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The influence of immunosuppressants on the morphology, proliferating cell nuclear antigen (PCNA) and apoptosis in the rat ventral prostate

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Summary. Aim: Analysis of the impact of immunosuppressants on apoptosis and PCNA in the rat ventral prostate. Method: The studies were performed on 48 male Wistar rats. The animals were divided into a control group and 7 experimental groups. For 6 months, the rats were administered drugs such as: rapamycin (Rapa), cyclosporin A (CsA), tacrolimus (Tac), mycophenolate mofetil (MMF) and glucocorticosteroids (GS). During section of the rats, prostate ventral lobes were obtained. Morphological evaluation (HE, PAS), TUNEL assay, PCNA expression analysis and quantitative image computer analysis were performed. Results: The highest percentage of apoptosis in epithelial cells was observed in groups which received two combinations of drugs: (V) CsA, MMF, GS and (VII) Tac, MMF, GS. A much lower percentage of apoptotic epithelial cells was found in the groups where the treatment schemes included rapamycin throughout the duration of the study. Interestingly, the conversion of the treatment to rapamycin caused a significant reduction of apoptosis in epithelial cells as well as in proliferation in both epithelial and stromal cells. Conclusions: On one hand, the obtained results may explain the anticancer activity of rapamycin in reducing the proliferation of epithelial cells, and on the other hand the adverse effect of rapamycin in the form of reduced regeneration of these cells. Taking into account the prostate in isolation from other organs/systems, the dosage scheme with Rapa, Tac and GS would appear to be the most favorable, due to the smallest morphological changes.

Key words: Rat prostate, Apoptosis, PCNA, Immunosuppressants

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Introduction

Currently, organ transplantation is a procedure widely used around the world to save lives and improve the health of patients. Along with development in surgical techniques, substantial progress in transplantation was made possible, for example thanks to the discovery of new drugs and biologically active substances (Gummert et al., 1999; Cruzado et al., 2009; Mahmud et al., 2010). These include immunosuppressive drugs, used to minimize the rejection of vascularised transplanted organs (Gummert et al., 1999; Flechner et al., 2007; Mahmud et al., 2010). An important factor is the choice of an appropriate immunosuppressive treatment scheme that will inhibit the immune system with a low rate of adverse effects

(Thomson and Forrester, 1994; Mahmud et al., 2010). This is all the more important as the substantial decrease in the number of rejected transplants has been accompanied by an increased occurrence of infections and malignancies (Gummert et al., 1999).

The previously published studies relate mostly to the influence of single drugs on transplanted organs and tissues (Ogawa et al., 1998; Westrhenen et al., 2007; Xiao et al., 2011). However, the discovery of a number of substances with various immunosuppressive properties is an opportunity to use them in various combinations to block different pathways in the immune system (Gummert et al., 1999). In addition, immunosuppressants may affect the metabolism of each other, and numerous systemic side effects caused by immunosuppressants have been reported (Marinari et al., 1977; Kay et al., 1991; Gummert et al., 1999; Cruzado et al., 2009; Chen et al., 2013). In the literature, there are many examples of the adverse effects of these drugs on the male reproductive system, and in particular on testis (Seethalakshmi et al., 1988; Srinivas et al., 1998; Masuda et al., 2003; Chen et al., 2013). Unfortunately, very few articles have been published on the impact of individual immunosuppressive drugs on the prostate (Freitas et al., 2012, 2013). Moreover, the effects of different combinations of immunosuppressants on this gland have not been discussed at all in the literature. Therefore, studies on the influence of immunosuppressive drugs on the histological structure of the prostate seem to be appropriate and justified.

Apoptosis and proliferation processes constitute an important element in the development of chronic graft dysfunction. Similarly, the normal structure and function of the prostate depends on the balance between these two processes (Harriss and Savill, 1995). Programmed cell death maintains the homeostasis of glandular epithelium, protecting these cells from both hyperplasia and neoplasia, and allowing for the selection of these cells (Wyllie, 1993; Harriss and Savill, 1995). A potential predictive factor and a background for the development of prognostic models for cancers and benign prostate hyperplasia (BPH) is proliferating cell nuclear antigen (PCNA), an important marker for proliferation (McNeal et al., 1995; Bierhoff et al., 1996; Peng et al., 2013). Determination of the degree of apoptosis and proliferation can be an important factor in the selection of appropriate immunosuppressive regimen, as well as allowing evaluation of the effectiveness of the applied therapy.

The aim of the study was a clinical assessment of the adverse influences of different combinations of immunosuppressive drugs in wide use on the morphology, immunolocalization and immunoexpression of proliferating cell nuclear antigen (PCNA), and the occurrence of apoptosis in the cells of the ventral lobe of the rat prostate.

Materials and methods

Animals

The studies were performed on 48 adult three-month

Table 1. Scheme of the experiment with group descriptions: control (I) and experimental groups (II – VIII). Drugs given to rats in accordance with schemes usually applied in clinical practice.

