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CONTENTS

CUPRINS

MARKERS OF ANGIOGENESIS IN RECTAL CANCERS

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ABSTRACT

Surgery reach its limits in treating rectal cancers. Old fashion chemotherapy has so many adverse effects and the survival rates became steady for the last decades. Efforts were made in two other directions prevention of rectal cancers by large population screenings, and the other was research of intimate mechanism of rectal cancer progression and identifying new markers for better targeted new anti-cancer drugs. We intend to emphasize the role of angiogenesis markers in actual stage of research in order to settle new goals for our research. We identified three possible paths in evaluation of the rectal cancer angiogenesis suitable for our research: microvessel density quantified by CD34 and CD105, morphometric analysis of the intratumor and peritumor vessel marked with CD34 and CD105 in order to achieve new patterns of progressing for rectal cancer, and a combined analyses of VEGF and podoplanin.The biological behaviour of rectal cancers is tailored by many factors, most of them activating different pathways of vascular growth. Reasearch should focus on the relation between these factors and finding new ways of targeting multiple factors during chemotherapy in order to restrict tumor blood supply by slowing the angiogenesis.

Keywords: rectal cancer, angiogenesis markers, CD34, CD105, microvessels

INTRODUCTION

Surgery reaches its limits in treating rectal cancers. Old fashion chemotherapy has so many adverse effects and the survival rates became steady for the last decades. Efforts were made in two other directions prevention of rectal cancers by large population screenings, and the other was research of intimate mechanism of rectal cancer progression and identifying new markers for better targeted new anti-cancer drugs. We intend to emphasize the role of angiogenesis markers in actual stage of research in order to settle new goals for our research. We identified three possible paths in evaluation of the rectal cancer angiogenesis suitable for our research: microvessel density quantified by CD34 and CD105, Morphometric analysis of the intratumor and peritumor vessel marked with CD34 and CD105 in order to achieve new patterns of progressing for rectal cancer, and a combined analyses of VEGF and podoplanin.

Immunohistochemistry assay and evaluation of the impact of microvessel density in rectal cancers

CD34 is an endothelial marker used in immunohistochemistry testing of the blood vessel both in normal and tumor tissues. Microvessel density (MVD) represents one of the markers for the blood vessel of the rectal malignant tumors. Its expression was studied intense and quantified for colon cancers and colon cancer metastasis, but not so often for rectal cancers. The study of CD34 in rectal tumors was associated with local inflammation and tumor intravascular emboli. Though, being a marker for all endothelial cells, malignant and benign, without specifity for malignant tissue blood vessel, its expression did not gain general acceptance as a major prognostic factor for the evolution and treatment of rectal cancers [1].This is the reason why CD34 usage as a marker for rectal cancer needs association of another marker, which should be more specific in assessing activated endothelial cells from the tumor vessels. Today, the most frequently used marker for quantifying activated endothelial cells of the malignant tumors, and obtaining MVD at this level, is endoglin (CD105). CD105 has an expression at the level of activated endothelial cells of the tumor, but no expression in the endothelial cells of the normal tissue [2]. Endothelial cells have many markers with variable specificity and sensibility, depending on the development stage of these cells, pathological or physiological state, and on the local organ environment. When analyzed separately these markers did not show any prognosis importance, but we believe that a combined analyze of both markers might lead to an improvement in their role as prognosis factors.

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MVD CD34 for the tumoral tissue is reported to have elevated numbers in literature [3, 4]. MVD CD34 use as a single marker for rectal cancer and its metastasis is not accepted anymore [5]. We supposed that analyzing the MVD CD34 ofintratumor (IT) area and peritumor(PT) area and associating these values with TNM staging might bring some better results in assessing MVD CD34 role in rectal cancer. Differences between MVD IT and PT in rectal cancers and association of these with TNM staging was based upon ultrasound methods of determining the tumor blood supply, showing that MVD CD34 did not correlate with any TNM parameters [6]. Others demonstrated that there is a correlation between MVD value and N parameter [7]. Zhou et al showed that vascular spreading with CD34 and CD105 positive vessels was a factor of bad prognosis for rectal cancers in the first and second TNM stages [8]. Li et al determined the plasmatic of CD105 and correlated these levels with MVD CD 105 finding a statistical significance between tissue expression, plasmatic levels and survival rates after Duke classification showing once more that CD105 is an independent prognosis factor for survival in rectal cancer [9].

