Original paper

The effect of immunosuppressive drugs based on calcineurin inhibitors on cell apoptosis and junctions between epithelial cells of rat's jejunum

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Abstract

Introduction: Long-term immunosuppressive therapy in organ recipients reduces the frequency and severity of graft rejection. However, it can also produce various side effects. Clinical evidence indicates that immunosuppressive drugs can have deleterious effects on the structure and function of the gastrointestinal epithelium, making it more susceptible to infectin, inflammation, and damage. It has also been documented that calcineurin inhibitors such as cyclosporine A (CsA) and tacrolimus (TAC) can have several effects on the morphology of various tissues.
Aim of the research: The aim of the study was to evaluate the effects of chronic treatment of adult male rats with a three-drug regimen based on calcineurin inhibitors (CNIs) – cyclosporine A and tacrolimus in combination with rapamycin and glucocorticoid – on the morphology and function of the mucosal epithelium of the jejunum.
Material and methods: Paraffin-embedded jejunum samples from 14-week-old male rats were analysed. The rats received immunosuppressive regimens based on calcineurin inhibitors with rapamycin and prednisone for 6 months. Histological (H-E) and immunohistochemical staining (annexin V, occludin, E-cadherin, vinculin) were performed. Digital image and histomorphometric analyses were conducted. The results underwent statistical evaluation using appropriate parametric and non-parametric tests.
Results and conclusions: After 6 months of treatment with calcineurin inhibitors, a reduction in epithelial cell height was observed, along with increased apoptosis of cells in the apical surface of the jejunal villi, as well as changes in the immunoexpression levels of intercellular tight and adhesion junction proteins. This effect was more pronounced in the TRG group of rats.

Introduction

Immunosuppressive drugs (ID) are used in clinical therapy for the treatment of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematous, and inflammatory bowel disease, but primarily to suppress the immune system to prevent solid organ rejection after transplants [1, 2]. The use of immunosuppression impairs the immune system, often increasing the risk of infections or malignancies [3]. Additionally, it has some side effects, such as nephrotoxicity [4, 5] or hepatotoxicity [6–8]. Calcineurin inhibitors (CNIs), such as cyclosporin A (CsA) and tacrolimus (TAC), both from natural product resources, selectively inhibit calcineurin, which is essential for activating T-cells, and both of them prevent organ rejection. Additionally, they are considered the basis of triple-drug (CsA, TAC, rapamycin, glucocorticosteroid) immunosuppressive treatment therapeutic regimen [9]. They are also considered as the support of maintenance immunosuppressive regimens [9].

The mechanism of cyclosporin A action is based on its potent immunomodulatory and immunosuppressive effect by blocking the activation of lymphocytes, particularly T lymphocytes. In the cytosol of the cells, CsA binds to the cyclophilin (also known as immunophilin), and the formation of this complex results in the binding of, and inhibits the activity of, calcineurin, a calcium ion-dependent phosphatase, and inhibits its activity. Inhibition of calcineurin activity blocks the transcription of genes for various cytokines, including IL-2, which prevents T-lymphocyte proliferation, and it prevents these cells from carrying out an immune response. Cyclosporin A, by acting on mitochondria and inhibiting the release of cytochrome C, accelerates the process of apoptosis of these cells [10].

Metabolic breakdown of cyclosporin A is performed through cytochrome P (CYP)450A3, a specialised enzymatic system in the gastrointestinal tract and kidney. This results in the formation of metabolites, some of which exhibit poor pharmacological activity [11].

Tacrolimus, on the other hand, exerts its immunosuppressive effect by binding with the cytosolic tacrolimus-binding receptor protein 12 (FKBP12; FK506 binding receptor) [10]. FK506 binding protein 12, a member of the immunophilin family, which mediates the immunosuppressive action of the drugs [12]. The complex formed binds to calcineurin, inhibiting its enzymatic activity, and it prevents T-cell activation and proliferation by blocking the expression and transcription of genes for selected cytokines, including IL-2 [10]. Tacrolimus as the calcineurin inhibitor with an effect many times stronger than cyclosporine [13], and like CsA, forms the basis of triple immunosuppressive treatment regimens [14]. Calcineurin inhibitors have also been shown to not only inhibit cytokine production by CD4+ T cells but also prevent naive T cells (CD4+) from differentiating into cytokine-producing immune memory cells [15].

Literature data indicate that long-term immunosuppression can affect apoptosis pathways in epithelial cells in several ways, particularly in the context of organ transplantation, chronic inflammatory diseases, or long-term treatment with immunosuppressive drugs [9]. The disruption of this process can underlie pathological conditions that may be closely related to chronic organ dysfunction and/or contribute to morphological changes [8]. Data on the effects of immunosuppressive drugs on the cell apoptosis and morphology of many organs are generally available. However, little is known about the effect of including an immunosuppressive three-drug regimen based on calcineurin inhibitors on the integrity of the epithelial barrier of the intestine, especially the small intestine, where terminal food digestion processes and nutrient absorption are continued. The effects of immunosuppression on the gastrointestinal tract can include impaired regenerative function, immune response, disruption of mucosal integrity, as well as increased risk of certain *de novo* malignancies and certain infections [16].

The cells of this epithelium synthesise and release a number of biologically active and antimicrobial substances [17]. Goblet cells and dendritic M cells as well as intraepithelial gamma/delta T lymphocytes are present between the enterocytes. In addition, intestinal stem cells, Paneth cells, and enteroendocrine cells, which release tissue hormones, are localised in the lower parts of the intestinal crypts [18].