GROUP (n)	Given drugs	Abbreviation	Pharmaceutical forrm (name, manufacturer)	Dose (mg/kg of body weight/d)	Feeding time (months)
I (6)	-	-	-	-	-
II (6)	Rapamycin	Rapa	Rapamune, Pfizer	0.5	6
	Tacrolimus	Tac	Prograf, Astellas	4	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
III (6)	Rapamycin	Rapa	Rapamune, Pfizer	0.5	6
	Cyclosporin A	CsA	Sandimmum-Neoral, Novartis	5	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
IV (6)	Rapamycin	Rapa	Rapamune, Pfizer	0.5	6
	Mycophenolate mofetil	MMF	Cellcept, Roche	20	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
V (6)	Cyclosporin A	CsA	Sandimmum-Neoral, Novartis	5	6
	Mycophenolate mofetil	MMF	Cellcept, Roche	20	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
VI (6)	Cyclosporin A	CsA	Sandimmum-Neoral, Novartis	5	First 3 months
	Mycophenolate mofetil	MMF	Cellcept, Roche	20	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
	Rapamycin	Rapa	Rapamune, Pfizer	0.5	Next 3 months
VII (6)	Tacrolimus	Tac	Prograf, Astellas	4	6
	Mycophenolate mofetil	MMF	Cellcept, Roche	20	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
VIII (6)	Tacrolimus	Tac	Prograf, Astellas	4	First 3 months
	Mycophenolate mofetil	MMF	Cellcept, Roche	20	
	Glucocorticosteroids	GS	Encorton, Polfa	4	
	Rapamycin	Rapa	Rapamune, Pfizer	0.5	Next 3 months

old male Wistar rats. The animals were housed in standard cages (6 rats in a cage) in ventilated rooms in a 12h light/12h dark cycle. The animals were divided into 8 groups – a control group (I) and 7 experimental groups (II-VIII). The rats in the control group received placebo. The animals in the experimental groups were administered drugs such as: rapamycin (Rapa), cyclosporin A (CsA), tacrolimus (Tac), mycophenolate mofetil (MMF) and glucocorticosteroids (GS), in their pharmaceutical oral form (in bread pellets) for a period of 6 months. The drug doses were chosen on the basis of available literature (Katz et al., 1991; Ogawa et al., 1998; Jolicoeur et al., 2003; Westrhenen et al., 2007). The study scheme is presented in Table 1.

In the experiment conducted on these Wistar rats, it was intended to create a model of the action of the immunosuppressive drugs which could be compared with chronic immunosuppressive therapy in humans. A "physiological" form - that is enteral (neither subcutaneous nor intravenous) - of drug administration had been chosen. The extended experimental time of 6 months was chosen to reflect an approximately twelve to fifteen years of human life for the average two or three-year living rat. Typical three-drug regimens of immunosuppression most commonly used in a clinical practice were used. The applied doses of immunosuppressive drugs allowed obtaining concentrations in the blood of the animals within a therapeutic range, rather than toxic levels as in articles by other researchers (Rovira et al., 2008; Sánchez-Pozos et al., 2010; Shing et al., 2012).

46 rats completed the study (2 rats in group III died). After the experiment, all animals were anesthetized with intraperitoneally administered ketamine hydrochloride (50 mg/kg). During the section, the ventral lobes of the prostates were obtained.

We conducted our research on the ventral lobe of a rat prostate due to the fact that most studies of apoptosis in the rodent prostate concerned mainly the ventral lobe (Banerjee et al., 1995, 2000). Similarly to other lobes, it consists of a stroma and a secretory part (acini) as well as a system of branching excretory ducts reaching the urethra (Lee and Holland, 1987; Hayasi et al., 1991). Moreover, the changes in this lobe are more intense and violent than in the case of the dorsal or lateral lobe (Banerjee et al., 1995, 2000).

All experiments were performed in accordance with the principles and procedures of the Local Commission of Ethics for the Care and Use of Laboratory Animals (No. 23/2009, 10.07.2009).

Morphological studies

After the end of the experiment, the ventral lobes of the rat prostates were obtained. Next, they were routinely fixed in 4% buffered paraformaldehyde and embedded in paraffin for evaluation in a light microscope (BX 41, Olympus Optical Co., Ltd., Tokyo, Japan). The paraffin blocks were sliced into 3 μm sections, which were then attached to poly-L-lysine-coated slides (ThermoScientific, Germany). The sections of the prostates were stained by standard methods (HE, PAS) (Bancroft and Gamble, 2002).

Hematoxylin and eosin stain (H&E)

After deparaffination, the sections of the prostates were stained with Mayer's hematoxylin and washed in running water. The sections were then stained with eosin. Afterwards, they were washed in distilled water, dehydrated and coverslipped in histokit (Bancroft and Gamble, 2002).

Periodic acid Schiff (PAS)

After deparaffination, the sections were placed in 0.5% acid solution, washed in running water, and next in Schiff solution. The sections were counterstained with Mayer's hematoxylin, dehydrated and coverslipped in histokit (Bancroft and Gamble, 2002).

Histochemistry

Identification of apoptosis with the use of TUNEL reaction (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling). The tissue sections of the ventral lobe were deparaffinized, rehydrated and digested with proteinase K (Dako, Denmark). The activity of endogenous peroxidase was inhibited with Peroxidase Blocking Solution (Dako, Denmark) for 10 min. Next, the sections were incubated with terminal deoxynucleotidyl transferase - TdT (ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit; Millipore™, USA) for 60 min in a humid chamber at 37°C. After that, an anti-digoxygenin antibody conjugated with peroxidase was applied for 30 min in a humid chamber at room temperature. Next, diaminobenzidine - DAB (Dako, Denmark) was used to visualize the reaction. The sections were counterstained with hematoxylin, dehydrated and coverslipped. After each step of the procedure described above, the sections were rinsed with PBS. A negative control was also made. The sections were examined in a light microscope (BX 41, Olympus Optical Co., Ltd., Tokyo, Japan).