Morphometric analysis of the intratumor and peritumor vessel marked with CD34 and CD105

MVD was previously a widely used parameter for the study of microscopic tumor angiogenesis in various types of tumors. The method used for the first time by Weidner et al [10] has proved to be insufficient over time in the evaluation of different types of tumor angiogenesis in the tumor. For this reason to the assessment of tumor vessels was attempted the addition of other parameters, such as the study of endothelial cell proliferation [11], microscopic analysis of the bifurcation of the tumor vessels, and the vascular area [12]. The combination of all these factors appears to have a significant correlation with clinical and biological factors and prognosis in various tumor types. Quantification of endothelial area is a useful parameter in the evaluation of tumor vessels, but at the same time is an extremely laborious method despite the existence of computerized systems for such procedures. For this reason, studies which include the assessment of tumor vessels are sparse in thecolo-rectal lesions, which are nominated in less than 120 articles. Furthermore, correlations with TNM system parameters, as well as tumor grade or the presence of anemia and leukocytosis are extremely rare and only sporadically being mentioned as studies conducted only in a few countries.Wedid not find a comparative analysis of vascular area between CD34 and highlighted vessels identified by endoglin (CD105).For the above reasons we considered useful reassessment of the tumor vessel area highlighted with CD34 in rectal tumors and more, a comparison between vascular area identified by CD34 and CD105, given that endoglinintratumoral and peritumoral highlights activated vessels.

Usually the study of tumor angiogenesis is achieved by using a single marker for quantifying endothelial MVD, the CD34. Currently there are only 9 studies reporting of a single marker to quantify MVD strictly for rectal tumors (without including those of the colon). We recommend the use of two comparative quantificationswith CD34 and CD105 in order to identify patients who may apply for individualized therapy. For rectal tumors there are currently in the literature studies that compare the MVD measured by CD34, and CD 105 in close interrelation with the expression of VEGF, suggesting that VEGF inhibition is effective in reducing the vascular density [13]. We believe that the method of quantifying the positive CD105 vessel area can be a useful parameter for determining the optimal dose of humanized anti CD105 that could be applied to rectal tumors in the future given that currently we did not identified in the literature data on the use of this therapy in rectal cancers and to a lesser extent in the colon. This couldlead to a tumor vasculature original analysis of rectal tumors with a direct impact on targeted therapy using humanized monoclonal antibodies. Reverse significant correlation between CD34 positive vessels area and the CD 105 positive vessels in the area adjacent to rectal tumors supports the use of anti CD105 rectal tumors because it can target tumor vessels with minimal side effects on normal tissues. One study by Takahashi et al demonstrated that serum levels of endoglin in patients with metastatic colorectal cancer was significantly increased compared with the group of patients with the same tumor type, but without metastases. This suggests that endoglin is a systemic factor involved in hematological and especially inflammatory tumor lesions (14).

Factors that influence the mechanisms of the angiogenesis in rectal tumors

VEGF is a therapeutic target in many types of metastatic cancer, bevacizumab therapy was applied as first line treatment in metastatic colorectal cancer [15]. Despite approval of bevacizumab as adjuvant therapy in colorectal cancer it is still controversial and its effectiveness depends on a number of factors based on age [16] and ending with survival and disease-free interval of patients who benefited from such therapies [17, 18]. Polymorphism of VEGF gene in colorectal cancers partly explains the controversial results regarding the impact of bevacizumab on prognosis and survival in these patients [19, 20]. Most studies quantify the expression of VEGF often limited to tumor cells without having to quantify the expression of VEGF and inflammatory infiltration associated with the malignancies. Currently in the literature there are only five possible studies of VEGF expression in