The balance between proliferation and differentiation of intestinal stem cells is essential for maintaining intestinal epithelial homeostasis [19]. Therefore, these cells are characterised by high plasticity and the ability to self-renew, not only under normal homeostatic conditions, but also after damage for regeneration [20]. Intestinal stem cells (ISCs) reside within the crypts and deliver differentiated progenitor cells from the crypts to the villi through mitotic divisions. Most of the differentiated progenitor cells undergo transformation within 3–5 days [21]. The differentiated epithelial cells die by apoptosis in the upper part of the villi [22].

Continuous turnover of the epithelial cells in the small intestine is essential to provide an effective barrier for nutrient absorption and protection from the external environment. In addition, the barrier separates the contents of its lumen from underlying tissues [23].

The structural integrity of the epithelium and its barrier properties are maintained by the adhesion of epithelial cells to each other, sealing the intercellular space and their attachment to the basement membrane. Intestinal epithelial cells form intercellular junction complexes, which include the following: (i) tight junctions (TJ) or zonula occludens; (ii) adherens junctions (AJ) zonula adherens, desmosome, and hemidesmosome; and (iii) gap or communicating junctions (GJ) [23].

Tight junctions (TJs) play a crucial role in maintaining cellular polarity and the integrity of tissue architecture. The proteins involved in the formation and function of tight junctions are primarily located at the apical region of the cell, just below the apical membrane. Some of the key tight junction proteins include transmembrane proteins as Claudins, the core structural components of tight junctions, occludin that interacts with claudins and other cytoplasmic proteins, and AMs (junctional adhesion molecules) that also contribute to tight junction formation. Additionally, TJs are also associated with cytoplasmic proteins as ZO proteins (Zonula Occludens – ZO-1, 2, and 3), cingulin, and afadin, which help organise the junctional complex and link them to the actin cytoskeleton. The TJs play a crucial role in maintaining cellular polarity and the integrity of tissue architecture. The proteins involved in the formation and function of tight junctions are primarily located at the apical region of the cell, just below the apical membrane [24].

Adherens junctions (AJs) are specialised structures that mediate cell-cell adhesion and are crucial for maintaining tissue integrity and cell signalling. Key components of adherens junctions include cadherins, with the most common being E-cadherin (epithelial cadherin), whose extracellular domain binds to cadherins on neighbouring cells, while the cytoplasmic domain interacts with intracellular proteins. Catenins include α-, β-, and γ-catenins. The α-catenin links cadherins to the actin cytoskeleton, the β-catenin interacts directly with the cytoplasmic domain of cadherins, while the γ-catenin also interact with cadherins and the actin cytoskeleton. In addition, P120-catenin, as a regulator of cadherin stability in the plasma membrane and actin cytoskeleton, co-form AJs [24].

Table 1. Administration of immunosuppressive drugs to rats during the experiment

Group	Cyclosporin A 5 mg/kg b.w./day	Tacrolimus 4 mg/kg b.w./day	Rapamycin 0.5 mg/kg b.w./day	Glucocorticosteroid 4 mg/kg b.w./day
Control *n* = 6	–	–	–	–
CRG *n* = 6/4	+	–	+	+
TRG *n* = 6	–	+	+	+

CRG – cyclosporine A + rapamycin + glucocorticosteroid, TRG – tacrolimus + rapamycin + glucocorticosteroid, n – number of animals per group.

Gap junctions are specialised intercellular connections that allow direct communication between adjacent cells. The type of junction is formed by the alignment of connexons from two adjacent cells. Each connexon is composed of six protein subunits called connexins. Connexons from adjacent cells align to create a pore that bridges the gap between the two cells, allowing molecules to pass through [24].

Abnormalities in the expression of proteins involved in junctions between intestinal epithelial cells can lead to impaired barrier function, loss of tissue integrity, and altered cellular communication [25], which can be reflected in its morphology. Additionally, long-term immunosuppression can affect apoptosis pathways in intestinal epithelial cells in several ways. Apoptosis is a critical process for maintaining cellular homeostasis, eliminating damaged or infected cells, and preventing cancerous growth. In epithelial cells, proper regulation of apoptosis is essential for tissue integrity and function

Aim of the research

Immunosuppressive drugs, which are used to treat autoimmune conditions, organ transplant recipients, or inflammatory diseases, can influence the function of the intestinal epithelium, the present study aimed to evaluate the effects of chronic treatment of rats with the three-drug regimen immunosuppression based on the calcineurin inhibitor group as CsA and TAC, combined with rapamycin and glucocorticosteroid, on the morphology and function of the epithelium in the jejunum mucosa.

Material and methods

The study was conducted on archival material stored at the Department of Nephrology, Transplantation, and Internal Medicine of the Pomeranian Medical University in Szczecin in the form of paraffin blocks containing embedded fragments of the jejunum – a part of the small intestine. The collected material was obtained from sexually mature male rats (14 weeks old) during a formerly conducted experiment. The study is a continuation of a previous study devoted to analysis of immunolocalisation of metalloproteinase-2 and its inhibitor in the wall of the jejunum [26]. Pieces of jejunum taken from rats in the control group and two experimental groups were used, in which rats were administered panels of three immunosuppressive drugs according to a regimen based on calcineurin inhibitors, combined with rapamycin and prednisone as glucocorticoid. The experiment used the following doses of immunosuppressive drugs with calcineurin inhibitor group: (1) cyclosporin A (Sandimmun-Neoral) C – 5 mg/kg b.w./day; (2) tacrolimus (Prograf); T – 4 mg/kg b.w./day; (3) rapamycin (Rapamune); R – 0.5 mg/kg b.w./day; and (4) prednisone (Encorton); G – 4 mg/kg b.w./day. Animals of the experimental groups – CRG and TR – were administered immunosuppressive drugs for 6 months, while 6 rats of the control group were not treated during the experiment (Table 1).

