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ORIGINAL RESEARCHES

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Immunoexpression of matrix metalloproteinases MMP-1, MMP-2, MMP-9 and MMP-14 in extragenital endometriosis and eutopic endometrium

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Abstract

Background: Matrix metalloproteinases are proteolytic enzymes responsible for the disorder of extracellular matrix modeling in endometriosis and their involvement in the invasion process. The aim of this study was to evaluate the immunohistochemical expression of matrix-metalloproteinases MMP-1, MMP-2, MMP-9 and MMP-14 in surgical excision specimens, collected from women with extragenital endometriosis compared to their expression in the normal endometrium.
Material and methods: The study included 40 female patients diagnosed with extragenital endometriosis. The used methods consisted in processing the specimens by classical histological technique with paraffin inclusion and enzymatic immunohistochemical technique for the detection of metalloproteinases MMP-1, MMP-2, MMP-9 and MMP-14.
Results: The expression of matrix metalloproteinases MMP-2, MMP-14 was significant in glandular cells from endometriotic lesions, while MMP-9 was evident in both stromal and glandular cells in these lesions. The expression MMP-1 was not present. Normal endometrial tissue showed high reactivity for MMP-14 and low reactivity for MMP-2 and MMP-9.
Conclusions: This study reveals some aspects related to the morphological and clinical features of extragenital endometriosis with different locations and the correlation between the clinical evolution and some immunohistochemical markers with potential prognosis regarding the aggressiveness of such lesions.
Key words: endometriosis, matrix metaloproteinases, invasiveness potential.

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Introduction

Endometriosis is a comon, benign, inflamatory pathology, represented by the ectopic location of functional endometrial glands and stroma outside the uterine cavity [1, 2].

Most sites of implantation involve the ovaries and Fallopian tubes, as well as the zones between the uterus and the anterior and posterior cul-de-sacs, the uterine ligaments and the pelvic wall. In other patients, the endometriotic implants spread beyond the inner genital organs and involve other organs in the peritoneal cavity, such as the intestines, the bladder or the lower parts of the ureters. Implants of ectopic endometrium have also been found in extragenital sites, such as surgical scars, the lungs or even the brain [3, 4].

The prevalence rate of symptomatic endometriosis is estimated to be 10% with an incidence of about 2-7/1000 women per year and a further 11% of undiagnosed cases, although there are only a few studies with well-estimated prevalence and incidence of endometriosis in the general population and some suggesting that many, if not all, women have endometriosis as a transient phenomenon [5]. Endometriosis is associated with a range of symptoms, including chronic pelvic pain, dysmenorrhea and infertility, all of which can have a strong adverse effect on the physical and mental health of the patient. While endometriosis is assumed to be a benign disease from histological standpoint, it bears a certain resemblance to malignant tumors due to the characteristic of infiltration. While the development of endometriosis has been shown to be a complex process involving interaction between genetic and environmental factors, the etiology and pathology of endometriosis remain poorly understood [6].

Numerous theories were proposed to explain etiology of endometriosis.

Matrix metalloproteinase (MMP) represents a large family zinc-dependent endopeptidases, involved in the degradation of the extracellular matrix in the process of en-

dometrial cell implantation and are classified by their substrate specificity [7, 8]. Several subtypes of MMPs are distinguished, depending on their substrate-specificity and localization: collagenases, gelatinases, stromelysins, matrilysins and membrane-type metalloproteinases. MMPs play a crucial role in numerous physiological processes, for instance: bone remodeling, angiogenesis, inflammation, ovulation and embryogenesis. What is more, MMPs are involved in cyclic changes of endometrium structure and thickness in the steroid hormones concentration levels. MMPs are expressed in both epithelial and stromal cells.

Numerous MMPs are additionally involved in many pathological processes, such as: fibrosis, weakening of matrix (e.g., in aortic aneurysm or dilated cardiomyopathy) or tissue destruction (e.g., cancer invasiveness, also endometrial carcinoma invasiveness, and ability to metastasize) [9].

Matrix metalloproteinases (MMPs) are essential in orchestrating proper physiological functioning of the endometrium; hence, alteration of MMP activities is considered as a critical factor for the development of endometriosis. MMPs are involved in the cellular event of epithelial-mesenchymal transition [10, 11].

Objective of the research was to determine the activity of MMP-1, MMP-2, MMP-9 and MMP-14 in endometriotic lesions of women with endometriosis and compare with normal endometrium.

Material and methods

The study group included 40 female patients diagnosed with extragenital endometriosis from 2010–2017, diagnosed and surgically treated at the Department of Surgery, Obstetrics and Gynecology of *Gherghe Paladi* Municipal Clinical Hospital, *Sfantul Arhanghel Mihail* Municipal Clinical Hospital (Chisinau, the Republic of Moldova) and Emergency County Hospital (Craiova, Romania). Endometrial samples of normal proliferative endometrium from women who underwent hysterectomy for benign conditions were also included for comparison. The investigation was performed by using the archival paraffin-embedded tissue blocks corresponding to these cases, preserved at the Morphopathology Departments of hospitals. Hematoxylin and eosin slides were reviewed by 3 pathologists to confirm the diagnosis. Pertinent clinical and demographic information was recorded for all study cases.

The research protocols were approved by the Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy, based on the patients informed written consent for the usage of the biologic material for research.

For immunohistochemistry, we used the paraffin blocks archived in the Laboratory of Pathology of the same Hospital. From these, 4 µm thick seriate sections were cut, which were further dewaxed, clarified and hydrated. Then, endogenous peroxidase was blocked with hydrogen peroxide (0.3%) for 15 minutes, at room temperature, and as antigen retrieval, we used microwaving for 20 minutes in 0.1 M citrate buffer of pH 6 (with the exception of fibronectin for which we used enzymatic retrieval with proteinase K, for 15 minutes, at 37°C). To avoid the nonspecific binding, the slides were covered with 2% bovine serum albumin (BSA) for one hour, at room temperature. Subsequently, the slides were incubated overnight, at 4°C, with the primary antibodies whose characteristics are presented in Table 1. Then, we used an amplification based on labeled Strep-tavidin–Biotin 2 (LSAB2) enzyme detection system and the correspondent Dako kit (Redox, Romania – K0675).

As chromogen, we used the 3.3'– Diaminobenzidine (DAB, Dako, K3468) and the Mayer's Hematoxylin kit (Tunic, Bio-Optica, Romania – M06002) for counter-staining. As negative internal controls, we used the same slides and procedures, but omitting the primary antibody.

The interpretation of the immunohistochemical reactions aimed first of all at highlighting the chromogen at the level of the antigenic targets and then at quantifying both the intensity of this signal and the proportion of the immunoreactive cells. The intensity of the reactions was graded using a scale with 4 degrees: 0 – for the absence of reactivity; 1 – for a light intensity; 2 – for moderate intensity and 3 – for high intensity (intense reaction). The reactivity of the inflammatory infiltrate (neutrophils, macrophages) was used as internal control to evaluate the intensity of these markers, this being considered the maximum reactivity.

A personalized score has been used to evaluate MMP-1, MMP-2, MMP-9 and MMP-14 expression: the percentage

Table 1

Antibodies used in immunohistochemical study

Antibody	Type	Clone	Producer	Catalog number	Dilution	Antigen retrieval	External positive control
MMP-1	Mouse monoclonal	6A5	Acris	AM06648SU-N	1:200	0.1 M Citrate, pH 6	Granulation tissue
MMP-2	Mouse monoclonal	OTI4A11	OriGene	TA806846	1:100	1mM EDTA in 10mM Tris buffer (pH8.5)	Granulation tissue
MMP-9	Mouse monoclonal	5G3	OriGene	AM06662SU-N	1:200	0.1 M Citrate, pH 6	Granulation tissue
MMP-14	Mouse monoclonal	113-5B7	OriGene	AF8410	1:100	0.1 M Citrate, pH6	Granulation tissue

of tumoral positive cells (P) and the intensity of staining (I), obtaining a P×I final score.

The proportion of positive cells was also estimated on a 4-degree scale: 0 – no positive cells in any microscopic field; 1 – <10% of positive cells; 2– between 10-50% of positive cells and 3 – > 50% of positive cells (the examination being done at the x10 objective, respectively on 10 fields). The final score (SIHC) was obtained by multiplying the 2 scores, respectively the qualitative one (intensity) and the semiquantitative one (the proportion of reactive cells) and was graded as follows: negative (-) for 0; weakly positive (+) for 1; moderately positive (++) for 2; and intensely positive (+++) for scores 3. In positive immunochemistry, the maximum score was 9 and minimum – 1. In our score system, we established a threshould value of 4, considering a value >4 as a high score, and a value ≤4 as a low score.

Results

The patients' group consisted of 42 women with endometriosis, age range of 21-63 years (median 40). Location included: the anterior abdominal wall after caesarean operation – 20, inguinal hernia – 7, umbilical hernia – 4, perineal region – 1, appendix – 4, colon – 5, and ileum – 1case. The classical histopatological examination of the general expression of MMP-1, MMP-2, MMP-9 and MMP-14 revealed a diverse variability. For MMP-2, MMP-9, MMP-14 markers, the reaction positivity was variable not only from one case to another but also within each case, the latter being characterized by the identification of different areas within endometriosis. Consequently, the complex staining patterns were homogenous and heterogenous, as a refletion of the endometriotic cells equal and variable reaction capacity in diferent neighboring territories. We should also note that the glandular cell deposit in the center expressed the investigated molecules compared to a weak expression in the peripheral focus of endometriosis.

Our data resulting from the immunohistochemical evaluation of MMP-1, MMP-2, MMP-9, MMP-14 respectively, based on the quantification of immunopositive tumoral cells count and staining intensity, and expressed as individual scores, are summarized in tab. 2.

The expression of matrix metalloproteinases MMP-2, MMP-14 was significant in glandular cells from endometriotic lesions, while MMP-9 was evident in both stromal and glandular cells in these lesions. The expression MMP-1 was absent in wall cases of endometriosis and in normal tissue. Normal endometrial tissue showed high reactivity for MMP-14 and MMP-9 and low reactivity for MMP-2.

The reactivity of MMP-2, MMP-9 and MMP-14 metalloproteinase in endometriotic lesions was higher than in eutopic endometrium. MMP-2 and MMP-9 IHC staining was performed, and endometriosis-associated fibrosis and lymphocytic infiltrate were evaluated with MMP-1. Nuclear staining of MMP-2 for glands and both membranous and

Table 2

Characteristics of MMP-1, MMP-2, MMP-9 and MMP-14 immunoexpression of endometriotic tissue and eutopic endometrium

Location	MMP-1					MMP-2					MMP-9					MMP-14				
	% of positive cells	P score	Staining intensity	I score	Final score	% of positive cells	P score	Staining intensity	I score	Final score	% of positive cells	P score	Staining intensity	I score	Final score	% of positive cells	P score	Staining intensity	I score	Final score
Anterior abdominal wall after caesarean operation	0%	0	0	0	0	10%	1	+	1	1	80%	3	+++	2	6	90%	3	+++	3	9
Inguinal hernia	0%	0	0	0	0	15%	2	+	1	2	60%	2	+++	2	4	75%	3	+++	3	9
Umbilical hernia	0%	0	0	0	0	10%	1	+	1	1	55%	2	+++	2	4	60%	3	+++	3	9
Perineal region	0%	0	0	0	0	0	0	-	0	0	20%	2	++	1	2	15%	2	+++	3	6
Gastrointestinal truct	0%	0	0	0	0	20%	2	+	2	4	90%	3	+++	3	9	95%	3	+++	3	9
Endometrium (proliferative phase)	0%	0	0	0	0	10%	1	+	2	2	80%	3	+++	3	9	90%	3	+++	3	9

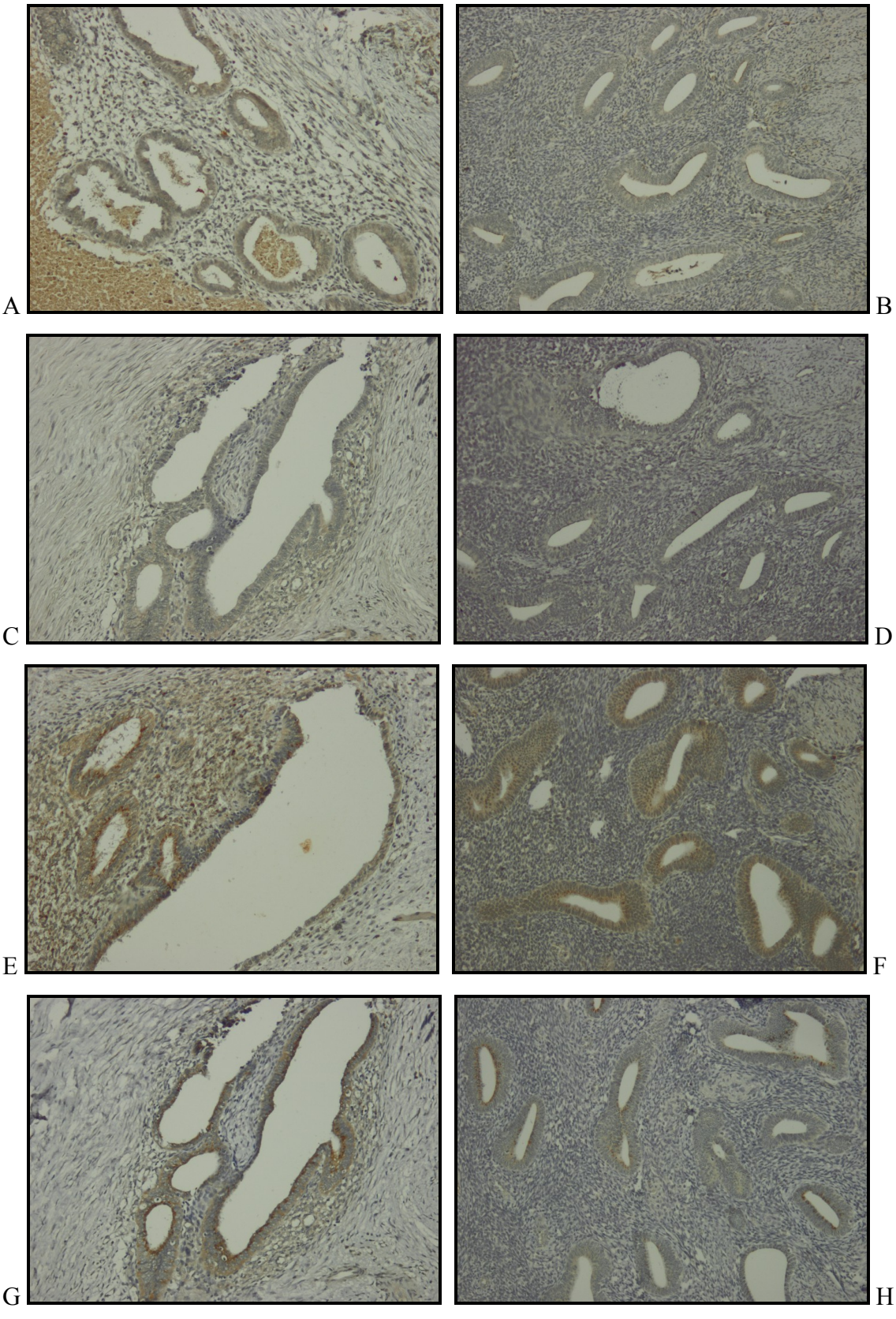

Fig. 1. Immunohistochemical reaction interpretation.

Negative reaction for MMP-1 in (A, B), moderate positive reaction for MMP-2 in (C, D), strong positive reaction for MMP-9 in (E,F) and MMP-14 in (G, H). IHC, objective 10 × /0.30.

cytoplasmic staining of MMP-9 for stroma were considered positive. In cases with intestinal endometriosis staining was diffuse and intensity was strong (3+), in abdominal wall endometriosis intensity was moderate (2+) while eutopic endometrium staining was diffuse and intensity was weak to moderate (1-2+) in all cases. MMP-14 was positive and intensity was moderate in both cases. The expression of MMP-1 on the examined tissues, was absent in endometriosis and in cases of eutopic tissue.

Immunohistochemical analysis has demonstrated the significant enhance of MMP-9 and MMP-14 expressions in endometriosis and in endometrium. The distinctive feature of MMP-9 and MMP-14 expression in endometriosis was considerable increase of its activity precisely on the border of endometriotic lesion and the peritoneum. Elevation of MMP-9 and MMP-14 activity was also observed in the stroma of the ectopic endometrium bordering on the underlying stroma (fig. 1, E, F, G, H). Enhanced activity of MMP-9 in the stroma of the ectopic endometrium bordering on the underlying stroma was accompanied by the formation of macrophage-lymphocyte infiltrates, (fig. 1, E), that evidenced the implication of immune cellular components into the inflammation zone. Thereby, the obtained research findings allow to suggest, that elevation of MMP-9 activity in the sites of endometriotic lesion on the border with the underlying stroma promotes the invasiviness of ectopic endometrium by remodeling of the underlying stroma and infiltration of endometrial cells into the peritoneum. Increased activity of MMP-2 was observed in stroma of the ectopic endometrium bordering on the underlying stroma as well (fig. 1, C). The intensified MMP-9 expression in normal endometrium was found to prevail in outer membranes of endometrial cells (fig. 1, F) while the expression of MMP-14 was found in intern. Thus, the obtained findings are indicative of the increased MMP-9 and MMP-14 activity in the sites of endometriotic lesion.

Discussion

Endometriosis is one of the most common diseases affecting women of reproductive age. So far, the pathogenesis of this disease remains poorly understood. Endometriosis is considered a benign disease that nontheless has the property of tissue invasion [6].

Metalloproteinases, enzymes that are important for extracellular matrix turnover, have recently been implicated in invasion and development of endometriosis. MMPs appear to be overexpressed in endometriotic lesions and contribute to establishment of endometrial glands and stroma at ectopic sites [12, 13].

MMP-1 (interstitial or fibroblast collagenase or colagenase-1) is the major collagenase able to degrade native fibrillar collagens type III, I, and II, at a specific site three fourths from the N-terminus. MMP-1 is produced by various types of cells: fibroblasts, keratinocytes, endothelial cells, macrophages, hepatocytes, chondrocytes and asteoblasts [14].

MMP-2 (gelatinase-A, 72-kDa type IV collagenase) cleaves collagen type IV, the major BM constituent, as well as degraded collagen and some noncollagenous ECM glycoproteins. MMP-2 also degrades native type I collagen. TNF-α and β stimulate MMP-2 production and early conceptus (blastocytes) and IFN-τ repress MMP-2 production. MMP-2 is expressed by various cell types, including fibroblasts, keratinocytes, osteoblasts, and monocytes [15].

MMP-9 (gelatinase-B, 92-kDa gelatinase) cleaves N-terminal telopeptide of type I collagen in an acidic environment, playing a role in the remodeling of collagenous ECM. MMP-9 is produced by normal alveolar macrophages, polymorphonuclear leukocytes, osteoclasts, keratinocytes, and invading trophoblasts [16].

MMP-14 is a trans-membrane type-1 protease capable of degrading different extracellular matrix components, such as collagen type I, II, and III as well as fibronectin and laminin. The main interest in this enzyme is due to its ability to activate different proteases, particularly MMP-2, MMP-9 and MMP-13 at the cell membrane. MMP-14 plays an important role in angiogenesis and is expressed by dermal fibroblasts, and osteoclasts [21].

The presence and role of MMPs in endometriosis is unquestionable, however the exact sequence of events is unclear. It remains obscure, whether changes in their activity occur primarily in uterine cavity, that due to cellular memory allows endometrium fragments implantation into peritoneum, or whether specific conditions in peritoneal cavity (inflammation or other immunological processes) change MMPs activity in endometrial cells discarded from uterus during retrograde menstruation. Moreover, some balance switches in matrix remodeling in eutopic endometrium can be periodic or temporal and vanish just after lesion constitution on ectopic site. What is more, changes of MMPs, demonstrated in uterine endometrium at women with endometriosis may also occur after implantation of ectopic lesions, under the influence of processes taking place in peritoneum. Solution of this riddle seems to be hard to find, as it would require prospective study based on endometriotic biopsies in asymptomatic population of women and long-term observation for endometriosis onset [9, 17].

In the literature, there is no agreement on differences in the expression of MMP-2 among endometriotic tissues and eutopic endometrium at women affected or unaffected by endometriosis. However, endometriotic tissues seem to express more MMP-2 than eutopic endometrium [18-21].

Conclusions

The MMP-9 and MMP-14 activity significant elevation is established on ectopic endometrium of women with endometriosis. Study of MMP-1, MMP-2, MMP-9 and MMP-14 activities in endometriotic lesions at women with endometriosis is perspective for further investigation in order to determine a possible role of matrix metalloproteinases in the development of invasiviness process in case of extragenital endometriosis.

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Authors' contribution

EC designed the trial, interpreted the data, wrote the manuscript, revised and approved the final version of the manuscript;
EZ conducted and conceptualized the project; CM conducted the laboratory work, revised the manuscript critically; RP collected material and performed the laboratory work; RN collected material and performed the laboratory work. All the authors revised the final version of the manuscript.

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Ethics approval and consent to participate

The study protocol was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova (proceeding No 63/58, 16.03.2017). Written informed consent was obtained from all participants in the study.

Conflict of Interests

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Stability studies of isohydrafural and fluocinolone acetonide combined ointment

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Abstract

Background: The isohydrafural and fluocinolone acetonide combined ointment contributes to the diversification of the treatment of dermatitis and psoriasis associated with bacterial infections. Complex stability studies were performed to ensure the quality of it during the shelf life. The objective of this study was to determine the stability and shelf life of the combined ointment containing isohydrafural and fluocinolone acetonide.
Material and methods: Three series of ointment were tested by the real-time method (temperature 25± 2°C; relative humidity 60 ± 5%) over a period of 30 months, periodically determining the appearance, homogeneity, pH, viscosity, identity, purity and assay. OHAUS DV215 CD electronic balance, Shimadzu LC-20 A HPLC, Consort C861 pH meter and Fungilab rheometer were used to fulfil the study.
Results: At 24 months after storage, the three series of the ointment proved to be homogeneous, with a pH between 5.76 and 5.53. The rheograms showed a pseudoplastic behavior, with a slight thixotropy, with a viscosity close to 70 cP. The active substances were detected at the characteristic retention times: isohydrafural – 3 minutes, fluocinolone acetonide – 5.9 minutes and also no additional peaks occurred. The content of active substances was within the permitted limits: 0.098-0.11% (m/m) for isohydrafural and 0.0248-0.025% (m/m) for fluocinolone acetonide.
Conclusions: The combined ointment containing isohydrafural and fluocinolone acetonide was found to be stable under the storage conditions stipulated in the quality specification. The established shelf life is 24 months.
Key words: combined ointment, stability, shelf life.

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Introduction

Currently, the combination of several active substances with different mechanisms of action in a single drug presented interest in medical practice [1-3]. In recent years, combinations of medicinal substances have been developed for different purposes, such as achieving synergism, reducing antibiotic resistance, reducing side effects, lower prices and increasing the treatment compliance [2, 3]. Currently, there is an interest in local combination therapy in dermatology [4]. There are already multiple combinations of antibiotics, topical corticosteroids, local anesthetics for external use [4-6]. However, the biggest challenge of dermatological drug combinations remains the simultaneous treatment of antibiotic-resistant bacterial infections and skin diseases [7].

In recent years, the development of antibiotic resistance has led to use antibacterial drugs, such as nitrofuran derivatives [8]. Researchers have also focused on modernizing the chemical structure of existing antibacterial substances to obtain new, less toxic molecules [9]. A similar problem was resolved by obtaining isohydrafural (isonicotinoyl hydrazone of 5-nitro-2-furan aldehyde) by a group of researchers from the Republic for Moldova [9, 10]. It has significant bactericidal activity in concentrations between 1.25-5.0 μg/ml, of all investigated strains of *Staphylococcus* genus [9, 10]. Isohydrafural is also less toxic (LD_{50}=990 mg/kg) than nitrofural (LD_{50}=166.7 mg/kg) and effective for topical application [9, 10]. Fluocinolone acetonide is a topical glucocorticoid, used in various dermatological diseases, such as dermatitis, psoriasis, lupus erythematosus etc. It has already existed in combined dosage forms, mixed with neomycin sulphate [11]. The combination of isohydrafural and fluocinolone acetonide in the same ointment dosage form, contributes to the diversification of the treatment of dermatitis and psoriasis associated with bacterial infections.

Stability of a drug is an important indicator of physical, chemical, therapeutical and microbiological safety. For the semisolid dosage forms the most important causes of instability are the chemical interactions and also the conferring by the excipients of a favorable environment for bacteria growth [12-14]. Stability studies are performed to prevent or eliminate these undesired causes. Stability studies are required in the process of developing a new drug before the elaboration of Analytical and Standardization Documents in order to establish its shelf life [12-14]. For semisolid dermatological dosage forms, such as ointment, the ICH guideline recommends determining the stability and shelf life by real-time (long-term) method, which consists in analysis of the quality parameters corresponding to the dosage form at

certain time intervals, by storage at temperature 25±2°C and relative humidity 60±5% [12]. According to the ICH guideline, the shelf life is considered to be established when there has been a decrease in the concentration of active substances of 90% [12-14].

The combined ointment containing isohydrafural and fluocinolone acetonide was elaborated within the research laboratories of *Nicolae Testemitanu* State University of Medicine and Pharmacy. Complex stability studies are necessary to ensure the quality of the combined ointment containing isohydrafural and fluocinolone acetonide during its shelf life. As a consequence, the objective of this study was to determine the stability and shelf life of the combined ointment containing isohydrafural and fluocinolone acetonide.

Material and methods

Ointment preparation. The pure substances, isohydrafural, (synthesized at the Department of Organic Chemistry, Chisinau State University of Moldova, concentration 99.9%) and fluocinolone acetonide (Sigma Aldrich, 99.9% concentration) were used. The excipients: propylene glycol, cetostearyl alcohol and petroleum jelly were used according to specifications of pharmacopoeia. All other materials were of analytical grade. Extemporaneous preparation of ointment was done by heating 3.0 g cetostearyl alcohol at 50-55^{0}C, cooling at 35^{0}C and then adding 91.875 g of petroleum jelly with stirring (lipophilic phase). Then 0.025 g of fluocinolone acetonide was dissolved in 5.0 g of propylene glycol and the obtained solution with 0.1 g of isohydrafural was added to lipophilic phase with stirring. The ointment was transferred to a suitable dark glass container.

Stability studies and shelf life determination. According to ICH Harmonised Tripartite Guideline Topic *Q1C*, 3 series of ointment were tested by the real-time method (temperature 25± 2°C; relative humidity 60±5%) over a period of 30 months, periodically determining the appearance, homogeneity, pH, viscosity, identity, purity and assay [12-14].

Appearance and homogeneity were tested by visual observation. *pH measurement* was determined by using the pH meter Consort C861 and the solution prepared by dissolving 1.0 g of ointment in 25 ml of deionized water and heating at 37^{0}C, cooling at room temperature and then filtering. The measurements were carried out in triplicate.

Rheological behavior was tested by using Rotational Viscometer Multi Visc Rheometer, Fungilab (Spain). The viscosity was analyzed at a fix shear rate of 3 rpm at 20±2^{0}C, which is a typical determination for ointments [12, 14]. The rheograms of shear stress as a function of shear rate during the period of 30 months of storage at 20±2^{0}C were recorded in order to determine the rheological behavior of the combined ointment.

Identity, purity and assay of isohydrafural and fluocinolone acetonide from the ointment were performed by HPLC method, which has been previously developed and validated. For chromatographic separation, the Shimadzu HIGH Performance liquid chromatograph LC-20 A and Nucleosil C18 chromatographic column, with dimensions 5x300 mm, particle size 2.6 µm were used. The mixture of acetonitrile and purified water in the ratio 40:60 was selected as the mobile phase. UV-VIS detection was performed at a wavelength of 260 nm. The flow rate of the mobile phase was 0.4 ml/minute. The temperature of the chromatographic column was maintained at 30°C.

Preparation of the isohydrafural standard solution: 0.05 g (exact mass) of isohydrafural standard was transferred into a 50 ml volumetric flask, then 20 ml of mobile phase was added and stirred until the substance dissolved. Then it was made up to the mark with mobile phase. Further 1 ml was transferred into a 10 ml volumetric flask and was made up to the mark with mobile phase.

Preparation of the fluocinolone acetonide standard solution: 0.05 g (exact mass) of fluocinolone acetonide standard was transferred into a 100 ml volumetric flask, then 30 ml of mobile phase was added and stirred until the substance dissolved. Then it was made up to the mark with mobile phase. Further 0.5 ml was transferred into a 10 ml volumetric flask and was made up to the mark with mobile phase.

Preparation of the sample solution: about 2.5 g of ointment was accurately weighed and transferred into a porcelain cup, to which 10 ml of mobile phase was added and heated in a water bath at 30°C until the ointment was melted. The sample was allowed to cool at room temperature and was filtered and collected into a 25 ml volumetric flask. The extraction was repeated twice with each 5 ml mobile phase. The obtained samples were added to the first extraction solution and were made up to the mark with mobile phase.

The concentration of isohydrafural in the combined ointment was calculated by the formula 1.

$$X = \frac{A_x \cdot 25 \cdot m_{st}}{A_{st} \cdot m_x \cdot 50 \cdot 10} \cdot P, \quad \text{in which:} \quad (1)$$

X – the content of isohydrafural in the ointment, g;

A_x – the area of the isohydrafural peak from the chromatogram of the sample solution;

A_{st} – the area of the isohydrafural peak from the chromatogram of the standard solution;

m_x – the amount of ointment taken for analysis, g;

m_{st} – the amount of isohydrafural standard, g;

P – total ointment mass, g.

The concentration of fluocinolone acetonide in the combined ointment was calculated by the formula 2.

$$X = \frac{A_x \cdot 25 \cdot m_{st} \cdot 0.5}{A_{st} \cdot m_x \cdot 100 \cdot 10} \cdot P, \quad \text{in which:} \quad (2)$$

X – the content of fluocinolone acetonide in the ointment, g;

A_x – the area of the fluocinolone acetonide peak from the chromatogram of the sample solution;

A_{st} – the area of the fluocinolone acetonide peak from the chromatogram of the standard solution;

m_x – the amount of ointment taken for analysis, g;

m_{st} – the amount of fluocinolone acetonide standard, g;

P – total ointment mass, g.

For measurements was used OHAUS DV215 CD electronic balance.

The shelf life of the combined ointment was determined in months and was based on a limit of 10% degradation of active substances in accordance with the recommendations of ICH guideline [12, 13, 14].

Statistical analysis. All values were reported as mean ± standard deviation. Statistical measurements were carried out by using the Statistical Package for the Social Sciences (IBM SPSS Statistics) 10.5 software.

Results and discussion

The stability studies of the combined ointment containing isohydrafural and fluocinolone acetonide were investigated to improve its quality and to predict the period of its stable storage [14].

Appearance and homogeneity. Appearance and homogeneity are very important parameters for the stability studies, the appropriate characteristics contributing to easier packaging and application on the skin, while any noticeable change shows the degradation of the drug [12-14].

Determinations made on the day of preparation showed that the combined ointment containing isohydrafural and fluocinolone acetonide was smooth, yellow, odorless and homogeneous. During 24 months, the combined ointment showed no change in appearance. At 30 months after storage, an intensification of the color and loss of homogeneity were observed.

pH measurement. pH is a quality parameter necessary to be studied in determining the stability of a dermatological drug, indicating its compatibility with the pH of the skin. The deviation from the limits (between 4.0 and 6.0) indicates the degradation of the drug [12-14].

For the combined ointment, pH values were recorded compatible with skin application over the period of 30 months, although a slight decrease in values was observed (fig. 1).

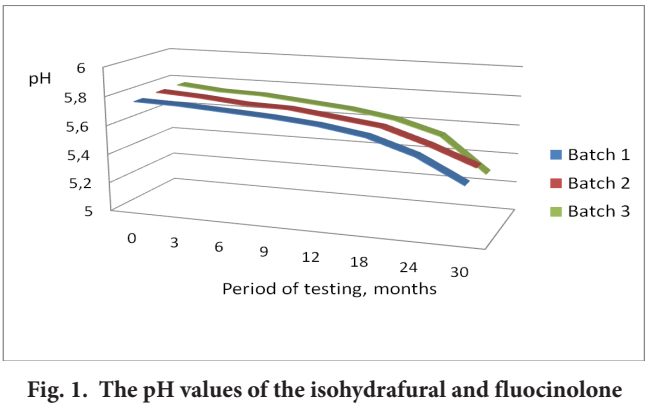

Fig. 1. The pH values of the isohydrafural and fluocinolone acetonide combined ointment

Rheological behavior. In the process of developing a new ointment it is essential to create an optimal viscosity by determining the amounts of viscosity enhancer at the preformulation and formulation stages of the drug, in order to prevent further instability. For the combined ointment containing isohydrafural and fluocinolone acetonide, the cetostearyl alcohol excipient was used as an enhancer, its amount being determined to provide an optimal viscosity close to 70 cP [12, 14]. A significant change of the ointment viscosity value in the stability study process is a reliable indication of drug degradation [12-14]. During the period of 30 months the viscosity values of all three batches of the combined ointment were close to 70 cP, being compatible for application on the skin (tab. 1).

Table 1

The viscosity values of the isohydrafural and fluocinolone acetonide combined ointment

Period of testing, months	Viscosity, cP (mean±standard deviation, 3 determinations)		
	Batch 1	Batch 2	Batch 3
0	73.5±0.6	73.8±0.5	73.1±0.1
3	71.0±0.1	71.0±0.2	71.0±0.1
6	70.5±0.4	70.2±0.1	70.2±0.1
9	69.7±0.1	69.9±0.1	69.9±0.1
12	69.1±0.1	69.1±0.1	69.1±0.1
18	68.9±0.1	68.9±0.1	68.9±0.1
24	68.2±0.1	68.2±0.1	68.2±0.1
30	69.4±0.2	71.1±0.1	71.1±0.1

The rheograms of shear stress as a function of shear rate during the period of 30 months of storage at 20±2^{0}C, indicate that the combined ointment showed a non-Newtonian, pseudoplastic, characteristic for ointments behavior, although a slight decrease in values was observed (fig. 2.1, 2.2., 2.3).