inflammatory cells describing the effect of bemacizumabtherapy on the factors secreted by the stromal cells, especially macrophages [21]. Accordingly to these studies blocking VEGF enhances an inflammatory pathway and suggest that the use of combined therapies, anti-inflammatory and bemacizumab together would be more beneficial in optimizing the effect of neoadjuvant treatment. A recent study by Caramanolis et al suggest that rectal tissue after irradiation had a decrease of VEGF expression with MVD growth that was attributed to inflammation that appeared frequently post irradiation and this is considered an adverse effectradiotherapyon rectaltissue. These data were reported in radiation proctitis [22]. Correlation of VEGF expression with clinical and biological factors in rectal tumors suggest their usefulness in quantifying serum of patients correlated with the amount of leukocytes, platelets and carcinoembryonic antigen [23]. Kwon et al demonstrated increased serum levels of VEGF in patients with colorectal cancer preoperatively and reported that this increased levels were a factor of poor prognosis for overall survival of these patients [24].

These results obtained in 2010 were validated a year ago by Wang et al after a metaanalysis, they certified the use of VEGF as prognostic biomarker, and also of the MVD proving that they have a major impact on survival and prognosis in other types of cancer [25]. Based on the above we decided that in our study we should quantify the expression of VEGF differentiated between distance and near normal rectal mucosa adjacent to the tumor and inflammatory infiltrate tumor bores of rectal tumors included in the study. We also wanted to identify the impact of your MVD VEGF cuantifiacte by two markers (CD34 and endoglin).

All these data we considered to be useful for a better quantification of angiogenesis in rectal cancers. The blood vessels are not the only way of metastasingfor rectal tumors. Lymphatic vessels are another way of disseminating tumor cells. Factors influencing the progression and metastasis on the lymphatic pathways are not fully studied rectal cancers. Except limphatic vessels MVD in rectal cancer, lymph podoplanin quantification was not a research priority. It is well known that podoplanin is expressed differently in different tumor cell types [26] and is a factor that favors tumor progression and metastasis [27, 28]. Podoplanine study in tumor lesions was centered more on expression in myofibroblasts type stromal cells which were demonstrated to have an increase in local invasion leading to a decrease in survival [29].

Podoplaninhas become a therapeutic target in studies on tumor tissues using antibodies antipodoplanine and there are reportsshowing significant inhibitory effects on reducing tumor growth without harming normal tissues [30]. Podoplanin concordant expression in tumor cells in esophageal carcinomas reported it suggested that positive interaction between stromal cells and tumor cells is an independent prognostic factor in tumor biology of this type [31]. This support once again as a potential therapeutic target podoplanine use. For this reason and due to lack of data on podoplanin expression in tumor cells in rectal cancer we consider that its quantification should be correlated with that of VEGF.

CONCLUSION

The biological behaviour of rectal cancers is tailored by many factors, most of them activating different pathways of vascular growth. Reasearch should focus on the relation between these factors and finding new ways of targeting multiple factors during chemotherapy in order to restrict tumor blood supply by slowing the angiogenesis.

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MARKERI AI ANGIOGENEZEI ÎN CANCERELE RECTALE

REZUMAT

Chirurgia a ajuns la unpunct critic in tratarea cancerelor rectale. Chimioterapia prezinta numeroase efecte adverse, iar rata de supravietuire a atins un prag stabil, fara modificari in ultimele decenii. Pentru preventia cancerelor colorectale sunt depuse eforturi in doua directii, prin testari screening pe grup epopulationale mari si prin cercetarea mecanismelor de progresie a cancerelor rectale si identificarea unor noi markeri pentru o mai buna tintire a noilor agenti anti-tumorali. In acest studiu ne propunem sa subliniem rolul markerilor angiogenici la nivelul actual al cunoasterii, pentru a stabili noi obiective ale cercetarii. Am identificat trei posibile cai de evaluare a angiogenezei in cancerele rectale, care sunt utile cercetarii desfasurate prin acest studiu: densitatea microvaselor cu antificata prin expresia CD34 si CD105, analiza morfometrica a vaselor intratumorale si peritumorale, marcate cu CD34 siCD105, pentru evaluarea modelelor de progresie a cancerelor rectalesi analiza combinata a VEGF si podoplaninei. Comportamentul biologic al cancerelor rectale este influentat de factori multipli, majoritatea activand diferite cai implicate in dezvoltarea vasculara. Cercetarile ar trebui directionate spre relatia dintre acesti factori si spre identificarea unor noi modalitati de tintire multifactoriala cu ajutorul chimioterapiei, pentru a restrictiona aportul sanguin la nivel tumoral, prin incetinirea procesului de angiogeneza.