Immunohistochemistry

The sections of the ventral prostate were deparaffinized and hydrated. Next, the sections were boiled in Target Retrieval Solution (DAKO, Denmark) at pH 9.0. The activity of endogenous peroxidase was blocked by Peroxidase Blocking Solution (Dako, Denmark) for 10 min in a humid chamber. Subsequently, the sections were incubated in a humid chamber with mouse monoclonal primary antibody - Anti-Proliferating Cell Nuclear Antigen (clone PC10) (Dako, Denmark) for 30 min. Next, the sections were incubated with a complex containing secondary antibody conjugated with

horseradish peroxidase (Dako REAL™ EnVision™ Detection System Peroxidase/DAB+, Rabbit/Mouse; Dako, Denmark). For visualization, immunohistochemical reaction DAB (Dako, Denmark) was used. The sections were counterstained with hematoxylin, dehydrated and coverslipped. Between each step of the procedure described above, the sections were rinsed with PBS. They were examined under a light microscope (BX 41, Olympus Optical Co., Ltd., Tokyo, Japan).

Quantitative image computer analysis of histochemistry and immunohistochemistry

All preparations were scanned with a ScanScope AT2 scanner (Leica Biotechnologies), at ×200 magnification (resolution 0.25μm). The appropriate level of background light intensity was calibrated automatically by the scanner, based on a pre-scan procedure. After scanning, the digital images of the slides were viewed and analyzed on a computer screen using an ImageScope viewer (version 11.2.0.780, Aperio Technologies, Inc). For the automatic computer analysis of nuclear histochemical apoptosis and immunohistochemical PCNA reaction, Nuclear v9 Algorithm (version 11.2.0.780, Aperio Technologies, Inc) was used. Other parameters were set in such a way as to achieve compliance with the visual evaluation of color intensity, taking into account the threshold for a positive result, namely a brown color of the reaction in the cell nucleus. Using Nuclear v9 Algorithm, the percentage of the positive and negative nuclei (those not showing color of the nuclear reaction) was calculated. In order to obtain reference values, the total number of positive nuclei was counted in 10 random fields with an average area of 0.26 mm^2 (for the glandular part) and 0.08 mm^2 (for stroma), in six slides for each group (I-VIII).

Statistical analysis

Statistical analysis was performed using the program Statistica 8.0 for Windows (StatSoft, Poland). For quantitative variables in each group, minimum and maximum values, the arithmetic mean and standard deviation were calculated. In order to assess the significance of differences between the values obtained in each of the group, a Kruskal-Wallis test was performed. To assess the significance of differences between the values obtained for the stroma and the glandular part for each group, a Mann-Whitney U test was carried out.

Results

The performed experiment permitted a response to the question of how different combinations of immunosuppressive drugs used in a clinic affect the histological structure of the middle part of a rat ventral lobe. A quantitative evaluation of TUNEL-positive and PCNA-positive cells in the glandular epithelium and stroma of rat ventral lobes was performed under conditions of exposure to the tested immunosuppressive drugs. The prostates of the animals from the experimental groups (II-VIII) and the control group (I) were compared with each other.

General morphology

Group I (control)

The acini were lined with a cubical (Fig. 1A) or a columnar epithelium, and PAS-positive eosinophilic secretion (Fig. 1B) was observed in their lumen. Significant changes were not found in the glandular epithelium.

Group II (Rapa, Tac, GS)

In the glandular part, the acini were lined with a simple cubical or columnar epithelium. In this group the smallest changes were found in the glandular epithelium.

Group III (Rapa, CsA, GS)

In most of the acini, a normal simple cubical as well as a columnar epithelium was observed. Also, in some acini, a distinctly atrophic epithelium was found (Fig. 1C).

Group IV (Rapa, MMF, GS)

The acini were lined with a simple cubical or columnar epithelium. In this group a large number of cells with a shrunken nucleus and distinct lumps of chromatin were observed. This may indicate that the process of apoptosis was initiated in these cells (Fig. 1D). Plasma cells infiltration accompanied the described changes. In some acini, a focal hyperplasia of glandular epithelium was also observed.

Group V (CsA, MMF, GS)

In the acini, a simple cubical or columnar epithelium was observed. A focal hyperplasia of glandular epithelium was observed on the papillary infoldings protruding into the acini lumen, formed by a connective tissue core and covered with glandular epithelium (Fig. 1E). This may indicate the existence of BPH in the ventral lobe of the rat prostate. In this group, as in group IV, a large number of cells with a shrunken nucleus and distinct chromatin were found.

Group VI (CsA, MMF, GS/Rapa)

A simple cubical or columnar epithelium was found. A focal hyperplasia was observed in the form of a stratification of the epithelial cells, whereas the stroma had not participated in the hyperplastic process (Fig. 1F). Cytological abnormalities in the cell morphology were found.