Three months after the beginning of the experiment, the animals were weighed again, and the drug doses were adjusted according to their body weight. During the experiment, two rats in the CRG group did not survive, so the group had a final count of four animals [5].

Morphological studies

Serial slides of 3–4 µm thickness were prepared from paraffin blocks and used for histochemical staining and immunohistochemical reactions.

Histological staining

After dewaxing and rehydration, the intestinal specimens were stained with haematoxylin and eosin (H&E) using freshly prepared reagents. This is the most commonly used method for review staining to assess the morphology of the tissue.

Histomorphometric analysis

Measurements of the height of the epithelium covering the intestinal villi in animals from both the control group and the experimental CRG and TRG groups were performed on slices stained with haematoxylin and eosin. Measurements were conducted using a Leica DM5000B microscope with software (Wetzlar, Germany), with an objective magnification of 40×. The results were expressed in micrometres (µm) (Table 2).

Immunohistochemistry (IHC)

Intestinal epithelial cell apoptosis was assessed by immunohistochemistry using a primary antibody

Table 2. Intestinal epithelial cell height of control and experimental groups of rats

Parameter	Control $n = 60$	CRG $n = 60$	TRG $n = 60$
Epithelial cell height [µm] Mean ± SD	20.17 ±2.06	17.16 ±1.92*	14.61 ±2.43*

*Values correspond to mean ± SD, SD – standard deviation, n – number of measurements, CRG – cyclosporine A + rapamycin + glucocorticosteroid, TRG – tacrolimus + rapamycin + glucocorticosteroid, *p < 0.05 vs. Control.*

against annexin V- Rabbit polyclonal anti-annexin V antibody (1 : 500, Abcam, Cat. No. ab14196; Abcam plc, 330 Cambridge, UK).

To assess the expression of intercellular junction proteins between jejunal epithelial cells, immunohistochemical reactions were performed using primary antibodies directed against junction proteins such as occludin, cadherin E, and vinculin.

The following primary antibodies were used: Mouse monoclonal antibody against occludin at a dilution of 1:250 (E-5: sc-133256; Santa Cruz Biotechnology, Inc., Heidelberg, Germany); Mouse monoclonal antibody to E-cadherin, diluted 1:250 (G-10: sc-8426; Santa Cruz Biotechnology, Inc., Heidelberg, Germany); and Mouse monoclonal antibody to vinculin at 1 : 300 dilution (V9131 Sigma-Aldrich; Merck Life Science Sp.z.o.o. Poznan, Poland).

All antibodies used in IHC were diluted with antibody diluent (Agilent Dako EnVision, Denmark).

Description of the procedure for IHC reactions

Slides with 4 µm-thick sections of jejunum from control and experimental animals, intended for immunohistochemical reactions, were placed in a dish filled with xylene in a heated chamber for 2 hours to deparaffinise. The dewaxed sections were then rehydrated by running them through a series of ethanol solutions at decreasing concentrations. Next, the slides were placed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave for 10 minutes to expose the antigen. In the next step, endogenous peroxidase activity was blocked by incubating the sections in Dual Endogenous Enzyme Block (Agilent Dako EnVision, Denmark) for 10 minutes. The sections were then incubated for 30 minutes at room temperature with primary antibodies to visualise the expression of the proteins of interest. The resulting product of the immunohistochemical reaction was identified using the EnVision+ Dual Link System-HRP kit for two-step staining, utilising horseradish peroxidase-labelled polymer (Labelled Polymer HRP; Agilent Dako EnVision, Denmark) conjugated with secondary antibodies. Sections were incubated for 30 minutes, followed by a 10-minute incubation with a substrate-chromogen complex containing 3,3'-diaminobenzidine (DAB+) (Agilent Dako EnVision, Denmark) to visualise the reaction product with a brown colour, indicating the location of the antigen. The tissues were then washed with distilled water, and contrast staining with haematoxylin (Sigma-Aldrich) was performed. The slides were evaluated under a microscope (Leica DM5000B, Wetzlar, Germany) [27].

Digital image analysis

For digital image analysis, slides were scanned using a 3DHISTECH Panoramic MIDI II scanner (Sysmex Polska Sp. z o.o. Warsaw, Poland), at a lens magnification of 20´, obtaining images with a resolution of 0.17 µm/pixel. Digital image analysis was performed using Pattern Quant and Cell Quant software from 3DHISTECH (3DHISTECH Kft. Budapest, Hungary). This module allows the initial segmentation of tissues and identification of several tissue structures, and it identifies stained tissue elements based on structure, colour characteristics, and intensity.

For quantitative digital image analysis of the studied tissues, intestinal slides were used to assess the immunoexpression of annexin V and selected proteins, involved in the formation of intercellular junctions.

The following parameters were determined: area and intensity of staining. The obtained results, expressed in pixels, were statistically analysed, and they are presented in Table 3.

Statistical analysis

To carry out statistical analysis of the results obtained from the quantitative digital evaluation of the tissues studied, Statistica 13 software was used. The results in the table are expressed as arithmetic means ± standard deviation and median. Normality was determined using the Shapiro-Wilk test.