Cuvintecheie: cancer rectal, markeri angiogenici, CD34, CD105, microvacularizație

BACTERIOLOGICAL PROFILE OF NOSOCOMIAL INFECTIONS IDENTIFIED IN THE ORTHOPEDIC WARD OF COUNTY CLINIC EMERGENCY HOSPITAL OF BRAŞOV. CORRELATIONS WITH THE MICROORGANISMS FROM HOSPITAL ENVIRONMENT

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ABSTRACT
The purpose of this paper is to implement in the orthopaedic ward of County Clinical Emergency Hospital of Brasov various activities and means leading to decreased incidence of nosocomial infections. The target population is the inpatients on the orthopedic ward, in 2011-2013, a total of 7551 patients. We aimed a longitudinal, analytic cohort and retrospective study through which to highlight the possible correlations between circulating flora from the orthopedic ward with the one identified in reported nosocomial infections or in wound infections detected passively. The measure association was shown by the size of the correlation coefficient. Germs circulating in the ward and with potential of nosocomial infections were identified; the first is Staphylococcus aureus, which is found in the etiology of nosocomial infections from the ward, with Acinetobacter, followed by Escherichia coli, Klebsiella and Acinetobacter with Enterococcus.
Key-words: nosocomial infections, circulating flora, microbial resistence

INTRODUCTION

Over time, nosocomial infections have been established in an important chapter of infectious pathology that has constantly developed along with the diversity of medical procedures provided to healthy and ill people [2, 4, 5]. They appear due to changes occurring in the human body as a result of abusive use of antibiotics, the upward trend for the prevalence of immunocompromised hosts, the disturbance of relations between different categories of pathogens, the decrease of general resistance of important population groups, socio-economic justified, and due to the use of risk practices [1, 3, 6, 7].
The purpose of this paper is to implement, within the ward of orthopedics, different activities and means that lead to decreased incidence of nosocomial infections. As objectives we mention highlight the associations of most common germs from the orthopedic ward and the study of their degree of resistance.

MATERIAL AND METHODS

The target population is the inpatients on the orthopedic ward of County Clinical Emergency Hospital of Brasov, in 2011-2013, a total of 7551 patients. In the first stage of research we have established the study group as reported nosocomial infection cases that are confirmed clinical, laboratory and epidemiological according to case definitions agreed at European level. The data from our study were collected from the observation charts of inpatients. Additionally, the records of surveillance of wound nosocomial ininfection from the department of prevention and control of the hospital were analyzed.
We consulted the laboratory data on the etiology of wound infection detected and the degree of microbial resistance to isolated germs and the data regarding the circulating flora from the ward within the surveillance bacteriological activities of hospital environment. All data were recorded in the registry books of the hospital laboratory and stored by the program WHO / Net.
We aimed a longitudinal, analytic cohort and retrospective study to highlight the possible correlations between circulating flora on hospital environment from the orthopedic ward with the one identified in reported nosocomial infections or in wound infections detected passively, in order to control and reduce the nosocomial infections in this ward. The measure association was shown by the size of the correlation coefficient (r). The r values of 0.3-0.4, a weak correlation is estimated at 0.5-0.7 values showed an average correlation and a value of over 0.7 shows a correlation of high significance.

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RESULTS

7 cases of nosocomial infection have been reported in 2011, 5 of them were surgical wound infection, one septicemia and one urinary infection. In 2012 and 2013 all infections were wound infections. During 2011-2013, from the analysis of unreported cases of nosocomial infections, identified from the medical records on epidemiological, clinical and laboratory criteria - 101 cases, we found a similar distribution as the location of the reported cases (Table I).