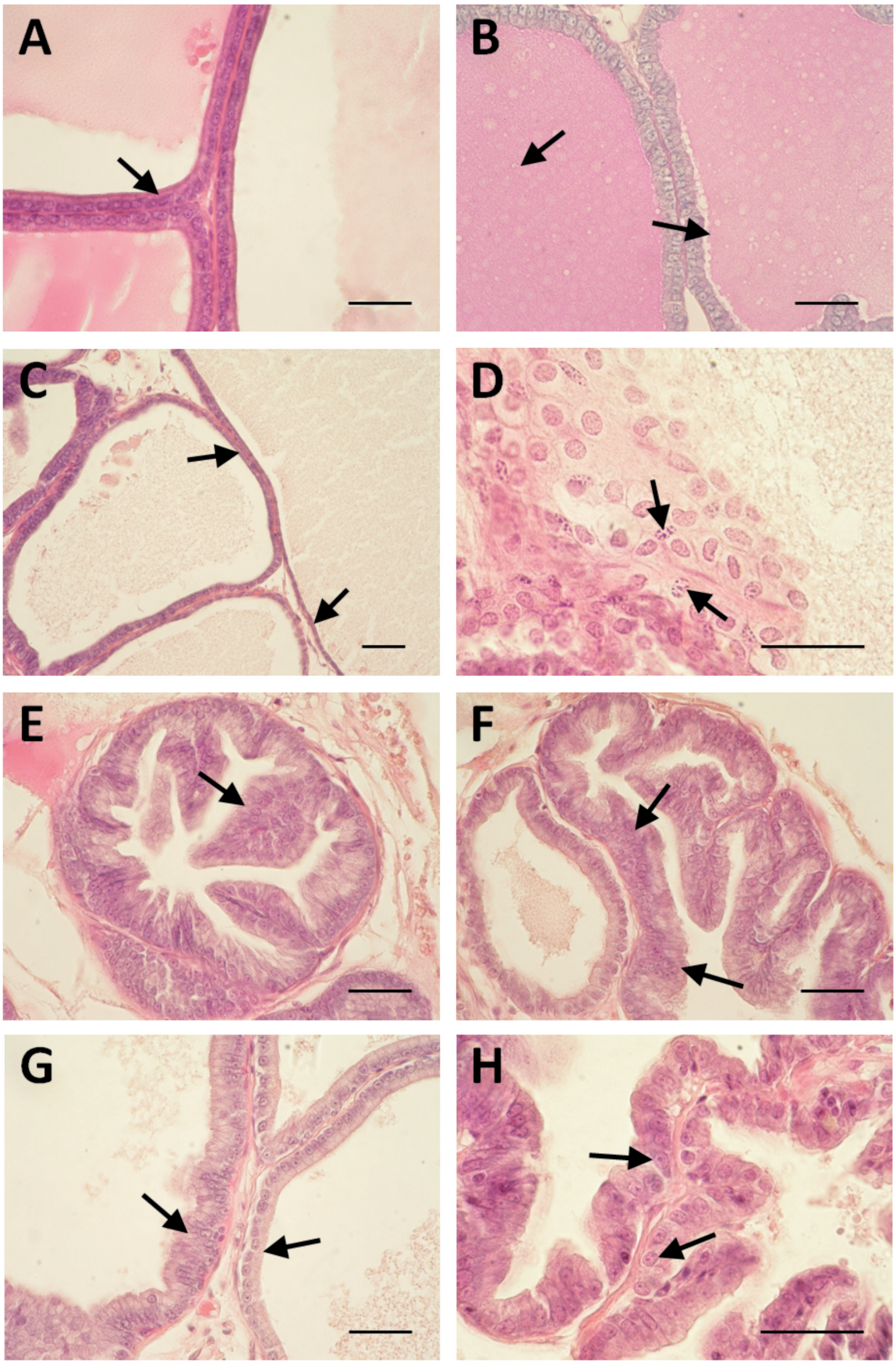

Fig. 1. Morphology of the ventral lobe of a rat prostate. Epithelial cells (arrow) **(A)** and PAS-positive **(B)** secretion in the lumen of acini (arrows) of the control group (I). HE **(A)** and PAS staining **(B)**. **C.** Simple squamous epithelium, distinctly atrophic (arrows). Group III, HE staining. **D.** Cells with shrunken nucleus and distinct lumps of chromatin (arrows). Group IV, HE staining. **E.** Papillary infoldings protruding into the acini lumen (arrow), distinct hyperplastic epithelium. Group V, HE staining. **F.** Focal hyperplasia in the form of the stratification of the epithelial cells (arrows), the stroma has not participated in the hyperplastic process. Group VI, HE staining. **G.** Disorder of the normal structure of the epithelium. Irregularly contracting nuclei of the epithelial cells, cells of different height (arrows). Group VII, HE staining. **H.** Cells with a thickened nuclear envelope, characterized by the presence of lumps of chromatin and a distinct nucleolus within the nucleus (arrows). Group VIII, HE staining. Scale bar: 50 μm.

Group VII (Tac, MMF, GS)

In the acini a simple cubical or columnar epithelium was observed. In this group a large number of disorders in the normal structure of the epithelium were found (Fig. 1G). Cytological abnormalities in cell morphology in the form of changes in the ratio of the nucleus to the cytoplasm and distinct lumps of chromatin within the nucleus were observed in some acini. What is more, a focal hyperplasia of the glandular epithelium was found.

Group VIII (Tac, MMF, GS/Rapa)

In the acini, a columnar or simple cubical epithelium was found. The epithelium showed disorders over a small area, manifested by a thickened nuclear envelope, the presence of lumps of chromatin and a distinct nucleolus within the nucleus (Fig. 1H). The above changes meet part of the criteria of prostatic intraepithelial neoplasia within the prostate - PIN. In a small number of acini, a focal hyperplasia of glandular epithelium was observed.

No important morphological changes were revealed in the stroma of the rat ventral prostate in the experimental groups (II-VIII).

Apoptosis

The TUNEL method used for the identification of apoptosis is based on the detection of cells with DNA fragmentation (Negoescu et al., 1996). In the rat prostate, a positive result of a reaction was observed in the form of brown-stained cell nuclei in both the glandular epithelium and stroma (Fig. 2A,C,E).