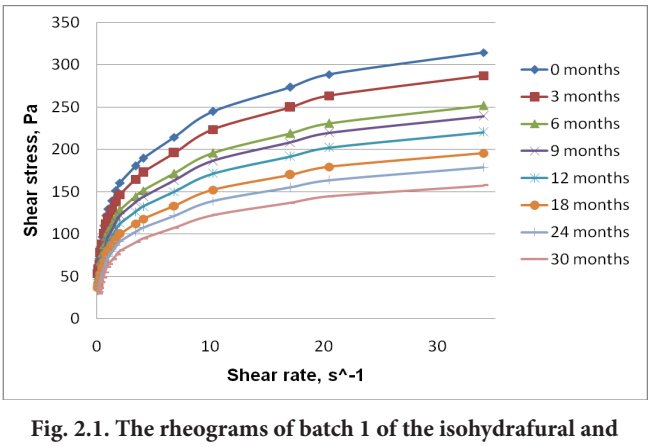

Fig. 2.1. The rheograms of batch 1 of the isohydrafural and fluocinolone acetonide combined ointment

Identity and purity. Identity and purity quality parameters demonstrate that no degradation products have appeared as a result of hydrolysis, oxidation, reduction, decomposition etc. of active substances and excipients [12-14].

The chromatograms of the three batches of the combined ointment corresponded to the quality parameters identity and purity during the period of 24 months, both active substances being detected, while the retention time values were included within the permissible limits: isohydrafural

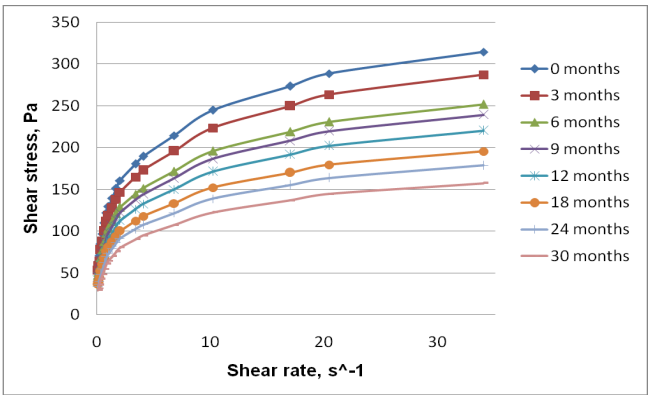

Fig. 2.2. The rheograms of batch 2 of the isohydrafural and fluocinoloneacetonide combined ointment

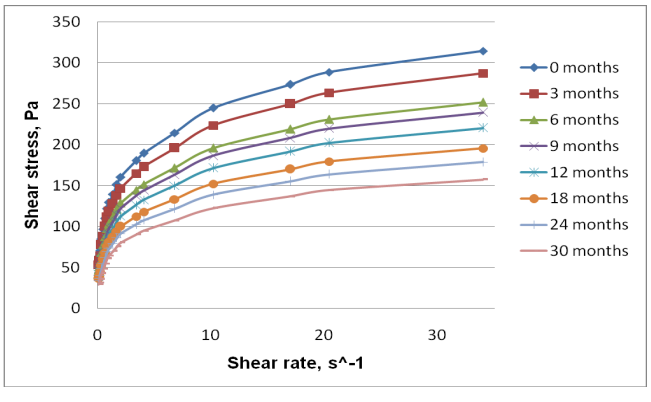

Fig. 2.3. The rheograms of batch 3 of the isohydrafural and fluocinolone acetonide combined ointment

– 3 minutes, fluocinolone acetonide – 5.9 minutes. At 30 months after storage, there were changes of the retention time values specific to fluocinolone acetonide, indicating its degradation (tab. 2).

Table 2

The retention time values of active substances from the isohydrafural and fluocinolone acetonide combined ointment

Period of testing, months	Retention time, minutes					
	Batch 1		Batch 2		Batch 3	
	IHF	FLAc	IHF	FLAc	IHF	FLAc
0	3.112	5.997	3.112	5.996	3.112	5.997
3	3.110	5.995	3.112	5.998	3.112	5.996
6	3.112	5.997	3.113	5.997	3.111	5.998
9	3.113	5.997	3.112	5.998	3.112	5.994
12	3.112	5.996	3.110	5.997	3.112	5.997
18	3.111	5.998	3.112	5.997	3.111	5.998
24	3.110	6.011	3.111	6.014	3.110	5.998
30	2.897	6.277	2.877	6.401	2.884	6.371

Note: IHF – isohydrafural; FLAc – fluocinolone acetonide.

Assay. Assay analysis of the dosage form is essential, because a change of the concentration of active substance can influence the efficacy of the drug. Also, the drug can be considered unstable if it retained less than 90% of in initial concentration of active substance [12-14].

During the period of 24 months, both active substances were detected on the chromatograms of all three series of the combined ointment in concentrations within the permitted limits: 0.098 – 0.110 g/100 g ointment for isohydrafural and 0.0248 – 0.0250 g/100 g ointment for fluocinolone acetonide. At 30 months after storage, the concentration of fluocinolone acetonide was below 0.0248 g/100 g ointment, indicating its degradation (tab. 3).

The stability profile curves of all three batches of the combined ointment, obtained by the real-time method, indicate a reducing up to 90% of fluocinolone acetonide concentration at 30 months of testing, that undergoes faster degradation than isohydrafural (fig. 3.1, 3.2, 3.3).

Table 3

The content of active substances of the isohydrafural and fluocinolone acetonide combined ointment

Period of testing, months	Content, g/100 g ointment					
	Batch 1		Batch 2		Batch 3	
	IHF	FLAc	IHF	FLAc	IHF	FLAc
0	0.10013	0.02504	0.10001	0.02501	0.09996	0.02500
3	0.09996	0.02500	0.09992	0.02499	0.09988	0.02498
6	0.09984	0.02498	0.09985	0.02498	0.09978	0.02497
9	0.09976	0.02495	0.09975	0.02496	0.09965	0.02494
12	0.09960	0.02493	0.09960	0.02493	0.09965	0.02492
18	0.09946	0.02492	0.09949	0.02491	0.09954	0.02491
24	0.09932	0.02490	0.09936	0.02488	0.09943	0.02490
30	0.09872	0.02268	0.09905	0.02267	0.09915	0.02262

Note: IHF – isohydrafural; FLAc – fluocinolone acetonide.

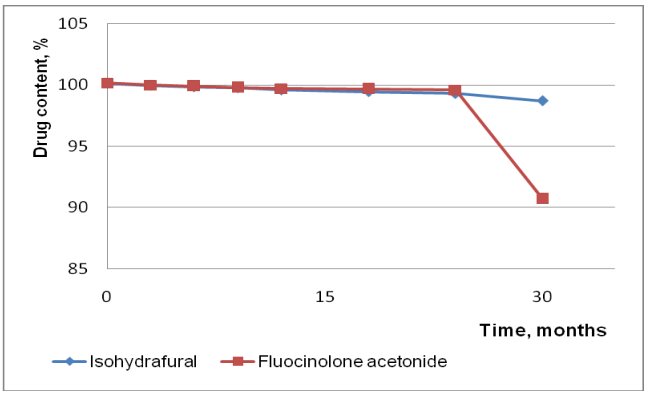

Fig. 3.1. Stability profile curves of batch 1 of the isohydrafural and fluocinolone acetonide combined ointment

Shelf life. The results of stability testing of the combined ointment containing isohydrafural and fluocinolone acetonide indicate a shelf life of 24 months at temperature 25±2°C and relative humidity 60±5%, time required for fluocinolone acetonide concentration to reduce up to 90%. During 24 months of real-time testing, the combined ointment presented no physical instability and underwent no significant changes of pH, viscosity, retention time and concentration of the active substances, the values being within the admissible limits (tab. 4).

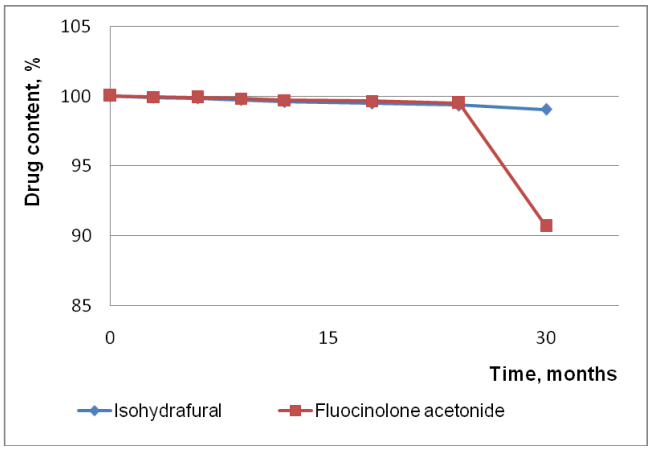

Fig. 3.2. Stability profile curves of batch 2 of the isohydrafural and fluocinolone acetonide combined ointment

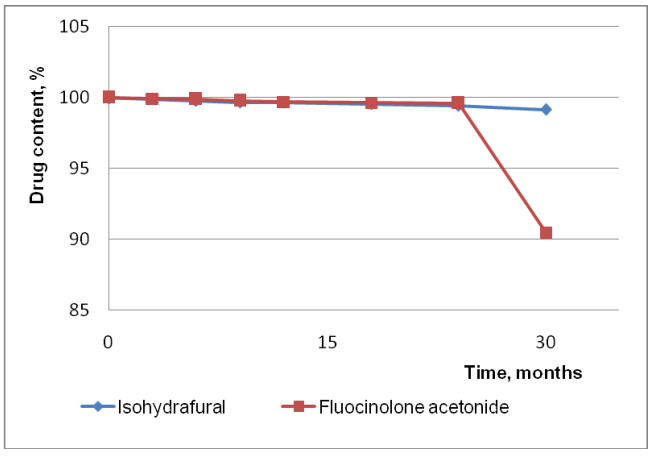

Fig. 3.3. Stability profile curves of batch 3 of the isohydrafural and fluocinolone acetonide combined ointment

Table 4

The result of real-time stability studies of the isohydrafural and fluocinolone acetonide combined ointment

Periodicity of testing, months	Analyzed parameters and admissibility conditions					
	Appearance and homogeneity smooth, yellow, odorless and homogeneous	pH (mean±standard deviation, 3 determinations) 4.0–6.0	Viscosity 65-75 cP	Identity and purity, HPLC Isohydrafural 2.995-3.115 Fluocinolone acetonide 5.994-5.998 minutes	Assay HPLC g/100 g ointment	
					Isohydrafural 0.09995-0.09930	Fluocinolone acetonide 0.02505-0.02485
Batch 1						
0	Corresponds	5.76±0.01	Corresponds	Corresponds	0.10013	0.02504
3	Corresponds	5.75±0.01	Corresponds	Corresponds	0.09996	0.02500
6	Corresponds	5.73±0.01	Corresponds	Corresponds	0.09984	0.02498
9	Corresponds	5.71±0.01	Corresponds	Corresponds	0.09976	0.02495
12	Corresponds	5.68±0.01	Corresponds	Corresponds	0.09960	0.02493
18	Corresponds	5.63±0.01	Corresponds	Corresponds	0.09946	0.02492
24	Corresponds	5.53±0.02	Corresponds	Corresponds	0.09932	0.02490
30	Does not correspond	5.37±0.01	Corresponds	Does not correspond	0.09872	0.02268
Batch 2						
0	Corresponds	5.77±0.02	Corresponds	Corresponds	0.10001	0.02501
3	Corresponds	5.75±0.01	Corresponds	Corresponds	0.09992	0.02499
6	Corresponds	5.72±0.01	Corresponds	Corresponds	0.09985	0.02498
9	Corresponds	5.71±0.01	Corresponds	Corresponds	0.09975	0.02496
12	Corresponds	5.67±0.01	Corresponds	Corresponds	0.09960	0.02493
18	Corresponds	5.63±0.01	Corresponds	Corresponds	0.09949	0.02491
24	Corresponds	5.53±0.01	Corresponds	Corresponds	0.09936	0.02488
30	Does not correspond	5.41±0.1	Corresponds	Does not correspond	0.09905	0.02267
Batch 3						
0	Corresponds	5.77±0.01	Corresponds	Corresponds	0.09996	0.02500
3	Corresponds	5.74±0.01	Corresponds	Corresponds	0.09988	0.02498
6	Corresponds	5.73±0.01	Corresponds	Corresponds	0.09978	0.02497
9	Corresponds	5.70±0.01	Corresponds	Corresponds	0.09965	0.02494
12	Corresponds	5.67±0.01	Corresponds	Corresponds	0.09965	0.02492
18	Corresponds	5.62±0.01	Corresponds	Corresponds	0.09954	0.02491
24	Corresponds	5.53±0.01	Corresponds	Corresponds	0.09943	0.02490
30	Does not correspond	5.29±0.01	Corresponds	Does not correspond	0.09915	0.02262

Conclusions

The results of stability testing of the combined ointment containing isohydrafural and fluocinolone acetonide, obtained by the real-time method, indicate that until 24 months the dosage form maintained its quality parameters, while at 30 months after storage, an intensification of the color, loss of homogeneity were observed and also inappropriate values of identity, purity and assay were recorded. The stability profile curves indicate that during the testing period isohydrafural is more stable in combined ointment than fluocinolone acetonide, which concentration reduced up to 90% at 30 months of storage.

The stability studies, performed by the real-time method, also allowed to determine the shelf life of the combined ointment at room temperature (25±2°C) and a relative humidity of 60±5%, which is 24 months. Therefore, it is recommended that the combined ointment containing isohydrafural and fluocinolone acetonide should be kept in cool place, protected from light in a dark container made from physically and chemically inert material that meets the pharmacopoeial standards.

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ED performed the technological part, interpreted the data, drafted the first manuscript, performed the analytical part of the laboratory work; VV interpreted the data, revised the manuscript; LU designed the study, conducted the laboratory work and revised the manuscript. All the authors revised and approved the final version of the manuscript.

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Identifying levels of professional communication language skills training

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Abstract

Background: The characteristic of the levels of professional communication skills is used by foreign medical students in the formation of the specialized language in Romanian. The professional communication is presented by the criteria and the levels of training of the professional communication skills.
Material and methods: The method of official documents analysis, scientific observation, case study, questionnaire method, tests provided with the examination and evaluation of the situation in the field of professional communication skills at foreign medical students were elaborated. Based on the specialized text, the low, medium or high levels of training of the professional communication skills of the specialized language were identified.
Results: The experiment was conducted at *Nicolae Testemitanu* State University of Medicine and Pharmacy, 72 second-year foreign medical students were involved, General Medicine study program. The levels of training of professional communication skills of the specialized language were identified through the integrated set of knowledge, skills and attitudes for each level. The production of oral / written messages and the ability to form sentences, texts in specialized language, in social and professional interactions, in order to perform service tasks were determined by the system of knowledge, skills and attitudes.
Conclusions: Based on the questionnaires and the compared values of the evaluation test, the training levels of the professional communication skills of the specialized language at the foreign medical students were identified.
Key words: specialized language, knowledge, skills, attitudes.

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Introduction

To have professional skills means to have a set of specific features and characteristics: to research and examine different professional situations, to relate a general principle to a particular case, to put into practice the specialized knowledge, to use specific skills, to collaborate with people in the group, to clarify an unpredictable problem or situation, to inform or to send some information etc.

Academic knowledge in a scientific educational field and knowledge of academic or empirical institutions in a professional field [1], an interdisciplinary approach, a medical education [2], clearly play an important role in the reception and comprehension of texts in a foreign language [3], relating to these fields [4], in the formation and analysis of medical terms.

All this would allow to obtain the competencies resulting from expressing a field according to a certain level; thus, we can identify the following characteristics of professional communication competence:

1. To communicate in the specialized language;
2. To put knowledge into practice through professional treatment of the field;
3. To present special features and characteristics based on a learned subject;
4. To render and explain by appropriate means the content of a communication, sources, notes, indications, etc .;
5. To inform problems, solutions, information, etc. both teammates and the patients he will treat;
6. To develop learning skills in order to continue their studies in the following cycles (secondary, residency, doctorate, etc.).

According to Sorin Cristea [5] "The superior pedagogical stake consists in creating favorable premises for the development of communication skills in a foreign language at the level of plurilingual and pluricultural competence [6] necessary in the perspective of present and future evolutions of the postmodern, informational, knowledge-based society."

The components of the professional communication competence express languages and terminology [7], knowledge, research, examination of professional situations, specific delimitation of professional activity, importance of the Romanian medical lexicon in the terminological content [8-10] etc. In order to identify the level of training of the professional communication competence, in our case of the specialized language in Romanian for foreign medical students; the experiment allowed us to assess the level of communication at the socio-cultural and linguistic level [11]

of foreign students according to the Common European Framework of Reference for Languages; we identified the needs and priorities for the formation of the specialized language in Romanian for foreign students; we have developed criteria for evaluating the level of training in professional communication skills, etc.

Material and methods

In order to establish the level of linguistic communication in Romanian for foreign medical students, we used applied scientific methods such as: test, questionnaire, opinion poll, etc.

Scientific documentation is manifested by researching bibliographic sources and gathering information in the field, examining specialized sites; recording and summarizing some fundamental principles, theses; research, essentialization, confrontation and verification of sources, information, data; systematic review of the examined content.

The biographical method (anamnesis) was put into practice in the pre-experimental stage, in order to know the level of possession of the Romanian language, the significance of knowing the Romanian language, communication within the institution, with colleagues, in society, clinics, hospitals, etc. before starting to study specialized language.

The scientific observation manifested itself as evidence of reflection, description and analysis of the researched field. In the experiment we capitalized on findings at the pre-experimental stage, which allowed us to classify, complete, rectify, modify some actions at the finding stage; the experimental evaluation was performed at the training stage; by comparing the results we managed to carry out the control experiment, which contributed to the systematization of the research results and the formulation of conclusions and recommendations.

The interview method allowed us to obtain information from the foreign students investigated to identify and collect data, visions to the research problem. Through individual and group interviews, an inventory was drawn up containing a set of established problems / difficulties that foreign medical students encounter in the formation of professional language; elaboration, correction, completion and development of research tools and strategies.

The test method (individual, group test) allowed us to evaluate and establish the level of linguistic communication skills in Romanian of foreign medical students.

The questionnaire method was put into practice in order to test the opinions, interpretations and convictions of foreign medical students regarding the formation of the specialized language in Romanian. Oral (direct) and written (indirect) questioning in combination with individual and group discussion allowed us to identify the motivation, levels of knowledge and training of specialized language for foreign medical students.

Data processing or statistical method consisted of testing and validating the hypothesis and the representative sample; the longitudinal analysis followed the evolution of the experimental group; data collection and processing, analysis, presentation in the form of tables, diagrams, graphs, formulation of conclusions gave precision and rigor to the researched topic.

The graphical representation of the statistical data included the combination of two basic methods: numerical and graphical. The numerical method allowed us to calculate the mean deviation variability of the researched situation. The graphical method visually identified the calculated data: the histogram graph, the frequency polygon, the bar graph, the PIE graph were useful tools in the concrete exemplification by providing valuable information.

The experiment was performed at *Nicolae Testemitanu* State University of Medicine and Pharmacy, Department of Romanian Language and Medical Terminology. 72 second-year medical students, study program General Medicine (35 students – experimental group, 37 students – control group) were involved in the experiment. The experimental research was carried out at several stages during three academic semesters: the second semester, the academic year 2015-2016 and the first and second semesters of the academic year 2016-2017: the pre-experimental experiment, the finding experiment, the training experiment, the control experiment. Foreign students were involved in the experiment, individual and group studies were combined; according to the conditions of realization it is a natural experiment; according to the mode of intervention it is a provoked and invoked experiment; according to the treated issue it is a pedagogical experiment; according to the number of variables it is a multivariate one; according to the research level it is a transversal and longitudinal type; according to the spent time, it is of average duration, it took place over a period of a year and a half (17 months, three semesters).

Results and dicussion

To identify the level of training of professional communication skills in medical students by comparing the results recorded by the two groups in the experiment: the experimental group and the control group, there were elaborated:

- The initial questionnaire to identify the level of professional communication competence;
- The test for assessing the level of linguistic communication competence in Romanian B2-C1;
- The questionnaire for identifying the communication needs of foreign students in the specialized language;
- The grid with the indicators for evaluating the level of professional communication competence of medical students;
- Criteria for assessing the level of training of professional communication skills of medical students.

The test for assessing the level of linguistic communication competence A2 and the test for assessing the level of linguistic communication competence in Romanian B2-C1, was aimed to establish the level of linguistic competence in communication for foreign medical students. The evaluation test was elaborated based on the paper Evaluation tests in Romanian [12].

The test on determining the level of linguistic communication competence comprises 5 components: Listening comprehension (20 points), Comprehension of a written text (20 points), Grammar and vocabulary (20 points), Creative text production (20 points), Oral examination (20 points).

The evaluation process showed the profile of the experimental and control samples, which can be characterized according to several criteria. Based on the accumulated score, they were identified in the following grades: unsatisfactory, satisfactory, good, very good and excellent.

In this regard, we established the level of linguistic communication competence in Romanian for foreign medical students, developed on the basis of the distribution tables.

The research on the level of identification was expressed based on knowledge, skills and attitudes [13-18].

Characteristics of the levels of training of professional communication skills of the specialized language in Romanian at foreign medical students

Low level Knowledge

The foreign medical students possess minimal knowledge, do not possess / do not know about decoding a sent message and understanding the content of a specialized text proposed for audition; identification or discovery according to certain particularities of the characteristic features of the audited text; identification of grammatical categories in written texts; vocabulary, knowledge of new terms, and their delimitation from different medical contexts; production of oral messages in specialized language, in social and professional interactions, in order to perform service tasks. They have insufficient information about the studied subject and correct usage of the specialized vocabulary; production of specialized written messages, in order to perform professional activities.

Intermediate level Knowledge

The foreign medical students have partial, incomplete knowledge of decoding a sent message and understanding the content of a specialized text proposed for audition; identification or discovery according to certain particularities of the characteristic features of the audited text; identification of grammatical categories in written texts; vocabulary, knowledge of new terms, and their delimitation from different medical contexts; production of oral messages in specialized language, in social and professional interactions, in order to perform service tasks. They have insufficient information about the studied subject and correct usage of the specialized vocabulary; production of specialized written messages, in order to carry out professional activities; knowledge of the usual expressions frequently encountered on topics that have professional relevance or about professional activity, as they do not know enough about the field; intentionally simulating to reproduce a situation model from medical practice; participation in discussions in medical practice; production of oral messages in specialized language, in social and professional interactions, in order to perform service tasks; production of specialized written messages, in order to carry out professional activities.

High level Knowledge

The foreign medical students have extensive, deep, complete, consistent knowledge about decoding a sent message and understanding the content of a specialized text proposed for audition; identification or discovery according to certain particularities of the characteristic features of the audited text; identification, of grammatical categories in written texts; vocabulary knowledge of new terms, and their delimitation from different medical contexts; production of oral messages in specialized language, in social and professional interactions, in order to perform service tasks; production of specialized written messages, in order to perform professional activities; knowledge of the usual expressions frequently encountered on topics that have professional relevance, or about professional activity, but do not know enough about the field; intentionally simulating to reproduce a situation model from medical practice. They are ready for the participation in discussions in medical practice; production of oral messages in specialized language, in social and professional interactions, in order to perform service tasks; production of specialized written messages, in order to perform professional activities; distinguishing and admitting the particular point of view of the one who produces a specialized audio text; lack of mistakes / quality of writing correctly in medical thematic contexts; presentation of a real fact, a communication situation from medical practice; they show through arguments an integral and detailed skill of the subject and elaborate texts.

Low level Capacities

The foreign medical students possess minimum capacities, do not have capacities in reading various medical texts on professional topics; participation in discussions of personal and professional interest; identifying the general and particular meaning of the specialized text; assimilation of textual units depending on communication; learning to form specialized statements using medical terms; the use of medical terms with the knowledge of the specialized lexicon; initiating and holding a specialized conversation; understanding and producing specialized written texts; elaboration and explanation of professional communication situations.

Medium level Capacities

The foreign medical students have partial, incomplete abilities in reading various texts on professional topics; participation in discussions of personal and professional interest; identifying the general and particular meaning of the specialized text; assimilation of textual units depending on communication cues; learning to form specialized statements using medical terms; the use of medical terms with the knowledge of the specialized lexicon; decoding medical terms from specialized texts; initiating and holding a specialized conversation; understanding and producing specialized written texts; elaboration and explanation of professional communication situations; formulating questions in order to find an answer; examination and wide exposure

of the information presented in the specialized text; producing, writing and reacting in writing to information transmitted orally, etc.; capturing and recording information from the audited text by performing the exercises.

High level Capacities

The foreign medical students possess large, deep capacities, which express, complete, consistent proficiency in reading various texts on professional topics; participation in discussions of personal and professional interest; identifying the general and particular meaning of the specialized text; assimilation of textual units depending on communication cues; learning to form specialized statements using medical terms; the use of medical terms with the knowledge of the specialized lexicon; decoding medical terms from specialized texts; initiating and holding a specialized conversation; understanding and producing specialized written texts; elaboration and explanation of professional communication situations; formulating questions in order to find an answer; examination and wide exposure to the information presented in the specialized text; producing, writing and reacting in writing to information transmitted orally, etc.; capturing and recording information from the text heard by performing the exercises; arguing one's own opinion in relation to writing a specialized text; associating the specialized text with a case, a socio-professional situation; explaination and comment on the issuer's opinion; selecting information from the specialized text to create and communicate new ideas.

Low level Attitudes

The foreign medical students do not express, do not apply, do not understand, do not present arguments in proposing solutions to a particular problem with a professional context; critical attitude and arguments in order to convince; decisions in solving a health problem; curiosity for understanding messages heard on specialized topics; willingness to communicate using specialized language in professional contexts.

Intermediate level Attitudes

The foreign medical students express partially or incompletely on the basis of arguments their interest in proposing solutions to a particular problem with a professional context; critical attitude and arguments in order to convince; decisions in solving a health problem; curiosity for understanding messages heard on specialized topics; willingness to communicate using specialized language in professional contexts; interest in conversations about problems, health recommendations.

High level Attitudes

The foreign medical students demonstrate on the basis of arguments and prove interest in proposing solutions to a particular problem with a professional context; critical attitude and present arguments in order to convince; decisions in solving a health problem; curiosity for understanding audited messages on specialized topics; willingness to communicate using specialized language in professional contexts; interest in conversations about problems, health recommendations; willingness to examine several opinions on a medical case, deliberations; conversations between doctor and patient, etc.; collaboration in professional contexts with specialists in the medical field.

Conclusions

The competence in the educational, instructional process becomes a component part of the educated one and belongs to a category with an elaborated educational capacity, has a triadic structure, is externalized in different levels of development, depending on the age and orientation in a certain field. However, the competence with the greatest influence on the future professional activity, even the didactic one, of the students studying this discipline, remains the transfer of the competences of learning foreign languages to the study of the specialized disciplines. Based on the evaluation tests of the investigative-experimental approach to the formation of students' medical language, we developed the study on the levels of the formation of professional communication skills in the specialized Romanian language for foreign medical students.

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Authors' contribution
AB established well-defined models; VV formatted specialized language of foreign medical students as effective benchmarks in substantiating and learning the medical language by foreign medical students. Both authors revised and approved the final version of the manuscript.

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Determination of carotenoids in extracts from species of *Tagetes* and *Calendula*

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Abstract

Background: Carotenoids have played a significant role in reducing the risk of chronic diseases. The most studied in this regard is β-carotene, present in species of *Tagetes* and *Calendula* genus. Objective of the study: Comparative analysis of β-carotene content in liquid and dry flowers extracts of *Tagetes* and *Calendula* species, cultivated in the collection of the Scientific Center for the Cultivation of Medicinal Plants of *Nicolae Testemitanu* SUMPh.
Material and methods: Dry extracts of flowers harvested in the budding-flowering phase, were obtained by repeated maceration and rotary evaporation, subjected to phytochemical evaluation by thin-layer chromatography (TLC) and UV-VIS spectrophotometry, equivalent to β-carotene.
Results: Beta-carotene was identified by TLC in hexan-ethyl acetate (50:50, v/v), retention factors were established. Caratenoid content (mg%) varied as follows: in *T. patula* L. (75.34±2.15), *T. erecta* L. (21.97±0.84), *C. officinalis* L. variety Natali (13.09±3.23), *C. officinalis* L. variety Diana (12.39±1.98), *C. officinalis* L. local population (10.99±0.06). The carotenoids content ranged in the dry extracts as well, determined in the highest amount in *T. patula* L. flowers (137.87±2.18 mg%).
Conclusions: This study demonstrated the opportunity for further research of *Tagetes* and *Calendula* varieties that could serve as sources of carotenoids for obtaining antioxidant phyto-pharmaceuticals.
Key words: carotenoids, vegetal products, dry extracts, spectrophotometry.

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Introduction

Carotenoids are natural pigments responsible for many of the red, orange and yellow hues of plant leaves, fruits and flowers, as well as the colors of some birds, insects, fish and crustaceans [1]. Carotenoids are synthesized in plants but not in animals. They are localized in subcellular organelles (plastids), *i.e.* chloroplasts and chromoplasts. In chloroplasts, the carotenoids are chiefly associated with proteins and serve as accessory pigments in photosynthesis, whereas in chromoplasts they are deposited in crystalline form or as oily droplets [2]. Carotenoids play crucial roles in photosynthesis, photoprotection, development, as stress hormones and signaling molecules in plants. In addition, these colors serve to attract pollinating and seed dispersal agents. More than 600 carotenoids have been identified so far in nature. About 40 carotenoids are present in the typical human diet and only 20 of them have been found in human blood and tissues, close to 90% of the carotenoids in the diet and human body are represented by β -carotene, α-carotene, lycopene, lutein and zeaxanthin [3].

Several carotenoids act as precursors of vitamin A, which is an efficient antioxidant and is important for human nutrition. The provitamin A carotenoid, β-carotene, is a significant source of vitamin A [4]. In human body, β-carotene is broken down by β-carotene dioxygenase in the mucosa of small intestine into two retinyl molecules, which are later reduced to vitamin A (retinol). Beta-carotene is a colored red-orange pigment and widely found in plants and fruits, especially in orange fruits such as cantaloupe, mangoes, pumpkins and papayas, and orange root vegetables such as carrots and sweet potatoes [5].

Lycopene is an unsaturated acyclic carotenoid with open straight chain hydrocarbon consisting of 11 conjugated and two unconjugated double bonds. Lycopene has no provitamin A activity due to the lack of terminal β-ionic ring as the basic structure for vitamin A. The red color of lycopene is mainly due to many conjugated carbon double bonds, as it absorbs more visible spectrum compared to other carotenes [2]. Over the last decade, there has been increased recognition that lycopene plays an important role in preventing the development of coronary disease and retarding the progression of atherosclerosis. The antioxidant activity of lycopene is almost twice as high as that of β-carotene and has the greatest synergism with vitamin E. Aside from the popular

tomato, other sources of lycopene include red grapefruit, watermelon and apricots [5].

Lutein and zeaxanthin belong to the class of carotenoids called xanthophylls, they are the major constituents of macular pigment, a compound concentrated in the macula region of the retina that is responsible for fine-feature vision. Given their accumulation in the retina, has been investigated how consumption of these carotenoids may prevent and/or slow the progression of age-related macular degeneration, the leading cause of blindness in older adults [6].

Nowadays, many of ongoing research has focused on the identification of foremost sources of carotenoids for the use in ophthalmology for the treatment of age-related ocular diseases**.** Genus *Tagetes* (Asteraceae) is considered an important source of carotenoid pigments, especially of the yellow carotenoids (α-, β-carotenes) and xanthophylls (lutein, zeaxanthin, violaxanthin) [7]. Genus *Tagetes* contain about 50 species of annual or perennial herbaceous plant, native to Central and South America and naturalised elsewhere in the tropics and subtropics [8]. Some species such as *Tagetes erecta* L., *Tagetes patula* L. and *Tagetes tenuifolia* Cav., are cultivated as ornamental plants, while *Tagetes minuta* L. has become a noxious plant [9]. *T. erecta* L. has been used as coloring agent and nutritional supplement in a wide range of foods and beverages in levels ranging from 2 to 330 mg/kg for lutein and 0.5 to 70 mg/kg for zeaxanthin [10].

Genus *Calendula* (Asteraceae), native to the Mediterranean Basin, includes approximately 25 herbaceous annual or perennial species, most common being *Calendula officinalis* L., *Calendula arvensis* L., *Calendula suffruticosa* Vahl., *Calendula stellata* Cav., *Calendula alata* Rech. and *Calendula tripterocarpa* Rupr. [11]. Among the various species of the genus *Calendula*, *C. officinalis* L. is the only one which is extensively used clinically throughout the world. The inflorescence of *C. officinalis* L. has abundant amount of carotenoids that give flowers their yellow-orange color and the color shade depends on pigment content and pigment profile. Its yellow flower petals contain 19 carotenoids and orange flower contains 10 unique carotenoids. The main carotenoids present in the petals and pollens are flavoxanthin, luteoxanthin, auroxanthin, 9Z-antheraxanthin, neoxanthin, lutein and its Z-isomers, mutatoxanthin, violaxanthin, 9Z-neoxanthin, 9Z-violaxanthin, α- and β-carotene, and α- and β-cryptoxanthin with higher quantity of lycopene in petals [12]. *C. officinalis* L. is considered to offer protection against some cancers, UV-induced skin damage, coronary heart disease, cataracts and molecular degeneration [13].

The aim of the present study is to investigate and compare the β-carotene content in liquid and dry flowers extracts of *Tagetes* and *Calendula* species, cultivated in the collection of the Scientific Center for the Cultivation of Medicinal Plants of *Nicolae Testemitanu* State University of Medicine and Pharmacy (SUMPh) by thin-layer chromatography (TLC) and UV-VIS spectrophotometry.