Table I. The types of nosocomial infection on the orthopedic ward

Nosocomial infection diagnosis - Reported (R) or unreported (UR)	Frequency 2013		Frequency 2012		Frequency 2011	
	R	U	R	U	R	U
bronchopneumonia						
Clostridium difficile infection						
deep surgical wound infection	4	29	3	22	3	28
superficial surgical wound infection			1	8	2	14
septicemia					1	
urinary infection					1	
Total	**4**	**29**	**4**	**30**	**7**	**42**

We mention that of the 116 possible cases of nosocomial infections only 15 (10%) were reported by physicians, the remaining 101 were lost for various reasons, fear of sanction or indifference to thick criteria of hospital infections (clinical, epidemiological or laboratory - microbiological).

In 2011, in the orthopaedic ward from the circulating flora the largest share has MRSA, 45% of all strains followed by the Escherichia coli (15%), Klebsiella (9%), Enterococcus (9%), Acinetobacter (9%), Enterobacter (4%), Proteus (4%), Pseudomonas (3%), Streptococcus spp (1%) and Citrobacter (1%). For 2012, the share of staph remains unchanged (45%); Escherichia coli, Enterococcus and Klebsiela also have a stationary tendency 15% and 9% respectively. Frequency for Acinetobacter has a slightly growing trend to 11%. In 2013, the circulating flora in the orthopaedic ward has a slightly upward trend for MRSA at 48%, the frequency drops to 11% for Escherichia coli, Acinetobacter and Enterococcus remain at 10% and the share for Klebsiella type strains drops to 8%.

In 2011, 72% of nosocomial infections are due to Gram-positive flora: Staphylococcus, Enterococcus, Clostridium dificille) circulated by medical personnel or patients colonized and disseminated by nosocomial risk medical practices. 28% is due to Gram negative flora (Proteus, Escherichia coli, Acinetobacter, Klebsiella, and Pseudomonas) present in the ward due to moderately effective cleaning and disinfection (Figure 1).

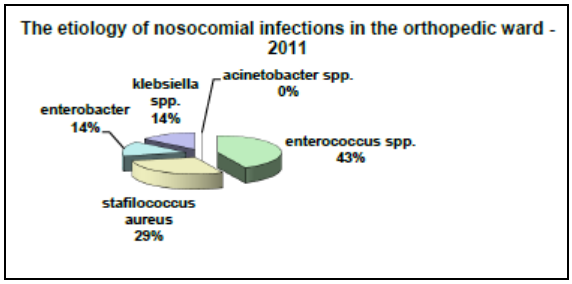

Fig. 1. The etiology of nosocomial infections in the orthopedic ward in 2011

In 2012 the situation on the etiology of nosocomial infections in orthopedic ward radical changes, incriminated germs being Gram negative, probably due to transmission through contaminated objects (Figure 2). In 2013 the etiology of nosocomial infections is 50% Gram negative and 50% Gram positive, which is due both staff and patient colonization and possibly to contaminated objects (Figure 3).

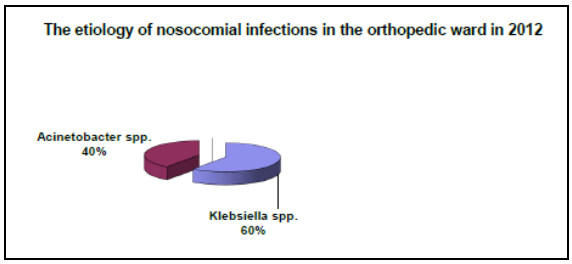

Fig. 2. The etiology of nosocomial infections in the orthopedic ward in 2012

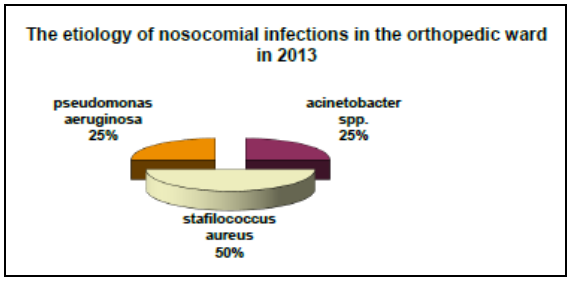

Fig. 3. The etiology of nosocomial infections in the orthopedic ward in 2013

In our study we tried to measure the association between circulating germs and nosocomial infections both in the hospital and in the orthopedic ward. For the year the processed data showed the size of the correlation coefficient between the germs isolated from hospital and those from the nosocomial infections of the entire unit 0.158. This demonstrates a lack of correlation; the infections acquired in our hospital as a result of medical

maneuvers are not due to environmental contamination and to the lack of cleaning or disinfection, but rather are associated with endogenous factors of the patient.
If we measure the same combination in orthopedic ward we already observed an average correlation of 0.5326 between circulating germs and those involved in nosocomial infections.