The percentage of apoptosis in the individual groups (I-VIII) in the glandular and stromal parts are presented in Table 2 and in Fig. 3. The percentage of apoptosis in the glandular epithelium of the control group I differed statistically significantly in comparison to all other groups II-VIII ($p=0.007$, $p=0.013$ and $p<0.001$ respectively). The highest percentages of TUNEL-positive cells in the glandular epithelium were found in group V ($p<0.001$ vs control), where cyclosporin A, mycophenolate mofetil and glucocorticosteroids had been administered, and in group VII ($p<0.001$ vs control), where tacrolimus, mycophenolate mofetil and glucocorticosteroids had been administered. The lowest percentage of TUNEL-positive cells in the epithelium was found in the control group, whereas within the experimental groups (II-VIII), the lowest percentage was noted in group III ($p<0.013$ vs control), where rapamycin, cyclosporin A and glucocorticosteroids had been administered. The percentage of apoptosis in the stroma of group I differed statistically significantly in comparison to groups III-IV ($p<0.001$ and $p=0.005$ respectively). The highest percentages of TUNEL-positive cells in the stroma were found in groups III ($p<0.001$ vs control) and IV ($p<0.001$ vs control), where rapamycin, cyclosporin A and glucocorticosteroids in group III and rapamycin, mycophenolate mofetil and glucocorticosteroids in group IV had been administered. The lowest percentage of TUNEL-positive cells in the

Table 2. The percentage of apoptosis in the epithelial and stromal cells in a rat prostate ventral lobe in control (I) and experimental groups (II – VIII).

Group Given drugs		I -	II Rapa, Tac, GS	III Rapa, CsA, GS	IV Rapa, MMF, GS	V CsA, MMF, GS	VI CsA, MMF, GS/Rapa	VII Tac, MMF, GS	VIII Tac, MMF, GS/Rapa
Apoptosis in epithelial cells	min-max	2.9-13.5	20.3-27.4	19.6-28.4	30.0-41.3	37.8-46.8	28.3-40.9	32.2-43.0	26.5-36.5
	X ± SD	7.5±3.0	24.4±1.7*	24.2±2.1**	35.8±3.0*	43.1±2.4*	36.7±3.0*	39.1±2.6*	31.3±2.9*
Apoptosis in stromal cells	min-max	0.2-10.3	3.0-11.0	9.5-17.2	11.1-17.0	3.0-15.1	4.2-16.6	1.2-10.8	0.1-7.4
	X ± SD	4.9±2.7	6.5±1.8	14.1±1.9*	14.1±1.7*	7.8±3.1*	9.7±2.6*	5.6±2.4	3.3±1.8

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

Table 3. The percentage of PCNA-positive epithelial and stromal cells in a rat prostate ventral lobe in control (I) and experimental groups (II – VIII).

Group Given drugs		I -	II Rapa, Tac, GS	III Rapa, CsA, GS	IV Rapa, MMF, GS	V CsA, MMF, GS	VI CsA, MMF, GS/Rapa	VII Tac, MMF, GS	VIII Tac, MMF, GS/Rapa
PCNA-positive cells in epithelium	min-max	16.4-29.7	13.3-28.9	21.0-38.7	26.8-42.9	16.7-37.2	6.8-24.2	17.0-36.3	10.4-29.6
	X ± SD	22.1±4.4	22.4±4.3	29.8±4.2*	38.2±4.0*	22.8±4.1	14.6±3.7*	24.8±3.5	16.0±3.9*
PCNA-positive cells in stroma	min-max	9.1-23.0	10.0-23.0	10.5-25.9	10.8-24.9	10.7-25.9	4.2-18.6	9.9-22.0	5.0-20.8
	X ± SD	16.5±3.9	16.8±3.9	18.4±4.1	18.3±3.6	16.8±4.1	12.2±4.2*	16.6±3.3	12.1±3.8*

*p<0.01 vs control.