Material and methods

Plant material. Flowers of the species *Tagetes patula* L., *Tagetes erecta* L., *Calendula officinalis* L. and the varieties of *Calendula officinalis* L. Diana and Natali were collected, in the complete flowering phase, from the collection of the Scientific Center for Cultivation of Medicinal Plants of Nicolae Testemitanu SUMPh. The vegetal products were dried in natural conditions in the shade, in a well-ventilated place. The Natali and Diana varieties of *C. officinalis* L. were obtained by scientists from the Institute of Genetics, Physiology and Plant Protection.

Extraction procedure. 5.0 g of crushed vegetal product was placed in a 100 ml flask, added 70 ml of hexane and heated in a water bath at 60°C for 5 minutes. The extracts were filtered into a 100 ml flask. The extraction was carried out twice with hexane for 30 ml for 5 minutes in a water bath at a temperature of 50-60°C. The extracts after cooling were filtered and their volume was made up to 100ml with hexane.

Thin layer chromatography (TLC) assay. The identification of β-carotene in the studied vegetal products was performed by thin layer chromatography on chromatographic plates "Sorbphil" (Krasnodar). For the chromatographic separation of β-carotene three mobile phases: hexane-ethyl acetate (50:50 v/v), hexane-ethyl acetate (80:20 v/v), hexane-ethyl acetate-propanol-2 (75:18:7 v/v/v) were used. As a control served β-carotene (Sigma-Aldrich). The analyzed solutions were obtained by mixing 0.5 g of vegetal product (*flores*) with 15 ml of hexane. The extractive solutions and the solution of the reference substance (β-carotene) were applied on the start line of the chromatographic plates [14].

Determination of total carotenoid content. The determination of the total carotenoids in flowers of *Tagetes* and *Calendula* species was performed spectrophotometrically on a Metertech UV/VIS SP 8001 spectrophotometer. 10 ml of the obtained solution was passed into a 25 ml volumetric flask, brought to the level with hexane. The optical density of the solution was determined at λ= 450 nm in a 10 mm thick cuvette. Reference solution – hexane. The carotenoid content (mg%) in the β-carotene equivalent was calculated according to the formula:

$$X = \frac{A * 0.00208 * 100 * 100 * 25 * 100}{A_0 * a * 10 * (100 - W)}$$

$$X = \frac{A * 0.00208 * 100 * 100 * 25 * 100}{A_0 * a * 10 * (100 - W)}$$, where:

A – the absorbance of the analyzed solution; A_0 – the absorbance of the standard solution; m – the mass of vegetal product (g); W – the weight loss on drying (%); 0.00208 – the amount of β-carotene, which corresponds to 1 ml of standard potassium dichromate solution (mg).

Preparation of the potassium dichromate solution: 0.0900 g of K_2CrO4 pass into a 250 ml volumetric flask, dissolve in water, make up to the mark with the same solvent. The solution obtained by color corresponds to 0.00208 mg of β-carotene in 1 mg.

Obtaining of dry extracts. The dry extracts were ob-

tained by the fractional maceration method with stirring from 5 g of dry vegetal products, treated 4 times with 100 ml of hexane, with an extraction cycle of 30 min. The extractive solutions of 4 fractions were combined and kept cold for 24 hours to sediment the resins, then filtered through the Buchner funnel. The concentration of the extractive solutions was performed on the rotating system Laborota 4011 – digital at 60°C.

Sample preparation for spectrophotometric analysis. 0.05 g of dry extract was placed in a 50 ml flask and diluted with 30 ml of hexane, then the solution was made up to the level with the same solvent. The optical density was determined at λ= 450 nm, in a 10 mm thick tank. The carotenoid content (mg%) in the dry extracts was calculated according to the formula [15]:

$$X = \frac{A*V*50*100*1000}{2592*m*(100-W)} \quad X = \frac{A*V*50*100*1000}{2592*m*(100-W)}$$, where:

A – the absorbance of the analyzed solution; V – the volume (ml) ; m – the mass of dry extract (g); W – weight loss on drying (%); 2592 – the absorbance of β-carotene at λ= 450 nm.

Statistical analysis. The average of multiple measurements (triplicates) are listed and expressed with the standard deviations. Statistical analysis was performed using Excel 2017 software package.

Results and discussion

The TLC assay revealed the presence of β-carotene under the described chromatographic conditions through the determination of retention factors. The results of the qualitative study of the analyzed vegetal products are presented in *table 1*. Following the analysis of visible light chromatograms, were observed in all studied products yellow spots, where the retention factors (Rf) corresponded to the Rf of the reference substance β-carotene. It was shown that the clearest separation of β-carotene in hexane solutions from *Tagetes* and *Calendula* flowers occurred in the mobile phase hexane:ethyl acetate (50:50 v/v). The migration of the chromatographic systems was 10 cm.

Total carotenoid content of the extraction samples, obtained from the under consideration vegetal products was evaluated spectrophotometrically. The highest level of carotenoid content was identified in *T. patula* L. flowers (75.34±2.15 mg%) and with a slighter quantity in *T. erecta* L. (21.97±0.84 mg%). Scientific studies show that the carotenoid content differs a lot, depending on the geographical area, climatic conditions, as well as species, genetics and variety. In some studies, done by Akshaya et al. (2017) *T. patula* L. has the highest content of carotenoids compared to yellow colored flowers of *T. erecta* L., but lesser compared to those of dark orange hue. Among the *Tagetes* genotypes studied, the total carotenoid content ranges from 19.61 mg/100g fresh weight to 525.68 mg/100g fresh weight of petals. Maliugina et al. (2013) determined, that the most contents of biologically active carotenoids exist in the inflorescences of the low-growing cultivars of the genus *Tagetes*, such as Gold kopfen (159.25±15.93 mg%), Orange flamme (56.0±15.61 mg%), Carmen (144.4±14,5 mg%) and Fiesta (143.5±14.33 mg%).

The study of total carotenoid content for the *Calendula* species, revealed a top content in *C. officinalis* L. variety Natali (13.09±3.23 mg%), followed by *C. officinalis* L. variety Diana (12.39±1.98 mg%) and *C. officinalis* L. (10.99±0.06 mg%). In some studies Toiu et al. (2016) observed high variations in carotenoid concentrations in some analyzed varieties from Romania: *Calendulae flores* contains 0.99-1.32 mg carotenoids/g dried flowers and *Tagetes flores* contains 0.52-3.72 mg/g.

It is important to note that flowers from *Tagetes* species, grown in the Republic of Moldova have been determined to have the higher concentration of carotenoids, compared to varieties of *Calendula*. Among the varieties of *Calendula* the maximum concentration was demonstrated in flowers of *C. officinalis* L. variety Natali.

Since carotenoids are widely utilized in pharmaceutical industry as dry extracts we also aimed to obtain and evaluate the total carotenoid content in the dry extracts. The highest content (fig. 1), was identified in the extract obtained from flowers of *T. patula* L. (137.87±2.18 mg%), followed by *T. erecta* L. (57.88±7.14 mg%). In the extracts obtained from marigold flowers, the caratenoid content varies as follows: *C. officinalis* L. variety Natali – 39.98±0.93 mg%, *C. officinalis* L. variety Diana – 34.05±5.34 mg%, *C. officinalis* L. – 27.38±2.02 mg%. Furthermore, the results show that the total carotenoids content differs statistically significant both for liquid extracts obtained from the flowers of the marigold varieties and for dry extracts as well ($p<0.05$).

Table 1

Values of Rf of β-carotene spots separated by TLC in the extractive solutions from *Tagetes* sp. and *C. officinalis* L. varieties

No	Extractive solutions obtained from vegetal product – flores / reference substance	Mobile phases/Rf of β-carotene		
		Hexane-ethyl acetate (50:50)	Hexane-ethyl acetate (80:20)	Hexane-ethyl acetate propanol-2 (75:18:7)
1	*T. patula* L.	0.90	0.77	0.89
2	*T. erecta* L.	0.90	0.76	0.89
3	*C. officinalis* L.	0.90	0.76	0.87
4	*C. officinalis* L. variety Diana	0.89	0.77	0.86
5	*C. officinalis* L. variety Natali	0.90	0.77	0.87
9	β-carotene – reference substance	0.90	0.75	0.87

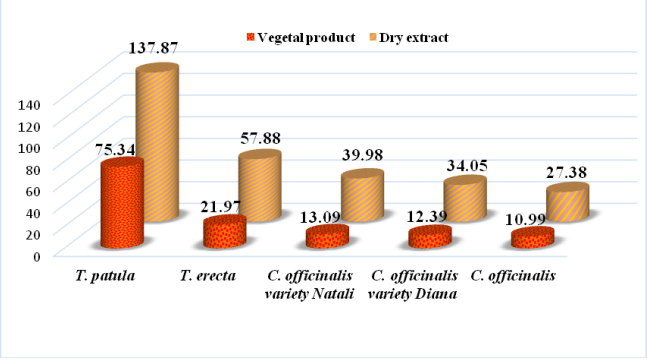

Fig. 1. The total carotenoid content, equivalent to β-carotene, in vegetal products (*flores*) and in dry extracts

The species of *Tagetes* and *Calendula* are cultivated as vegetable products with high carotenoids content. Concomitant with the pharmaceutical industry, where carotenoids are used as compounds with anti-inflammatory and antioxidant properties, they are processed in cosmetics and food production as natural dyes and preservatives. *Calendula officinalis* L. is listed in German Commission E, European Scientific Cooperative on Phytotherapy, British Herbal Pharmacopoeia, World Health Organization monographs for wound healing and anti-inflammatory actions [11], whereas species of the genus *Tagetes* are not found in the reference Pharmacopoeia, being used in folk medicine and often as ornamental plants. Different parts of these plants including flowers are used traditionally to cure various diseases, as the leaves are reported to be effective against piles, kidney troubles, muscular pain, ulcers, wounds and earaches [8]. The results of the present study showed, that carotenoid content, especially of β-carotene, from *Tagetes* genus is a source that needs further validation for correlation to biological activity and elaboration of normative documentation.

Conclusions

In this work, during the phytochemical evaluation, we have determined that the richest in carotenoids are the dark orange inflorescences of the species *T. patula* L., followed by *C. officinalis* L. varieties Natali and Diana, which recommends them to be grown for medicinal use.

The results indicate that the flowers of *Tagetes* species and *C. officinalis* L. varieties, cultivated in the Republic of Moldova, can be used as vegetal products with high carotenoid content in the pharmaceutical, cosmetic and food industries.

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Authors' contributions: AB designed the study, performed the laboratory work and drafted the first manuscript; CC interpreted the data, revised the manuscript; MC-T revised the manuscript; NC conducted the laboratory work, revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Macrophages and dendritic cells density correlates with depth of invasion in the prostate carcinoma

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Abstract

Background: Immune cells interact not only with tumor cells but also with stromal cells facilitating the progression of neoplasia. The ongoing battle between immune cells and the tumor is an important factor influencing the clinical course and outcome of treatment in various types of cancer. The aim of the study was to identify the prognostic value of dendritic cells and macrophages in prostate carcinoma.
Material and methods: This retrospective study analyzed 73 samples of prostate cancer. The macrophages and dendritic cells have been evaluated using the immunohistochemical methods with CD68 (macrophages) and S100 (dendritic cells). Macrophages were quantified as intratumoral and peritumoral, and dendritic cells – intraepithelial and stromal. The results were analyzed statistically.
Results: For evaluation of the prognostic impact of immune cells was accomplished a correlation between the total number of CD68+/S100+ cells and the Gleason score. Thus, statistically significant correlations were obtained both for CD68+ cells (intratumoral p=0.008, peritumoral p=0.001), and for S100+ cells (intraepithelial p=0.036, stromal p=0.042). In addition, a statistically significant positive linear correlation was observed between the density of intraepithelial S100+ cells and intratumoral CD68+ cells (p=0.018).
Conclusions: The increase in the density of S100+ and CD68+ cells, as well as the significant association of their density with the histological degree of the tumor allow us to consider these cells as predictive biomarkers in prostate carcinomas.
Key words: prostate cancer, dendritic cells.

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Introduction

Frequently, the prostate benign and malignant proliferations are accompanied by inflammatory processes. The link between inflammation and cancer, studied very intensely in the last years, was identified 150 years ago, when in 1863, Virchow, noticed that cancer tends to occur in sites of chronic inflammation. While recently, increased the number of researches which demonstrate that acute inflammation contributes to cancer regression [1], there are multiple epidemiological studies claiming that chronic inflammatory diseases are frequently associated with an increased risk of cancer [1-3]. Inflammatory foci are dominated by multiple immune cells, such as: macrophages, lymphocytes, plasma cells, mast cells, etc.

From this wide range of cells, macrophages represent an important component of the tumor inflammatory infiltrate [2, 4]. Macrophages, as well as other inflammatory cells generate a large amount of growth factors, cytokines and chemokines that can cause irreversible DNA damage. Moreover, the macrophages can act as pro-inflammatory or anti-inflammatory cells, depending on the type of stimulus received from the neighboring microenvironment. In general, two major macrophage phenotypes are identified: M1 and M2. The M1 macrophages, or classically activated macrophages, are aggressive cells, intensively involved in phagocytosis, and promotion of Th1 responses. Also, they are important inflammatory cytokine secreting cells, such as IL-12, IL-18 and IL-23. In tumors, classically activated macrophages play an important role in the recognition and destruction of cancer cells, and their presence in the tumor mass is usually related to a favorable disease prognosis. M2 macrophages or alternatively activated macrophages are anti-inflammatory cells, actively involved in the angiogenesis and tissue regeneration. They secrete increased amounts of IL-10 and other anti-inflammatory cytokines [5]. Depending on secreted molecules, are identified several subsets of M2 macrophages.

These are: 1) M2a macrophages – called profibrotic, which secrete IL-4 and IL-13; 2) M2b macrophages – contain IC or TLR/IL1-R ligands and are involved in the regulation of immunity and, often, are called regulatory cells; 3) M2c macrophages – secrete IL-10 and TGF-β, and sometimes are described as inactivated cells, which are involved in suppressing immunity, tissue regeneration and matrix re-

modeling; 4) M2d macrophages or tumor-associated macrophages (TAM) – increase tumor progression and growth by promoting the process of neo-angiogenesis [5].

The tumor microenvironment, significantly affects the polarization of macrophages. Growth factors, such as CSF-1 (colony stimulating factor 1) and VEGF (vascular epithelial growth factor), MCP-1 (chemotactic protein-1 monocyte), as well as several chemokines CCL can induce monocyte chemotaxis in the tumor microenvironment [6]. Neutralization of MCP-1 completely blocks the accumulation of macrophages in the tumors [7]. VEGF, in addition to its powerful angiogenic role, recruits monocytes into the tumor microenvironment. VEGF blockade leads not only to the reduction of vascular density, but also to the reduction of macrophage infiltrate [8]. Multiple studies have shown that the invading property of tumor cells depends largely on macrophages. The invasion of tumor cells, mainly, is accompanied by directed migration of macrophages [9].

Dendritic cells (DC) represent the most efficient antigen-presenting cells, which have the function of tissue sentinel. They belong to monocyte-macrophage cell lines that strongly express the protein S100 [10]. As immature cells, they take up the antigens from peripheral tissues, process them, and then expose antigen molecules to the membrane surface as molecules of the class I and II histocompatibility complex [11, 12]. During antigen processing, the dendritic cell undergoes the maturation process, which determines its migration into the secondary lymphoid organs, where it becomes a competent cell in presenting of antigen to T lymphocyte. Thus, dendritic cells initiate the special antigen-specific immune response [13]. Another functional characteristic of dendritic cells is their effective ability to increase the immunomodulatory and cytotoxic potential of NK cells, which essentially contribute to the removal of tumor cells [14-16]. Furthermore, DCs can also directly mediate tumor-targeted cytotoxicity [17].

The progression of prostate cancer is accompanied by a marked suppression of local immunity, which includes the apoptotic death of dendritic cells [18]. Tumor cells can significantly inhibit the monocyte conversion into the dendritic cells. It is also recognized that prostate cancer not only destroys mature dendritic cells, but also inhibits their genesis and maturation, leading to decreased production of antigen-presenting cells and inhibition of their functional activity [18-20]. The rapid-growing tumors are usually poorly infiltrated by DC and unable to trigger DC recruitment and activation that result in delayed or insufficient antitumor immune responses. However, the mechanisms regulating migration and homeostasis within the tumor are not well understood.

In this study, we examined the cell population composition of CD68+ macrophages and S100+ dendritic cells. We also examined the prognostic value of these cells and their correlation with the proven parameter of the biological aggressiveness of prostate cancer: degree of tumor differentiation. The obtained results could lead to improved therapeutic modalities in patients with prostate cancer.

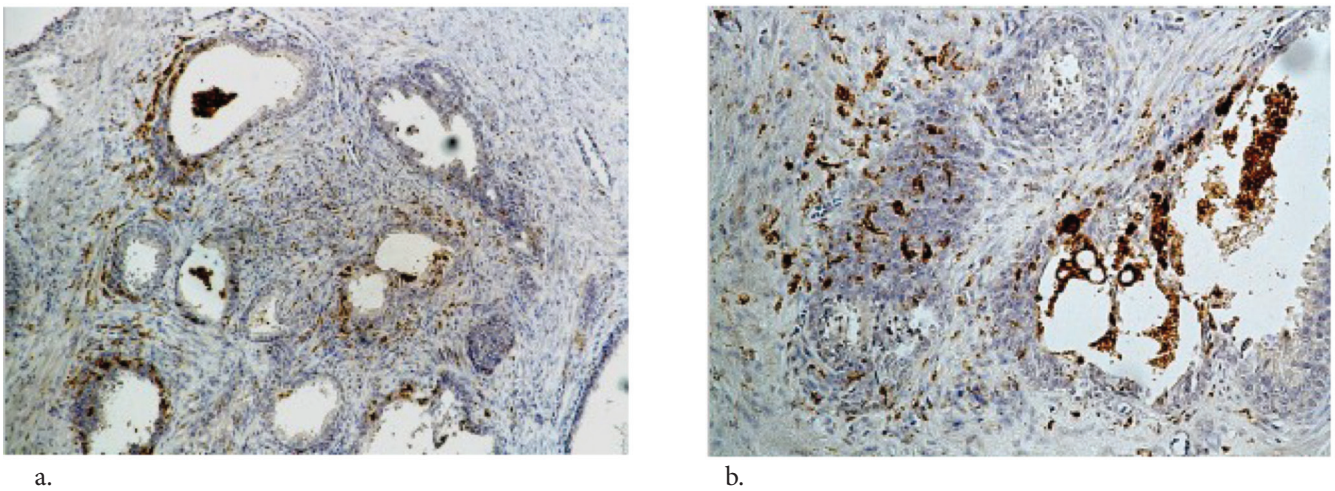
Material and methods

The study included 73 cases of prostate carcinoma. Histological grading of prostate carcinoma is an important step in defining the diagnosis and prognosis. Thus, the prostate cancer specimens were divided into 2 groups: acinar and non-acinar carcinomas. For the histological differentiation of acinar adenocarcinomas, the Gleason score was used. The adenocarcinoma specimens were grouped in: *well-differentiated (Gleason score 2-5), medium-differentiated (Gleason score 6-7) and poorly-differentiated (Gleason score 8- 10)*. Non-acınar carcinomas were considered poorly differentiated cancers. Due to early lysis of the study material, the histological pieces were rapidly harvested. The biopsy fragments, after fixation in 10% buffered formalin, were primarily processed following the standard procedures. Sections 5 µm thick were sliced off each block, which were mounted on histological and silanized slides. Histopathological profiling was performed on hematoxylin-eosin stained sections.

The immunohistochemical study included 2 monoclonal antibodies: anti-CD68 *(clone 514H12, predilute, Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK)* and anti-S100 *(polyclonal, predilute, Leica Biosystem Newcastle Ltd, Newcastle Upon Tyne, UK)*. The application of primary antibodies was preceded by the exposure to Bond Epitope Retrieval Solution 2 (20 minutes) for anti-CD68 and Bond Enzyme 1 (10 minutes) for anti-S100. Incubation with primary antibodies was 20 minutes, compatible working system was Bond Polymer Refine Detection System (Leica Biosystems, Newcastle Upon Tyne, UK) and 3.3" diaminobenzidine was the chromogen used. Counterstaining was performed with modified Lille's hematoxylin. The entire immunohistochemical technique was performed with DakoCytomation Autostainer. The final product of the reaction entailed staining cell brown. CD68+ macrophages were quantified in the peritumoral and intratumoral areas of the stroma, and S100+ dendritic cells were counted in both the stroma and intraepithelium. Microscopic examination was performed using the Nikon Eclipse E600 microscope.

Assessment of CD68+ macrophages was performed by the modified hot spot method. Initially, the highest cell density field was identified in the studied stromal areas, at the microscopic magnification x100, subsequently immunoreactive cells were counted in 3 fields, at the microscopic magnification x400. The average value of the three fields was used as data for analysis. CD68+ macrophages located near areas of necrosis or associated with inflammatory infiltrate were excluded from the evaluation.

The modified hot spot method was used for the quantitative evaluation of S100+ dendritic cells. S100 was also expressed by glial cells, and the presence of the chromogenic signal in nerve structures was considered as a positive internal control. Only S100+ dendritic cells, which had cytoplasmic extensions, were included in the evaluation. After identifying the field with the highest cell density in the studied areas, at x100 microscopic magnification, the immunoreactive cells were counted in 5 visual fields, at x400 microscopic magnification.



a. b.

Fig. 1. Distribution of CD68+ cells in prostate carcinoma, ×10; anti-CD68 immunoreaction, DAB

Statistical analysis was performed using SPSS13.0 and Microsoft Excel 2010 software. Images were taken and processed using Lucia G system.

Results

Immunohistochemical analysis revealed the non-homogeneous and heterogenic character of CD68+ and S100+ immune cells distribution. CD68+ cells were preferentially located: along the tumor invasion border, in the stroma of tumor foci, including in the lumen of transformed acini (fig. 1).

The numerical distribution of CD68+ cells varied in the studied areas, the highest density being for intratumoral areas (tab. 1). Except non-acinar carcinomas, where the great density of CD68+ cells was recorded in the peritumoral areas.

Table 1

The mean density of CD68+ and S100+ cells in the prostate carcinoma specimens and comparison of cellular density between the studied areas

n*	CD68+ macrophages		S100+dendritic cells	
	Intratumoral	Peritumoral	Stroma	Intraepithelial
73	**72.7±4.7**	44.7±5,0	16.0±1.1	**22.4±1.3**
	t = 4.456 p=<0.001		*t = 3.963 p=<0.001*	

*n – the number of cases included in the study, p – value obtained by Student test

In relation to the histological types of adenocarcinomas was observed a linear increase of CD68+ macrophages, both intratumoral and peritumoral (fig. 2). The density of CD68+macrophages was relatively uniform, both intratumoral and peritumoral, in most of well- and medium-differentiated adenocarcinomas. Except four cases (two well-differentiated and two medium-differentiated carcinomas), where the number of intratumoral macrophages ranged between 101-186 cells/field. In poorly-differentiated adenocarcinomas, the numerical distribution of immunoreactive macrophages was much more heterogeneous. It was observed that in five cases (17%) the density of CD68+ cells in both studied areas was identical.

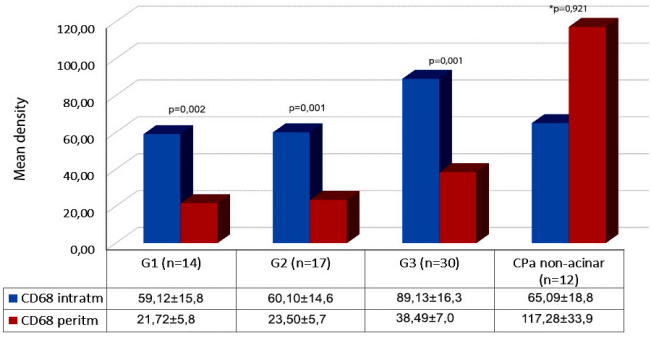


Fig. 2. The mean density of CD68+ cells in prostate carcinoma stroma, where:

G1 – well-differentiated adenocarcinoma, G2 – medium-differentiated adenocarcinoma, G3 – poorly- or undifferentiated adenocarcinoma, CPa – non-acinar carcinoma,

*no true differences were established between the compared groups

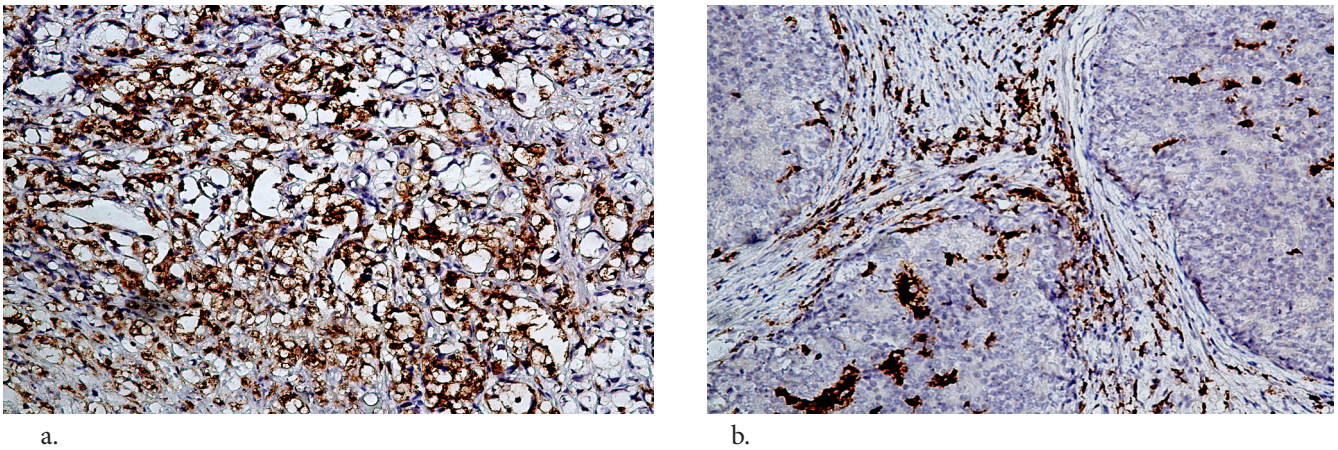
The stromal CD68+ macrophages of prostate adenocarcinomas showed morphological heterogeneity. Thus, two distinct cell populations were highlighted:

1. Small-sized cells (mostly) – intensely branched, with thin and long cytoplasmic extensions, intensely and weakly granulated cytoplasm, and round, or oval to elongated shape;

2. Large-sized cells – characterized by round shape, rare, short cytoplasmic extensions, sometimes polynucleated, and highly granular cytoplasm.

The distribution of small-sized CD68+ macrophages frequently had an infiltrative character, in contrast to large-sized cells, which were often located alone among stromal cells or formed small cell groups in the tumor mass (fig. 3).

Also, numerous CD68+ cells have been observed in: most of intravascular tumor emboli and peripheral areas



a. b.

Fig. 3. Heterogeneous character of CD68+ cell populations distribution in the stroma of malignant hyperplastic lesions: a) infiltrative features of small-sized CD68+ cells; b) comparative distribution of small-sized versus large-sized cells; x40; anti-CD68 immunoreaction, DAB

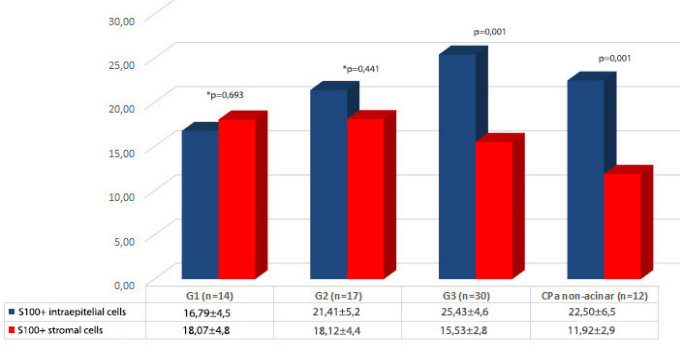
of inflammatory infiltrates. A particular aspect was the circumscription by the CD68+ cells of prostatic sympexions, indirectly, that suggest about the changes in the composition of the glandular secretion. In relation to blood vessels, CD68+ macrophages were located in the thickness of the vascular wall, less often among endothelial cells.

Comparing density of macrophages from peritumoral areas with intratumoral areas was obtained a partial correlation. In order to evaluate the prognostic impact of macrophages in adenocarcinomas, it was considered appropriate to achieve the correlation between the total number of CD68+ cells and the Gleason score, thus obtaining a statistically significant correlation (p=0.001). Splitting down the correlation on studied areas were obtained statistically significant correlations for both intratumoral – p=0.008, and peritumoral – p=0.001.

Distribution of S100+ cells has been studied both intraepithelial and stromal. Distribution of immunoreactive cells was non-homogeneous in both areas. However, in most of prostate carcinomas the high density was recorded in the intraepithelial areas (tab. 1). Evaluation of S100+ dendritic cells variation in carcinoma groups revealed the heterogeneous character for well-differentiated adenocarcinomas and the relatively homogeneous character for poorly differentiated adenocarcinomas and small cell carcinomas. It was observed that starting with the medium-differentiated carcinomas, the density of S100+ cells was raised for the intraepithelial areas (fig. 4).

However, in 13 cases of prostate carcinoma: 7 medium-differentiated adenocarcinomas (41.2%), 5 poorly-differentiated adenocarcinoma – 16.7%), one case of non-acinar carcinoma (8.3%)), high density of dendritic cells was recorded for the stromal areas. Also, we noticed that in three cases of the total samples with adenocarcinoma (4.1%), the density of S100+ cells in both studied areas was identical.

It was interesting to observe some S100+ tumor cells, especially in the tissue samples of poorly-differentiated and non-acinar carcinomas – 16.4% (n = 12), (fig. 5). An extremely important fact was the observation of S100+ tumor



	G1 (n=14)	G2 (n=17)	G3 (n=30)	CPa non-acinar (n=12)
S100+ intraepitelial cells	16,79±4,5	21,41±5,2	25,43±4,6	22,50±6,5
S100+ stromal cells	18,07±4,8	18,12±4,4	15,53±2,8	11,92±2,9

Fig. 4. Comparative aspect of intraepithelial and stromal positive S100+ dendritic cells distribution in prostate carcinomas, G1 – well-differentiated adenocarcinoma, G2 – medium-differentiated adenocarcinoma, G3 – poorly or undifferentiated adenocarcinoma, CPa – non-acinar carcinoma or small cell carcinoma, p – value obtained by Student test,

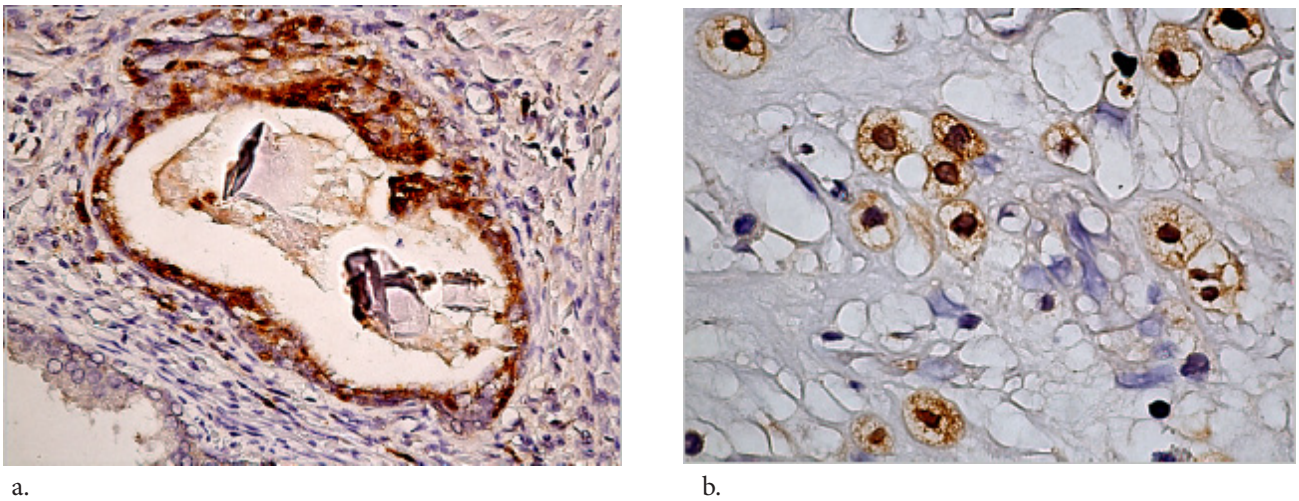
*no true differences were established between the compared groups

cells in the lumen of blood vessels, as well as at the site of tumor metastasis into vessel.

In most of specimens, S100+ cells formed the cell clusters, much rarely were located isolate. Frequently, the dendritic cell clusters were followed by polymorphonuclear cells infiltrate. A particular aspect observed was the presence of single dendritic cells in the lumen of glandular acini and ducts.

S100+ dendritic cells could be characterized as a heterogeneous cell population. Heterogeneity is determined either by the intensity of immunolabeling (thus, are cells that intensely express the marker (mostly) and cells with low-intensity) or by cell size (thus, are small and large- sized (dominant) cells) (fig. 6).

In order to evaluate the prognostic impact of dendritic cells in adenocarcinomas, it was considered appropriate to achieve the correlation between the total number of S100+ cells and the Gleason score, thus obtaining statistically significant correlations both intraepithelial (p=0.036) and stromal (p=0.042). In addition, to highlight the interrela-



a. b.

Fig. 5. Expression of the S100 marker of tumor cells, ×10, x40; anti-S100 immunoreaction, DAB.