CONCLUSIONS

There were identified germs circulating in the orthopedic ward and with potential for nosocomial infections, in first are Staphylococcus aureus, which is found in the etiology of nosocomial infections from the ward with Acinetobacter, followed by Escherichia coli, then Klebsiella and Acinetobacter with Enterococcus.
On the orthopedic ward we observe an average correlation of 0.5326, between circulating germs and those involved in nosocomial infections.

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PROFILUL BACTERIOLOGIC AL INFECTIILOR NOSOCOMIALE DEPISTATE IN SECTIA DE ORTOPEDIE A SPITALULUI CLINIC JUDETEAN DE URGENTA BRASOV. CORELATII CU FLORA CIRCULANTA DIN MEDIUL DE SPITAL

REZUMAT

Scopul acestei lucrări este de a implementa, în cadrul secţiei de ortopedie a Spitalului Clinic Judeţean de Urgenţă Braşov diferite activităţi şi mijloace ce duc la scăderea incidenţei infecţiilor nosocomiale. Populaţia ţintă este reprezentată de bolnavii internaţi pe secţia clinică de ortopedie, în perioada 2011-2013, in total 7551 pacienţi. Ne-am propus un studiu longitudinal, analitic, de cohortă, cu caracter retrospectiv, prin care să evidentiem corelatiile posibile intre flora circulanta din sectia de ortopedie cu cea identificata in infectiile nosocomiale declarate sau in infectiile de plaga depistate pasiv. Măsura asocierii a fost redată prin mărimea coeficientului de corelaţie. S-au identificat germenii circulanţi pe secţie şi cu potenţial de nosocomialitate, pe primul loc se situează Staphylococcus aureus, care se regăsește și în etiologia în infecțiile nosocomiale de pe secție împreună cu Acinetobacter, urmat de Escherichia coli, Klebsiella și Acinetobacter cu Enterococ.

Cuvinte cheie: infectii nosocomiale, germeni circulanți, rezistență microbiană.

TEMPORAL ASPECTS OF FULL-FIELD ERG IN PATIENTS WITH DIABETES WITHOUT DIABETIC RETINOPATHY

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ABSTRACT

Aim: To evaluate implicit time changes of full-field ERG in patients with type 2 diabetes without diabetic retinopathy. **Material and Method:** The prospective study included 11 diabetic patients, aged between 50 and 80 years old, without diabetic retinopathy and 14 aged-matched controls. All of the participants underwent full-field ERG and ophthalmologic examination to exclude any ophthalmologic pathology. The ERGs were recorded with Metrovision MonPackOne system, which has the same stimulus parameters as the ISCEV standard. The implicit times were analyzed for "a" and "b" waves in dark-adapted 0.01 ERG, dark-adapted 3 ERG, dark-adapted oscillatory potentials, light-adapted 3.0 ERG, and 30Hz flicker ERG, and compared between diabetic patients and healthy subjects. **Results**: The significantly delayed responses, between diabetic patients and healthy subjects older than 50 years, were the dark-adapted oscillatory potentials N2 (21.91 ± 0.85ms versus 22.45 ± 1.02ms, p=0.044) and P2 (25.49±1.01ms versus 26.13±0.94ms, p=0.027), dark-adapted, scotopic 3 "b" wave (42.89±2.74ms versus 44.99±3.16, p=0.015) and light adapted, photopic 3 "b" wave (31.62±1.62ms versus 32.72±1.36ms, p=0.014). **Conclusion**: The electrophysiological findings from the present study showed that, apart from oscillatory potentials changes, reported in previous studies, there was a significant delay in the cone system "b" wave, which indicates that the functional integrity of the inner retina is compromised before the appearance of clinical changes.