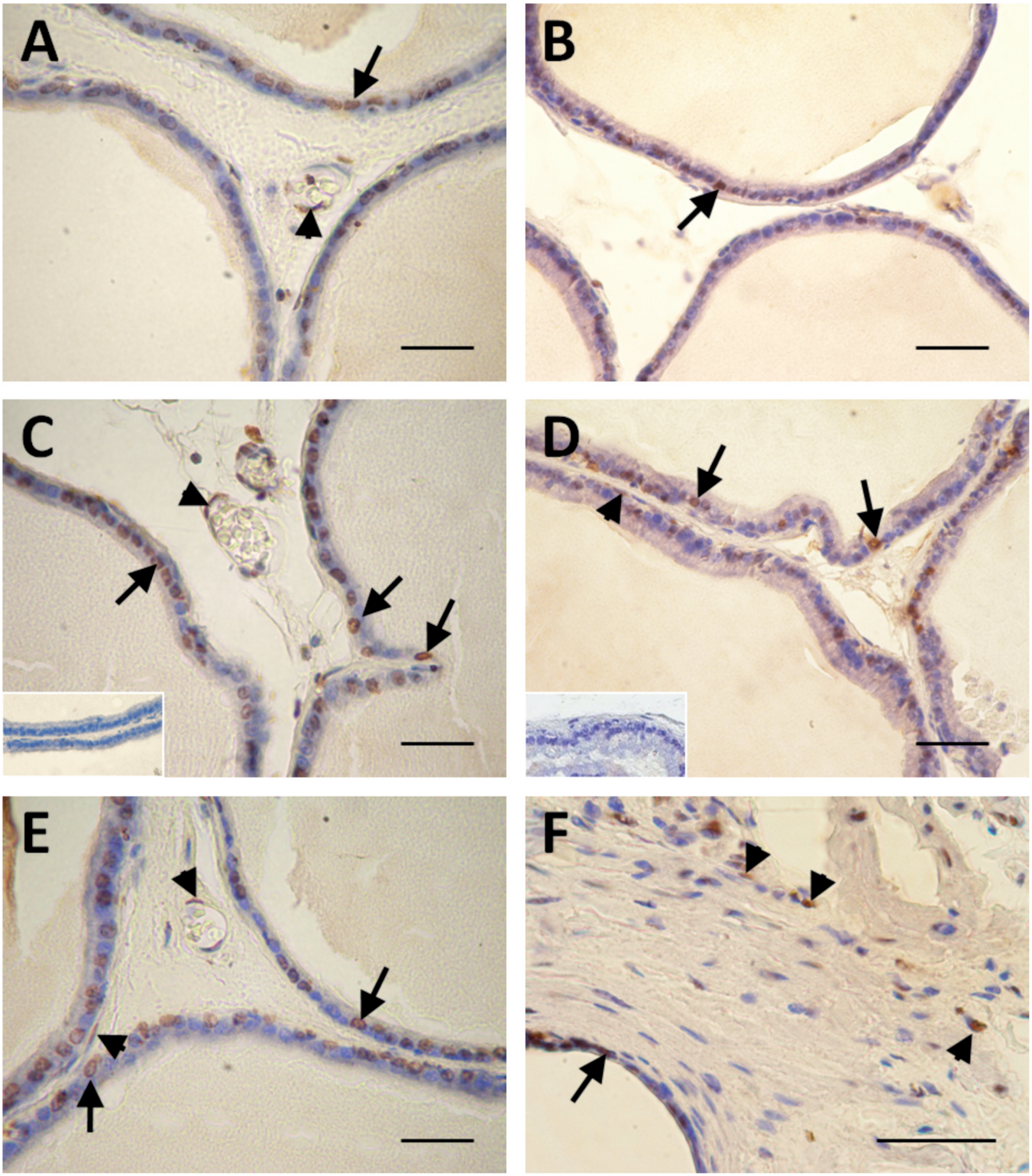

Fig. 2. Histochemical reaction TUNEL **(A, C, E)** and immunohistochemical reaction on the immunolocalisation and immunoexpression of PCNA **(B, D, F)** in the ventral lobe of a rat prostate. Insert represents a negative control of histochemical **(C)** and immunohistochemical **(D)** reaction. **(A, C, E)** TUNEL-positive cells of the glandular epithelium (arrows) and stroma (arrowheads). **A.** Group I - control. **C.** Group IV. **E.** Group VI. **B, D, F.** PCNA-positive cells of the glandular epithelium (arrows) and stroma (arrowheads). **B.** Group I - control. **D.** Group VII. **F.** group III. Scale bar: 50 μm.

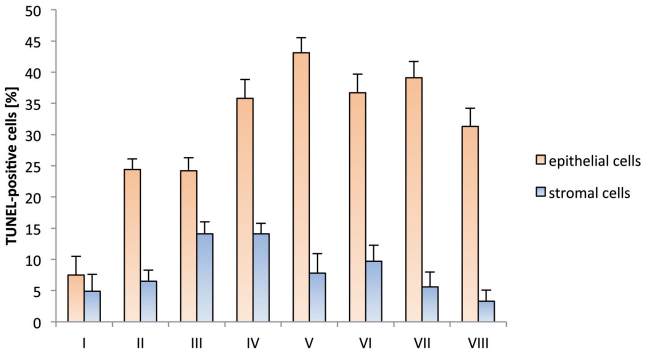

Fig. 3. The percentage of apoptosis in the epithelial and stromal cells in a rat prostate ventral lobe in control (I) and experimental (II – VIII) groups.

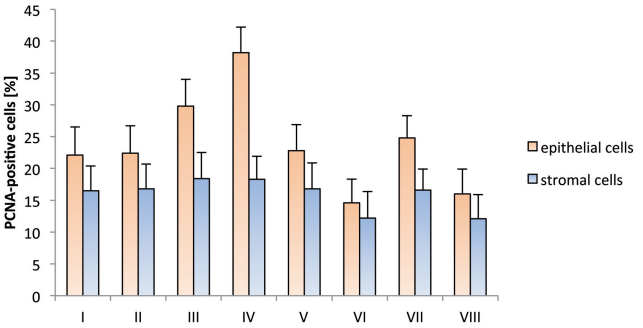

Fig. 4. The percentage of PCNA-positive epithelial and stromal cells in a rat prostate ventral lobe in control (I) and experimental (II – VIII) groups.

stroma was noted in group VIII (not statistically significant), where for the first 3 months tacrolimus, mycophenolate mofetil and glucocorticosteroids had been administered, and for the following 3 months only rapamycin had been administered. The percentage values of apoptosis for the epithelial part in comparison to the values for the stroma in each group demonstrated statistically significant differences ($p<0.001$).

Immunohistochemistry

In the rat prostate, PCNA-positive cells, both in the glandular epithelium and the stroma, were characterized by a brown-stained nucleus (Fig. 2B,D,F).

The percentages of PCNA-positive cells in individual groups (I-VIII) in the glandular and stromal part are presented in Table 3 and in Fig. 4. The percentage of PCNA-positive cells in the glandular epithelium of group I differed statistically significantly in comparison to groups III, IV, VI and VIII ($p<0.001$). The highest percentage of PCNA-positive cells in the glandular epithelium was found in group IV ($p<0.001$ vs control), where rapamycin, mycophenolate mofetil and glucocorticosteroids had been administered. The percentage of PCNA-positive cells in the stroma in group I differed statistically significantly in comparison to groups VI and VIII ($p<0.001$). The highest percentage of PCNA-positive cells in the stroma was found in group III (not statistically significant), where rapamycin, cyclosporin A and glucocorticosteroids had been administered. The lowest percentage of PCNA-positive cells both in the epithelium and the stroma was found in group VI ($p<0.001$ vs control), where for the first 3 months cyclosporin A, mycophenolate mofetil and glucocorticosteroids had been administered and for the following 3 months only rapamycin had been administered, and in group VIII ($p<0.001$ vs control), where for the first 3 months tacrolimus, mycophenolate mofetil and glucocorticosteroids had been administered and for the following 3 months only rapamycin had been administered. The percentage of PCNA-positive cells in groups I for the epithelial part in comparison to the values for the stroma in each group demonstrated statistically significant differences ($p<0.001$ and $p=0.005$ respectively).