For data that exhibited a normal distribution, the Fisher test was employed, and the Tukey test was utilised for post hoc comparisons. Conversely, for data that did not exhibit a normal distribution, the Kruskal–Wallis test was used, followed by Dunn's post hoc test for comparisons between groups. Differences were considered statistically significant when the p-value was < 0.05.

Results

Histological staining

On slides, stained with H-E, the morphology of the small intestine mucosa, both the villi and the lamina propria, were visualised (Figure 1). After analysis of the mucosa, there were no detectable changes in overall intestinal morphology between the control and experimental groups, both CRG and TRG.

Table 3. Statistical analysis of the results obtained from the IHC quantitative digital evaluation of three parameters as area and intensity in the control and experimental groups [mm^2]

Parameter	Group		
	Control (C)	CRG	TRG
Annexin V			
		Area	
n Mean ± SD Med.	1232 8.41 ±77.90 5.78	167 49.78 ±333.21 33.87	437 223.02 ±32.72*C 121.01
		Intensity	
n Mean ± SD Med.	1232 154 ±23 153	167 140 ±20*C 143	437 184 ±2.69*C, CRG 179
Occludin			
		Area	
n Mean ± SD Med.	964 34.81 ±356.21 54.6875	872 76.83 ±1608.703 6.25	352 159.48 ±2.87 43.75
		Intensity	
n Mean ± SD Med.	964 81 ±12 80.0	872 199 ±3.13884*C 194	352 151 ±2.1454*C, CRG 156.0
E-cadherin			
		Area	
n Mean ± SD Med.	127 144.22 ±1.131 103.12	1482 21.2 ±245.98*C 43.75	127 506.62 ±3.06*CRG 175
		Intensity	
n Mean ± SD Med.	127 101 ±1.14288 100	1482 111 ±29*C 122.0	127 182 ±3.21*C, CRG 199
Vinculin			
		Area	
n Mean ± SD Med.	731 24.71 ±121.48 100	1543 14.32 ±75.98*C 34.37	797 97.87 ±2.41 71.87
		Intensity	
n Mean ± SD Med.	731 68 ±14 65	1543 138 ±37*C 125.0	797 187 ±2.21*C, CRG 193.0

*n – number of measurements, CRG – cyclosporine A + rapamycin + glucocorticosteroid, TRG – tacrolimus + rapamycin + glucocorticosteroid. Values correspond to Mean ± SD (standard deviation) and Median (Med.), *p < 0.05 vs. Control or CRG.*

Histomorphometric analysis

Slides stained with H-E were used to assess the intestinal epithelial height from the basement membrane to the outer surface of the microvilli. The results showed that the height of the intestinal epithelium in both experimental groups was reduced compared to the control group (Table 1).

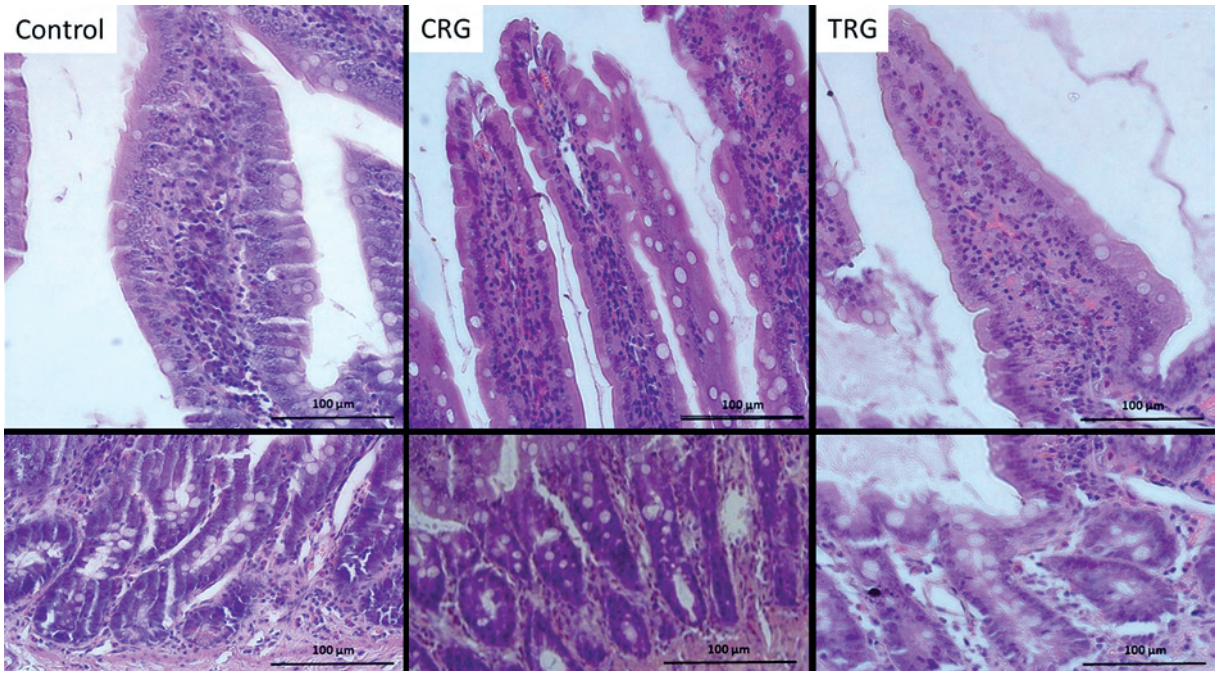

Figure 1. Jejunum – villi and crypts in the mucosal lamina propria of rats from the control group and two experimental groups: CRG (cyclosporine A + rapamycin + glucocorticosteroid); TRG (tacrolimus + rapamycin + glucocorticosteroid); Haematoxylin-eosin (H-E) 40×; bar 100 µm

Quantitative digital image analysis – immunohistochemistry

Digital analysis was performed on slides showing the immunoexpression of annexin V and proteins involved in the formation of junctions between epithelial cells.