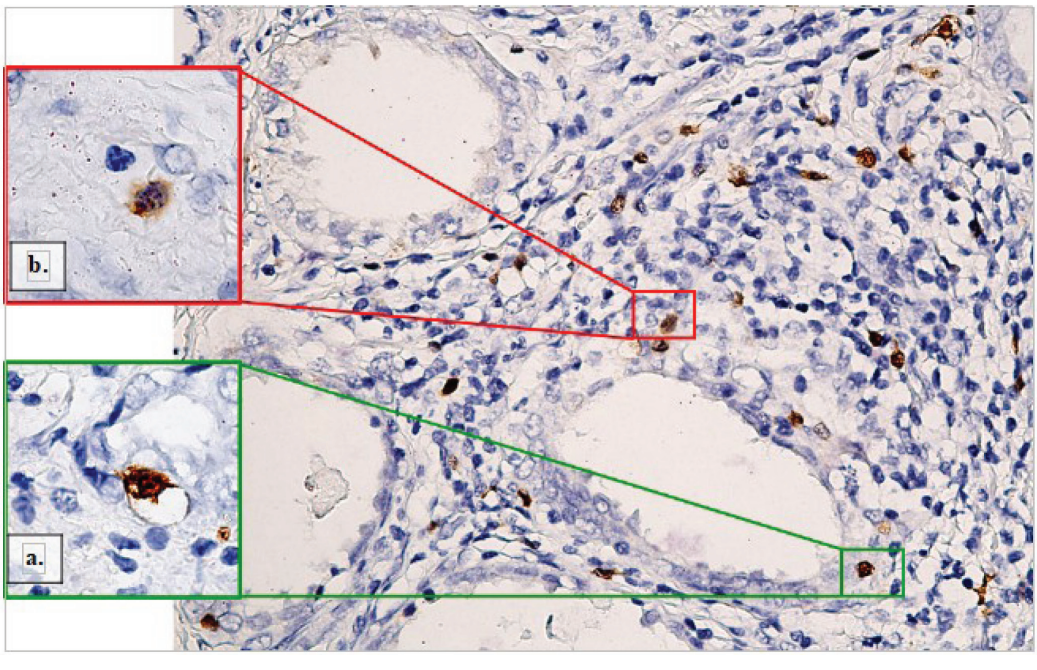


Fig. 6. Identification of the heterogeneous character of the immunolabeling expression.

a) cells that highly express the marker S100, b) cells with low-intensity of immunolabeling, ×40; anti-S100 immunoreaction, DAB

tionships between the immune cells in the adenocarcinoma stroma, there were correlated the densities of dendritic cells and macrophages, thus, was obtained a statistically significant correlation (p=0.018) between S100+ intraepithelial dendritic cells and CD68+ intratumoral macrophages.

Discussion

Tumor cells secrete several pro-inflammatory cytokines, which promote the infiltration of the tumor microenvironment with various immune cells, such as macrophages, neutrophils, NK cells, dendritic cells, mast cells, T and B-lymphocytes. Subsequently, the tumor microenvironment facilitates the angiogenesis, proliferation and invasion of carcinoma. According to our data, in prostate carcinoma the number of macrophages and dendritic cells is increased compared to normal prostate tissue. Distribution of macrophages CD68+, especially for intratumoral areas, was uneven, which indirectly demonstrates the active involvement of macrophages in the process of tumor initiation and progression. The high amount of macrophages was largely achieved due of intratumoral macrophages. In addition, a linear increase of CD68+ macrophages was noticed in relation to the histological grade of adenocarcinomas, in both intratumoral and peritumoral.

In the literature, there are studies that present similar data about the number of macrophages. Thus, Gollapudi et al. have demonstrated increased levels of TAM in prostatic intraepithelial neoplasia compared to those from benign le-

sions tissues [21]. At the same time, they have reported that patients with high Gleason score contain the higher number of TAM. Many clinical researches have shown that there are various correlations between TAM density and the tumor prognosis [22, 23]. It is also very important, that TAMs in different tumor compartments, apparently, have opposite effects on the progression of prostate cancer [22, 23].

Studying the morphological features of CD68+ cells in the prostate carcinoma samples, were observed two different cell populations. Some studies have described two different types of macrophages: M1 phenotype, which has an anti-tumor effect and M2 phenotype that promotes angiogenesis, tumor growth and metastasis. Analysis of various studies revealed that the density of the M1 macrophages could not be considered a prognostic factor, only the M1/M2 ratio could be a true and independent prognostic factor. Cellular and molecular interaction between the M1 and M2 population has an important determining role for prognosis in cancer patients. Tumor-associated macrophages (TAMs) mainly represent a variety of the M2 phenotype, although in some studies they have been described as a mix of M1 and M2 phenotype [24].

Being part of the tumor microenvironment, dendritic cells influence in positive or negative way the course of malignant disease. Several studies have pointed out the central role of the dendritic cells in antitumor immunity [25]. Our results have shown an increase of dendritic cells density in prostate carcinoma tissues compared to normal tissues. The numerical difference from the control group was significant. In this study, we noticed that the highest degree of infiltration with S100+ dendritic cells was associated with poorly-differentiated adenocarcinomas. Thus, our data demonstrate that the increase of the S100+ dendritic cells density in prostate adenocarcinoma is associated with an unfavourable prognosis (the evidence was to obtain a statistically significant correlation with the Gleason score).

Dendritic cells play a crucial role in many types of human cancers. Various studies have shown that the presence of high intratumoral DC density is associated with a favourable prognosis [26]. In addition, there is evidence related to the loss of T lymphocyte activation capacity after exposure to dendritic cells in contact with tumor cells [27] and/or related to the loss of their maturation capacity in the absence of migration into lymph nodes [28]. Thus, the increase of dendritic cells into tumor areas could be associated with an unfavorable prognosis. Our results are different from those reported about other tumors. So, in gastric and cervical carcinomas no significant associations were observed between the degree of dendritic cells infiltration and the degree of tumor differentiation [29, 30].

The correlation of increased S100+ dendritic cells density with a good prognosis has been proven in cases of colorectal, lung and esophageal cancers [31-33]. For other tumors (e.g. squamous cells laryngeal carcinoma) DC infiltration does not correlate with histological grade, tumor stage, or survival [34]. The prognostic value of S100+ cells for patients with renal and breast cancer remains limited [35, 36]. Studying the dendritic cells morphology in intraepithelial and stromal areas led us to assume that two different subpopulations of DC were highlighted, which differ both in maturation and functional status. In our opinion, a particular interest was represented by the quantitative difference of dendritic cells in two studied areas: stromal and intraepithelial. The increased dendritic cells total density was due to the increase of intraepithelial DCs number. Increased number of intraepithelial dendritic cells had a linear feature, which highlight a significant association between the DC density and histological grade of the tumor. Following the obtained results, we suppouse that S100+ stromal dendritic cells can be considered elements of the microenvironment with important anti-tumor effect, and the decrease of their number in prostate adenocarcinoma is an unfavourable prognostic factor. At the same time, the increase in the intraepithelial dendritic cells density can be associated with increased immune tolerance to antitumor mechanisms. Similar data were reported in 2013 by Doros et al. that associated the migration of stromal dendritic cells in the tumor areas of laryngeal carcinomas with a favourable prognosis [37]. Instead, Nagorsen et al. (2007) observed in colorectal carcinoma that better survival depends not only on increased stromal dendritic cell number from the tumor area but also on the increased amount of epithelial DC [38].

Existing vessels in peritumoral tissues can also promote tumor vascularization by co-optation – a process in which existing vessels are surrounded by tumor cells and used to vascularize the tumor. Moreover, our study showed that peritumoral macrophages have often been observed in close contact with the proliferating endothelial cells of capillaries and smooth muscle cells of arterioles and venules. Also, multiple data reported about involvement of dendritic cells in the process of angiogenesis. Depending on the specificity of the antigenic subset, localization, activation status and cytokines, dendritic cells can produce pro- and antiangiogenic mediators. Mostly, *in vivo* studies demonstrated that DC, especially immature DC, promote angiogenesis, while *in vivo* data about antiangiogenic activity of these cells are limited.

Analyzing comparatively the densities of these immune cells, we noted that the expression of CD68 (macrophages) was significantly higher than the expression of S100 (dendritic cells). In order to highlight the interrelationships between the immune cells of prostate carcinoma stroma, the densities of dendritic cells were correlated with those of macrophages. Thus, we obtained statistically significant correlations for both intraepithelial and stromal dendritic cells. The presence of increased densities of immune cells in the modified prostate tissues suggests that hyperplastic epithelial cells have sufficient immunogenicity to recruit immune cells. Antigens, such as PSA (prostate-specific antigen), prostate-specific genes C1, C2, C5, PAGE-1, and prostate stem cell antigen, can induce local activation and proliferation of immune cells [39]. In addition, tumor epithelial cells also produce multiple cytokines and adhesion molecules that recruit more immunocytes to cancer sites. The involve-

ment of epithelial cells in prostate malignancy processes is also supported by our study, which reported in 12 cases of prostate adenocarcinoma about tumor cells expression of S100 marker. Moreover, the presence of these S100+ tumor cells was observed in the areas of tumor invasion, as well as in the lumen of vessels.

Conclusions

In conclusion, the present study has shown that chronic inflammatory processes of the prostate, especially those involving the intratumoral infiltrates with macrophages and dendritic cells, are essential for tumor progression. Thus, the increase in the density of S100+ and CD68+ cells, as well as the significant association of their density with the histological degree of the tumor allow us to consider these cells to be predictive biomarkers of tumor grade, progression and aggressiveness.

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Authors' contribution
TG designed the study, conducted the laboratory work and performed its technological part, interpreted the data, drafted the first manuscript; LG conducted/performed the laboratory work and drafted the manuscript; VD conducted/performed the laboratory work; PG conducted/performed the laboratory work and drafted the manuscript; EP conducted/performed the laboratory work; LS revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate
No approval was required for this study.

Conflict of Interests
No competing interests were disclosed.

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Compatibility determination of potassium orotate with spironolactone by high-performance liquid chromatography

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Abstract

Background: Compatibility determination between active pharmaceutical ingredients (APIs) in fixed-dose combinations is an indispensable step in the elaboration. High-performance liquid chromatography provides information on possible interactions between APIs and their related interaction products. The purpose of the present study was to investigate the compatibility of potassium orotate in combination with spironolactone by a HPLC method.
Material and methods: The detection was carried out using Liquid Chromatograph Agilent 1100 with UV-VIS detector and a RP-18 reversed column (250*4 mm, 5 µm), mobile phase of acetonitrile: phosphate buffer solution (pH=4.0) with the ratio 1:49 and 1:1, at flow rate 1 and 1.5 mL/min, injection volume 20 µL; potassium orotate and spironolactone substances were provided by Sigma Aldrich, USA.
Results: Due to the developed method both separation and simultaneous qualitative and quantitative determination of APIs in the mechanical mixture were carried out. Spironolactone: retention time 6.9 min, concentration 98.1% (±0.21); potassium orotate: retention time 3.06 min, concentration 91.67% (±0.15). There were just well-separated symmetrical peaks of APIs and no additional peak in the chromatograms.
Conclusions: There is compatibility between APIs. Further studies will be performed by other methods (DSC, FT-IF Spectrometry) to confirm the obtained result.
Key words: HPLC, combination, potassium orotate, spironolactone.

Cite this article
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Introduction

Nowadays, the number of new fixed-dose combinations (FDCs) is rising significantly. FDC is a medicine that includes two or more active pharmaceutical ingredients (APIs) combined in a single dosage form. Due to this, the FDCs have several advantages over monocomponent medicine, such as potentiating the therapeutic efficacy, reducing the incidence of adverse effect of medicines, having pharmacokinetic advantage, better compliance by reducing the pill burden, reducing dose of individual medicines, decreasing development of resistance, ensuring cause treatment. No less important they are cheaper than individual medicine because of reduced cost from packaging to distribution. Nowadays, monotherapy is an unsuccessful treatment of many cases, particularly chronic diseases, such as hypertension, diabetes, immunodeficiency virus (HIV), tuberculosis as well as hypopotassemia [1, 2, 3].

Hypopotassemia is serum potassium level less than 3.6 mEq per L (3.6 mmol per L). It is a common electrolyte disorder, which occurs in up to 21% of hospitalized patients and 2% to 3% of outpatients [4, 5]. Hypopotassemia occurs in less than 1% of healthy individuals, but is present in up to 20% of hospitalized patients, 40% of patients taking diuretics, and 17% of patients with cardiovascular conditions [6, 7]. Furthermore, as many as 20% of hospitalized patients are found to have hypopotassemia but only in 4–5% this is clinically significant [8]. The high prevalence of hypopotassemia among patients with COVID-19 suggests the presence of disordered renin-angiotensin system activity, which increases as a result of reduced counter activity of angiotensin-converting enzyme 2, which is bound by severe acute respiratory syndrome coronavirus 2 [9].

Potassium (K^+) plays a key role in maintaining normal cell function [10, 11]. K^+ is the main intracellular cation and almost all cells have the pump called 'Na^+-K^+-ATPase', which pumps sodium (Na^+) out of the cell and K^+ into the cell leading to a K^+ gradient across the cell membrane (K^+ in > K^+ out), which is partially responsible for maintaining the potential difference across membrane [4, 11]. Many cell functions rely on this potential difference, particularly in excitable tissues, such as nerve and muscle. Two percent of K^+ exist in the extracellular fluid (ECF) at a concentration of only 4mEq/L [10, 12]. Potassium is the third essential ele-

ment in the human body after calcium and phosphorus and is the most abundant cation in the intracellular compartment, which facilitates nerve impulse conduction and the contraction of skeletal and smooth muscles, including the heart. It controls the formation and storage of glycogen and prevents the calcium loss through the urine. If there is a potassium lack, the lungs cannot remove the carbon dioxide and the kidneys cannot concentrate urine, resulting in excessive urination [13].

Hypopotassemia is frequently asymptomatic finding identified only on routine electrolyte screening. Clinical symptoms and signs of hypopotassemia depend on the rate of onset and severity. It can be associated with symptoms ranging from the confusion, disorientation, weakness and discomfort of muscles to the arrhythmias, muscle cramps (paralysis), respiratory failure and sudden death [8, 14]. Hypopotassemia may be iatrogenically caused by other medicines, such as furosemide, insulin, gentamicin, theophylline and salbutamol. Appropriately 80% of patients who are receiving diuretics (thiazide, loop) become hypopotassemic, while many of patients with hypopotassemia could also have an associated systemic disease [12, 13, 15]. Therefore with the application of loop diuretics and digoxin, hypopotassemia has become a frequent and feared side effect of treatment.

The correction of hypopotassemia depends on its severity: mild (plasma potassium 3.1-3.5mmol/L), moderate (plasma potassium 2.5-3.1mmol/L) and severe (plasma potassium <2.5mmol/L). Urgent treatment is warranted for patients with potassium levels less than 2.5mEq/L by intravenous potassium chloride at 10-20 mEq/hour applying ECG monitoring and continuously careful estimation of serum potassium levels [12]. Mild and moderate hypopotassemia may be corrected by using an oral potassium supplementation (potassium chloride, potassium orotate, potassium aspartate) [16]. The most important is to correct underlying causes, for example, thiazide and loop diuretics can be replaced with the potassium-sparing diuretics, such as spironolactone that leads to minimize potassium loss [17]. It is well-known that hypopotassemia can be induced by hypomagnesemia, therefore combination of potassium and magnesium aspartate provides potassium and magnesium correction, which resolves the cause of the imbalance [14, 18].

To gain the maximum benefit from treatment, we need to use fixed-dose combinations that improve the potassium supplementation and ensure etiological treatment in hypopotassemia. Unfortunately, there is a deficiency of a combined local pharmaceutical product on the pharmaceutical market of the Republic of Moldova. Therefore, the pharmaceutical product, which consists of potassium orotate, potassium and magnesium aspartate, spironolactone, is in the process of development at the Scientific Center of Medicine. Due to this complex composition this new pharmaceutical product can be applied not only to eliminate the symptoms of hypopotassemia (weakness, cramps), but also to ensure the causal treatment. Resulting from the fact, that combination of potassium and magnesium aspartate provides potassium and magnesium correction, because hypomagnesemia is linked to potassium imbalance. Hypopotassemia in individuals with high blood pressure, who require taking thiazide diuretics, may be improved by replacement or combination of it with a potassium-sparing diuretic (spironolactone). Very often potassium is used as an orotic acid salt that is a non-steroidal anabolic agent, which helps to normalize the external electrolyte balance of potassium through the stimulation of metabolic processes. Thus, due to these active substances combined in the single dosage form for oral administration, it can produce better effect in the treatment of hypopotassemia, than any of them taken separately. Therefore, it is now in the active process of development at the Scientific Center of Medicine [18, 20].

Definitely, various regulatory authorities, such as the Food and Drug Administration (FDA) and International Conference on Harmonization (ICH) require the pre-formulation studies on each new FDC. Pre-formulation testing is an important step in the development of new products or the reformulation of existing ones. It includes the chemical characterization of the medicine and analytical compatibility/stability tests. In the pharmaceutical industry, the active pharmaceutical ingredient is subjected to pre-formulation studies, which provide the necessary information for the development of a stable medicine formulation with adequate bioavailability [21]. Thus, it is designed to find out if the APIs have the potential to interact with each other or between APIs and excipient pharmaceutical ingredients (EPIs) [22, 23].

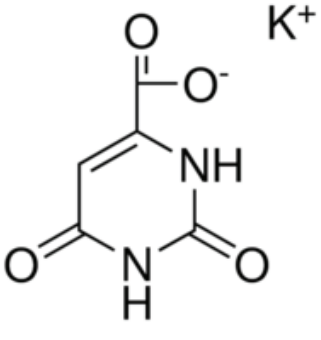

Fig. 1. Chemical structure of potassium orotate

Incompatibility is an undesirable chemical or physical reaction between the API and EPIs or between two or more APIs that could reduce their effectiveness or result in toxicity, increasing of minor or serious unexpected side effects. Physical and chemical interactions between APIs can affect the chemical nature, the stability and bioavailability of medicine products and consequently, their therapeutic efficacy and safety [24, 25].

Evaluation of possible incompatibilities between the APIs is an important part of the preformulation phase during the development of a dosage form. Successful compatibility studies require a good experimental design that furnishes the required information with the minimum of

experimental effort. Definitely, not all APIs interactions are so bad, but major of them are life-threatening, therefore it is so necessary to determine them at the beginning of the medicine development phase by applying sensible and modern methods. Despite the importance of compatibility test between APIs, there is no universal protocol for this study. It is a key step in determining the success of medicine development [26].

To investigate the compatibility of the components of a formulation, techniques, such as X-ray diffraction, Fourier-transform infrared spectroscopy (FT-IR spectroscopy), high-performance liquid chromatography (HPLC) and thermal analysis (especially differential scanning calorimetry – DSC) are used [27-30]. Although DSC is a fast and reliable technique and generally regarded as one of the methods of first choice in assessing pharmaceutical compatibility study, the evaluation of the curves is often difficult [31]. Therefore, this study was based on HPLC method, because it is easier than DSC and carries out qualitative and quantitative determinations of APIs and their related interaction products. Therefore, this method compares favorably with that commonly employed DSC and the values obtained by HPLC are of special importance.

The principal purpose of this study was to investigate the possible compatibility between the potassium orotate and spironolactone in the mixture by comparing the HPLC-obtained chromatograms. Consequently, this study serves to give information on a medicine's interaction, using HPLC method by providing not only qualitative but also quantitative results for pure APIs and their related interaction products. Analyses of HPLC chromatograms obtained on pure APIs and combinations of them made it possible to determine the compatibility between APIs by comparing the following values: the number, shape and size of peaks. Due to the chemical structure of potassium and magnesium aspartate and specifically to the lack of chromatophore groups they couldn't be investigated by HPLC. Thus, HPLC method was applied for spironolactone and potassium orotate.

Potassium orotate is chemical potassium 2.4-dioxo-1H-pyrimidine-6-carboxylate (potassium Uracil-6-carboxylate) (fig. 1). Its molecular formula is $C_5H_3KN_2O_4$ and its molecular weight is 194.19 g/mol. Potassium orotate is a bioavailable form of potassium (orotic acid helps it (K+) pass easily through cell membranes), that supports the nervous system, kidneys, cardiovascular health, bone strength, and encourages a positive response to stress and anxiety. Definitely, orotic acid is an intermediate in the pyrimidine biosynthesis, which is required for DNA and RNA synthesis.

Spironolactone is a synthetic 17-spironolactone corticosteroid with potassium-sparing diuretic, antihypertensive, and antiandrogen activities (fig. 2). Spironolactone competitively inhibits adrenocortical hormone aldosterone activity in the distal renal tubules (it actually works on aldosterone-dependent sodium- potassium exchange channels). This increases the excretion of water and sodium, while decreasing the excretion of potassium. The increased excretion of water leads to diuretic and also antihypertensive effects. Spironolactone has a fairly slow onset of action, taking several days to develop; similarly, the effect diminishes slowly.

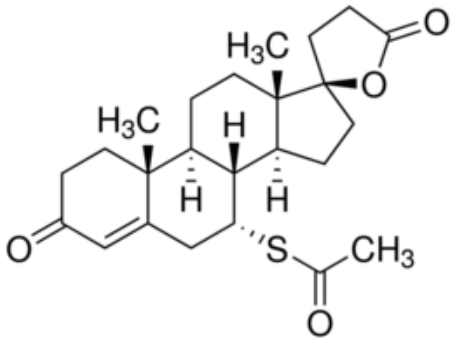

Fig. 2. Chemical structure of spironolactone

Material and methods

The laboratory analyses were performed in the Laboratory of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Graz, within the doctoral research mobility CIII-RO-0010-14-1920 – "Teaching and Learning Bioanalysis" under the leadership of professor Martin Schmid.

Materials

The HPLC-grade potassium orotate, HPLC-grade spironolactone, HPLC-grade acetonitrile (ACN) and analytical-grade potassium phosphate monobasic were provided by Sigma Aldrich (USA). HPLC-grade water was obtained from Fisher Chemical (Belgium).

Instrumentation and chromatographic conditions

The chromatographic analyses were performed by Liquid Chromatograph Agilent 1100 equipped with autosampler. The study was made on a RP-18 reversed column (250mm long by 4 mm internal diameter, particle size 5μm) and by an isocratic elution method.-

There are 2 elution techniques of pumping mobile phase through a column: isocratic and gradient methods. In the isocratic method, the composition of the mobile phase remains constant, whereas in the gradient method the composition changes during the separation process. The isocratic method is the simplest technique and should be the first choice when developing a separation. Therefore, isocratic method of compatibility determination was selected to determine compatibility of these studied 2 APIs in the mixture.

In order to select a proper mobile phase for the good separation and quantitative determination of potassium orotate and spironolactone simultaneously, the chromatographic conditions were selected after testing different parameters, such as diluents, buffer, buffer concentration, organic solvent for mobile phase, mobile phase composition, flow rate and temperature. Mobile phase selection was based on peak parameters (symmetry, tailing), run time, therefore two mobile phases were prepared.

Preparation of mobile phase N1: 2.72 g of potassium dihydrogen phosphate were weighed by balance (RAD-

WAG) and transferred to 1000 mL volumetric flask and dissolved by HPLC-grade water then completed to the mark by HPLC-grade water. The solution was adjusted to pH 4.0 with 2M orthophosphoric acid. HPLC-grade acetonitrile and obtained phosphate buffer solution 0.02M were mixed with (1:49) ratio. The mobile phase was degassed in an ultrasonic bath (GT SONIC Professional Ultrasonic Cleaner, China).

Preparation of mobile phase N2: 6.8 g of potassium dihydrogen phosphate were weighed by balance (RAD-WAG) and transferred to 1000 mL volumetric flask and dissolved by HPLC-grade water then completed to the mark by HPLC-grade water. The solution was adjusted to pH 4.0 with 2M orthophosphoric acid. HPLC-grade acetonitrile and obtained phosphate buffer solution 0.05M were mixed with (1:1) ratio. The mobile phase was degassed in the ultrasonic bath (GT SONIC Professional Ultrasonic Cleaner, China).

The study was performed on mobile phase N1 and at flow rate 1 mL/min, injection volume 20 µL, temperature 25°C. The detection was set at 254 nm for spironolactone and at 278 nm for potassium orotate using UV-visible absorption detector. Total run time was less than 20 min for each injection.

Preparation of standard and test solutions, made on mobile phase N1 (M/Ph. N1), in which potassium orotate is predominantly determined:

A standard and test potassium orotate solutions were prepared in a 10-mL volumetric flask by dissolving 15.00 mg of the substance to be examined and then diluting to volume with mobile phase. Then this obtained solution undergoes a sonication for 30 seconds and then it is completed to the mark by mobile phase. Dilute 1mL of this solution to 10.0mL with mobile phase. Place 1mL of the obtained solution in the 10-mL volumetric flask and make up to mark with mobile phase (the final solution contains 15 µg/mL). The solutions were scanned and found to have maximum absorption wavelength at 278 nm using mobile phase as blank.

A standard and test spironolactone solutions were prepared in the 10-mL volumetric flask by dissolving 2.40 mg of spironolactone and then diluting to volume with mobile phase. Then this obtained solution undergoes the sonication for 30 seconds and then it is completed to the mark by mobile phase. Place 1 mL of this solution in a 5-mL volumetric flask and made up to mark with mobile phase (the final solution contains 48 µg/mL). The solutions were scanned and weren't found to have maximum absorption wavelength at 240 nm using mobile phase as blank.

Test mixture preparation on M/Ph. N1: 2.40 mg of spironolactone and 15.00 mg of potassium orotate were weighed and mixed together with the ratio according to the amount of each APIs from the new fixed-dose combination, which is currently in progress. Whereupon, it was transferred into the 10-mL volumetric flask and then diluted to volume with mobile phase. The mixture was sonicated for a minimum of 30 seconds with intermittent shaking. Then the solution was brought back to room temperature and diluted to the mark with mobile phase. Dilute 1mL of this solution to 5 mL with mobile phase. Place 1mL of the obtained solution in the 10-mL volumetric flask and make up to mark with mobile phase.

As well as the study was performed on mobile phase N2 and at flow rate 1.5 mL/min, injection volume 20 µL, temperature 40°C. The detection was set at 254 nm for spironolactone and at 278 nm for potassium orotate using UV-visible absorption detector. Total run time was less than 10 min for each injection.

Preparation of standard and test solutions, made on mobile phase N2 (M/Ph. N2), in which spironolactone is predominantly determined:

A standard and test spironolactone solutions were prepared in the 10-mL volumetric flask by dissolving 2.40 mg of the substance to be examined and then diluted to volume with mobile phase. Then this obtained solution undergoes the sonication for 30 seconds and then it is completed to the mark by mobile phase. Dilute 1mL of this solution in the 5.0 mL with mobile phase. Place 1mL of the obtained solution in the 10-mL volumetric flask and make up to mark with mobile phase (the final solution contains 4.8µg/mL). The solutions were scanned and found to have maximum absorption wavelength at 240 nm using mobile phase as blank.

A standard and test potassium orotate solutions were prepared in a 10-mL volumetric flask by dissolving 15.00 mg of the substance to be examined and then diluting to volume with mobile phase. Then this obtained solution undergoes the sonication for 30 seconds and then it is completed to the mark by mobile phase. Dilute 1mL of this solution to the 5.0 mL with mobile phase. Place 1mL of the obtained solution in the 10-mL volumetric flask and make up to mark with mobile phase (the final solution contains 30µg/mL). The solutions were scanned and found to have maximum absorption wavelength at 278 nm using mobile phase as blank.

Test mixture preparation on M/Ph. N2: 2.40 mg of spironolactone and 15.0 mg of potassium orotate were weighed and mixed together with the ratio according to the amount of each API from the new fixed-dose combination. Whereupon, it was transferred into the 10-mL volumetric flask and then diluted to volume with mobile phase. The mixture was sonicated for a minimum of 30 seconds with intermittent shaking. Then the solution was brought back to room temperature and diluted to the mark with mobile phase. Dilute 1mL of this solution to 5 mL with mobile phase. Place 1mL of the obtained solution in the 10-mL volumetric flask and make up to mark with mobile phase.

Measurements were made on the standard and test solutions of spironolactone, standard and test solutions of potassium orotate and mixture of these APIs. Three replicated injections of each standard preparation and mixture were injected and analyzed. The peaks were detected at 254 nm for spironolactone and at 278 nm for potassium orotate and identified using reference standards of spironolactone and potassium orotate.

Three replicated injections of each standard and test preparation and mixture were injected and analyzed accord-

ing to the selected chromatographic conditions. The mean values of these three injections were used to evaluate retention time (t_R), area of peak (A), theoretical plate (N), resolution factor (Rs) and concentrations for APIs. The percent relative standard deviation (RSD, %), coefficient of variation (CV, %) values were used for evaluation of obtained values of spironolactone and potassium orotate separately and in the mixture. Concentrations of active substances in % were calculated according to the equation (1):

$$X\% = \frac{S_x * m_{st} * W_x * P_0}{S_{st} * m_x * W_{st}} * 100\%, \qquad (1)$$

where,

S_x – Average of area counts of principle peak obtained from the chromatograms of the test solution;

S_{st} -Average of area counts of principle peak obtained from the chromatograms of the standard solution;

m_x – mass of test substance, g;

m_{st} – mass of standard substance, g;

P_0 – Purity of reference standard (% w/w);

W_{st} *and* W_x – Dilution factor of standard and test solutions, respectively.

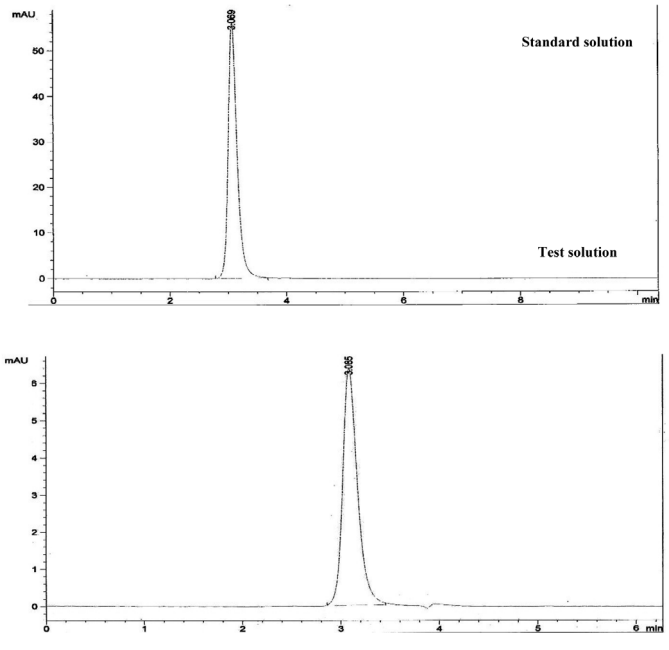

Fig. 1. Chromatograms of standard and test potassium orotate solutions at 278 nm on M/Ph No 1

Results and discussion

There were analyzed several methods to develop a suitable and effective HPLC method for both the compatibility determination of potassium orotate in the combination with spironolactone and quantitative determination of them.

Measurements were made on the pure substances, such as potassium orotate, spironolactone and mixture of them, using isocratic HPLC technique, carried out on the reversed phase chromatography and in the convenient chromatographic conditions. By accurate analyses of the obtained chromatograms for solutions of potassium orotate (fig. 1 and fig. 2), there were found the good shape of principal peak and its area, theoretical plate (N) and concentration, which are shown in table 1.

By accurate analyses of the obtained chromatograms for standard and test solutions of spironolactone (fig. 3), there were found the good shape of principal peak and its area, theoretical plate (N) and concentration, which are shown in table 2.

According to our preliminary data, it was found that the detection of potassium orotate is carried out at 278 nm with t_R 3.06 min and spironolactone at 240 nm with t_R 7.1min. Therefore, HPLC method for potassium orotate in combination with spironolactone was performed at different detection wavelengths, which are specific for each active substance.

By accurate analyses of the values obtained from the chromatograms for the mixture it was found that potassium orotate and spironolactone eluted out forming symmetrical peaks, which were well separated from each other, applying mobile phase No 2. There was no additional peak in chromatograms (fig. 4).

By comparing the obtained chromatograms, it was shown that the separation and simultaneous determination of two APIs on mobile phase N1 is difficult. Thus, it

Table 1

Evaluation data of potassium orotate chromatograms at 278 nm on M/Ph. N1 and No 2

	Retention time (t_R, min)		Theoretical plate (N)		Area of peak (A)		Concentration %	
	M/Ph. N1	M/Ph. N2	M/Ph. N1	M/Ph. N2	M/Ph. N1	M/Ph. N2	M/Ph. N1	M/Ph. N2
Potassium orotate	3.069	1.257	5976	2302	595.073	382.604	93.67	76.83
	3.083	1.267	6084	2312	595.553	385.362	93.38	76.69
	3.013	1.261	5709	2290	594.711	385.504	93.58	76.87
Mean. $\overline{XX}$	3.06	1.26	5923	2301	595.11	384.49	93.55	76.79
RSD. %	0.04	0.005	192.85	11	0.422	1.635	0.15	0.09
CV. %	1.21	0.40	3.26	0.47	0.07	0.43	0.16	0.12

Table 2

Evaluation data of spironolactone chromatograms on M/Ph N2

M/Ph N2	Retention time (t_R. min)	Theoretical plate (N)	Area of peak (A)	Concentration %
Spironolactone	7.019	16023	167.299	99.14
	7.147	15263	175.151	99.26
	7.148	16439	168.272	98.86
Mean. $\overline{XX}$	7.10	15908	170.24	99.09
RSD. %	0.074	596	4.28	0.20
CV.%	1.04	3.75	2.51	0.20

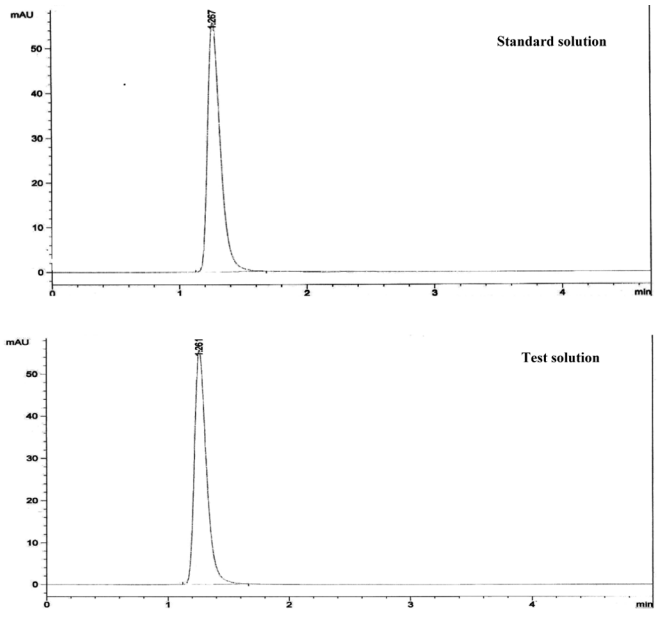

Fig. 2. Chromatograms of standard and test potassium orotate solutions at 278 nm on M/Ph No 2

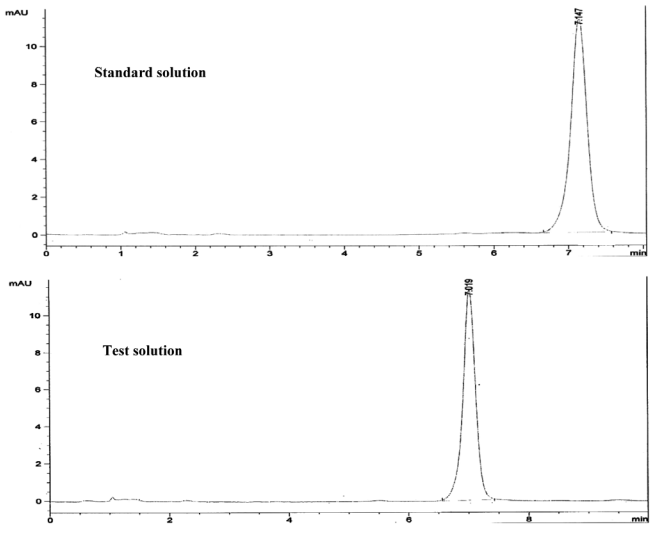

Fig. 3. Chromatograms of spironolactone standard and test solutions at 240 nm on M/Ph No 2

was concluded that the second mobile phase provided the good separation of potassium orotate and spironolactone.