Discussion

So far no studies have been encountered in the literature concerning the impact of different combinations of immunosuppressive drugs on the morphology, immunolocalization and immunoexpression of PCNA, as well as the occurrence of apoptosis in the ventral lobe of the rat prostate with consideration for the division between glandular and stromal parts. For this reason, our research can be regarded as pioneering in this field. In support of the presented experimental model, it can be pointed out that the drugs were administered to the animals for a lengthy period of six months. Therefore, the presented results refer to the long-term impact of various combinations of immunosuppressive drugs. By comparison, in many conducted studies concerning the influence of immunosuppressive drugs on different organs, the duration of administration of these drugs was relatively short (Masuda et al., 2003; Westrhenen et al., 2007; Munivenkatappa et al., 2010; Freitas et al., 2012).

Our morphological studies have demonstrated that in group III, where Rapa, CsA and GS were administered, the epithelium of the rat ventral lobe revealed distinct features of atrophy. Freitas et al. (2012, 2013) have shown that CsA treatment in the rat ventral prostate resulted in a significant reduction in the volume of both glandular epithelium and stroma. The authors claimed that as a result of administering CsA in the ventral lobe of the rat prostate, the ratio of the nucleus to the cytoplasm changed, the lumen of acini decreased, and the glandular epithelial cells were significantly fewer. Studies conducted by other authors (Masuda et al., 2003; Chen et al., 2013) have shown that CsA in rats caused some degenerative changes in the seminiferous tubules of the testis. The changes concerned, among others, the

impaired functioning of Sertoli cells and spermatid development, which led to the destruction of spermatogenesis.

The hyperplasia and atrophy of the glandular epithelium which was found in our study may be the result of oxidative stress which can be caused by the immunosuppressive drugs (Chien et al., 2001; Trapp and Weis, 2005; Kędzierska et al., 2011). It is known that oxidative stress is characterized by an excessive generation of reactive oxygen species (ROS), where cellular components (among others membrane lipids and nucleic acids) are damaged to a varying degree, contributing to the occurrence of metabolic dysfunction or mutations (Chien et al., 2001). The studies conducted by many authors (Deshpande et al., 2000; Lee et al., 2002; Devadas et al., 2003) have demonstrated that ROS exacerbate or activate apoptosis processes. Kędzierska et al. (2011) observed that drugs in combination - CsA, MMF and GS - caused a significant production of catalase (CAT) in rats. This serves as evidence for the existence of oxidative stress because the increased production of CAT occurs when glutathione peroxidase (GPx) is not produced in a sufficient quantity (Kędzierska et al., 2011). Moreover, Trapp and Weis (2005) observed that cyclosporin A and mycophenolate mofetil were immunosuppressive drugs which most commonly caused oxidative stress. Türk et al. (2007) confirmed that the damage to individual cells in rat testicular seminiferous tubules is associated with CsA-induced oxidative stress.

The TUNEL method is quite a common technique of identification of apoptosis in the cells of many organs (Negoescu et al., 1996; Pardo-Mindán et al., 1999; Grub et al., 2000; Munivenkatappa et al., 2010) including the male reproductive system (Laszczyńska et al., 2002; Freitas et al., 2012, 2013), therefore, it was used for the detection of apoptotic cells in the experiment performed in this study. The highest percentage of TUNEL-positive cells in the glandular epithelium of the rat ventral prostate was observed in group V ($p<0.001$ vs control), where the drugs CsA, MMF and GS were administered in combination. Apoptosis induced by CsA has been observed in many organs of the body, under both *in vitro* and *in vivo* conditions. In *in vitro* cultures, CsA induced a programmed cell death in rat hepatocytes (Grub et al., 2000), mouse thymocytes (Saiagh et al., 1994a,b) and human microvascular endothelial cells (HMEC-1) (Trapp and Weis, 2005). Moreover, there are several reports concerning the pro-apoptotic effect of CsA on kidney cells of various animals under *in vitro* conditions (Healy et al., 1998; Xiao et al., 2011), including proximal tubular epithelial cells, mesangial cells and vessels. *In vivo*, CsA-induced apoptosis was found in mouse thymocytes (Saiagh et al., 1994a,b) as well as in renal tubular epithelial cells (Thomas et al., 1998). However, despite the numerous examples of CsA-induced apoptosis in many organs of the body, other researchers (Freitas et al., 2012, 2013) have not observed statistically significant differences in apoptotic index in the ventral prostate of rats treated with CsA. Authors who had analyzed the effect of MMF on the process of apoptosis did not agree. Pardo-Mindán et al. (1999) demonstrated that MMF in a combination of CsA and GS inhibited apoptosis in renal transplant patients. Other authors (Cohn et al., 1999; Trapp and Weis, 2005), however, have shown the pro-apoptotic effect of MMF. In another study, Trapp and Weis, (2005), did not find a significant participation of glucocorticosteroids in the initiation of the apoptosis process. This may explain the common use of glucocorticosteroids in combination with other drugs due to its anti-inflammatory properties (Cupps and Fauci, 1982). Moreover, the conducted studies have demonstrated that glucocorticosteroids do not adversely affect patient fertility (McKay et al., 2005). In our experimental model, the lowest percentage of TUNEL-positive cells in the glandular epithelium of the rat ventral prostate was observed in group III ($p=0.013$ vs control), in which the drugs Rapa, CsA and GS were administered in combination. Cyclosporin A and glucocorticosteroids were common for both the group in which the most apoptotic cells were observed as well as the group with the lowest percentage of TUNEL-positive cells. Therefore, it can be assumed that the action of rapamycin resulted in the significant reduction of the amount of apoptotic cells. In support of this statement, the fact needs to be mentioned that the observed percentage of apoptosis in epithelial cells of group VI (where for the first 3 months CsA, MMF and GS were administered, and for the following 3 months only Rapa) was significantly lower ($p<0.001$) in comparison to group V (where CsA, MMF and GS were administered throughout the duration of the experiment). The results have a similar distribution to the comparison of groups VII and VIII, both in epithelial and stromal cells. In group VIII (where for the first 3 months Tac, MMF and GS were administered, and for the following 3 months only Rapa) the percentage of TUNEL-positive cells was significantly lower ($p<0.001$ for epithelial cells and $p=0.037$ for stromal cells) in comparison to group VII (where Tac, MMF and GS were administered throughout the duration of the study). Moreover, in our research, in the glandular epithelium, in all the groups in which Rapa in combination with other drugs was administered, the percentage of apoptosis was statistically lower in comparison to the groups where this drug was not administered (II, III vs V, VII $p<0.001$; IV vs V $p<0.001$). In the stroma, the results were similar (III, IV vs V, VII $p<0.001$). The anti-apoptotic effect of Rapa has also been observed by other researchers (Yang et al., 2006). Yang et al. (2006) have shown that Rapa decreased apoptosis in the kidney of rats where long term ischemia/reperfusion was induced. The lower percentage of apoptotic cells in the stroma may result from the fact that the pharmacologically induced apoptosis process in the muscle cells of the prostate stroma was inhibited by insulin-like growth factor (IGF) (Grant et al., 1998).