Annexin V

The results of the analysis showed that the largest area of positive immunohistochemical reaction was identified in the jejunum from the TRG group, while the smallest was seen in the control group (Table 3). Furthermore, between the aforementioned groups, the differences were statistically confirmed ($p < 0.01$) (Figure 2, Table 3). Considering the intensity of staining (Figure 2), the protein in the TRG group stained most intensely and the CRG group stained least intensely. The differences in staining intensity were statistically confirmed between the control and CRG groups, and between the TRG and CRG, and TRG and control groups (Table 3).

Occludin

Analysis of the immunoexpression of occludin showed that the largest area of positivity reaction was seen in the epithelium of rats' jejunum from the CRG group (76.83 mm^2) and the smallest in the TRG group (159.48 mm^2). Considering the intensity of staining (Figure 3), the protein in the CRG group was the most intensely stained, and the control group it was the least stained. The differences in staining intensity were statistically confirmed between the control and CRG groups, and between the TRG and CRG, and TRG and control groups (Table 3).

E-cadherin (epithelial cadherin)

The largest area occupied by the colour IHC product visualising E-cadherin immunoexpression was recorded in the jejunal epithelium of animals in the control group, while the smallest was seen in the TRG group (Figure 4).

As for the intensity of the reaction, the most intense was in the intestine epithelium of the TRG group, while the lowest was recorded in the intestines of the control group animals. The mean values were 182 and 101 pixels, respectively. A statistically significant difference was also confirmed between the CRG and control groups, and between the TRG and CRG and control groups (Table 3).

Vinculin

The largest area occupied by the IHC-positive product was in the epithelium of rats from the control group, while the smallest was in the TRG group (Figure 5). A statistically significant difference between control and CRG groups was also confirmed (Table 3). As for the intensity of the reaction, the most intense