Keywords: ERG, Diabetes Mellitus, ERG implicit time

INTRODUCTION

Diabetes mellitus is one of the most serious global health problems and is associated with increased levels of visual impairment after adjusting for the effects of age, sex, race, educational level, blood pressure, smoking and body mass index [1]. The incidence of type 2 diabetes is increasing worldwide and hence the risk of developing complications [2]. The prevalence of visual impairment in adults with and without diabetes vary from 3.8 to 13% and from 1.4 to 2%, respectively, and the relative risk of blindness is 5.2 times higher in patients with diabetes than in those without diabetes [3].

Diabetic retinopathy (DR), the most common complication in diabetes, is the fifth leading cause of blindness worldwide and the leading cause of visual impairment in adults of working age in industrialized countries [4]. It is not uncommon in clinical practice that DR is already present at the first visit after diagnosis of type 2 diabetes [5].

Classically, DR is described as a microangiopathy, affecting the perycites and endothelial cells [6], caused by the metabolic effects of hyperglycemia. In recent years, more attention has been focused on the neurodegenerative aspects of DR [2]. Barber was the first who observed that one month after inducing diabetes in rats, using streptozotocin there was a high rate of apoptosis in the neuroretina without a significant apoptosis in endothelial cells [7]. Glial cell activation is another phenomenon that occurs in diabetic retina, apart from apoptosis in the neuroretina. In other words, an early event in the pathogenesis of DR which participates in the microcirculatory abnormalities is the retinal neurodegeneration [7].

Since DR constitutes a major cause of visual impairment and blindness in the world, is extremely important to assess the retinal function in patients with diabetes (X). Color vision defects, reduced contrast sensitivity and visual field alterations have been reported in diabetic individuals with minimal or no retinopathy. All these are subjective tests. Thus, it is necessary to evaluate the retinal function in diabetes in an objective way, using electrophysiological tests [5].

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Although, clinical exams focus on the visualization of retinal lesions in diabetic patients, electrophysiological changes have been shown to occur before the onset of clinically visible retinopathy. Hence, an electrophysiological assessment of retinal function could be a valuable monitoring method for retinal health in diabetic patients [8].

Standard full-field ERG is an objective method which reflects the function of the whole retina. In recent years, a big number of studies showed the ERG changes in individuals with diabetic retinopathy, but these studies mainly focused on patients with long duration diabetes [2]. It is already known, that the oscillatory potentials are reduced in amplitude and delayed in diabetic patients without retinopathy, but it is not clearly shown what other components of full-field ERG are modified in this group of patients [8]. As we mentioned above, there can be retinopathic changes in the retina in non-diabetic subjects, especially due to aging. The aim of our study was to evaluate the ERG implicit time changes, in patients with type 2 diabetes, without visible diabetic retinopathy and to eliminate the changes that may be due to aging by comparing them to an aged-match healthy group of subjects.

MATERIAL AND METHOD

Subjects

Full-field ERGs were recorded for 28 eyes from 14 healthy subjects aged 50 to 80 years old (63.00 ± 11.62) who had no history of ophthalmologic disease, normal findings on eye examination, normal visual acuity, clear optic media, no history of diabetes mellitus or other vascular or neurologic disease which could influence the ERG. The ERGs were also recorded for 22 eyes of 11 diabetic patients without diabetic retinopathy, aged between 50 and 80 years old (63.18 ± 7.09). All these patients were seen by an ophthalmologist and recruited from the Diabetic Eye Department. The inclusion criteria for these patients were: clinical diagnosis of type 2 diabetes mellitus for less than ten years, no other vascular or neurologic diseases, no history of ophthalmological disease, normal findings on eye examination without any lesion of diabetic retinopathy on photo fundus, normal visual acuity, clear optic media.

The study was performed according to the tenets of the Declaration of Helsinki and was approved by the University Ethics Committee. All the subjects were fully informed about the possible consequences of the research protocol, and they sign their approval for participation.

Recording protocol

The ERGs were recorded at Ophthalmological Research Centre “Ocularius”, Craiova, under an agreement with the University of Medicine and Pharmacy of Craiova. We used the MonPackOne System (Metrovision, Perenchies, France), which has stimulus parametres according to the ISCEV standards. Recording electrodes were HK loop type ("Hawlina- Konec loop") and the reference and ground ones were Ag-AgCl cup type.