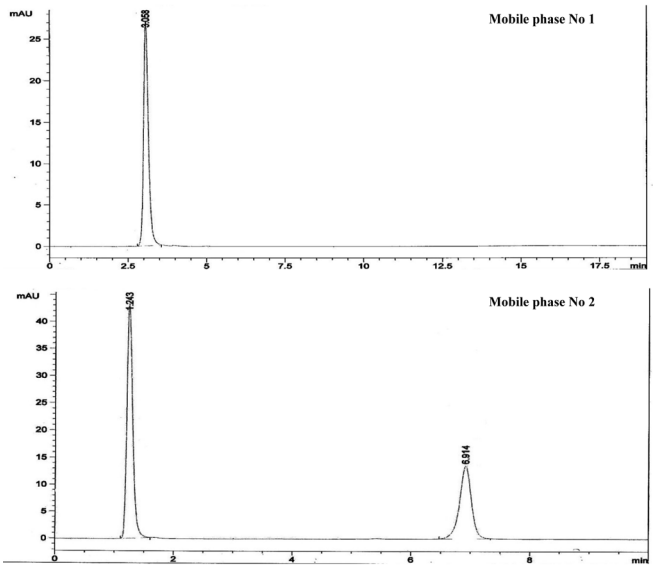

Fig. 4. Chromatogram of mixture at 278 nm and 240nm simultaneously M/Ph. N1 and M/Ph No 2

Furthermore, there were found the good shape of principal peaks and good resolution (Rs 36.04), which are shown in table 3.

The mixture of potassium orotate with spironolactone was evaluated in terms of concentration of each APIs from the mixture, according to the equation (1), relative standard deviation (RSD) and coefficient of variance (CV), which are shown in table 4 and table 5.

The chromatographic techniques developed and reported in this study will serve as a basis for selecting the efficient method for separation and quantitative determination of both APIs simultaneously.

Therefore, it was determined that the concentration of spironolactone is 98.1% (RSD 0.21, CV 0.2%), obtained using mobile phase N2, and the concentration of potassium orotate is 91.68% (RSD 0.15, CV 0.16%), obtained using mobile phase N1. By undertaking of both chromatograms (fig.4) it was found that potassium orotate showed the good resolution from spironolactone on mobile phase, in which spironolactone is predominantly determined.

The method was found to be rapid as potassium orotate and spironolactone eluted out at 1.24 and 6.9 minutes respectively, which is important for routine analysis.

Table 3

Evaluation data of peaks from the mixture on mobile phase No 2

	Theoretical plate (N)		Symmetry (S)		Width (min)		Resolution factor (Rs)	
	Orotate K	Spironolac.	Orotate K	Spironolac.	Orotate K	Spironolac.	Orotate K	Spironolac.
	2259	17147	0.65	0.97	0.1046	0.2112	35.92	
	2266	17713	0.65	0.96	0.1044	0.2072	36.27	
	2272	17227	0.66	0.96	0.1044	0.2101	35.93	
Mean. $\bar{X}\bar{X}$	2266	17362	0.65	0.96	0.10	0.21	36.04	
RSD. %	6.16	306.05	0.004	0.005	0.0001	0.002	0.20	
CV.%	0.27	1.76	0.62	0.49	0.11	0.99	0.56	

Table 4

Evaluation data of mixture on mobile phase No 1

	Retention time (t_R)	Area of peak (A)	Concentration of Orotate K, %
	3.06	1121.683	91.80
	3.058	1118.193	91.514
	3.059	1120.589	91.71
Mean. $\bar{X}\bar{X}$	3.06	1120.16	91.68
RSD. %	0.00	1.79	0.15
CV.%	0.03	0.16	0.16

Table 5

Evaluation data of mixture on mobile phase No 2

	Retention time (t_R)		Area of peak (A)		Concentration %	
	Orotate K	Spironolac.	Orotate K	Spironolac.	Orotate K	Spironolac.
	1.243	6.914	295.94	188.808	75.29	98.15
	1.242	6.894	295.413	189.018	75.15	98.26
	1.244	6.894	296.097	188.269	75.33	97.87
Mean. $\bar{X}\bar{X}$	1.24	6.90	295.82	188.70	75.26	98.10
RSD. %	0.001	0.01	0.36	0.39	0.09	0.20
CV.%	0.07	0.17	0.12	0.20	0.12	0.20

Conclusions

In the present study the compatibility between potassium orotate and spironolactone in the mixture was carefully investigated by applying HPLC method.

Results demonstrated that there was no interaction between potassium orotate and spironolactone in the mixture, due to the absence of any additional peak in the chromatograms, which were investigated by HPLC method. Measurements were performed on RP-18 reversed column (250*4 mm, 5 μm), using mobile phase of acetonitrile: phosphate buffer solution with the ratio 50:50 (pH=4), at flow rate 1.5 mL/min, injection volume 20 μL, temperature 40°C. By analyzing obtained HPLC chromatograms of the potassium orotate in the combination with spironolactone there were shown just 2 principal peaks at 1.24 min with the 278 nm detection wavelength, which is specific for potassium orotate and at 6.9 min with the 240 nm detection wavelength, which is specific for spironolactone. Moreover, potassium orotate showed a good resolution from spironolactone: Rs 36.04. Potassium orotate did not react with spironolactone, therefore the interaction wasn't observed by HPLC. There was found the concentration of spironolactone (98.1% ±0.21), applying mobile phase N2, and the concentration of potassium orotate (91.68% ± 0.15), applying mobile phase N1.

Thus, due to this method a conclusion was made that potassium orotate is compatible with spironolactone. Furthermore, these results were confirmed by FT-IR spectroscopy.

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The role of endothelin-1 in the doxorubicin cardiotoxicity

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Abstract

Background: The cardiotoxicity of doxorubicin (Dx), an antineoplastic drug, is imposed by the development of cardiomyopathy and heart failure. The expression of endothelin-1 (ET-1) in myocardium under the action of Dx, directly correlates with the degree of cardiac dysfunction, mediated by endothelin A (ETA) receptor.
Material and methods: For prospective randomized study 2 groups of white rats (experimental group n=9, control group n=9) were used. During 2 weeks in the control group was administrated Dx (i/p, 4mg/kg in one dose, twice/week), cumulative dose – 16 mg/kg. The ET-1 effects were estimated at its peak action in concentration 10^{-7} M (mol), reproduced after 30 sec of endothelin stimulation.
Results: The functional parameters of isolated heart perfused in physiologic regime and in condition of volume and resistance overload under the ET-1 action in the group with Dx compared with the control one, were reduced considerably, namely: cardiac output (CO); left ventricle systolic pressure (LVSP); left ventricle end-diastolic pressure (LVEDP).
Conclusions: Under the ET-1 action on the isolated heart perfused in physiologic regime in the group with Dx – the LVSP and CO were reduced determining negative inotropic effect. At the volume overload test, under the ET-1 action, the diastolic impairment was more evident in the group with Dx, due to increased LVEDP. At the resistance overload test under the ET-1 action, the CO was reduced indicating the depreciation of myocardial contraction capacity.
Key words: doxorubicin cardiomyopathy, endothelin-1, coronary flow, heart reactivity.

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Introduction

Doxorubicin cardiotoxicity (doxorubicin, the antineoplastic drug efficient in treatment of leukaemia, lymphomas and sarcomas) is characterized by rapid evolution of cardiac failure imminent to doxorubicin (Dx) cardiomyopathy, inclusively at a young person, that is imposed as a cause of death or cessation of drug administration to oncologic patients. So, over several decades the researching of pathogenetical mechanisms of doxorubicin cardiomyopathy represents the main goal of cardiology and of course of oncology. Although many studies have been realized, till our days the pathophysiological prerogatives in conceptual plan have not been well established, the attempts to elaborate pathogenic therapy have at the base experimental values [1-3].

In this context, it is important to mention some successful mechanisms:

1. Development of myocardial energy deficiency is caused by endangerment of mitochondrial respiration and of oxidative phosphorylation [4]. The depletion of ATP reserves directly correlates with the severity of contractile dysfunction of myocardium and inability of it to realize the heart adaptation to hemodynamic and neuroendocrine efforts. A. Murabito et al. (2020) consider that doxorubicin (Dx) is accumulated in cardiomyocyte and binds to one of the phospholipids of mitochondrial membrane, cardiolipin, that in turn affects the electron transport and increases the membrane permeability for cytochrome C, responsible for activation of caspase 9 and initiation of apoptosis process [5]. Moreover, Dx reduces the ratio Bcl2/Bax (cardiac anti- and proapoptotic factors).

2. The activation of oxidative stress is due to exaggerated production of reactive oxygen species and depreciation of antioxidant system. It has been established that accumulation of Dx into the mitochondria in concentration more than 100 μM, represents the threshold level for the onset of lipid and protein peroxidation in cardiomyocytes. The administration of antioxidants, such as vitamin C and coenzyme Q10, in the experimental model of Dx cardiomyopathy in rats, has shown the improvement of left ventricle function at the effort and resistance [6, 7].

3. The impairment ratio between collagen type I and type III, caused by extracellular matrix metalloproteinase activation, determines one of the causes of pathologic myocardial remodeling with the eccentric pattern and dilation of left ventricle (LV), responsible for the disturbance of lusitropy function of the heart [8]. The evaluation of circulating level of different types of metalloproteinases (e.g. MMP-2

and MMP-9) has the predictive value of the risk of extracellular matrix remodeling of myocardium and exacerbation of heart failure.

4. The activation of inflammatory response, according to the results of several fundamental researchers, is one of the important mechanisms leading to dysfunction of heart inclusively doxorubicin cardiotoxicity [9-11]. In this context X. Xinyong et al. (2020) have demonstrated the role of transmembrane receptors, Toll-like, in the onset of inflammatory response of cardiomyocyte and interstitial macrophage to the action of signal molecules derived from damaged cardiac cells – Damage Associated Molecular Pattern (DAMP), resulting in increased production of cytokines [12]. The expression of Tumor Necrosis Factor-α (TNF-α) in murine myocardium subjected to action of Dx is increased in direct ratio with the cumulative dose of anthracycline. In one of previous fundamental researches, we have demonstrated that administration of monoclonal antibody of TNF-α attenuates the cardiotoxicity of doxorubicin, manifested by the improvement of inotropy of the heart [13].

5. The activation of neuro-endocrine system is characterized by initial activation of sympathetic adrenal system followed by excessive synthesis of endothelin-1 (ET-1). The expression of ET-1 in myocardium subjected to action of doxorubicin (Dx) correlates directly with the severity of structural disturbances, remodeling and degree of cardiac dysfunction, effects of this oligopeptide is mediated by ETA receptor [14, 15]. The functional activity of ET-1 is characterized by increasing concentration of calcium into the cardiomyocyte and smooth vascular muscle, which denotes the increased cardiac contraction and constriction of coronary arterioles. Evidently, that inotropy stimulation of the myocardium requires also additional energy expenditure, which can compromise the heart adaptation to the action of stressful and effort factors, especially those, compared with catecholamine and angiotensin II (Ang II), the time of ET-1 metabolisation is much longer. Furthermore, in the myocardial interstitium there are granules that can store ET-1 formed by endothelial coronary and cardiac cells and which are realized in huge amount at the paracrine and endocrine actions, triggered by hypoxia, ischemia, energy depletion, oxidative stress, etc. Thus, ET-1 imposes notable pathogenetic contribution in the onset and exacerbation of cardiac failure, but the particularities of its action on the heart functionality in doxorubicin affection are still poorly explained.

Material and methods

The doxorubicin cardiomyopathy of the heart has been reproduced on the white rats by administration of Dx intraperitoneal (cumulative dose 16 mg/kg during 2 weeks, 2 injections/per week in one dose 4.0 mg/kg). Rats have been sacrificed by euthanasia (thiopental sodium, 0.4 mg/kg) after 10 days from the last injection of anthracycline, because doxorubicin is a drug that due to reduced clearancei s accumulated in the body.

The isolated heart has been perfused in working regime according to the Neely-Rovetto method, functional indices of left ventricle (LV) were estimated by the technical registration device of the parameters in real time "Bio-Shell" (Australia) or by recorder Linearcorder Mark WR3101 (Germany) connected to mechanical sensor.

The effort reactivity of the heart has been studied by the increasing filling pressure of the left atrium to the value of 25cm H_2O (volume overload) or of the pressure in aorta to the value of 120 cm H_2O (volume overload). The effects of endothelin-1 (ET-1) were estimated at the peak of its action in concentration of 10^{-7} M (mol) or, when the action of ET-1 during 30 sec on the isolated heart preceded the manoeuvres of volume and resistance overload. At the same time, the premedication of isolated isovolumic heart with ET-1 in concentration of $5x10^{-7}$ M during 30 sec, gave the opportunity to appreciate the impact of ET-1 on the myocardium tolerance to ischemia-reperfusion stress (30 min ischemia and 45 min reperfusion).

The obtained data exposed by value of M±m (mean and standard error) were compared and statistically analysed according to t-Student criteria in relation to the records of the control group (intact rats) or in relation with indices estimated before the effort test. Margin of error less than 5 % was considered admissible, but deviation from the reference value – statistically significant ($p<0.05$).

Results

The estimation of functional indices of isolated heart in condition of physiologic effort perfusion (pressure in the left atrium and aorta is 15 and respectively, 80 cm H_2O) already has shown serious dysfunction of left ventricle (LF) in the group with doxorubicin (tab. 1).

Table 1

The values of functional indices of isolated heart perfused in physiologic regime

Functional indices	Groups		
	Control (n=9)	Dx (n=9)	p
Aortic jet velocity (AJ), ml/min	21.5±1.4	12.8±0.8 -40.47%	<0.01
Cardiac Output (CO), ml/min	37.4±1.9	23.6±1.3 -36.90%	<0.01
Left ventricular systolic pressure (LVSP), mm Hg	143.5±8.2	106.2±6.3 -25.99%	<0.05
Heart rate (HR), 1/min	289±13	245±11 -15.22%	<0.05
Left ventricular enddiastolic pressure (LVEDP), mm Hg	4.7±0.26	12.6±0.78 +168%	<0,01
Diastolic Stiffness of LV, mm Hg/ml	29.8±1.7	62.5±4.4 +109.73%	<0.01
+dP/dTmax, mm Hg/sec	8565±208	6320±165 -26.21%	<0.05
-dP/dTmax, mm Hg/sec	6710±174	5045±120 -24.81%	<0.05

Note: p – value of significance vs control; ± – relative deviation from the control group.

So, the main indices of pumping function of left ventricle (AJ and CO) are by 40.47% reduced from the control group, but systolic pressure generated by LV reaches the averages of 75% from control values of parameter. Also, is remarked the evident decline of diastolic relaxation of LV, being given increased LVEDP and diastolic stiffness by 168% and, respectively 109.73%.

Also, it is attested the disturbance of contraction and isovolumetric relaxation of the heart, and main parameters that characterized these important phases of cardiac cycle (e.g. +dP/dTmax and -dP/dTmax) are decreased significantly by 26.21% and respectively 24.81% compared with the control group.

There are important evidences that reflect the inotropy response of isolated heart perfused in physiologic regime under the action of ET-1 (tab. 2).

Table 2

The values of functional indices of isolated heart perfused in physiologic regime under the action of ET-1

Indices/lot	Action ET-1 (10^{-7} M)	
LVSP (mm Hg)	**Initial**	**Stimulation**
Control	141.7±8.2	177.3±8.6 +25.1%vs initial
Dx	104.9±6.4*	95.4±6.6* -9.1%vs initial
CO (ml/min)	Initial	Stimulation
Control	36.9±1.9	42.7±2.2 +15.7%vs initial
Dx	23.6±1.3*	21.5±1.5* -9%vs initial
HR (1/min)	Initial	Stimulation
Control	284±11	297±15 +4.6%vs initial
Dx	243±10*	250±16* +2.9% vs initial
LVEDP (mm Hg)	Initial	Stimulation
Control	4.9±0.29	6.1±0.55 +24.5%vs initial
Dx	12.5±0.87*	17.7±1.26* +41.6% vs initial

Note: * - p<0.05 vs control

The obtained results describe 3 important features:

First of all, inotropic response of the heart in the doxorubicin disorder is compromised. Unlike the control heart, it is manifested by the positive inotropic effect, being given by the increased value of left ventricular systolic pressure (LVSP) at the peak of stimulation by 25.1%, but in the Dx group it has been reduced by 9.1% that denotes the negative inotropic effect. As a result, cardiac output increased in case of positive inotropic effect with the ratio of 15.7%, but decreased cardiac output (CO) compared with initial value – by 9% that is characteristic for negative inotropic effect.

Secondly, ET-1 has increased the diastolic rigidity in both groups, fact that explains the imminent effect of this oligopeptide for increasing the concentration of calcium in cardiomyocyte, but unlike catecholamines it doesn't stimulate analogical lusitropy function of the myocardium. However, in the group of Dx the value of left ventricular enddiastolic pressure (LVEDP) has increased more at the peak of stimulation by about 70% compared with the control one: 41.6% vs 24.5%.

Thirdly, for the action of ET-1 it is not characteristic the notable chronotropic effect, but heart rate (HR) increased up to 4.6% from the initial values, both in the control and the Dx groups.

The detrimental effects of ET-1 have been manifested especially in the context of adaptive processes of the heart in the condition of volume and resistance effort, because both tests require from the heart the engaging of intrinsic mechanisms of contraction and relaxation capacity.

The premedication of the isolated heart with ET-1 has reduced the increasing rate of CO in the condition of increasing left atrial pressure up to 25 cm H_2O (tab. 3).

Table 3

ET-1 effect on the adaptive capacity of the isolated heart in the condition of effort with volume overload

Indices	Effort with volume overload without ET-1 (n=9)		Effort with volume overload preceded by ET-1 (n=9)	
	Control	**Dx**	**Control**	**Dx**
CO, ml/min	54.4±3.6 +47.43%	32.5±2.9 +37.71% p<0.01	51.3±4.7 +39.02%	28.6±2.6 +21.19% p<0.001
LVEDP, mm Hg	6.8±0.52 +38.78%	19.3±1.83 +54.4% p<0.01	7.5±0.72 +53.06%	24.8±2.44 +98.4% p<0.001

Note: +% – relative increment vs initial index; p – significance vs control

At effort test with volume overload of LV, the cardiac output and LVEDP are the main important parameters that estimate functional feasibility of the heart for adaptive processes. According to the physiologic entity of the test with volume overload, the values of these indices are increased in both groups. So, CO has increased in the control group by 47.43%, but in the group of Dx the rate of rise was lower – 37.71%. On the other hand, elevation of control LVEDP came to 38.78%, but in doxorubicin affection it was considerably more – 54.4%, that indicates the depreciated diastolic relaxation capacity.

In case of premedication with ET-1 the heart response was limited, especially in the group with Dx. The increasing rate of CO has been reduced by 43.8% (from 37.71 up to 21.19%), while in the control group it decreased only by 17.73% (from 47.43% up to 39.02%). This phenomenon is determined by the more pronounced alteration of diastole by ET-1 in the Dx group, because LVEDP increased by 98.4%, while in the control group the increasing of this index was 53.06%. As a result, the absolute mean value of LVEDP in doxorubicin disorder has become above 3.3 times greater than that from the control group (24.8 vs 7.5 mm Hg).

Premedication of the heart with ET-1 has compromised essentially the adaptive ability in the condition of increasing peripheral resistance by elevation pressure in aorta up to 120 mm Hg (tab. 4).

Table 4

Effect of ET-1 on the isolated heart adaptation in the condition of effort with resistance

Indices	Effort with resistance without ET-1		Effort with resistance preceded by ET-1	
	Control (n=9)	Dx (n=9)	Control (n=9)	Dx (n=9)
LVSP, mm Hg	176.4±11.3 +22.9%	126.3±11.5 +20.4% p<0.05	172.8±14.7 +20.42%	111.5±10.6 +6.29% p<0.001
CO, ml/min	29.2±2.4 -21.92%	16.3±1.2 -30.93% p<0.01	25.4±2.23 -32.08%	11.4±1.22 -51.7% p<0.001

Note: +% – relative increment vs initial index; p – significance vs control

The increasing rate of LVSP in the condition of effort with resistance without ET-1 premedication didn't differ considerably in both groups (from 20.4% in Dx up to 22.9% in the control group), although the recoil of absolute index compared with the control one was considerable, 28.4% (126.3±11.5 vs 176.4±11.3 mm Hg).

When the test with effort and resistance was reproduced under the action of ET-1, the contraction capacity of the myocardium in the Dx group reduced more considerably. So, the increasing rate of LVSP in the control group depreciated from 22.9% to 20.4%, but in the Dx group – from 20.42% to 6.29%. In this context it is mentioned that the difference of absolute value of LVSP increased from 28.4% up to 35.47%.

The disturbance of heart contractility under the action of ET-1 in the Dx group manifested by decreasing much more significantly CO at elevation of pressure in aorta. So, depreciation of CO in the control group was 52.08%, but in doxorubicin disorder – 51.7%. As a result, the cardiac output in the Dx group was depreciated by 55.12% compared with control index.

Therefore, the ET-1 action has manifested the negative inotropic effect in doxorubicin disorder of the heart and has been imposed by the exhaustion of adaptive capacity of the myocardium in the condition of effort with volume and resistance.

Premedication with ET-1 depreciated more notably the heart tolerance in the Dx group compared to the control one, at the action of ischemia (30 min), as so reperfusion (45 min), attested by the evaluation of enddiastolic pressure of LV (tab. 5).

The LVEDP value at 30 min action of ischemia has increased by 21.2% in the Dx group, in case the premedication of isovolumic heart with ET-1. It is important to mention that in the control group the elevation of LVEDP in similar condition constituted only 11.8%. So, ET-1 increased the

Table 5

Value LVEDP of isovolumic heart in ischemia and reperfusion

Group	Ischemia (min)		Reperfusion (min)	
	30	Deviation ET-1 (%)	45	Deviation ET-1 (%)
Control (n=9)	41.7±2.3		14.9±1.1	
ET-1 + control (n=9)	46.6±3.6 p1<0.05	+11.8%	20.2±1.8 p1<0.05	+35.6%
Dx (n=9)	63.7±4.4 p2<0.05		22.8±1.4 p2<0.05	
ET-1 + Dx (n=9)	77.2±6.9 p1<0.05 p2<0.01	+21.2%	35.7±2.7 p1<0.05 p2<0.01	+56.6%

Note: p1 – significant vs index before ET-1 action; p2 – significant vs control

difference of LVEDP between the Dx and the control groups from 52.76% up to 65.67% that was appreciated at 30 min of ischemia action. ET-1 affects the functional recovery of myocardium after reperfusion in both groups, being given elevation of LVEDP. However, the rising of this index was greater in the Dx group: 56.6 vs 35.6%. As a result, the value LVEDP estimated at 45 min of reperfusion increased in the Dx group compared with the control one by 76.73% (initial increment constituted 53.02%).

Discussion

The repeated action of doxorubicin in cumulative dose of 14 mg/kg during 14 days leads, in our study, to the myocardial contractility disturbance, especially in effort test with resistance, manifested by increasing decline of cardiac output and systolic pressure of LV compared with the control group. Impairment of pumping function of LV explains the phenomena of doxorubicin cardiotoxicity.

The recent reported data have shown that Dx can induce sarcopenia caused by disturbed synthesis of sarcomere contractile proteins, and concomitantly with detrimental effect on ATP synthesis, it can be a serious cause of functional incompetence of heart for adaptation to hemodynamic and neuroendocrine efforts [16, 17]. The action of ET-1 on the isolated heart perfused in physiologic regime has manifested in the group with Dx by decreasing of systolic pressure of left ventricle and of cardiac output at the peak of stimulation, that from pathophysiologic point of view is described as negative inotropic effect.

Endothelin-1 (ET-1), as angiotensin II (Ang II) or adrenergic agonists, is one of the important natural stimulators of myocardial inotropy, which in the control group has been imposed by elevation of LVSP, followed by increasing of aortic jet velocity and cardiac output. The negative inotropic effect specific for ET-1 can be recorded as notable pathogenetic mechanism of onset and exacerbation of

heart failure in patients with doxorubicin cardiomyopathy, because hemodynamic effort or homeostasis disturbances (e.g. hypoxia, ischemia, oxidative stress, augmentation of immune-inflammatory response, hyperglycemia, acidosis, etc.) lead to excessive realizing of ET-1.

A. Luu et al. (2018) consider that limitation of endothelial dysfunction is a conclusive benefit in doxorubicin disorder of the myocardium, due to limitation of the factors that stimulate realizing of ET-1, or by the factors that decrease detrimental effect of it, such as nitric oxide, prostacyclin, antioxidant enzymes, etc [18]. In pathophysiology of doxorubicin cardiomyopathy, endothelin 1 is viewed as neuroendocrine factor with mitogenic properties, such as growth factor, prooxidant, proinflammatory, etc.

Through the mechanisms of negative inotropy of ET-1in Dx affection can be underlined the coronaroconstriction action superior to reactivity of the intact heart, demonstrated in recent researches [19]. The diminished coronary functional reserve increases energy depletion and cardiomyocyte overloading with calcium that is detrimental to diastolic relaxation and realizing of Starling law. The effect of cardiomyocyte overload with calcium, specific for doxorubicin cardiotoxicity has been demonstrated by us through decreasing of myocardial tolerance to the ischemia-reperfusion impact [13].

It is remarkable that premedication of the isolated heart with ET-1 even 30 sec before the hemodynamic effort, limited the adaptive-compensatory capacity, in this way underlining the role of diastolic relaxation disturbance in heterometric regulation of heart in volume overload, as the role of compromised myocardial inotropy in homeometric regulation of heart in resistance overload.

The obtained data indicate the key mechanisms of diastolic and systolic disturbance in doxorubicin disorder, these being determined by significant decreasing the velocity of relaxation(-dP/dTmax) and isovolumetric contraction (+dP/dTmax) of the heart. The functional component of these phases is orchestrated mainly by the energetic potential, necessary for adequate turnover of the calcium cations, and it is dominant in the intrinsic system of the heart, such as: (1) increasing cardiac output in heart overload with volume by the formation of filling pressure for realizing Starling mechanism; (2) reaching the "mechanical stress", by the accumulated kinetic energy, sufficiently to overcome increased peripheral resistance in effort with resistance.

In estimation of functional severity and prognostic of heart failure, the values, such as isovolumic relaxation time and isovolumic contraction time of the heart are used as variables for echocardiographic appreciation of *Tei* index, considered as an index of precocious evaluation of cardiac dysfunction based on reduced ejection time of LV, due to increasing relaxation time and isovolumic contraction time, or decreasing values +dP/dTmax and -dP/dTmax [20].

W. Border et al. (2020) consider in this context that echocardiographic indices of isovolumic relaxation and contraction of the heart are important functional predictors of precocious heart disorders in oncologic children who take anthracycline [21]. Thus, the effort tests which underline the functional dysregulation of isovolumic relaxation and contraction of the heart, which are absent in rest condition, have a great diagnostic value in doxorubicin cardiotoxicity.

The drugs that modulate activity of ET-1 (nonspecific blockers of receptors ETA/ETB and inhibitory of ET-1 conversion enzyme) are used in the treatment of cardiac failure, and especially in pulmonary arterial hypertension, in which pathogenesis of the ET-1 has crucial role, promoting the von Euler reflex [22, 23]. So, it is a great benefit for modulation the activity of ET-1 in patients who administrate doxorubicin, especially due to the fact that the level of circulated ET-1 elevates with increasing cumulative dose of Dx and directly correlates with severity of injury and cardiac dysfunction [24].

Conceptually the ET-1 effect is important for reducing myocardial tolerance under the action of ischemia-reperfusion syndrome. The impact of this is imposed by cardiomyocyte overload with calcium and excessive realizing of oxygen free radicals. The premedication of the heart with ET-1 potentiated these mechanisms and in such a way limited the capacity of intrinsic system of the myocardium to diminish impact of calcium overload and oxidative stress. It is important to mention, in this context, that myocardial ischemia increases the level of ET-1 realized by interstitial granules and expression of vasoconstrictor receptors [25]. On the other hand, cardiotoxicity of Dx also is imposed by the increasing ET-1 expression and disturbance of coronary reactivity depending on the endothelium [13]. Another mechanism is determined by the decreasing expression of NO under the action of ET-1, that compromises myocardial resistance to ischemic reperfusion impact [26].

Conclusions

Cardiotoxicity of doxorubicin is characterized by significant decreasing of contraction and relaxation velocities of left ventricle, as well as by negative inotropy effect under the ET-1 action *in vitro*, which disturbs heterometric and homeometric cardiac regulation, and myocardial tolerance to ischemia and reperfusion.

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LT reviewed the scientific literature, designed the study, interpreted the data, performed the analytical part of the laboratory work and interpreted the data.

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Particularities of ensuring with medicines the patients with rare diseases in the Republic of Moldova

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Abstract

Background: To ensure rare disease patients with medicines is a challenge for the health system. Treatments for rare diseases are very expensive, so the main financial burden for providing patients with medicines lies with the state. The objective of the study was to identify the aspects related to the optimization of process of assurance with medicines the patients with rare diseases.
Material and methods: Retrospective study was performed using systemic analysis methods. Data were collected and processed with reference to health programs in the field of rare diseases and centralized public procurement.
Results: The mechanism by which "rare" patients are provided with medicines and major problems related to was analyzed. The National Program "Combating rare diseases" represents the first stage by identifying the rare diseases that most frequently affect patients in the Republic of Moldova. Treatment options and annual medicines requirements are set in accordance with national clinical protocols and international guidelines recommendations, the number of patients on the doctor's records, as well as statistical data.
Conclusions: Major problems in ensuring medicines to patients with rare diseases have been identified. The need of medicines is ensured by centralized public procurement, from the financial resources of the state budget, which were analyzed for the period of years 2018-2020.
Key words: rare diseases, health programs, public procurement.

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Introduction

Rare diseases, by definition, affect few people, but have a major impact on public health, being highlighted by the lack of relevant treatments or their cost-inefficiency, becoming financially unviable for health domain [1, 2]. However, the health care of all patients is a value element of the right to health protection, being so a fundamental principle in a modern state. [3]. Thus, the problem of medical and pharmaceutical assistance for people suffering from rare diseases has become one of the most difficult in the modern medicine. In these circumstances, rare diseases are recognized as an important issue in the field of public health and in the budget allocation in healthcare. So, over time, the need to ensure an equitable access to patients' treatment, regardless of the prevalence of the disease, has become a fundamental premise in creating policies and strategies supporting the multilateral development of the field [4-7].

Material and methods

The objective of the study was to identify the aspects related to the optimization of process of assurance with medicines the patients with rare diseases in the Republic of Moldova. For data collection, a retrospective study using systemic analysis methods was performed. Data were collected and processed with reference to health programs in the field of rare diseases and centralized public procurement. The National Program "Combating Rare Diseases" was identified and analyzed according to the following criteria: rare diseases included, medicines selected for the treatment of patients, the need of medicines for the years 2018-2020 and procurement through tenders.

Results and discussion

Organizing medical and pharmaceutical assistance for patients with rare diseases in the Republic of Moldova remains difficult because of the lack of a patient's registry, standards for the diagnosis and treatment of patients and inaccessibility of medicines. Therefore, many patients suffering from rare diseases cannot receive the necessary care. Also, it should be pointed out that medicines for the treatment of rare diseases are very expensive and the main financial burden for providing patients with the necessary medicines lies with the state.

The State guarantees the health protection of citizens, regardless of gender, age, presence of diseases, conditions, property and official status, the residence and other circumstances. Under the program of "State guarantees", citizens

are provided with free healthcare, according to the list of diseases, whose treatment is reimbursed by the state.

The obligation to provide the rare disease patients with costless medicines is assigned to the component entities of the State, which are authorized to organize the medical care provided by the legislation of the Republic of Moldova for certain categories of citizens. They facilitate the accessibility of medicines to citizens and organize the supply of medicines included in the lists and registers of people entitled to receive state assistance as provided by law.

National programs are an organized set of activities and services, established by law in order to prevent and treat diseases with serious consequences for the health of population and, in some cases, with increased epidemiological risk. Prophylactic activities and the specific treatment of these diseases are financed from the state budget.

