyclosporine A was noted on the weight of rats in this model while rapamycin or tacrolimus treatment led to body weight loss in the animals which is consistent with the observations of other researchers (Lloberas et al., 2008; Rovira et al., 2008). Mycophenolate mofetil had no significant effect on the body weight of the study animals.

There was no significant difference in the concentration of creatinine and GFR between the study groups and the control group. When different immunosuppressant regimens were analysed it was observed that rats receiving MMF had lower creatinine and higher GFR than the rats of the groups not treated with MMF. This observation has been verified in the multiple regression model which confirmed the positive effect of MMF on creatinine clearance. In our study only MMF exerted a significant effect on creatinine clearance this being consistent with other research. They proved the beneficial effect of MMF treatment on GFR both in renal transplant patients (Heemann et al., 2012) and experimental animals (Tian et al., 2007).

Preparations made from rat kidney stained with HE and PAS method were evaluated in search of potential acute or chronic inflammatory lesions within the kidney

tubules, glomeruli and vessels. In the experiment, there were no statistically significant pathological changes in the kidney that were visible in a light microscope study. For this reason further analysis using TUNEL was made to assess the apoptosis intensity in the tested material.

In the study it was found that the groups CRG, MRG and CMG have significantly more pronounced apoptosis in distal nephron tubules compared to the control group. In the proximal tubule a higher apoptosis rate in CMG group was also observed than in the control group. In rats treated with CsA-containing regimens there were significantly more apoptotic nuclei in the cells of the distal tubules. The rats receiving MMF also had a higher percentage of apoptotic nuclei in the proximal tubule than rats not receiving the drug. In the distal tubule of the tacrolimus-treatment rats there was a lower percentage of apoptotic nuclei and a lower percentage of apoptotic cells in the tubules compared to rats not receiving the drug.

The influence of rapamycin-based models was analysed on the apoptosis phenomenon in the proximal tubules and the percentage of apoptotic nuclei in proximal tubular cells. It was found that they were significantly lower than in the groups not treated with rapamycin. There were also less apoptotic nuclei in the distal tubules in those animals. The results of our study are again consistent with those obtained by other researchers. Evidence for the influence of CsA on renal tubular cell apoptosis comes from in vivo (Xiao et al., 2013) and in vitro (Xiao et al., 2011) studies. Cyclosporine A may affect apoptosis through various mechanisms. The most important are: oxidative stress within the mitochondria and the endoplasmic reticulum, the effect on NO synthase and increased expression of Fas/Fas-L, as well as a reduction in Bcl-2/Bcl-XL which leads to increased caspase 1 and 3 activity. A decrease in catalase and NADPH expression, as well as the release of cytochrome c into the cytoplasm are the main symptoms of oxidative stress in mitochondria. In our experiment mycophenolate mofetil did not protect proximal tubular cells from apoptosis. In a number of studies it has been shown that MMF causes apoptosis, but this was carried out with the use of myeloid cells. In those studies MMF contributed to anaemia and leukopenia development in patients treated with this drug (Pile et al., 2011). In previous studies it was found that in patients who underwent renal transplantation MMF in combination with cyclosporine and prednisone could reduce the rate of apoptosis in the proximal tubules of nephrons (Pardo-Mindán et al., 1999).

Rapamycin may also cause apoptosis in proximal tubular epithelial cells and podocytes and thereby contribute to delayed graft function (Munivenkatappa et al., 2010). In experiments on rats after unilateral nephrectomy where ischemia-reperfusion phenomenon was induced in the remnant kidney a decrease in apoptosis and caspase-3 activity in proximal tubules of the groups treated with tacrolimus, rapamycin and MMF was observed. In the CsA treated group apoptosis

intensity was increased (Yang et al., 2006). Other investigators emphasise that a rapamycin-dependent decrease of the apoptosis rate may be a secondary effect of tubular epithelial cells autophagy induction (Jiang et al., 2012).

In summary the present study shows that in models with no rapamycin and/or tacrolimus apoptosis was more pronounced, both in the proximal and in distal tubules. It seems that apoptosis intensity may be the indicator of the on-going repair process which starts as a response to damage. Conversely, if apoptosis is inefficient, the repair processes may become abnormal leading to pathological accumulation of cells and their products (ECM) with further adverse consequences, such as the development of fibrosis. There is also another possible interpretation of this observation. It is probable that regimens containing cyclosporine and/or MMF are more harmful to the kidney tubules, hence the increase in tubular cell apoptosis rate may be evidence of the nephrotoxicity of immunosuppressive regimens. Therefore models based on rapamycin and/or tacrolimus seem to be less nephrotoxic.

### *Conclusions*

Apoptotic changes found in nephron tubules under the influence of immunosuppressants are not accompanied by any histopathological changes observed in light microscopy (eg. fibrosis, inflammatory infiltration, tubular atrophy, vacuolation of the tubular cells). Apoptosis is still very difficult for interpretation. In everyday practice histopathological assessment is used in graft quality evaluation. Our study indicates that immunosuppressants may induce changes which are not expressed in light microscopy and require more detailed evaluation. One question remains; is histopathological examination a good method for drug nephrotoxicity assessment, or does it just show current irreversible damage? Further studies should show the proper direction of diagnostics and will probably indicate new methods which would be useful to evaluate the early stages of graft damage.

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