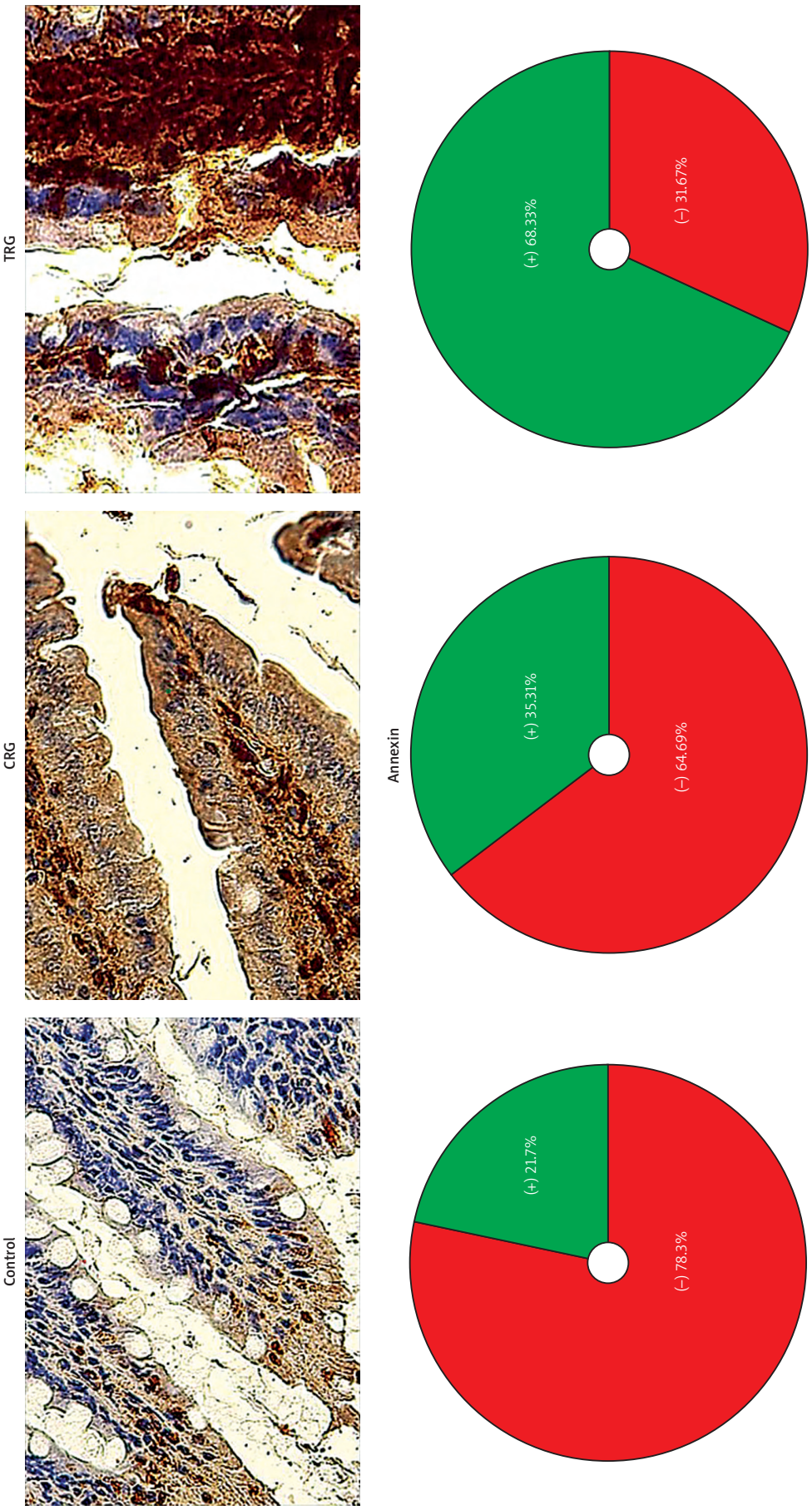

Figure 2. Quantitative digital analysis of annexin V immunoexpression indicating cells apoptosis in the jejunum of Control, CRG (cyclosporine A + rapamycin + glucocorticosteroid) and TRG (tacrolimus + rapamycin + glucocorticosteroid) treated rats. Percentage of area occupied by IHC product reaction (green +)

Figure 3. Quantitative digital analysis of occludin immunoexpression in the jejunum epithelium of rats from the control group, CRG (cyclosporine A + rapamycin + glucocorticosteroid) and TRG (tacrolimus + rapamycin + glucocorticosteroid) treated rats. Percentage of area occupied by IHC product reaction (green +)

Figure 4. Quantitative digital analysis of E-cadherin immunoexpression between jejunum epithelial cells of the control group, CRG (cyclosporine A + rapamycin + glucocorticosteroid), and TRG (tacrolimus + rapamycin + glucocorticosteroid) treated rats. Percentage of area occupied by IHC product reaction (green +)

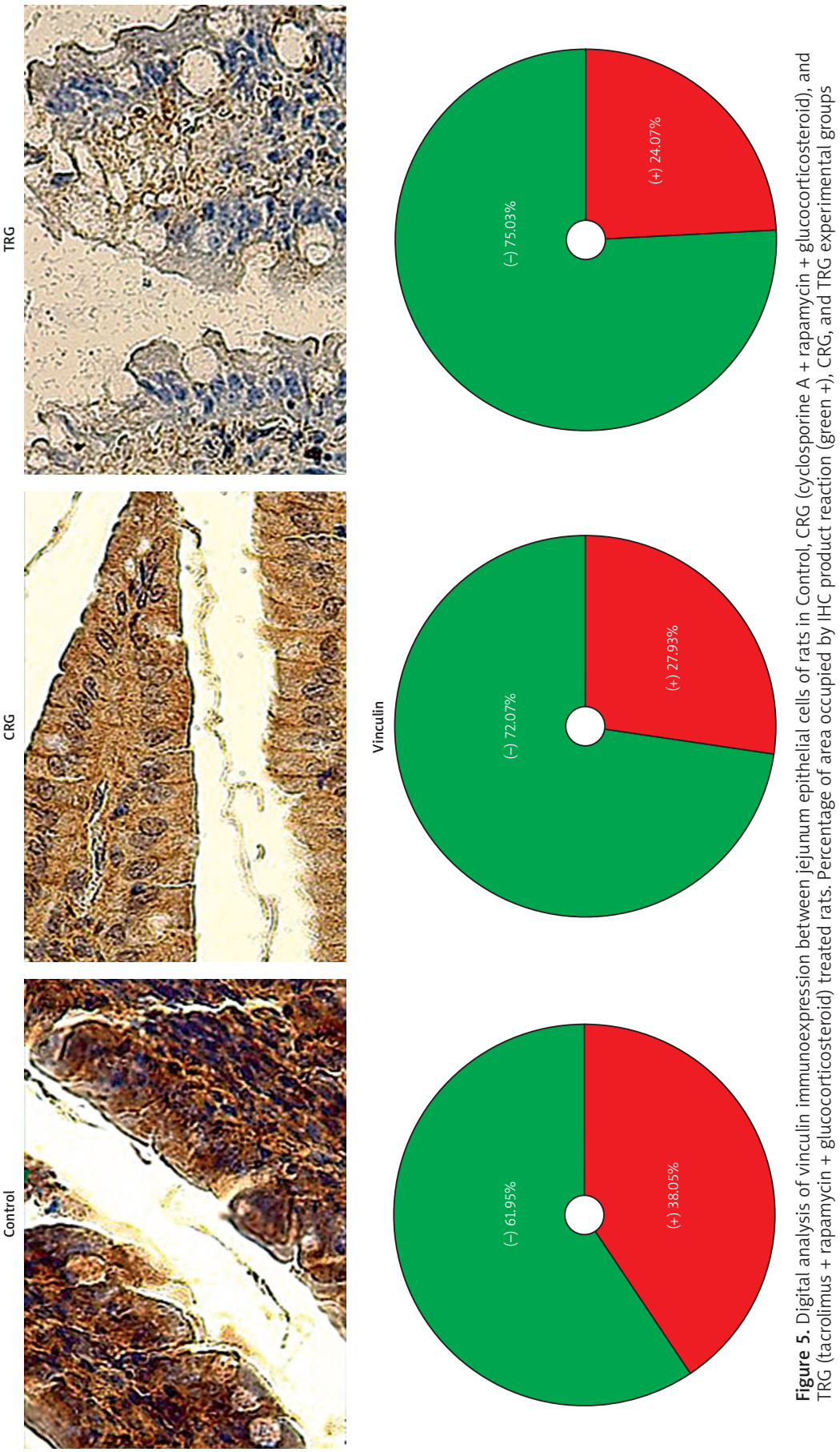

Figure 5. Digital analysis of vinculin immunoexpression between jejunum epithelial cells of rats in Control, CRG (cyclosporine A + rapamycin + glucocorticosteroid), and TRG (tacrolimus + rapamycin + glucocorticosteroid) treated rats. Percentage of area occupied by IHC product reaction (green +), CRG, and TRG experimental groups

was seen in the jejunum of the TRG group, while the lowest was recorded in the intestines of the control group animals (Figure 5). A statistically significant difference was also confirmed in the colour intensity between the groups: CRG and control, and TRG vs. CRG, and TRG vs. control.