The national programs are designed, implemented and coordinated by the Ministry of Health, Labor and Social Protection. Their objectives are established by the same institution together with the National Health Insurance Company, representatives of professional scientific medical associations, university clinics, research units and others.

The national program for rare diseases could solve many problems of the targeted patients. In this context, the Government of the Republic of Moldova adopted Decision no 636 of December 11, 2019, on the approval of the Government Action Plan for the years 2020-2023, in which to the part no VI "Social protection and health protection", was included the point 6.13 "Intensification of measures to prevent communicable and non-communicable diseases", listing the development and approval of a new National Program for Rare Diseases, to be completed by September 2021 [8].

Likewise, in 2019, the Ministry of Health, Labor and Social Protection ordered the list of rare diseases, current for the Republic of Moldova.

At present, there are 12 health programs at the national level, including the National Program "Combating Rare Diseases", which aim to significantly improve the quality of care for "rare" patients and to solve the rare disease problems in the Republic of Moldova. In order to carry out the health programs, the Ministry of Health, Labor and Social Protection together with the Center for Centralized Health Procurement organize tenders for the procurement of specific medicines and sanitary materials for consumption in hospitals and outpatient, in compliance with legal provisions on public procurement.

The National Program "Combating Rare Diseases" has been running for several years. The treatment of rare diseases began to be partially reimbursed from the budget of national programs in 2012. Then, 2 million 291 thousand lei were allocated for patients diagnosed with Wilson-Konovalov, cystic fibrosis, phenylketonuria and pituitary insufficiency diseases. In the subsequent years, the treatment for patients suffering from ß-thalassemia, early puberty, bullous epidermolysis and juvenile arthritis was also reimbursed. The volume of financial sources, allocated for the rehabilitation of the patients, also increased to over seven million lei in 2017 and 12 million lei in 2018. In 2020, for the diagnosis and treatment of rare diseases was planned over 39 million lei from the state budget, which is 15 million lei more compared to the amount provided for 2019 [9-10].

In 2020, the National Program "Combating Rare Diseases" includes 15 rare diseases, such as Wilson-Konovalov, phenylketonuria, juvenile arthritis, ß-thalassemia, hemophilia, pituitary insufficiency, early puberty, bullous epidermolysis, epilepsy, diabetes insipidus, Addison's disease, nonspecific ulcerative colitis / Crohn's disease, pulmonary hypertension, Duchenne muscular dystrophy and infectious diseases as cholera, malaria, toxoplasmosis.

Table 1

Medicines introduced in the National Program "Combating Rare Diseases" and selected according to the approved national clinical protocols

No	National clinical protocol	Recommended medicines to be used in the treatment of the rare disease	The presence of medicine in the list of essential medicines
1.	PCN-7 Idiopathic juvenile arthritis [11]	Tocilizumab, 80 mg/4 ml, vial	Section 2.4. Antirheumatics
2.	PCN-243 ß-thalassemia in children [12]	Deferoxamine, 500 mg, lyophilized powder	Section 4.2. Non-specific medicines used in intoxications
3.	PCN-108 Hemophilia in children [13]	Coagulation factor VIII, 500 IU, lyophilized powder	Section 11. Blood products and plasma substitutes
		Coagulation factor IX, 500 IU, lyophilized powder	
4.	PCN-191 Hemophilia in adults [14]	Coagulation factor VIII, 1000 IU, lyophilized powder	
		Coagulation factor IX, 1000 IU, lyophilized powder	
5.	PCN-290 Epilepsy in adults [15]	Levetiracetam, 500 mg, tablets	-
		Levetiracetam, 250 mg, tablets	-
6.	PCN-258 Wilson's disease in children [16]	D-Penicillamine, tablets	-
		Zinc Sulphate, tablets	-

Clear and detailed scales are needed to make clinical decisions about diagnosing and treating such diseases. These are implemented by elaboration of clinical protocols that have a double beneficial effect: on the one hand it gives patients a quality and optimal standard, and on the other hand, it gives the doctor protection in the decision-making process. The national clinical protocols are developed on the basis of international guidelines based on evidence of clinical and economic efficacy.

The list of rare diseases included in the National Program "Combating Rare Diseases" contains only six diseases for which national clinical protocols are developed, this representing the clinical basis in the process of establishing the diagnosis and choosing the treatment. Approved clinical protocols were consulted in the following rare diseases (tab. 1).

Also, in table 1 it is highlighted that the medicines selected for the listed diseases, except Levetiracetam, D-Penicillamine and Zinc Sulphate, are included in the list of essential medicines, approved by the Order of the Ministry of Health of the Republic of Moldova No 162 of April 23, 2007 and amended by the Order of the Ministry of Health of the Republic of Moldova No 144 of February 28, 2011, which ensures the access to healthcare [17, 18].

In other cases, international guidelines and protocols were consulted to determine the medication. So, the list of medicines for the treatment of patients with rare diseases is elaborated according to the national clinical protocols and international guidelines and protocols, and the need for medicines to be purchased is calculated based on statistical data on the number of "rare" patients registered. Thus, in accordance with the provisions of the Order of the Ministry of Health, Labor and Social Protection No 948 of August 10, 2018 on the organization of centralized procurement, the Ministry of Health, Labor and Social Protection submits to the Center for Centralized Health Procurement the need of medicines for the implementation of National Programs and treatment of rare diseases, according to the established diseases list.

According to the comparative analysis of the results of tenders organized for the procurement of medicines within the National Program "Combating rare diseases", for the years 2018 - 2020 (the first 5 months of the year), the following data were obtained:

1. For 50 patients with Wilson's disease, during the analyzed period, constant quantities of medicines according to the protocol were purchased;

2. 123 patients with phenylketonuria were provided with increasing amounts of nutrients free of phenylalanine and low in protein. Thus, in 2020, phenylalanine-free protein substitutes were purchased 3 times as much as in 2018, low protein pasta and flour 4 times and, respectively, 6 times as much as in 2018. In 2020, 4 new positions of nutrients were purchased (tab. 2).

3. Medicines for the treatment of juvenile arthritis, hemophilia in children, pituitary insufficiency, early puberty and diabetes insipidus were purchased annually in unchanged quantities or slightly fluctuating quantities as in the case of bullous epidermolysis;

Table 2

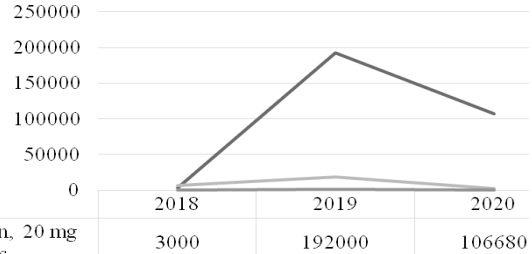
Nutrients purchased for patients with phenylketonuria

No	Nutrients	Contracted quantity (kg)		
		2018	2019	2020
1.	Phenylalanine-free substitutes with different protein content	436.5	986.0	1344.0
2.	Low protein flour	327.0	1890.0	2262.0
3.	Low protein pasta	500.0	2160.0	2145.0
4.	Low protein rice	-	-	900.0
5.	Milk substitute, low protein drink	-	-	260.0
6.	Low protein biscuits	-	-	1296.0
7.	Low protein egg substitute (powder)	-	-	162.0

4. In the case of thalassemia, there is a considerable decrease of about 800% in the amount of Deferoxamine purchased and a decrease of about 600% in the amount of Levetiracetam purchased in the years 2019-2020, for the treatment of patients with epilepsy;

5. In adult hemophilia, coagulation factor VIII was achieved in 2019 1.5 times as much as in 2020, and the quantity of coagulation factor IX – decreased by 2 times;

6. Medicines for the treatment of Addison's disease were purchased in quantities that differ from year to year, with a maximum in 2019 (fig. 1);



	2018	2019	2020
Hydrocortison, 20 mg tablets	3000	192000	106680
Fludrocortison, 100 mcg tablets	7000	18800	1800
Hydrocortison, injectable suspension 25 mg/5 ml, ampoules	0	1075	0

Fig. 1. Medicines purchased for the treatment of patients with Addison's disease, 2018-2020

7. Larger quantities of medicines were purchased for the treatment of pulmonary hypertension in 2020 compared to 2019, which is explained by the increase in the number of patients;

8. The biologic medicine Golimumab (300 pre-filled syringes for 30 patients with nonspecific ulcerative colitis) was purchased for the first time in 2020, as well as medicines for patients with Duchenne muscular dystrophy and for the treatment of infectious diseases such as cholera, malaria and toxoplasmosis.

The procurement of medicines within the program is done through tenders, announced by the order of the Ministry of Health, Labor and Social Protection. The Center for Centralized Health Procurement organizes public ten-

ders in order to conclude a state contract for the supply of medicines as a part of the implementation of the decision. The evaluation of the offers and the award of the contract is done by each position of medicine at the lowest price without VAT.

According to the results of the tenders published on the website of the Center for Centralized Health Procurement, for the concluded contracts, the following amounts were allocated: for 2018 year – 5 million 895 thousand lei, for 2019 – 5 million 194 thousand lei, and for 2020 – 28 million 678 thousand lei (calculated for the first 5 months of the year).

In the treatment of rare diseases are used both medicines that have the status of orphans and authorized medicines, without orphan designations, but which have included in the indications a rare disease.

Following this aspect, it was performed a comparative analysis of the list of medicines for the treatment of rare diseases, authorized in the European Union and the list of medicines proposed for the treatment of "rare" patients in the Republic of Moldova. Therefore, the medicines included in the National Program "Combating rare diseases", which are also found in the European list, such as tocilizumab, coagulation factor VIII and IX, somatropin, levetiracetam, golimumab, sildenafil, bosentan, iloprost and hydrocortisone were highlighted.

Conclusions

To facilitate the access to orphan medicinal products or medicines with rare disease indications and to build an infrastructure with all the necessary elements to support affected patients, it is necessary to develop, adopt and implement strategies and policies at national level, which, from the experience of other states, have positive results. In the Republic of Moldova, the State guarantees the protection of the health of patients with rare diseases through the National Program "Combating Rare Diseases". Patients suffering from the rare diseases included in the list of diseases of the Program and who are registered by a specialist, receive free medicines, reimbursed from the state budget, within the limit of purchased quantity.

However, during the study, major problems were stressed in the process of providing medicines to patients with rare diseases, such as:

– The lack of necessary medicines in the list of current rare diseases, as well as the absence of a common register of patients affected by rare diseases, making it impossible to analyze priorities in selecting diseases for inclusion in the National Program, which could so deprive other patients of free treatment;

– The absence of national clinical protocols for diseases included in the National Program, which aim to provide patients with qualitative and optimal treatment;

– The list of essential medicines that need regular updating and the assessment of the possibility of including several medicines for the treatment of rare diseases, thus ensuring safe access to care with effective and harmless medicines.

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EZ conceptualized the idea, collected the data, wrote the manuscript, revised and approved the final text.

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The influence of entomological preparations on oxidative stress in subacute inflammation

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Abstract

Background: It was found out that the development of oxidative stress in the inflammatory processes is determined by the action of harmful factors, as well as by the activity of leukocytes, macrophages, monocytes with the production of reactive oxygen species. Preparations of entomological origin have revealed antioxidant effect in various pathological processes. Therefore, in the present study, we determine the influence of imuheptin and imupurin on the evolution of oxidative stress parameters during subacute inflammation.
Material and methods: Subacute inflammation was induced in 40 rats. Imupurin, imuheptin and dexamethasone were administered daily for seven days. Malondialdehyde (MDA), total antioxidant activity (TAA) superoxide dismutase (SOD) activity, pro-oxidant antioxidant balance (PAB), native and total thiols in the serum were measured on the 7th day. One-way ANOVA followed by Bonferroni's post-hoc comparisons tests were performed.
Results: Imuheptin produced non-essential reduction of MDA (15.9±2.4 μM/L), native thiol (84.1±18.04 μM/L) level and a tendency to increase SOD (1033.6±171.4 u/c) activity, compared to the control group ($p>0.05$). Imupurine decreased MDA (14.6±2.0 μM/L), total thiol (85.9±14.7 μM/L) and native thiol (78.36±12.4 μM/L), also restored SOD activity (1117.6±103.7 u/c), increased TAA (0.41 ± 0.02 mM/L, $p<0.05$) compared with the control group. PAB was more influenced by imuheptin (325.82 ± 57.82 HK) than imupurin (340.14 ± 37.09 HK).
Conclusions: Imupurine and imuheptin have shown a tendency to reduce the intensity of free-radical generation from membrane lipids and to restore antioxidant capacity.
Key words: imupurin, imuheptin, malondialdehyde, superoxide dismutase, total antioxidant activity, thiol.

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Introduction

Inflammation is a natural defense mechanism against pathogens and it is associated with many pathogenic diseases such, as microbial and viral infections, exposure to allergens, radiation and toxic chemicals, autoimmune and chronic diseases, obesity, consumption of alcohol, tobacco use etc. The acute inflammatory response is started by immune cells enabling secretion of various cytokines and chemokines in order to recruit immune cells, macrophages, neutrophils. Neutrophils are the first to adhere to endothelial cells, and they begin to migrate across the vascular wall and also secrete vasoactive and pro-inflammatory mediators. Most of the early vascular changes observed in acute inflammation are due to inflammatory mediators that are released by inflammatory cells at the site of injury. These mediators, including histamine, platelet-activating factors, bradykinin, and thrombin, increase vascular permeability followed by edema and leukocyte extravasation. Accumulation of activated macrophages at the site of injury is the characteristic feature of inflammatory diseases. There are two distinct populations of macrophages: classically activated macrophages, produce excessive oxidative stress and secrete pro-inflammatory cytokines TNFα, IL-1, and IL-6, which contribute to tissue injury by releasing large amounts of highly reactive cytotoxic oxidants to destroy pathogens and alternatively activated macrophages secreting anti-inflammatory cytokines IL-4, IL-10, and IL-13 which suppress inflammation and help in wound resolution by phagocytizing dead neutrophils and synthesizing molecules that are responsible for tissue remodeling. Inflammatory process in many diseases linked with higher production of reactive oxygen species (ROS) induces oxidative stress and reduces cellular antioxidant capacity. Intensive research into the mechanisms of inflammation in the last decade has described the complicated relationship between oxidative stress and inflammation. ROS are key signaling molecules that play an important role in the progression of inflammatory disorders [1-3]. An enhanced ROS generation by polymorphonuclear neutrophils (PMNs) at the site of inflammation causes endothelial dysfunction and tissue injury. The vascular endothelium plays an important role in passage of macromolecules and inflammatory cells from the blood to tissue. Under the inflammatory conditions, oxidative stress produced by PMNs leads to the opening of inter-endothelial junctions and promotes the migration of inflammatory cells across the endothelial barrier [4]. ROS are generated as by-products of cellular metabolism through the electron transport chain in mitochondria as well as via the cytochrome P450. The other major source, where ROS are not produced

as by-products, are the NADPH oxidases that are present in a variety of cells, especially the phagocytes and endothelial cells, which are central to the genesis of the inflammatory response. Overproduced free radicals react with cell membrane fatty acids and proteins impairing their function permanently. Damage of oxidative stress, such as oxidized proteins, glycated products, and lipid peroxidation results in degeneration of cell membrane and tissue. In addition, free radicals can lead to mutation and DNA damage that can be a predisposing factor for many disorders. Furthermore, free radicals are generally too reactive and have a half-life too short to allow direct measurement in cells, tissues, or body fluids. Because molecular products formed from the reaction of free radicals with biomolecules are generally considered more stable than free radicals themselves, most commonly, free radicals have been tracked by measuring stable metabolite concentrations of their oxidation target products, such as malondialdehyde (MDA) etc. To prevent the damaging effects of oxidants, cells have evolved an array of antioxidant defense systems that function to remove ROS. The antioxidant enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), peroxiredoxins, and thioredoxins are classified as ROS scavengers. While it is clear that ROS are important to the pathogenesis of inflammation and tissue injury, much remains to be learned about how ROS function physiologically and how they contribute to the mechanism of inflammation and tissue injury [5-7].

Material and methods

Anti-inflammatory activity was evaluated in felt pellet-induced granuloma formation. 11 rats were left as the intact group (no manipulations). The others were divided into four groups (n=10). After shaving the fur, the rats were anesthetized and two 26±1 mg of sterile felt pellets were surgically inserted in the groin region. The right pellet was impregnated with Freund's adjuvant. Imuheptin (500 mg/kg, p.o.), imupurin (500 mg/kg, p.o.), dexamethasone (2.5 mg/kg, intraperitoneally) or saline (0.9% NaCl) solution (intraperitoneally) were administered for 7 consecutive days from the day of the felt pellet implantation. The animals were anesthetized on the 8th day and the pellets were extracted together with the granulation tissue formed, blood was collected for biochemical investigations. The level of malondialdehyde (DAM), the content of native and total thiol, the total antioxidant activity (AAT), the activity of superoxide dismutase (SOD) and pro-oxidant antioxidant balance were determined in serum.

Albino rats (160–250 g) were purchased from the Animal House of *Nicolae Testemitanu* State University of Medicine and Pharmacy. The animals were allowed standard access to food and water. Rats were housed at room temperature under conditions of 12 h of light and 12 h of the dark. The experimental procedures involving rats were approved by the Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy.

The entomological preparations obtained from insects of the order *Lepidoptera*, the genus *Lymantria* at the pupal stage (imupurin) and at the egg and pupae stage (imuheptin) were produced by Arena Group SA, Romania. Dexamethasone was purchased from KRKA d.d., Slovenia.

Oxidative stress assessments were performed in the Biochemistry Scientific Laboratory of *Nicolae Testemițanu* State University of Medicine and Pharmacy.

The level of malondialdehyde (MDA) was dosed according to the method described by Galaktionova L.P. et al. (1998) [8] with amendments [9, 10]. The method is based on the spectrophotometric determination of the trimetinic colored complex formed from the MDA interaction with thiobarbituric acid. The DAM content was calculated based on the molar absorption coefficient $\Sigma=1.56 \cdot 10^5$ mol·cm^{-1} and was expressed in μmol/L blood serum.

The determination of total and native serum thiol was performed according to the method described by Erel O. and Neselioglu D. [11] with modifications [9, 10]. In the first phase the amount of native thiol groups was measured after the addition of formaldehyde. For total thiol assay, disulfide bonds were reduced with NaBH4 to free thiol groups, the unused reductant remnants were completely removed by formaldehyde. Mercaptoethanol solutions were used as calibrators.

The determination of total antioxidant activity (TAA) by the ABTS method was performed according to the method described by Re R., et al. [12] with modifications [9, 10]. Antioxidant capacity was estimated in terms of radical scavenging activity using the pre-formed radical monocation of 2.2-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$). Scavenging of the ABTS radical was monitored by measuring the decrease in absorbance at 734 nm. Trolox® was used for the calibration of the method. Inhibition of absorbance versus Trolox® concentration curve was used to express the plasma antioxidant capacity in Trolox equivalent (μmol Trolox® equiv./l).

Superoxide dismutase (SOD) **activity** was estimated according to the method described by Galaktionova L.P., et al. [8] with modifications [9, 10]. The method is based on inhibition of the nitroblue tetrazolium reduction (NBT) in the system containing phenazine methosulfate and NADH under the action of SOD. The degree of inhibition depends on the activity of the enzyme. The activity of the enzyme is expressed in conventional units. The amount of enzyme required for 50% inhibition of the NBT reduction is taken as the unit of SOD activity. Enzyme activity is related to 1L of blood serum.

The determination of the pro-oxidant-antioxidant balance (PAB) was estimated according to the method described by Alamdari DH. et al. [13] with modifications [9, 10]. The assay is based on 3,3',5,5'-tetramethylbenzidine and its cation, used as a redox indicator participating in two simultaneous reactions. PAB is expressed in arbitrary units calculated based on the standard curve and in Hamidi-Koliakos units (HK) based on the percentage of hydrogen peroxide evaluated in the standard solution.

Statistical analysis. The results were presented as mean

Table 1

The influence of entomological preparations on the parameters of the oxidative and antioxidant system in serum in subacute inflammation

Parameters	DAM, µM/L	TAA, ABTS mM/l Trolox® equiv./l	SOD, u/c	Total thiol, µM/L	Native thiol µM/L	PAB, HK units
1. Intact animals (no manipulations)	15.2±2.2	039±0.01 P_{1-5}<0.05	1064.9±170.1	84.19±9.4	76.12±9.03	303.4±49.57
2. Control saline (0.9% NaCl) solution	16.8±1.3 P_{1-2}>0.05	0.395 ±0.02 P_{1-2}>0.05	999.1±115.9 P_{1-2}>0.05	93.97±12.75 P_{1-2}>0.05	90.13±14.11 P_{1-2}>0.05	348.63± 43.79 P_{1-2}>0.05
3. Dexamethasone 2.5 mg/kg, 7 days	16.1±1.0 P_{1-3}>0.05 P_{2-3}>0.05	0.397 ±0.01 P_{1-3}>0.05 P_{2-3}>0.05	1120.4±123.5 P_{1-3}>0.05 P_{2-3}>0.05	96.5±8.1 P_{1-3}>0.05 P_{2-3}>0.05	82.52±8.05 P_{1-3}>0.05 P_{2-3}>0.05	216.92± 28.54 $P_{3-1,3,4,5}$<0.05
4. Imuheptin 500 mg/kg, 7 days	15.9±2.4 P_{1-4}>0.05 P_{2-4}>0.05 P_{3-4}>0.05	0.39±0.01 P_{1-4}>0,05 P_{2-4}>0,05 P_{3-4}>0,05	1033.6±171.4 P_{1-4}>0.05 P_{2-4}>0.05 P_{3-4}>0.05	94.3±19.2 P_{1-4}>0.05 P_{2-4}>0.05 P_{3-4}>0.05	84.1±18.04 P_{1-4}>0.05 P_{2-4}>0.05 P_{3-4}>0.05	325.82± 57.24 P_{1-4}>0.05 P_{2-4}>0.05 P_{3-4}>0.05
5. Imupurin 500 mg/kg, 7 days	14.6±2.0 P_{1-5}>0.05 P_{2-5}>0.05 P_{3-5}>0.05	0.415± 0.02 P_{1-5}<0.05 P_{2-5}<0.05 P_{3-5}>0.05	1117.6±103.7 P_{1-5}>0.05 P_{2-5}>0.05 P_{3-5}>0.05	85.9±14.7 P_{1-5}>0.05 P_{2-5}>0.05 P_{3-5}>0.05	78.36±12.4 P_{1-5}>0.05 P_{2-5}>0.05 P_{3-5}>0.05	340.14± 37.09 P_{1-5}>0.05 P_{2-5}>0.05 P_{3-5}>0.05

± standard deviation (± SD). Statistical significance of the differences was evaluated using one-way ANOVA with Bonferroni post hoc testing. The difference was considered statistically significant when P<0.05.

Results

The animals from the control group with subchronic inflammation expresed an imbalance between the prooxidant and antioxidant system revealed by increasing the DAM level from 15.2 ± 2.2 µM/L to 16.8 ± 1.3 µM/L (P_{1-2}> 0.05), total thiol from 84.19 ± 9.4 µM/L to 93.97 ± 12.7 µM / L (P_{1-2}> 0.05), native thiol from 76.12 ± 9.03 µM/L to 90.13 ± 14,11 µM/L (P_{1-2}> 0.05) and prooxidant-antioxidant balance (PAB) from 303.4 ± 49.57 HK to 348. 63 ± 43.79 HK (P_{1-2}> 0.05). At the same time, the TAA did not change significantly – 0.39 ± 0.01 mM/L vs. 0.40 ± 0.02 mM/L, P_{1-2}> 0.05, but the SOD activity decreased from 1064.9 ± 170.1 u/c. up to 999.1 ± 115.9 u/c. (P_{1-2}> 0.05). Dexamethasone resulted in an insignificant reduction in DAM, a significant reduction in PAB (216.92 ± 28.54 HK) and a restoration of SOD activity (Table 1). Imuheptin after 7 days of administration decreased the DAM level from 16.8 ± 1.3 µM/L to 15.9 ± 2.4 µM/L (P_{2-4}> 0.05) and PAB from 348.63 ± 43.79 HK to 325.82 ± 57.82 HK (P_{2-4}> 0.05). The entomological preparation contributed to the increase of SOD activity from 999.1 ± 115.9 u/c. up to 1033.6 ± 171.4 c.u. (P_{2-4}> 0.05) and total thiol from 93.97 ± 12.75 µM/L to 94.3 ± 19.2 µM/L (P_{2-4}> 0.05). Imupurin caused a more pronounced decrease in DAM level (14.6 ± 2.0 µM/L) and increased SOD activity (1117.6 ± 103.7 u/c.), but had less influence on PAB (340.14 ± 37.09 HK) compared to imuheptin. The total and native thiol in the serum of rats given imupurine practically did not change compared to the intact group (tab. 1).

Discussion

Inflammatory process, linked with higher production of ROS, induces oxidative stress and reduces cellular antioxidant capacity. This study revealed that dexamethasone had decreased lipid peroxidation and products, such as MDA and restore antioxidant enzyme (SOD) activity. It was shown in previous studies that dexamethasone significantly decreased formation of the inflammatory exudates and weight of granulation tissue, increased the percent of neutrophils, decreased leucocites and lymphocyte number and TNF-alpha, IL-1-beta, IL-6 levels [14].

Glucocorticoids play an important role in regulating the inflammatory and immune response by acting on almost all types of immune cells. Glucocorticoids can regulate the phenotype, survival, and functions of monocytes and macrophages; exhibit anti-apoptotic effects promoting the survival of anti-inflammatory macrophages; improve the phagocytic activity of macrophages; stimulate the clearance of neutrophils; inhibit the release of various pro-inflammatory mediators (cytokines, chemokines etc.) and ROS; can regulate the maturation, survival, and migration toward the lymph nodes and motility of dendritic cells. Corticosteroids inhibit transcription factors that control synthesis of pro-inflammatory mediators, including macrophages, eosi-

nophils, lymphocytes, mast cells, and dendritic cells. Another important effect is inhibition of phospholipase A2, which is responsible for production of multiple inflammatory mediators. Glucocorticoids inhibit genes responsible for expression of cyclooxygenase-2, inducible nitric oxide synthase, and pro-inflammatory cytokines, including tumor necrosis factor alpha and various interleukins. In contrast, corticosteroids initiate upregulation of lipocortin and of annexin A1, a protein that reduces prostaglandin and leukotriene synthesis and that also inhibits cyclooxygenase-2 activity and reduces neutrophil migration to inflammatory sites [15, 16].

Eicosanoids, which control many complex physiological and immunological functions in vertebrates and invertebrates, may be involved in the balance of the prooxidant and antioxidant system of insects. It was studied the influence of eicosanoids inhibitors, such as dexamethasone (0.001%), esculetin (0.001%) and phenidone (0.1%) on oxidative stress parameters (DAM level and glutathione S-transferase (GST) activity) in *Galleria mellonella* larvae on artificial diets containing 0.05% xanthotoxin (XA) for 2 days and supplemented with these inhibitors in concentrations mentioned above. Treating larvae of *G. mellonella* with XA induced lipid peroxidation as evident from the increased content of malondialdehyde (MDA) and antioxidative enzymatic response in a dose-dependent manner. Relative to control, eicosanoid biosynthesis inhibitors (EBIs) – esculetin, dexamethasone and phenidone also resulted in impaired MDA content and antioxidant enzyme activities. MDA and antioxidant enzymes – SOD, GST and glutathione peroxidase (GPx) activities exhibited an incremental increase while catalase (CAT) activity was decreased in the experimental larvae that had been reared on media amended with esculetin, dexamethasone and phenidone and then challenged with standard XA dose. This oxidative stress was associated with elicited antioxidative responses by increasing SOD, GST and GPx and decreasing CAT activities in hemolymph. From these findings it can be deduced that eicosanoids mediate the antioxidant enzymatic responses of insects to food pro-oxidants [17].

Lepidoptera, one of the most widespread and widely recognizable insect orders in the world is characterized by the life cycle of eggs, larvae, pupae, adults with a high content of lipids, proteins, carbohydrates, antioxidants, essential and non-essential amino acids [18-20]. The content at each stage of development depends largely on the feeding of the larvae with the assimilation of vegetal compounds with their subsequent transfer to pupae, adults and eggs. The larvae use the primary metabolites (carbohydrates, lipids, proteins) that participate in nutrition and essential metabolic processes, such as growth, development or reproduction, and the secondary metabolites, such as alkaloids, terpenoids and phenolic compounds. Phenolic compounds consist of flavonoids, tannins and phenolic acids and contain several phenolic rings (polyphenols) with several hydroxyl groups. The digestive tract of larvae ensures the assimilation of nutrients and the transformation of secondary vegetal compounds in various chemical and metabolic processes. During the pupae stage, the larval structures of the insect are slowly broken down, while adult structures (such as wings) are formed. Different types of flavones and flavonol glycosides have been detected in the pupae of butterflies, and these compounds are subsequently transferred to the wings of adult butterflies. Pupae are inactive and many *lepidopterans* produce a cocoon from fibrous protein, i.e., silk, as well as secondary compounds (alkaloids, flavonoids, amino acids, catechins, quercetin, etc.). Flavonoids can protect the cocoon from radiation or increase its antioxidant capacity. *Bombix mori* cocoon extracts have shown antimicrobial activity that is probably due to antimicrobial peptides. Most compounds are also detected in eggs with a protective role against the environment and possible predators [21-24]. Antioxidant defense components protect insects from oxidative stress by scavenging ROS. There were investigated the effects of organophosphorus insecticide, malathion in different concentrations on the activity of SOD and acetylcholinesterase (AChE), glutathione level (GSH) and DAM as biomarkers of oxidative stress in *Galleria mellonella* larvae. The diet with the lowest concentration of malathion (0.01 ppm) did not significantly influence the DAM content and AChE activity, but at 1.0 ppm caused a significant increase in DAM levels, decreased AChE and SOD activity and decreased GSH content [25]. It was examined the effect of long-term exposure to environmentally relevant concentrations of dietary fluoranthene (6.7 and 67 ng / g dry food weight) on defense mechanisms of the *Lymantria dispar*. The activities and expression of isoforms of SOD and CAT, the activities of GST and glutathione reductase (GR), and GSH were determined in the whole midgut and midgut tissue, while SOD and CAT activities were assessed in hemolymph of the larvae. It was shown significantly increased activity of SOD in the whole midgut and midgut tissue, also increased CAT activity in midgut tissue. Significantly decreased SOD activity and increased CAT activity in hemolymph of *L. dispar* larvae were recorded. The tissue-specific responses of enzymes to dietary fluoranthene enabled the larvae to overcome the pollutant induced oxidative stress [26]. CAT, Cu/ZnSOD and Mn-SOD, found in different tissues of *Lymantria dispar* larvae play an important role in the protection against oxidative stress of the environment. When treated with the pesticide avermectin in sublethal doses, a higher expression of CAT and Cu/Zn-SOD was found after 2 hours and of Mn-SOD after 6 hours. The cuticulas transcribed Cu/ZnSOD mRNA and Mn-SOD mRNA significantly higher than other parts of insect body after spraying avermectin of sublethal concentration. The results suggested that CAT and SOD are important antioxidant enzymes for defense against pesticide-induced stress in *Lymantria dispar*, and Cu/ZnSOD isoform has a faster and stronger response [27, 28].

Methanolic extracts from the *Chrysomya albiceps, Lucilia sericata* and *Musca domestica* larvae exhibited a higher antioxidant activity than water extracts, revealed by free radicals scavenging and increasing total antioxidant activity [29].

The products of some insects (bees, wasps, etc.), such as royal jelly, propolis are studied and widely used in traditional medicine, and recent research has shown that they contain a wide range of compounds with multiple pharmacological effects, including antioxidant. Experimental studies have shown that the antioxidant activity of royal jelly is due to increased GSH levels, reduced lipid peroxidation, free radical generation and DAM production, increased concentration of antioxidant enzymes – SOD, CAT, glutathione reductase (GR) and GPx. Royal jelly administration in radiation-induced lung and liver damage reduced oxidative stress and increased antioxidant properties, decreased nitric oxide (NO) and ROS, increased SOD activity and glutathione levels. Hydroxyl radicals and hydrogen-peroxide scavenging activity were verified with 29 antioxidant peptides isolated from RJ hydrolysate, in which 12 small peptides having 2–4 residues (Ala-Lys, Phe-Arg, Ile-Arg, Lys-Phe, Lys-Leu, Lys-Tyr, Arg-Tyr, Tyr-Asp, Tyr-Tyr, Leu-Asn-Arg, and Lys-Asn-Tyr-Pro) had the strongest activity. Moreover, three dipeptides (Lys-Tyr, Arg-Tyr, and Tyr-Tyr) in RJ indicate strong scavenging activity due to a donation of the hydrogen atom from their phenolic hydroxyl group [30].

Phenolic compounds (flavonoids and phenolic acids), substances that express the ability to scavenge free radicals, are mainly responsible for the antioxidant capacity of bee products. Flavonoids are plant derivatives with a polyphenolic structure comprising several subgroups, such as flavones, flavonols, flavanones flavanonols, flavanols (catechins), anthocyanins and chalcones, as well as isoflavones and neoflavonoids. The presence of phenol groups in flavonoid molecules gives them antiradical activity. Phenolic acids are compounds that possess carboxylic groups and phenol, which determine their antioxidant activities, including the prevention of oxidation and generation of oxygen species, as well as the chelation of prooxidant metals. Non-phenolic compounds also can be responsible for the antioxidant capacity of propolis. It was noted antioxidant activity exhibited by hydroxy dicarboxylic fatty acids with 8–12 carbon atoms in the chain and their derivatives. 10-hydroxydecanoic acid, 10-hydroxy-2-decenoic acid and sebacic acid were identified in royal jelly [31].

Terpenes are another class of compounds that have antioxidant action. Triterpenes have been described to act as free radicals scavengers (superoxide anion, hydroxyl radical), also they prevent lipid peroxidation and modify the activity of antioxidant enzymes (SOD, CAT, GPx) [32]. Alpha- and beta-amyrins, non-phenolic compounds found in propolis, belong to triterpenoids and revealed antiapoptotic, antioxidant, anti-inflammatory and antifibrotic, gastro- and hepatoprotective effects [31].