In a recent work by Kędzierska et al. (2014) it was

found that in rats treated with cyclosporin A, distal nephron tubules were characterized by more pronounced apoptosis. In tacrolimus-treated rats, a lower intensity of apoptosis was found in the distal tubules, while in rapamycin-treated rats the apoptosis was inhibited both in the distal and proximal nephron tubules. In MMF treated rats, intense apoptosis was observed in the proximal nephron tubules. It is worth noting that there were no significant changes in renal histopathology. These observations made in nephron epithelial cells are consistent with our results.

In our experiment, both in the glandular epithelium and stroma of the rat ventral prostate, the lowest percentages of PCNA-positive cells were observed in groups VI ($p<0.001$ vs control) and VIII ($p<0.001$ vs control), where for the first 3 months drug combinations without Rapa were administered, and for the following 3 months only Rapa. The percentage of PCNA-positive epithelial and stromal cells in group VI (where for the first 3 months CsA, MMF, GS were administered and for the following 3 months only Rapa) was significantly lower ($p<0.001$) in comparison to group V (where CsA, MMF and GS were administered throughout the duration of the study). On the basis of our results, it can be assumed that Rapa has antiproliferative properties. Moreover, during the comparison of groups VII and VIII, the results revealed a similar distribution. In group VIII (where Tac, MMF and GS were administered for the first 3 months, and for the following 3 months only Rapa) the percentage of PCNA-positive epithelial and stromal cells was significantly lower ($p<0.001$) in comparison to group VII (where Tac, MMF and GS were administered throughout the duration of the study). Studies conducted by other authors (Jolicoeur et al., 2003; Munivenkatappa et al., 2010) confirm that Rapa exerts an antiproliferative action. However, in groups III and IV, in which Rapa was administered in combination with other drugs throughout the duration of the study, a lower number of PCNA-positive cells had not been found in comparison to the groups in which this drug has not been administered (V and VII). This may indicate that Rapa exerts weaker effects on the ventral lobe of the rat prostate in combination with other drugs. Unfortunately, no articles have been found concerning the influence of immunosuppressive drugs on PCNA expression in the male reproductive system organs.

It has been observed that due to the use of immunosuppressive drugs, gonadal dysfunction usually persists for 6 months following a successful kidney transplantation, although, there is no evidence on whether fertility is fully recovered (McKay et al., 2005; Chen et al., 2013). Therefore, the drugs which are characterized by a low rate of side effects, particularly with regard to the male reproductive system, are currently being sought (Chen et al., 2013). This has a great importance in the light of fatherhood planning by male patients following transplantation (Xu et al., 2008; Chen et al., 2013).

Summation and conclusions

In the groups in which CsA, MMF and GS as well as Tac, MMF and GS were administered (V and VII groups, respectively), the highest percentage of apoptosis in epithelial cells was observed. A much lower percentage of apoptotic epithelial cells was revealed in the groups where the treatment schemes included rapamycin throughout the duration of the study (II, III, IV). Interestingly, the conversion of the treatment to rapamycin, which took place in groups VI and VIII, caused a significant reduction of apoptosis in epithelial cells as well as proliferation in both epithelial and stromal cells. The results concerning epithelial cells were differently distributed than the results for stromal cells.

The obtained results might be of significant importance in the application of rapamycin in clinical practice. The results may explain the anticancer activity of rapamycin in reducing the proliferation of epithelial cells and explain rapamycin's adverse effect in the form of reduced regeneration of these cells.

On the basis of the presented results, it is impossible to determine which of the schemes is "the safest" since an immunosuppressive treatment must be always individualized due to the different needs and coexisting diseases in a patient following an organ transplantation. Taking into account isolation of the prostate from other organs/systems, scheme II (rapamycin, tacrolimus, glucocorticosteroids) would appear to be the most favorable due to the smallest morphological changes, the low percentage of apoptosis and a proliferation degree most similar to the control group. In patients with prostate cancer, schemes VI and VIII containing rapamycin would be the most recommended.

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