Discussion

The use of immunosuppressive therapy is essential from the time of organ transplantation to suppress the immune response, reduce the frequency and severity of both acute and chronic rejection, and ensure long-term graft survival. Post-transplantation immunosuppressive treatment typically involves the concurrent use of several medications, tailored to specific regimens based on the type of transplanted organ and the degree of immunological risk [28]. The drugs used in immunosuppression are a factor that significantly modulates the body's immune response, and they can produce various side effects [29], among them gastrointestinal tract injury [30].

The intestinal mucosa contains well-developed diffuse lymphoid tissue [31], which helps explain the potential adverse effects of immunotherapy on intestinal morphology and function. Organ recipients with gastrointestinal complaints may experience symptoms related to the underlying disease that led to end-stage organ failure or as a result of the immunosuppressive treatment regimen used. Cyclosporin A and tacrolimus, as the principal immunosuppressive calcineurin inhibitors, are used in immunotherapy for maintenance [10].

The present study investigates the effects of chronic treatment of rats with the three-drug regimen immunosuppression based on calcineurin inhibitors – CsA and TAC – on the jejunum mucosa, with a focus on the simple columnar epithelium with microvilli, including apoptosis of epithelial cells and epithelial junctions.

As mentioned above, the resulting complex binds to calcineurin, inhibiting its enzymatic activity, and prevents T-cell activation and proliferation by blocking the expression and transcription of genes for selected cytokines, including IL-2 [10]. Due to the differential expression of CYP3A5 and P-glycoprotein, tacrolimus is most intensively absorbed in the distal segments of the intestinal tract. This relationship is reflected in the occurrence of diarrhoea in patients, caused by accelerated intestinal transit and increased concentrations of the drug in the blood [32].

Due to the properties and narrow therapeutic window of CNIs, individual dosing and treatment monitoring are used. However, these drugs induce toxic effects such as renal dysfunction, hypertension, neurotoxicity, and metabolic disorders. There is also documented clinical evidence that calcineurin inhibitor toxicity can cause changes in the morphology of various tissues [33].

In our studies, we observed histological changes induced by CNIs in the epithelium of the jejunum in experimental rats. These changes suggest that the immunosuppressive drugs used in the experiment negatively affect the height of enterocytes of the jejunum epithelium. The height of the epithelium was reduced in both experimental groups compared to the control group, as well as between the experimental groups. Additionally, the lowest epithelial height was observed in the group of rats treated with TRG. Similar results were obtained in study by Wilk *et al.* in 2024, in which immunosuppressive treatment of rats was carried out using calcineurin A and tacrolimus in combination with antimetabolite mycophenolate mofetil and glucocorticosteroid [26].

The effect of tacrolimus on morphological features of the lamina propria in a rat intestinal transplantation model was investigated by Takama *et al.* (2011). In recipients rats focal and minimal damage of epithelial cells in crypts was observed [34]. The best-documented morphological side effect of CNIs is nephrotoxicity, including constriction of the efferent and afferent arterioles or endothelial dysfunction. In addition, morphological changes also involve renal tubular epithelial cells, such as loss of microvilli and cell vacuolisation [35].

Apoptosis as a programmed, physiological manner of cell death plays an important role in tissue homeostasis, including gastrointestinal epithelium. Intestinal epithelial cells, after stem cell division, migrate along the crypt-epithelium axis towards the tip of the villi – sites to undergo apoptosis. The viability of epithelial cells is only 3–5 days before they are shedding. This process under normal and pathological conditions determines the maintenance of epithelial barrier function [36].

Increased apoptosis of epithelial cells is the most important pathological consequence of immunosuppression in the gastrointestinal tract. One of the markers of cell apoptosis is annexin V. Apoptosis, although a physiological process, can be triggered by a number of different factors, not only of internal origin, but also of external origin, such as chemical damage. An important signal that a cell undergoes the process of apoptosis is changes in the cell membrane, involving translocations of components of the cell membrane. Phosphatidylserine is translocated from the inner phospholipid layer to the outer phospholipid layer of the cell membrane. Annexin binds with high affinity precisely to the phosphatidylserine of the apoptotic cell, thus hindering their interaction with immune cells. Annexin binding with phosphatidylserine moves to the outer layer of the cell membrane during apoptosis [37]. Annexin V is considered an early marker of apoptosis, hence its selection to visualise this process in enterocytes of jejunum epithelium. In our study, conducted in male rats, it was shown that in two experimental groups the intensity of apoptosis in epi-

thelial cells was increased. A stronger proapoptotic effect was observed in the epithelium of rats in the TRG group; it has been documented, following computer analysis of immunolocalisation and annexin V immunoexpression, which allowed the determination of its staining intensity and other studied parameters.