Tenebrio molitor larvae have been consumed worldwide for their nutritional value, which includes high protein, mineral, and unsaturated fatty acids, and were officially registered as a new food ingredient in Korea in 2015 [33, 34]. In previous studies, analyses of their functional properties revealed that *Tenebrio molitor* larvae ethanol (EtOH) extract inhibited the expression of TNF-α and IL-6 in the inflammation-induced RAW 264.7 cell line and had a DPPH radical scavenging ability similar to that of blueberry extract [33, 35].

Sericin is a globular protein from silk fiber, it protects against unfavorable factors and the environment. The antioxidant potential of the sericin is related to its high content of amino acids with hydroxyl groups (mostly serine), which act as chelators [36]. Sericin protein consists of 18 different amino acids, most of them are characterized as polar and the differences observed among the authors regarding the amino acid proportion is due to the method of sericin extraction. Nevertheless, all authors agreed that serine was the most abundant amino acid, followed by aspartic acid and glycine [37]. Among the antioxidant mechanism of the sericin, the inhibition of the tyrosinase activity has been widely studied. Several studies have reported anti-tyrosinase activity of sericin from cocoons belonging to different strains and from different extraction methods as well. The hydrolyzed sericin had increased anti-tyrosinase activity and some authors suggest that the reason behind it is the metal-chelating ability. This effect is due to the presence of a high content of amino acids with hydroxyl groups, such as serine, asparagine and threonine acting as chelators [33, 36]. Likewise, biopeptides of sericin, obtained by enzymatic hydrolysis, exhibited a higher antioxidant potential compared to non-hydrolyzed sericin [37]. It was shown in several studies that active compounds, such as the essential amino acids arginine, histidine, lysine, methionine, cysteine and proteins have an antioxidant capacity, revealed by the ability to reduce iron, scavenging of oxygen and hydroxyl free radicals, as well as hydrogen peroxide scavenging activities. It was concluded that amino acids and proteins inhibit lipid oxidation by biologically designed mechanisms (antioxidant enzymes and iron-binding proteins) or by non-specific mechanisms [38-40].

Conclusions

The analysis of the results of our study allowed us to conclude that Freund's adjuvant produced an inflammatory process with an increase in the level of inflammation mediators, induced an imbalance between the pro- and antioxidant system; dexamethasone diminished the inflammatory process by simultaneously attenuating the formation of ROS and restoring the activity of antioxidant status; imuheptin reduced the level of DAM and increased SOD activity; imupurine decreased the level of DAM and thiol groups with the restoration of SOD and AAT activity. Entomological preparations showed antioxidant activity through their components: unsaturated fatty acids, proteins, peptides and amino acids (serine, arginine, histidine, lysine, methionine, cysteine, etc.); phenolic compounds (flavones, flavonols, flavanones flavanonols, flavanols (catechins), anthocyanins and chalcones, etc.), tannins (proanthocyanids, etc.), alkaloids, terpenoids; antioxidant enzymes (SOD, CAT, GPx, GST). Several mechanisms can be responsible

for antioxidant action of entomological preparations: the presence of hydroxyl groups, which confer the ability to stabilize unpaired electrons; protection of cells against lipid peroxidation; acting as hydrogen donor agents, singlet oxygen and superoxide radicals quencher; activating antioxidant enzymes and metal chelation; free radical scavenging (superoxide anion, hydroxyl radical); changes in the activity of antioxidant enzymes (SOD, CAT, GST, GPx) and the level of endogenous antioxidants (GSH).

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Author's contribution
IG designed the study, collected and interpreted the data, drafted the first manuscript.

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Ethics approval and consent to participate
The experimental procedures involving rats were approved by the Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 78 of 22.06.2015).

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Antimycotic activity of phenoxythiazolchloralum

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Abstract

Background: The therapeutic options in invasive candidiasis and aspergillosis are limited and don't provide expected results. Introducing a new drug in the therapeutic practice can improve the quality of life of immunocompromised patients. The aim of this research is to study the antimycotic activity of new substance phenoxithiazolchloralum (MF-0010) on *Aspergillus* spp., *Candida albicans*, and *Saccharomyces cerevisiae.*
Material and methods: Phenoxythiazolchoralum has been kindly offered by the Institute of Chemistry. The standards were offered by *Nicolae Testemitanu* State University of Medicine and Pharmacy. Antifungal activity of the phenoxythiazolchloralum against *Aspergillus* spp. was evaluated by microdilution method. The successive double dilution method was used for determination of *in vitro* susceptibility against *Candida albicans*, and *Saccharomyces cerevisiae.*
Results: For the first time, it was studied *in vitro* susceptibility of MF-0010 against *Aspergillus* spp., *Candida albicans, Saccharomyces cerevisiae*. With at least 0.05 μM/ml difference of MF-0010 MIC value from standards, it can be considered quite more potent than ketoconazole and bifonazole against *Aspergillus fumigatus, Aspergillus versicolor, Aspergillus ochramensis.* The MIC/MCF ratios of MF-0010 are lower than nistatine ones with 0.08 μMol for both pathogens: *Candida albicans*, and *Saccharomyces cerevisiae.*
Conclusions: All analyzed pathogens were susceptible to MF-0010. According to experimental data on *Aspergillus* spp., the antimycotic activity of MF-0010 is quite better than the standards one. The MIC/MCF ratios showed *in vitro* significant susceptibility of MF-0010 against *Candida albicans.*
Key words: Phenoxythazolchloralum, *Aspergillus* spp., *Candida albicans, Saccharomyces cerevisiae*, drug discovery.

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Introduction

Patients with AIDS, organ transplants, cancer, diabetes, autoimmune disorders, biomedical-assist devices, or long-term antibiotic therapy are at major risk of developing mycosis [1, 2]. Reports of invasive aspergillosis and candidiasis in immunocompromised patients have been increasing [3, 4]. *Candida* spp. can affect almost all organ systems in the human body. The genome plasticity and high reproductive capacity constitute a serious risk to human health [5, 6]. Nistatine in one of the few topical drugs for the treatment of cutaneous mycosis caused by this pathogen [7]. Reports of resistance to antimycotic agents have been reported for several years [8]. Thus, every year it is more difficult to predict clinical success /failure and duration of antimycotic therapy. The therapeutic options are quite limited and don't provide expected results [9, 10]. The introduction of new drugs could help to manage this critical situation.

The computer-aided study of novel 5-aryl-2thio-1,3,4-oxadiazoles reported *in vitro* anti-tubercular activity of phenoxythiazolchloralum (MF-0010) (fig. 1) against *Mycobacterium tuberculosis* $H_{37}Rv$ [11]. Pharmacophore groups –NH-, =C=O, =N-N=, -Cl produce three-dimensional arrangements that are required for anti-tubercular activity [12].

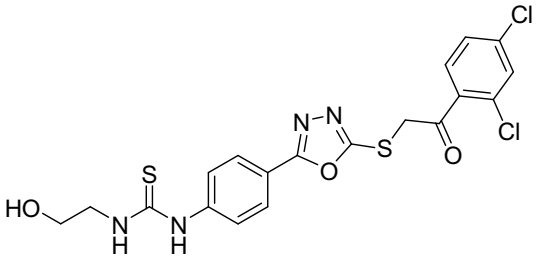

Fig. 1. The chemical structure of phenoxythiazolchloralum (MF-0010)

Acute toxicity tests reveal that MF-0010 in mice after intraperitoneal injection and intragastric administration for a single dose suggests that compound has minimal toxicity, and can be placed in the toxicity class 5 according to TG 423 Acute Toxic Class Method (OECD) [13]. Organoleptic characteristics, solubility, melting temperature and UV absorption spectra are described by O. Butescu [14]. It was observed that compound has poor solubility in polar solvents (water, methanol, ethanol, acetonitrile, acetone) and low bioavailability that limits the use as antitubercular agent. Following research of chemical structure showed that substance could have other biological activities. The

presence of the azole ring (pharmacophore group of azole antimycotic drug) suggests that the studied substance may have antimycotic activity.

The aim of this research is to study the antimycotic activity of MF-0010 on *Aspergillus* spp., *Candida albicans*, and *Saccharomyces cerevisiae.*

Material and methods

***Aspergillus* spp.**

For evaluating of the antifungal activity of the phenoxythiazolchloralum compound against *Aspergillus* spp. was used the microdilution method described by E. Stingaci et al. [15].

Candida albicans and Saccharomyces cerevisiae

For evaluating of the antifungal activity for *Candida albicans*, and *Saccharomyces cerevisiae* was used the successive double dilution method. For this, at the initial stage, 1 mL of Sabouraud broth for test fungus was introduced into a series of 10 tubes. Subsequently, 1 mL of the analyzed compound was dropped into the first test tube.

Then, the obtained mixture was pipetted, after that 1 mL of it was transferred to the next tube, so the procedure was repeated until the tube No 10 of the series. Thus, the concentration of the initial preparation decreased 2-fold in each subsequent tube.

At the same time, 24 hour test fungi of *Candida albicans* and *Saccharomyces cerevisiae* were prepared. Initially, suspensions of test fungi were prepared with optical densities (D.O.) of 0.5 according to the McFarland index. Subsequently, 1 mL of the obtained fungal suspension was dropped in a tube containing 9 mL of sterile distilled water. The content of the tube was mixed, after which 1 mL was transferred to the tube No 2 of the 5-tube series containing 9 mL of sterile distilled water.

From the 5-th tube of the series was taken 0.1 mL of the fungal suspension, which represents the seeded dose and added to each tube with titrated preparation. Subsequently, the tubes with titrated preparation and the seeded doses of the microorganisms were put in the thermostat at 35°C for 24 hours. On the second day, a preliminary analysis of the results was made. The last tube from the series in which no visible growth of microorganisms has been detected was considered to be the minimal inhibitory concentration (MIC) of the preparation.

For the estimation of the minimum fungicidal concentration (MFC), the contents of the test tubes with MIC and with higher concentrations were seeded on Sabouraud agar from Petri dishes with the use of the bacteriological loop. The seeded dishes were kept in the thermostat at 35°C for 24 hours. The concentration of preparation, which did not allow the growth of any colony of microorganisms, was considered to be the minimal fungicidal concentration of the compound [16].

Results

***Aspergillus* spp.**

For the first time, it was studied *in vitro* susceptibility of MF-0010 against *A. fumigatus, A. versicolor, A. ochramensis* and *A. niger.* The MIC and MFC values of MF-0010 against *Aspergillus* spp. ranged from 0.23 μM/ml – 0.62 μM/ml (fig. 2) and 0.62 μM/ml –1.24 μM/ml (fig. 3), respectively. The highest values of MIC and MCF of MF-0010 are related to *A. niger*. Thus, the use of MF-0010 against this pathogen is not appropriate.

The MIC values of MF-0010 on *A. fumigatus, A. versicolor, A. ochramensis* and *A. niger* are 0.23 μM/ml -0.31 μM/ml, which are lower than MICs of standards: ketoconazole (0.28 μM/ml-0,38 μM/ml) and bifonazole (0.32 μM/ml-0.48 μM/ml). With at least 0.05 μM/ml difference from standards, MF-0010 can be considered quite more potent than ketoconazole and bifonazole.

The MCF value of MF-0010 against *A. fumigatus, A. versicolor, A. ochramensis* and *A. niger* is 0.62 μM/ml, which is lower than MFC values of standards: ketoconazole – 0.94 μM/ml (exception is *A.ochramensis* – 0.38 μM/ml) and bifonazole – 0.64 μM/ml. With a 0.02 μM/ml difference from standards, MF-0010 has the same antimycotic activity as bifonazole, and better one than ketoconazole.

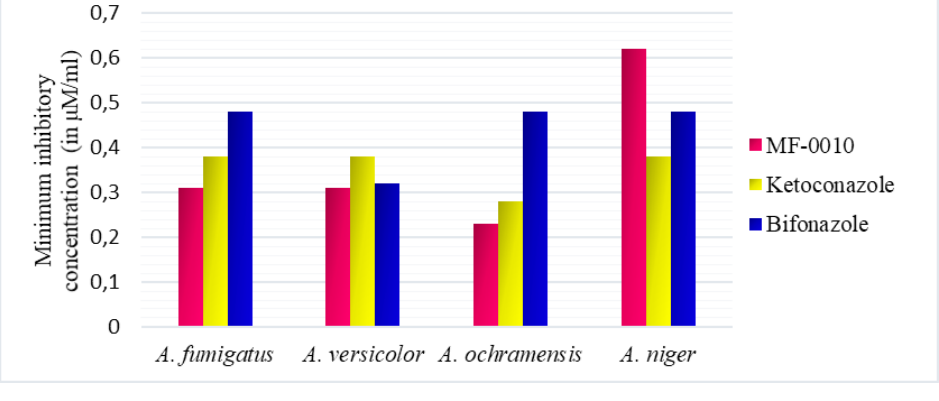

Fig. 2. The Minimum Inhibitory Concentration (MIC) of MF-0010, ketoconazole, bifonazole against *A. fumigatus, A. versicolor, A. ochramensis* and *A. niger*

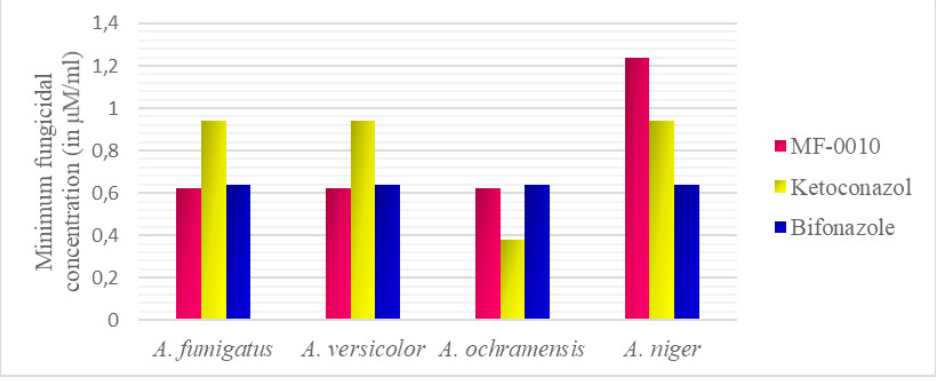

Fig. 3. The Minimum Fungicidal Concentration (MCF) of MF-0010, ketoconazole, bifonazole against *A. fumigatus, A. versicolor, A. ochramensis* and *A. niger*

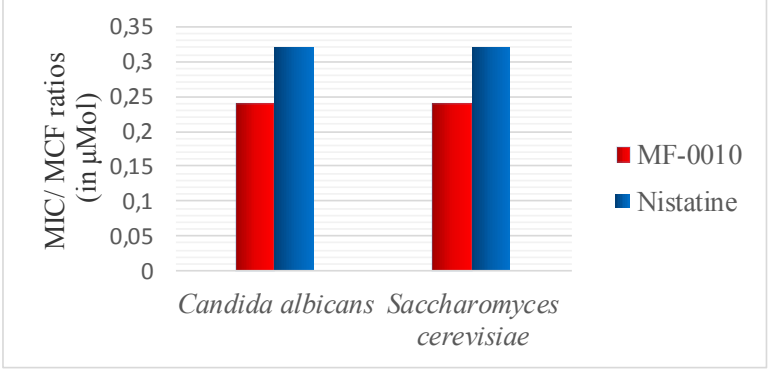

Fig. 4. MIC/MCF ratios of MF-0010, nistatine against *Candida albicans, Saccharomyces cerevisiae*

Candida albicans* and *Saccharomyces cerevisiae

For the first time, it was studied the *in vitro* inhibition potential of MF-0010 against *Candida albicans, Saccharomyces cerevisiae* (fig. 4).

The MIC/MCF ratios of MF-0010 for inhibition of *Candida albicans, Saccharomyces cerevisiae* are lower than nistatine ones with 0.08 µMol for both pathogens. Thus, we can conclude that MF-0010 is more potent active molecule than nistatine against *Candida albicans*. Further research is needed to create topical forms and confirm by clinical trial the pharmacological action, pharmacokinetic profile and safety of formulations with MF-0010. Several types of topical medication including creams, ointments, and pastes can be designed.

Conclusions

In this study, we found that all analyzed pathogens were susceptible to MF-0010. According to the experimental data, the antimycotic activity of MF-0010 is quite better than the standard one. The MIC and MCF values of MF-0010 show a good potency against *Candida albicans*, and new studies are warranted in order to design optimized formulations, to analyze *in vivo* the efficacy and quality assurance of formulations.

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Authors' contribution
AP interpreted the data, drafted the first manuscript. VV formulated the research hypothesis, revised the manuscript. SP synthetized MF-0010, performed the technological part of laboratory work. LL designed the study, conducted the laboratory work. AU performed the technological part of laboratory work. FM interpreted the data, revised the manuscript. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate
No approval was required for this study.

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No competing interests were disclosed.

REVIEW ARTICLE

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Diagnosis and management of ischemic stroke: time is critical

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Abstract

Background: It is predicted that stroke's incidence and impact will increase considerably over time. Proper management of stroke depends on a reliable and urgent diagnosis that includes patient / relative - emergency team - hospital chain, so the diagnosis begins with the recognition of the first signs of stroke. In order to act promptly in the acute period and subsequently assess the risk factors, the neurological service needs to be equipped with high-performance neuroimaging and clinical laboratory. The specific treatment of acute ischemic stroke is nowadays the reperfusion procedure, performed by thrombolytic therapy and, since 2015, by the endovascular treatment. Stroke is also a leading cause of severe long-term disability. The rehabilitation of post-stroke patients requires an interdisciplinary approach, in order to prevent recurrences, combat complications and reintegrate the patient into society.
Conclusions: Stroke remains one of the leading determinants of death and severe disability worldwide and the Republic of Moldova is not an exception. Considering the narrow window for recognition and administration of outcome-modifying treatment, the management of stroke focuses mainly on rapid reperfusion via intravenous thrombolysis and endovascular thrombectomy. The availability of this specialized treatment in the Stroke Unit could improve the patient's outcome and decrease the disability's level and economical burden. There is clear evidence that preventing a stroke is much more effective than treating it, so we should seize the opportunity and act involving not only the medical staff, but also the government, health decision makers, specialists in public health and international agencies.
Key words: ischemic stroke, pathogenesis, prevention, diagnosis, treatment.

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Introduction

Stroke is the second leading cause of death after heart diseases and the leading cause of death from neurological diseases. Stroke is estimated to become the leading cause of death worldwide by 2030, reaching approximately eight million deaths annually [1]. It is a serious problem, with socio-economic implications, because the surviving patients often remain with significant motor and cognitive sequelae, most of which are unable to resume their activity before the onset of the disease [2]. Like cardiovascular diseases, stroke is a suffering of elderly, but in the population of the Republic of Moldova it occurs at a much younger age. Many stroke patients do not have access to adequate, modern, highly specialized treatment and, as a result, the level of mortality at home from stroke in the Republic of Moldova is one of the highest in Europe and neighboring countries [1].

We searched in the PubMed Central and Google Scholar engines, in specialized books, guidelines and protocols, for the following keywords "ischemic stroke", "symptoms", "pathogenesis", "prevention", "diagnosis", "recovery" and "treatment". The materials were searched in English and Romanian.

At the same time, the experience and data gained within the Institute of Emergency Medicine, the Department of Neurology, Epileptology and Internal Diseases were included.

History of Stroke

The term "stroke", which is an acute event that leads to symptoms of neuronal dysfunction [3], is thought to have evolved from the ancient name "apoplexy", which also refers to a clinical concept characterized by rapid loss of consciousness and various manifestations of brain dysfunction. The concept of "apoplexy" was used to encompass various disorders, later identified as acute, vascular, and non-vascular cerebral events, as well as acute non-cerebral events [4, 5].

Since Hippocrates, or even earlier, many authors have dedicated their talent to studying "apoplexy." From Antiquity until the Renaissance, the definition was relatively stable, a concept for a wide range of conditions. The introduction in practice of autopsies in the Modern era has allowed the evolution of the concept of apoplexy. It was not until the mid-1600s that Jacob Wepfer discovered that patients who died of apoplexy had intracerebral hemorrhage or blockage of one of the cerebral blood vessels [6].

Medical science continued to study apoplexy, and in 1928 it was divided into etiological categories. This is how the term "stroke" came about. Stroke is often referred to as a "brain attack" due to the mechanism similar to a heart attack. The term "brain attack" also conveys to the general public a more urgent call for immediate action and emergency treatment.

Epidemiology. RES-Q Registry in the Republic of Moldova

Effective treatment of stroke exists, but the implementation of evidence-based treatment is limited. The main objective of the "Registry of Stroke Care Quality" (RES-Q) project is to improve the quality of healthcare provided to stroke patients by translating the data collected by RES-Q into effective health policies, both at national and European level, by collecting data over the course of one month, several years consecutively [7, 8]. The RES-Q registry was implemented in Moldova in 2016, when the pilot data collection of stroke patients in a single Stroke center took place, so that in February 2017 another 3 hospital centers would be co-opted [9], and in 2018 – other 11 from different regions of the Republic of Moldova. Thus, the number of patients admitted to the RES-Q increased from 251 in 2017 to 920 in 2019 [9,10]. In the Republic of Moldova there are only 3 Stroke Units (Institute of Neurology and Neurosurgery, Institute of Emergency Medicine, "Holy Trinity" Municipal Hospital), in which thrombolysis treatment is performed. Since 2018, thrombolysis treatment is performed in 3 more centers [10]. In 2018, the Institute of Emergency Medicine, and later, in 2019, the Institute of Neurology and Neurosurgery "Diomid Gherman" implemented the surgical method of treatment of ischemic stroke by thrombectomy.

The data obtained through the RES-Q registry were presented at the annual meetings organized by the European Stroke Organization (ESO). According to the latest report, presented in September 2019 at the Summit of member countries of the IRENE-COST program, under the auspices of ESO the distribution by stroke subtype in the Republic of Moldova was as follows: 66% – ischemic strokes, 13% – hemorrhagic strokes, including 2% – subarachnoid hemorrhages (SAH), 19% – strokes of undetermined origin. It is noteworthy that the high proportion of patients with stroke of undetermined origin, a phenomenon explained by the fact that 8 participating centers are not equipped with computer tomography (CT) / magnetic resonance imaging (MRI) devices. From the category of patients who underwent CT or MRI, only in 63% the investigation was performed within one hour of hospitalization. From the category of patients who suffered hemorrhagic stroke or SAH, only 25% underwent angio-CT examination [10].

In 2019 only 2.67% of patients received thrombolytic treatment and 0.49% of patients – thrombectomy, a phenomenon explained by the fact that recanalization treatment, at that time, was performed only in 3 centers in the Republic of Moldova [10]. 36.78% of patients benefited from rehabilitation measures during the treatment provided in inpatient conditions, the majority of patients with stroke being subsequently discharged at home (75%), only 5% of patients were transferred for rehabilitation purposes to another center, and 15% died (we notice a decrease in the number of patients who died compared to previous years: 23% of patients – in 2017, 17% of patients – in 2018) [10-12].

RES-Q allows the comparative analysis of the quality of healthcare at national and international level, and is a motivational tool to improve its quality. The participation in the project will allow the dynamic evaluation of results and will be an important decision factor on changing the strategy to combat vascular diseases in the country [8-10].

Stroke Risk Factors

Stroke is a heterogeneous syndrome that can occur due to many risk factors. The risk factors can be classified into modifiable and non-modifiable. Age, sex, race/ethnicity and family history of stroke are non-modifiable risk factors, while hypertension, diabetes, obesity, metabolic syndrome, atrial fibrillation, carotid artery atherosclerosis, smoking, diet and physical inactivity are the modifiable ones. Modifiable risk factors can be further subdivided into medical conditions and behavioral risk factors [13-15].

An international case-control study showed that 90% of all stroke cases are caused by 10 risk factors: hypertension, diabetes, cardiac causes, current smoking, abdominal obesity, hyperlipidemia, physical inactivity, alcohol consumption, diet and psychosocial stress/depression [16]. Risk factors can also be categorized as short-term triggers (e.g., infectious conditions, sepsis, stress), intermediate-term (e.g., hypertension and hyperlipidemia), and long-term triggers (e.g., sex and race) [13].

In the epidemiology of stroke, a new field of research has emerged that involves the determination of stroke triggers. Thus, one study reported that a recent hospitalization for an infection was associated with an increased risk of stroke [17]. Another study showed as well that severe sepsis is associated with *de novo* onset of atrial fibrillation, and thereby, with an increased risk of stroke [18]. Therefore, identifying a short-term condition with an increased risk of stroke af-

ter an acute infection might also have direct therapeutic implications. Other potential triggers of stroke include air pollution, which has been identified as a new risk factor for stroke [13].

There are also risk factors for stroke that are specific to a certain category of population, such as women. Differences in sex hormones, exogenous estrogens and pregnancy are considered unique risk factors for women [19].

Stroke is a devastating disease worldwide, but a better identification and understanding of the stroke risk factors is essential to improve primary and secondary stroke prevention and to reduce the consequences of stroke.

Etiology and Pathophysiology of Ischemic Stroke

Ischemic stroke develops through functional and anatomical damage of the brain tissue, leading to different degree of transient or permanent neurological damage [20].

The main mechanism of the ischemic stroke could be explained by the lack of blood supply of the brain tissue as consequence of several pathological processes that affect the cerebral and extracerebral vessels [21]. Although biochemical changers in ischemic brain are approximately similar, the etiological factors are different. Atherosclerotic and atherothrombotic stenotic vascular lesions of the extracranial cervical arteries and large basal cerebral arteries can cause critical hypoperfusions and distal high-grade stenoses. Intracranial vascular occlusions are produced by embolic mechanism, both arterio-arterial from atherothrombotic lesions and systemic emboli as well (from cardiac sources, such as valve prostheses, atrial fibrillation, intracardiac thrombi, dilated cardiomyopathies, recent myocardial infarction or shunts). Another factor is the small vessel lipohialinosis, which causes microangiopathic lacunar lesions [22, 23].

The most common cause of ischemic stroke is proven to be atherosclerosis of the large and small arteries (20%). Atherosclerosis of the proximal part of the aorta, is considered to be one of the sources of cerebral emboli. In large vessel atherosclerosis, stroke develops if the cerebral perfusion drops down, because of occlusive atherosclerotic stenoses and coexisting thrombosis or arterio-arterial embolism. Occlusive diseases of small penetrating vessels, such as micro-atheromas and lipohyalinosis, are main cause of small, subcortical infarcts in 25% ("lacunar stroke"). About 20% of ischemic strokes are caused by cardiogenic embolism induced by atrial fibrillation. Uncommon causes include cervical artery dissections, vasculitis or thrombosis secondary to coagulopathies, drug abuse, etc. (5%). In more than 30%, despite of a full assessment, the cause of strokes etiology remains to be undiscovered [24-26]. Thus, seeking of the pathogenetic cause of the stroke in some patients is quite difficult, but extremely important for choosing the optimal treatment and prevention strategy.

The degree of cerebral blood flow impairment and the time when cerebral perfusion was restored, define the severity of the neurological deficit caused by ischemic stroke. This could be explained by particularities of brain's metabolism which normally is constant and permanent, but demanding a continuous supply of glucose and oxygen [20, 21, 27]. Decreasing of blood flow from normal values (50 ml / 100g / min) to <10 ml / 100 g / min, triggers the ischemic cascade reaction, which comprises a series of biochemical reactions in the brain, which usually last for 2-3 hours, but they also can be extended for several days, even after normal blood flow is restored [28].

Acute oxygen distress, caused by blood flow drawback for more than 10 seconds, deprives neurons in the affected area of the ability to produce energy and triggers anaerobic pathway, with release of lactic acid. As consequence of that, the acid-base balance in the brain is disturbed, which causes the depolarization of the cell membrane with an influx of calcium and efflux of potassium ions. Elevated intracellular calcium triggers and the release of glutamate by stimulating the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) receptors lead to more calcium influx. All these processes lead to excess of intracellular calcium with cells overexcitation, activation of proteases and free radicals' releases due to the excitotoxicity process. Activated proteases and free radicals break down the cell membrane, promoting intracellular influx of other molecules and toxic substances. These processes affect the activity of mitochondria, and results in toxins and apoptotic factors release. Those are milestones of cell death, causing irreversible changes in brain tissue [21, 29].

At the early onset of the cerebral ischemia, we can differentiate few perfusion areas around ischemic nucleus, where blood flow is reduced, but maintained. This area is defined as penumbra. It suffers from hypoxia, but remains metabolically active, containing affected, but still viable brain tissue, blood supply being ensured through the collateral vessels. Exactly this region is the target of acute therapy [28, 30]. Important issue is that the penumbra is a dynamic, time-dependent area in which brain tissue will necrotize in a few hours or days due to poor perfusion and the cascade of biochemical events. Otherwise, if blood flow and oxygen supply are restored shortly after stroke onset, these cells can survive [23, 24].

Based on these biochemical processes and supporting MRI studies, the critical time of 4.5 hours for effective reperfusion was established. At the same time it is highlighted, that earlier blood flow restore will save more brain tissue. All this is the basis of the concept "time is brain" [26].

Symptoms of Stroke

Stroke syndromes are clinically presented as sudden onset neurological deficits. The symptoms depend on the affected region of the brain, which in turn is defined by the arterial anatomy involved. Although some features are more or less typical of hemorrhagic forms of stroke, as distinct from ischemic stroke, none are specific to allow clinical diagnosis of the stroke type. Therefore, in the acute phase of stroke, cerebral and neurovascular imaging is required. Common symptoms of stroke in the left hemisphere include aphasia, right hemiparesis and right hemianopia, and in the right hemisphere – left spatial hemineglect, left hemiparesis and left hemianopia. The majority (90%) of strokes are

supratentorial; as such, the public can be taught to recognize and act upon stroke using the acronym FAST, for facial droop, arm drop, speech disturbance and time. Posterior circulation or infratentorial stroke has a multitude of additional symptoms, including diplopia, bulbar palsies, dysphagia, unilateral dysmetria and incoordination, as well as reduced levels of consciousness. Stroke is typically painless. The most important historical feature of stroke is the suddenness of its onset. Identification of a stroke syndrome is relatively easy: sudden onset of acute neurologic symptoms, peaking within a few minutes, is deemed a stroke until proven otherwise. However, detailed diagnosis and management are highly dependent upon clinical assessment of the history and physical examination, because symptoms and signs vary tremendously according to the region of the brain that is affected [31].

Patients with acute ischemic stroke with larger infarct volumes have a higher risk of developing symptomatic intracranial hemorrhage and worse clinical outcome following intravenous thrombolysis [32, 33]. In addition to volume, the location of an infarct is linked to neurologic deficits. Some studies reported that ischemic infarcts in the insular ribbon, lentiform nucleus, and corona radiata are associated with poor prognosis in patients with stroke [34-36].

The Pathophysiological Mechanisms of Recovery

The degree of impairment depends on many factors, such as the extent of the infarct, the identity of the damaged region and the effectiveness of the early medical care. The functional status of stroke patients spontaneously improves over 6 months after onset. More specifically, rapid recovery is achieved during the first month [37].

The term brain plasticity defines all the modifications in the organization of neural components occurring in the central nervous system during the entire life span of an individual [38]. Such plastic phenomena involve particularly the perilesional tissue in the injured hemisphere, but also the contralateral hemisphere, subcortical and spinal regions. Functional improvement overlaps with motor learning in terms of underlying mechanisms [39]. Motor learning is associated with structural changes which we report below.

Neuroblasts usually originate from the subgranular zone in the dentate gyrus of the hippocampus and the subventricular zone. These migrated neuroblasts may replace injured neurons or glial cells, and help with remodeling and reorganization processes [40]. Newly formed blood vessels might help with augmenting nutrient supply and repair processes. Proangiogenic growth factors promote survival of the neuronal, glial and endothelial cells in the peri-infarct tissues, and transient neovascularization in the ischemic brain helps with the clearance of damaged tissues. Moreover, it may create a vascular niche for neuroblast migration [41]. Axonal sprouting is mainly driven by the balance between a growth-promoting status and reduction of growth-inhibitory environment. Axonal sprouting may alter cortical sensory or motor maps, and robust evidence exists to show that new connections are formed in peri-infarct cortex areas [42]. In humans, ipsilateral perilesional cortical activation including premotor or supplementary motor area is a common finding after primary motor cortex injury. Studies suggest that ipsilateral perilesional cortical activation is associated with functional recovery, at least in the acute period. In the recovery phase, the corresponding area in the contralateral cortex frequently shows coactivation. Therefore, a decrease in the activation in the contralateral cortex is observed in patients with better functional recovery. The underlying mechanisms of change in contralateral cortical activation share similar physiologic changes, such as unmasking of latent synapse, facilitation of alternating network, synaptic remodeling, and axonal sprouting [43].

Imaging

Non-contrast Computed Tomography

Nowadays, non-contrast CT remains the "golden standard" of imaging examination for the initial evaluation of patients with suspected stroke. The CT changes can be classified as: acute (less than 24 hours), subacute (24 hours to 5 days) and chronic (weeks) (Figure 1) [44]. Acute stroke represents cytotoxic edema that causes loss of the normal gray matter/white matter differentiation and effacement of the cortical sulci. A subacute stroke represents vasogenic edema, with greater mass effect, hypo-attenuation and well-defined margins. Mass effect and risk of herniation is the greatest at this stage. Chronic strokes have loss of brain tissue and are hypo-attenuating. A non-contrast head CT may identify the early signs of stroke, but most importantly will exclude intracerebral hemorrhage and stroke mimics, such as volume occupying lesion. Non-contrast CT is also used in the evaluation of acute intracranial hemorrhage as it produces good contrast between the high attenuating ("bright") clot and the low attenuating ("dark") cerebrospinal fluid (CSF) [45]. Non-contrast CT has also been used historically to exclude patients from receiving thrombolysis based on the extent of hypo-attenuation at presentation. This criterion has, however, been removed from the 2018 American Heart Association guidelines [46].