Both calcineurin inhibitors, cyclosporine A and tacrolimus, exert proapoptotic activity of cells because they have properties to induce endoplasmic reticulum (ER) stress. In *in vitro* studies by Yilmaz *et al.* (2022), comparison the influence of CsA and TAC, used in the same doses and incubated for the same time, on human embryonic kidney 293 (HEK 293) cells, primary human proximal tubule cells, freshly isolated rat human proximal tubule cells was performed. Obtained results with evaluation of proapoptotic markers showed that apoptosis was performed faster in cells treated with CsA than in those treated with Tc [38].

Furthermore, Hissong *et al.* (2022) in their study also addressed the effects of tacrolimus on the gut. They retrospectively identified colon samples from 20 patients receiving monotherapy with tacrolimus. They reviewed the records for symptoms, endoscopy test results, other drugs, and infections. Eighteen (90%) patients were recipients of parenchymal organ transplants. Seventeen (85%) had gastrointestinal symptoms, especially diarrhoea (75%). More than 50% had endoscopic colitis, and 15% had ulcers and/or erosions. The majority (90%) of cases showed regenerative epithelial changes; apoptotic crypt cells were present in 55% and numerous in 10% of cases [16].

Neutrophilic cryptitis was present in 60% of cases; 35% showed crypt destruction. Inflammation of the lamina propria rich in plasma cells and distortion of the crypts were observed in 40% and 25% of cases, respectively. These researchers did not find a correlation between the duration of therapy and features of chronic damage; instead, they concluded, based on the data obtained, that tacrolimus can cause symptomatic colitis, and that histologic abnormalities are often mild and include crypts – regenerative and scattered remnants of apoptosis. In another study by Pittman *et al.* (2017) also confirmed the negative effect of immunosuppression on possible inflammation within the intestine colon. They followed 51 transplant patients over a 15-year period. Eleven (22%) patients had infectious colitis, and 10 of them had acute colitis confirmed by biopsy. Another 17 (33%) patients were found to have damage related to the drugs based on the resolution of symptoms after drug withdrawal. The majority (53%) of these colon biopsies showed apoptosis of crypt epithelial cells and/or distorted architecture, although 41% were histologically normal. Four (8%) patients were diagnosed with a definitive form of inflammatory bowel disease after excluding other aetiologies; biopsies from these patients showed chronic active colitis or inflammatory bowel disease with plasma cell-rich plasma cell expansion of the lamina propria and basal lymphoplasmocytosis [39].

Adverse drug reactions such as calcineurin inhibitors, especially those taken chronically, can disrupt the function of the gastrointestinal tract, the site of drug absorption, which is reflected in the histological features of the organ [40]. The alteration of the intestinal barrier can be one of the side-effects of permanent immunosuppressive treatment. This barrier is formed by elements such as the mucus layer covering the epithelium, the intestinal epithelial cells, and the lamina propria. They constitute a physical barrier between the internal milieu and environmental factors. The intestine barrier integrity is maintained due to junctions between cells of intestinal epithelial cells [41]. A disfunction of the intestinal barrier, connected with altered expression of proteins participating in cell-to-cell adhesion, has been observed in the course of various diseases [25].

In our studies we observed that chronic treatment of rats with three-drug regimen immunosuppression based on calcineurin inhibitors showed changes in immunoexpression of proteins involved in the formation of junctions between enterocytes of the jejunal epithelium. Detailed computer analysis showed differences between the control and experimental groups, as well as between the experimental groups. These changes included both proteins that form thigh junctions – occludin, as well as adhesion junctions – E-cadherin and vinculin. In our study the highest intensity of occludin expression was noted in the CRG group. Studies by Kolonko *et al.* (2023) showed that treatment of 49 patients, kidney transplant recipients treated with different tacrolimus extended-release formulations (Advagraf and Envarsus), produced functional changes of the intestinal barrier. To assess the function of tight junction, inter alia, the plasma zonulin level was measured. The level of zonulin was higher in patients treated with Envarsus, an extended-release brand of tacrolimus [41].

There are not many studies devoted to disruption of the immunoexpression of individual enterocyte intercellular junction proteins as a result of immunosuppressive drug therapy. However, particular attention has been paid to the disruption of the intestinal barrier function involving the mucus layer, microvilli, and enterocytes, as well as transepithelial transport, following the effects of the drugs [42].

It should be taken under the consideration that, although the studies mentioned above were conducted on a rat model, single immunosuppressive drugs (frequently cyclosporine A, tacrolimus, mycophenolate mofetil, and others) were administered to the animals during the experiment [41] and not according to the three-drug regimen used in the present study. In studies by Malinowski *et al.* (2011), the immunosuppressive drugs used did not show direct effects on the gastrointestinal function of Wistar rats

when applied at therapeutic concentrations. However, they may lead to changes in the pathophysiology of the small and large intestinal barrier, as well as alterations in transport function [42].

Conclusions

Due to limited information regarding the effect of immunosuppressive drugs according to a three-drug regimen based on calcineurin inhibitors on the apoptotic process and the expression of intercellular junction proteins of the small intestinal epithelium, the results of our study contribute new knowledge and are of great cognitive importance. It should also be emphasised that the morphological effect of immunosuppressive drugs on the small intestinal epithelium may depend on the mode of therapy, i.e. monotherapy or another, including a three-drug regimen, but also on the form of the drug used, i.e. short- or long-lived.

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Ethical approval

Approval for the study was obtained from the Local Bioethics Committee

Conflict of interest

The authors declare no conflict of interest.

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