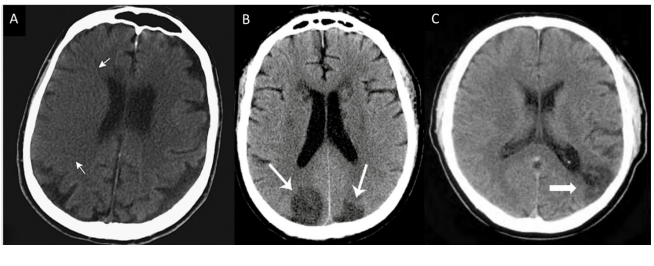

Fig. 1. Imagistic findings on brain CT in different stroke phases
Acute (A) Axial non-enhanced computer tomography demonstrates hypo-attenuating foci throughout the left-sided white matter (arrows) and sulcal effacement in the left middle cerebral artery (MCA) territory; subacute (B) "fogging effect" obtained at 36h with bilateral occipital hypodensities; chronic (C) encephalomalacia after left MCA stroke [47, 48]

Magnetic Resonance Imaging

Conventional brain MRI is not very good at detecting cytotoxic or intracellular edema that is seen in the acute or <24h phase of stroke. Standard MRI images (T1 and T2) can

help identify a subacute stroke. Fluid attenuated inversion recovery (FLAIR) sequences provide good sensitivity for acute subarachnoid hemorrhage, as compared to conventional T1 and T2 weighted images and are useful in the initial evaluation of the acute stroke patient suspected of having a subarachnoid hemorrhage. Subarachnoid hemorrhage appears bright on FLAIR images. The gradient recalled echo (GRE) sequence is also useful for the detection of blood products. Hypointensity due to paramagnetic effect of the hemosiderin, otherwise known as "blooming," affects the magnetic field and decreases the signal. Therefore, blood appears "black" on GRE images. MR diffusion is diffusion weighted images (DWI) and can be obtained within 10 minutes so the clinical determination of ischemic stroke can be confirmed quickly. DWI is used to detect early ischemic changes (acute stroke; early ischemic change; cytotoxic edema) with greater conspicuity than standard protocol. MRI with diffusion is quickly becoming the gold standard in acute stroke imaging. Once a hemorrhagic stroke has been excluded by CT, MR diffusion improves stroke detection from 50% to more than 95% [47-49]. Perfusion weighted imaging (PWI), just like CT perfusion, can identify the ischemic penumbra. The ischemic penumbra is the difference between the DWI defect (cytotoxic edema irreversible ischemia – the ischemic core) and the perfusion defect. The penumbra is the DWI/PWI mismatch. The accurate identification of this ischemic penumbra will help guide future ischemic stroke therapy and potentially aid in extending the time window for treatment [49].

Acute Treatment of Ischemic Stroke (Prehospital, Hospital, Thrombolysis, Stroke Unit)

Stroke is the main cause of long-term disability and one of the leading causes of death worldwide. About 31% of patients die during the first year, this index being higher in patients over 65 years. And concerning cost of care in the most European countries stroke has the leading position [50].

It is expected that the incidence and burden of stroke will increase considerably over time. The good news is that in the last decade was gained a substantial progress in the treatment of this devastating disease. Evidence-based treatments, aiming to restore blood flow to the affected area of the brain, such as intravenous thrombolysis and endovascular thrombectomy, show to improve functional abilities of the patients [51-54]. And the optimized operational algorithms allow to minimize "door-needle" time by rapid selection of patients, using detailed clinical evaluation as well as cerebral and vascular imaging [55-58].

A proper stroke management begins with the recognition of the first signs of the disease by the patient or family. People must be aware that "time is brain" and at first signs of a stroke to call immediately 112 for instant admission to the nearest Stroke Unit [59].

The ambulance team must be familiar with the signs of a stroke, they must perform initial assessment and stabilization of the patient and start initial management according to the protocol, hospital pre-notification is very important as well [50, 59]. Different hospitals have different stroke treatment programs; thus, time of symptoms onset is crucial point when the decision where patient will be referred to is taken [50, 60].

Presently, intravenous thrombolytic treatment with rtPA (recombinant tissue plasminogen activator) is the only approved pharmacological treatment in acute ischemic stroke. Intravenous thrombolysis is based on the concept that most ischemic strokes are of thrombotic or thromboembolic in origin. Thus, in order to benefit from thrombolytic treatment, patient is to fulfill all inclusion and exclusion criteria [59].

As it was mentioned above, recombinant tissue plasminogen activator (rtPA) was the only treatment approved for acute ischemic stroke. However, only 3-9% of patients with ischemic stroke benefit from rtPA [61-64], in part due to the narrow therapeutic window. In 2008, after publication of ECASS-3 study results, therapeutic window for rtPA was extended from 3 to 4.5 hours from symptoms onset, with additional exclusion criteria [65]. The extension of the therapeutic window led to 20% increase in the number of patients who profited from intravenous thrombolysis [66].

Subsequently, implementation in 2015 of endovascular treatment, allowed the extension of the intervention time to 7 hours from the onset of symptoms [67]. The positive result is defined by improving by 4 or more points of the neurological deficit according to the NIHSS scale. Much better functional results for severe ischemic strokes could be obtained by combination of the pharmacological thrombolysis and endovascular therapy, this strategy will also allow to get additional time for treatment [59].

Despite the risk of bleeding complications, patients with severe stroke treated with rtPA have better prognosis. The risk of intracerebral hemorrhage is linked with severity of the neurological deficit (measured by the NIHSS) and presence of cerebral edema on CT [68, 69].

But in reality, only 25% of stroke patients are admitted in a specialized medical facility in first 4.5 hours and less than 65% arrive within 8 hours of onset, what is behind therapeutic window [70]. In such cases, DWI MRI is recommended to accurately assess the penumbra area and viable brain tissue [71].

Approach to stroke management has undergone a lot of changes in recent years, with more patients receiving treatment avoiding long-term disability. A critical step forward has been the establishment of regional systems of care, which are capable rapidly to identify stroke patients and, using the decision support, to redirect them to appropriate centers with access to the state-of-the-art treatment. Modern medicine is constantly trying to improve the management of stroke patients. One of the options is telemedicine, which allows remote audio-visual connection between comprehensive stroke center and community hospitals [72]. This comprises detailed clinical and radiological evaluation with interpretation of these results by stroke expert, with further decision about patients' eligibility for intravenous thrombolytic and endovascular therapy.

Evaluation of patients with an acute stroke is also a good time for tracing of measures for secondary prevention.

Stroke constantly confirmed to be the second cause of

mortality worldwide [73]. Proper stroke management depends on accurate and rapid diagnosis. The Specialized Neurological Facilities should be equipped with neuroimaging techniques and a high-performance clinical laboratory. The treatment concept of cerebrovascular diseases is constantly evolving. It has already been proven that prompt response and effective treatment can protect large areas of brain tissue from irreversible damage. Also, the population must be aware that "time is brain" and with the first signs of stroke immediately call 112 for immediate admission to the nearest Stroke Unit.

Endovascular Treatment of Ischemic Stroke

The history of endovascular treatment of acute ischemic stroke began as early as the end of the last millennium. PROACT I and II trials investigated the effect of intra-arterial thrombolysis with pro-urokinase in the treatment of acute ischemic stroke [74, 75]. Pro-urokinase has not been approved by FDA (Food and Drug Administration) for the treatment of stroke, due to the lack of evidence.

After PROACT II, a new tool appeared – the MERCI retriever. This was the first instrument for mechanical thrombectomy (MT) approved by FDA. The effectiveness of this device was investigated in MERCI and MULTI MERCI trials [76, 77]. These studies showed good outcome of the treated patients, but the mortality was high. However, they proved the importance of early recanalization in patients with ischemic stroke, because the promptness of recanalization was shown to be related to good outcome.

Later, second-generation devices appeared, such as the Penumbra aspiration system that was studied in the Penumbra Pivotal Stroke Trial [78]. Compared to the MERCI device, Penumbra obtained higher recanalization rates, but good clinical outcome of patients (modified Rankin Score- mRS ≤ 2) was observed only in 1/4 with high rates of complications and all-cause mortality.

The third-generation devices are stent-retrievers. Solitaire and Trevo showed promising results in the rate of recanalization and good outcomes in SWIFT and TREVO trials [79, 80].

The short history of MT in stroke had ups and downs. In 2013, the results of 3 studies were published that did not provide sufficient evidence for this method. These were MR RESCUE, IMS III and SYNTHESIS. All these studies did not show better results compared to systemic intravenous thrombolysis [81-83].

But in 2015-2016 the picture changed. The results of several randomized clinical trials (MR CLEAN, ESCAPE, EXTEND-IA, SWIFT-PRIME, REVASCAT, THRACE) have been published, with promising results of endovascular treatment in patients with ischemic stroke: high degree of patient's independence at 3 months (mRS ≤ 2) and decreased mortality [57]. These trials avoided the bias of previous ones by standardizing the processes and selecting patients by detecting proximal occlusion at mandatory vascular imaging, emphasizing the importance of door-recanalization time and the use of new stent-retriever devices.

Another revolution came in 2018 with DAWN [54] and DEFUSE 3 [51] trials, which showed the efficacy of MT in patients with large vessel occlusion in the anterior circulation who were presented between 6 and 24 hours and between 6 and 16 hours, respectively, from the onset of stroke, using perfusion imaging. These studies have essentially replaced the "therapeutic window" with the "perfusion window".

Currently, endovascular therapy is the standard of treatment for patients with ischemic stroke caused by large vessel occlusion. Adequate selection of candidates and use of last generation devices give us good results in the rate of recanalization and good clinical outcome.

Stroke Prevention Strategies. Management of Stroke Risk Factors

The burden of stroke is of major importance for the global health [84], being the second leading cause of death and disability [85]. Currently, the global health system is facing a stroke pandemic, which mainly affects low and middle-income countries, where the incidence of strokes is constantly rising [86, 87]. The growing incidence of stroke and the persistence of alarmingly high rates in some parts of the world reflect significant gaps in the stroke prevention strategies [84]. The good part is that the individual risk of stroke can be reduced by about 80% [88] and the incidence of stroke by about 50% just by implementing lifestyle changes [89]. Therefore, there is an urgent need to increase the efforts towards the stroke prevention.

Currently, prevention is the main tool in the fight against stroke, whose purpose is to reduce the incidence of stroke by changing a single or several risk factors for stroke [13]. There are three levels of stroke prevention: primordial, primary and secondary prevention. The primordial prevention consists of implementation of general measures that are related to the healthy lifestyle and are applied at the population level. Primary prevention comprises the measures directed to improve the profile of cardiovascular risk factors that are present in people who have not suffered a stroke or a transient ischemic attack (TIA), in order to prevent a cerebrovascular event. Secondary prevention includes measures applied to people who have suffered a stroke or TIA, in order to prevent their recurrence [90, 91]. Primary and secondary prevention also cover the measures to control both behavioral and medical risk factors [13].

Strategies used to prevent stroke fall into 2 broad categories: strategies for people at high risk for cardiovascular diseases and population-based strategies [85]. Strategies for people at high risk for cardiovascular disease include interventions related to lifestyle changes and those related to pharmacological treatment. Lifestyle changes involve reduced salt intake, increased consumption of fruits and vegetables, physical activity, weight loss, smoking cessation, reduced alcohol consumption and management of psychosocial stress [85]. Regarding pharmacological interventions, in order to reduce the risk of cardiovascular and cerebrovascular diseases, the use of blood pressure and lipid lowering drugs together with antiplatelet agents has been suggested [85]. Both the measures related to lifestyle and pharmaco-

logical treatment have several limitations in their practical use: reduced ability to practically test the effectiveness of healthy lifestyle measures [85], practical difficulties in using cardiovascular risk scores, the need of laboratory tests for some of the risk scores, risk unawareness in rural areas, daily use of several drugs. But the major problem is that these measures are focused only on a certain category of population, failing to cover the majority of other populations [92] and even though several studies have shown their effectiveness, they are underused [88].

Population-based strategies comprise the entire population, with the aim of reducing cardiovascular risk [85]. They target several behavioral and lifestyle risk factors – tobacco use, unhealthy diet, physical inactivity, overweight, and alcohol abuse. Although these measures also prevent the risk of other diseases, such as heart diseases, lung diseases, diabetes, cancer, and dementia [85] and even though several studies have shown their effectiveness, unfortunately no country in the world has fully implemented these measures [88].

Stroke prevention is a complex medical and political issue but there is clear evidence that stroke prevention is effective and we need to seize the opportunity and act now [14, 93]. This health issue also requires the involvement and support of governments, health decision-makers, public health professionals and international agencies [84].

Post-Stroke Recovery and Rehabilitation

According to statistics, 10% of post-stroke patients recover almost completely, 25% have minimal functional impairment, 40% have moderate to severe functional impairment and need special care, 10% require special care at home or in long-term medical facilities, 15% die shortly after stroke, 14% of stroke survivors suffer repeated stroke in the first year [94]. Rehabilitation of post-stroke patients is a complex process that requires a multidisciplinary approach, consisting of neurologist, physiotherapist, social therapist, speech therapist, clinical psychologist, and other specialists, the main purpose being the application of contemporary recovery methods and techniques, preventing recurrences, combating complications and reintegrating the patient into society [95]. The objectives of neurorehabilitation are to minimize disability, increase the degree of functional independence, prevent recurrences and complications, help in social integration, increase the quality of life, reduce costs [96]. A crucial contribution to establishing the staging of rehabilitation by identifying deficiencies that cause disabilities is neurological assessment with a focus on functional testing, gait, balance, muscle strength, physiological and pathological reflexes, cognitive and psychoemotional status, and the degree of independence. It is also essential to use functional ability assessment scales, such as Barthel score, Rankin scale, ADL (Daily Living Activities) scale, FIM (functional independence measurement) scale, modified Ashworth spasticity scale, MRC (Medical Research Council) muscle strength scale, Mini Mental test for cognitive functions [97]. Cognitive and behavioral symptoms can be seen frequently in people who have had a stroke. Post-stroke depression is probably the most common emotional disorder and the most important long-term psychosocial consequence of a stroke, and has a negative effect on patients' functional outcome. As the functional status of patients improves rapidly in the early rehabilitation period, it is necessary to focus on the treatment of depression. Also, the support of family caregivers can improve the patient's psycho-emotional state, and later become a modifiable factor. Therefore, the rehabilitation team should take this into account and reflect this in planning a treatment program [98].

The patient's level of disability, concomitant illness, cognitive and mental functions can have a significant impact on both treatment decisions and outcomes. Psychosocial factors, such as the availability of support networks, financial resources, the patient's sense of control over the disease can affect the results and therefore must be evaluated. Stroke is a difficult living condition. Patients who have found an acceptable way to live with the disease are distinguished by the following: they dare to prepare for a more difficult future, they do not withdraw from the world of the healthy, they develop topics of interest other than stroke, they evolve towards a more conscious life, based on fundamental values [2].

Conclusions

Stroke remains one of the leading determinants of death and severe disability worldwide. It is a medical emergency with a narrow window for recognition and administration of outcome-modifying treatment in the emergency department, that's why the management of stroke focuses mainly on rapid reperfusion that can be achieved with intravenous thrombolysis and endovascular thrombectomy. Both methods reduce disability in patients after stroke but are time-critical. The key to maximizing the benefits of reperfusion therapies and to achieve good outcomes we need to improve our health system of care that will minimize the treatment delays and errors. For acute treatment of stroke, the intravenous thrombolysis will reduce disability only if it is administered within 4.5h of the onset of stroke. Thrombolysis also can benefit selected patients with evidence from perfusion imaging of salvageable brain tissue for up to 9h and in patients who have woken up stroke symptoms. The endovascular thrombectomy reduces disability in a broad group of patients with large vessel occlusion when performed within 6h of stroke onset and in patients selected by perfusion imaging up to 24h following stroke onset. Admission to specialized stroke units is associated with improved outcome in patients suffering from acute stroke.

Primary and secondary prevention of ischemic stroke has many common elements with cardiovascular risk management from other fields, including blood pressure control, cholesterol management and antithrombotic medications. Other preventative interventions are tailored to the mechanism of stroke, such as anticoagulation for atrial fibrillation and carotid endarterectomy for severe symptomatic carotid artery stenosis.

Most survivors of a stroke are left with chronic disability. Rehabilitation efforts during the initial three to six months after stroke should aim to maximize patients' physical, communicative, and cognitive functioning.

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SG conceptualized the project and designed the research; EZ, AB, AG, EM conducted literature review, EZ, AB, AG, EM, PL, DE, IC, DG, TB drafted the first manuscript; SG, EZ, AB, AG, EM revised the final version of the manuscript. All the authors approved the final version of the manuscript.

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Inflammatory glaucoma. Elements of etiology and pathology

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Abstract

Background: Inflammatory glaucoma, also known as uveitic glaucoma is a multifactorial process of inflammation that causes the rising of intraocular pressure (IOP) accompanied by morphological and physiological modifying similar to open angle glaucoma. The mechanisms by which ocular inflammation causes an increase of the IOP are not integrally comprehended, and many of the relevant pathways and features are still covered with a veil of mystery. The eyes of uveitic patients are defined by complex interactions between the angles (open or closed), trabecular outflow, fluctuations in the aqueous production and the response to steroids. Regarding the elevation of IOP in these individuals, it is mostly attributed to the increased outflow resistance, which distorts the equilibrium between aqueous production and outflow.

Conclusions: Inflammatory glaucoma is a multifactorial pathology that needs a careful diagnosis and therapeutical approach in order to obtain a good management of inflammatory process and intraocular pressure. There are a lot of pathological pathways that influence the evolution of inflammatory glaucoma and make treatment more difficult. Last years, glaucoma treatment has significantly improved, but to better understand pathogenesis of uveitic glaucoma more scientific studies are necessary.

Key words: inflammatory glaucoma, uveitic glaucoma, secondary glaucoma.

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Introduction

Inflammatory glaucoma, also known as uveitic glaucoma is a multifactorial process of inflammation that causes the rising of intraocular pressure accompanied by morphological and physiological modifying similar to open angle glaucoma. Glaucoma and uveitis were described together for the first time in 1813, by Joseph Beer. Later, in 1891, Priestley Smith proposed first modern classification of uveitic glaucoma [1].

The overall prevalence of glaucoma in eyes with uveitis varies from 10 to 20%, but it is much more common in chronic uveitis and can be as high as 46% [2]. , the prevalence of uveitis has been estimated at approximately 115 people per 100000 population. Approximately 20% of uveitis patients develop glaucoma. At internation scale, prevalence of uveitis has been estimated at 38-730 people per 100000 worldwide. Approximately 20% of uveitis patients develop glaucoma [3].

The mechanisms by which ocular inflammation causes an increase of the intraocular presure (IOP) are not integrally comprehended, and many of the relevant pathways and features are still covered with a veil of mystery. In contrast with primary glaucoma, where pressure-independent mechanisms may be related, uveitic glaucoma is usually correlated with increased IOP, although the elevation of IOP may happen intermittently. The eyes of uveitic patients are defined by complex interactions between the angles (open or closed), trabecular outflow, fluctuations in the aqueous production and the response to steroids. Regarding the elevation of IOP in these individuals, it is mostly attributed to the increased outflow resistance, which distorts the equilibrium between aqueous production and outflow [4].

The wide spectrum of variations in the underlying trabecular function in different individuals also adds to the perplexity of the uveitis. It is expected that the trabecular meshwork function may be affected while aging and therefore older patients are more susceptible to intraocular inflammation in comparison with younger individuals. Probably, the accumulation of pathological alterations secondary to the chronic inflammatory activity may also be relevant to the IOP rise in older uveitic patients. Interestingly, the management of uveitic glaucoma in our patients appears to be more difficult and challenging in the younger age group. Whereas, younger individuals may have a stronger optic nerve that can withstand high pressure for a more extended period of time, it appears that older patients develop severe optic nerve damage even during shorter intervals of raised IOP and consequently more visual disabilities [5, 6].

Factors increasing intraocular pressure:

1. Concentration of aqueous proteins, inflammatory cells, and debris
2. Trabeculitis
3. T-cells, IL, cytokines and other immune factors
4. Hypersecretion of prostaglandin
5. Genetic background
6. Corticosteroid-induced elevation of IOP
7. Vascula endothelian grown factor (VEGF) and iris neovascularization etc.

The levels of aqueous proteins have been found to be elevated in uveitic patients, indicating that they may be associated with the trabecular outflow. Previous studies have investigated the role of aqueous proteins during acute intraocular inflammation [7, 27–31]. However, due to the fact that these studies have explored acute uveitis, it was not feasible to define the long-term effect of elevated aqueous proteins in uveitic eyes. It is a well-established knowledge that the protein concentration in the anterior chamber is increased in acute uveitis, causing a drop of trabecular outflow. This could probably happen more extensively in individuals with clinical entities that present with the acute rise of IOP [4]. Though it must be underlined that in Posner-Schlossman syndrome, which presents with acutely raised IOP, the levels activity in the anterior chamber remain normally low, implying that other mechanisms (e.g., trabeculitis) contribute in the increased IOP. The increased number of trabecular precipitates that include proteins, inflammatory cells, and debris in the anterior chamber of uveitic eyes may decrease trabecular outflow by clogging of the trabecular meshwork. Evidence that inflammatory cells lead to clogging of the trabecular meshwork derive from studies that recorded acute rises in IOP after Nd: YAG laser capsulotomy. Moreover, there is evidence that the acute inflammatory processes that follow Nd: YAG laser can also lead to chronic ocular hypertension (OHT), underlining that the clogging of trabecular meshwork that occurs during an episode of intraocular inflammation may have long-term effects on trabecular outflow and subsequently on IOP. Gonioscopy of the trabecular meshwork can reveal its obstruction by inflammatory precipitates in several pathological entities, such as in Grant's syndrome, pseudoexfoliations, and pigmentary glaucoma. The inflammation of the trabecular meshwork, which is known as trabeculitis, may also cause a rise in IOP. Herpetic uveitis consists one of the most characteristic examples. Two older studies by Hogan et al. and Townsend et al. have described the histological alterations caused in enucleated human eyes and rabbits with herpetic inflammation, respectively [4, 7, 8].

Both studies highlighted that trabeculitis could play a critical role in the elevation of IOP in uveitis, especially when caused by *herpes simplex* virus (HSV). However, it is yet to be defined whether trabeculitis interferes in types of uveitic glaucoma with a more chronic course, such as those related to Fuch's heterochromic cyclitis or juvenile idiopathic arthritis (JIA) [4].

Interestingly, T-cells consist the largest cell population in the aqueous, vitreous, retina and the uveal tract of uveitic patients. More specifically, Th-1 cells might have a substantial contribution in the pathophysiology of uveitis, but their role in uveitic glaucoma remains uncertain [9]. A study by Murray et al. investigated the aqueous humor obtained from patients with and without uveitis during a cataract extraction surgery. Individuals with uveitis demonstrated an inflammatory response mediated by T-cells. There was a prevailing expression of IL-2 and IFN-γ, which are Th-1 related cytokines and their levels were significantly lower in non-uveitic individuals [10]. Nonetheless, the levels of proinflammatory cytokines have been associated with the activity of inflammatory activity in various types of uveitis. Ohira et al. analyzed the effects of factors on the levels of aqueous humor proinflammatory cytokines and growth factors in uveitic eyes. According to the results mean interleukin (IL)-6, IL-8, monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α and VEGF were found to be higher in cases with uveitic glaucoma than those in cataract (non-glaucomatous) cases. Additionally, IL-6, MCP-1, and VEGF were all higher in uveitic glaucoma than in patients with primary open angle glaucoma (POAG). The uveitic cases with a history of phacoemulsification indicated higher levels of IL-6, IL-8, MCP-1 and PDGF-AB/BB in comparison with the phakic eyes. Finally, the presence of cells in the anterior chamber was associated with higher levels of TNF-α, IL-8 and PDGF-AB/BB. As for PDGF-AB/BB level, it was found to be higher in infectious rather than in non-infectious uveitis [4, 11, 12].

Mechanisms of intraocular pressure elevation in secondary open angle glaucoma:

1. Trabecular meshwork obstruction is the most common mechanism and can be caused by: Disruption of the blood aqueous barrier, which allows entry of inflammatory cells into the aqueous humor and entrapment of normal serum components in the aqueous outflow system. Swelling of trabecular lamellae and endothelial cells with both a physical narrowing of trabecular pores and dysfunction also leads to aqueous outflow obstruction, ultimately leading to permanent damage and scarring of the trabecular meshwork [13-15].

2. Hypersecretion caused by PGE_1- and PGE_2-mediated increase in the rate of aqueous secretion or by a breakdown in blood-aqueous barrier (BAB), with an associated increase in aqueous protein concentration and thus aqueous viscosity [16].

3. Corticosteroid-induced elevation of IOP.

Steroid-induced glaucoma is a form of secondary open angle glaucoma that results from the use of steroids. Corticosteroids are believed to decrease outflow by inhibiting degradation of extracellular matrix material in the trabecular meshwork (TM), leading to aggregation of an excessive amount of the material within the outflow channels and a subsequent increase in outflow resistance. The amounts of glycosaminoglycans, elastin, and fibronectin have been shown to increase in tissue culture preparations in response to dexamethasone treatment while the levels of tissue plas-

minogen activator, stromelysin, and the activity of several TM metalloproteases have been shown to fall. Furthermore, excessive accumulation of glycosaminoglycans has been identified in human trabecular meshwork specimens obtained from steroid-responders, confirming similar findings in a rabbit model [17, 18]. In support of the evidence for extracellular matrix deposition, dexamethasone treatment has also been shown to inhibit TM cell arachadonic acid metabolism and reduce phagocytic activity. It is hoped that recent advances in novel molecular genetic methods will allow a better understanding of the mechanisms causing the steroid-induced glaucoma. By gene deletion or overexpression studies, the exact role of individual genes responsible for the modulation of meshwork extracellular material may be identified in the near future [19]. An increase in the IOP related to the corticosteroids that are used for the control of inflammatory reactions in uveitis has been recorded in 18–36% of patients; these individuals are described as steroid responders. Clinically, a response to steroids is expected to develop within 2 to 6 weeks after starting therapy, but can potentially happen at any time.

Shrestha et al. monitored and studied 116 consecutive new uveitic patients, recording the IOP at presentation, at 1 week, 3 and 6 weeks. They recorded that 20% of these eyes developed ocular hypertension, which was at a percentage of 64.5% attributed to corticosteroids (37.03% of the oral group, 14.28% of the posterior sub-tenon group and 8.57% of the topical group). The same study indicated that timely medical treatment might avert the necessity of early surgical intervention for the control of eye pressure [4].

Interestingly, as a response to decreased pressure gonocytes, which consist of fibro-elastic cells in the anterior chamber of the normal human eye secrete polymerized mucopolysaccharides. This results in swelling of the cells of the trabecular meshwork and a subsequent decrease in the trabecular outflow. To curtail these effects and reduce IOP hyaluronidase breaks down the polymerized mucopolysaccharides. However, the release of hyaluronidase may be restricted due to the use of steroids, causing inhibition of mucopolysaccharides depolymerization. Some studies have investigated the genetic background of steroid responders, showing that a gene that may play some role is responsible for a protein named Myocilin, which is produced by the cells of the human trabecular meshwork [20]. Despite the fact that there are not any known Myocilin mutations associated with steroid responders, it appears that the cells of human trabeculum increase the production of Myocilin in response to the administration of dexamethasone. Consequently, although genes might contribute in defining steroid responders more studies are required to confirm this hypothesis [4].

Pathogenesis of Neovascular Inflammatory Glaucoma (NVG) is unclear too. Extremely high levels of VEGF are present in patients with NVG [21]. In addition to VEGF, several other molecules have been associated with the development of NVG, including basic fibroblast growth factor, platelet-derived growth factor, insulin-like growth factor-1 and interferon-α [22]. Vascular proliferation first occurs with endothelial budding at the capillary level not only of the vasculature of the minor arterial circle of the iris but also the major arterial circle at the iris base. These endothelial buds progress to glomerulus-like vascular tufts, resembling renal micro-vasculature. The new vascular tissue is composed of endothelial cells without a muscular layer and with little adventitial or supportive tissue. The vessels are thin walled and tend to be located near or on the iris surface but can be seen histologically at any level within the iris. The fibrovascular membrane in neovasularization of the iris (NVI) also contains proliferating myofibroblasts with smooth muscle differentiation. This clinically transparent and contractile membrane causes a flattening and effacement of iris surface architecture, ectropion uveae, development of peripheral anterior synechiae (PAS) and subsequent secondary angle closure [23].

Most common causes of uveitic glaucoma:

1. Juvenile rheumatoid arthritis is an autoimmune disease typically affecting children under the age of 16 years and lasts more than six months. The uveitis is typically bilateral, nongranulomatous, asymptomatic anterior uveitis, usually preceded by arthritis [24]. It has been shown that individuals with persistent low-grade uveitis are at a higher risk of developing glaucoma. JIA-related glaucoma often occurs with open angles, but secondary angle-closure caused by pupillary block as a result of the formation of posterior synechiae is relatively common. Apart from glaucoma, the main complications that can lead to loss of vision are a cataract, band keratopathy, and cystoid macular edema [25]. Regarding medication, the therapeutic scheme includes a topical steroid, cycloplegics that may be followed by systemic steroid therapy and possibly regional injection of steroids. In persistent and more severe cases immunomodulation (e.g., methotrexate) can be incorporated in the management of the disease, resulting in low toxicity and high efficacy. According to recent studies adalimumab, which is an anti-TNF-α agent, has shown efficacy in treating refractory uveitis in multiple settings, including juvenile idiopathic arthritis [26-28]. With regard to the treatment of glaucoma patients are initiated on antiglaucoma medications, but in complicated and severe cases surgical treatment (e.g., trabeculectomy or tube shunt surgeries) may be unavoidable. Unfortunately, many of them might require medication even after surgery.

2. Fuch's heterochromic iridocyclitis was first described by Fuch in 1906, FHIC is an idiopathic, painless, chronic, low-grade iridocyclitis with heterochromia, due to iris stromal atrophy. The typical age of onset is 20 - 40 years of age, with men and women affected equally.

It is typically unilateral, but in 13% of the cases it has presented bilaterally [29, 30]. Infiltration of TM by mononuclear inflammatory cells, typically lymphocytes and plasma cells, causes rubeosis, trabeculitis, and collapse of the Schelmm's canal, leading to TM obstruction and rise in IOP. Interestingly, iris angiography can detect leakage of the iris vessels and ischemic alterations of the iris. Reported incidence of glaucoma varies from 13-59%, with higher fig-

ures seen on long-term follow-up. The glaucoma typically persists after uveitis has subsided and does not respond to steroids. Fuch's cyclitis rarely causes synechiae formation. Unless glaucoma develops, Fuch's cyclitis is a benign disorder and does not require therapy. Use of steroids may only accelerate PSC formation and increase IOP [31].

The glaucoma associated with FHI resembles primary open-angle glaucoma. Gonioscopic evaluation may reveal multiple fine blood vessels, arranged either radially or concentrically in the trabecular meshwork. Cataract is a constant feature of FHI, whereas glaucoma has been reported to occur in 6-47% of cases. Low-grade inflammation does not need treatment with anti-inflammatory or immunosuppressive agents [2].

3. Posner-Schlossman syndrome is characterized by a number of unusual features, including unilateral involvement, recurrent attacks of often very mild cyclitis, marked elevation of IOP, open angle, and occasional heterochromia. IOP may elevate up to 40 to 70 mm Hg during an acute episode. The condition typically affects individuals aged 20-50 years and resolves spontaneously regardless of treatment [2].

This rise in the eye pressure has been correlated with the aqueous levels of prostaglandins and usually resolves spontaneously. The pathogenetic mechanisms have not been clarified yet. Some of the possible etiologic factors include HSV and cytomegalo virus (CMV) infections, an immunogenetic factor that involves HLA-Bw gastrointestinal disease and several allergic factors (i.e., eczema, urticaria, asthma, rhinitis, contact dermatitis, angioneurotic edema, intolerance to aspirin and food allergies). The prognosis is benign, with only exception for the patients that develop glaucomatous alterations, which is recorded in about 25% of cases. Clinical findings include small, flat, fine, non-pigmented KP detected in the inferior corneal endothelium. In gonioscopy, the angle appears to be open with some occasional trabecular precipitates. The therapeutic approach includes steroids and antiglaucoma medication (beta-blockers and carbonic anhydrase inhibitors). It has been shown that oral indomethacin, prostaglandin inhibitors and subconjunctival polyphloretin (prostaglandin antagonist) are effective in lowering the IOP during acute attacks. Surgical treatment is indicated in patients that glaucoma persists after maximal medical treatment [4].

4. The development of secondary glaucoma consists the most common complication of herpetic uveitis. It has been reported that 28–45% of patients with HSV keratouveitis demonstrate transient increased IOP and 10–54% may present with uveitic glaucoma. Disciform keratouveitis and necrotic stromal keratitis are associated more commonly with elevated IOP than epithelial keratitis [32]. Active iridocyclitis accompanied by an acute increase in eye pressure are the main features of herpetic infection and in most cases, HSVs or VZVs are the etiologic factors. These spikes in the IOP occur as a result of the trabecular meshwork inflammation, similarly to hypertensive episodes of Posner-Schlossman syndrome that has been described above.

This explains why the IOP returns to normal levels while responding to topical corticosteroids. It must be underlined though, that the elevated IOP can occur secondarily to the obstruction and swelling of the trabecular meshwork. Episodes of herpetic uveitis are typically unilateral, acute and in severe cases may present with hyphema, hypopyon, fibrin deposition and formation of anterior synechiae [4].

Conclusions

1. Inflammatory glaucoma is a multifactorial pathology that needs a careful diagnosis and therapeutical approach in order to obtain a good management of inflammatory process and intraocular pressure.

2. There are a lot of pathological pathways that influence the evolution of inflammatory glaucoma and make treatment more difficult.

3. Previous years, glaucoma treatment has significantly improved, but to better understand pathogenesis of uveitic glaucoma more scientific studies are necessary.

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VC, NB and VC designed the study and drafted the first manuscript. VC, LD and LG revised the manuscript and completed the final design. All the authors approved the final version of the manuscript.

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