The research methodology consisted, for each subject, in:

A. Usual eye examination: visual acuity, biomicroscopy, intraocular pressure, fundus examination, refraction, color vision,
B. ERG recording according to ISCEV protocol:
    1. fully dilatated pupils using 1% tropicamide and 2.5% phenylephrine eye drops;
    2. skin cleansing with an abrasive paste and medicinal alcohol;
    3. 4% xiline in the lower conjunctival bag;
    4. electrods’ placement: the active electrodes were placed on the free edge of the lower eyelid for each eye; the reference electrodes were placed near each orbital rim, with the ground electrode placed on the vertex, using a conductive paste;
    5. dark adaptation for 20 minutes;
    6. dark adapted ERG recording:
        – rod response: the stimulus is a dim blue flash of 0.01 cd.s.m^{-2} , with an interval of 2 seconds between flashes;
        – combined rod-cone response: the stimulus is a white flash of 3.0 cd.s.m^{-2} , with an interval of 10 seconds between flashes;
        – oscillatory potentials: the stimulus is a white flash of 2.0 cd.s.m^{-2} , with an interval of 165 seconds between flashes.
    7. light adaptation for 10 minutes;
    8. light-adapted ERG recording:
        – single flash cone response: a 3.0 cd.s.m^{-2} stimulus, with an interval of 0.5 seconds between flashes and a background luminance of 30cd.m^{-2}
        – 30 Hz flicker: 30 stimuli of 3.0 cd.s.m^{-2} per second.

Statistical analysis

The analysis of the results of ERG recordings was performed using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), together with the XLSTAT add-on for MS Excel (Addinsoft SARL, Paris, France). We used the Anderson-Darling test to assess data normality. Because most of the data of diabetic patients did not follow a Gauss distribution, we had to use nonparametric tests (i.e. Mann-Whitney test) to compare data between the two groups.

RESULTS

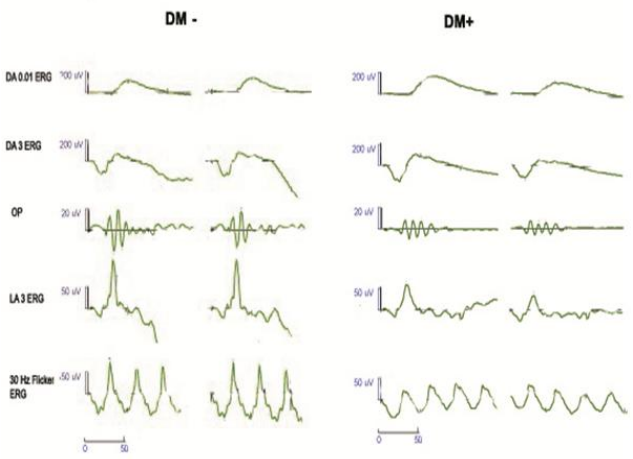

Fig. 1. Sample ERG recording for subjects without Diabetes Mellitus (DM-) and subjects with Diabetes Mellitus (DM+)

We computed mean values and standard deviations for the implicit times of all parameters described for the ERG responses, recorded according to the ISCEV standard procedure (Figure 1).

Table I. Comparison of mean implicit times between subjects without Diabetes Mellitus (DM-) and subjects with Diabetes Mellitus (DM+)

Parameter	DM-	DM+	p Mann-Whitney	Significance
Scotopic 0.01 ERG a	29.62 ± 2.29	30.31 ± 2.57	0.320	- NS
Scotopic 0.01 ERG b	61.27 ± 2.99	62.90 ± 2.99	0.061	- NS
Scotopic 3.0 ERG a	21.03 ± 2.43	21.40 ± 2.12	0.573	- NS
Scotopic 3.0 ERG b	42.89 ± 2.74	44.99 ± 3.16	0.015	- S
Photopic 3.0 ERG a	15.87 ± 1.10	15.82 ± 0.46	0.833	- NS
Photopic 3.0 ERG b	31.62 ± 1.62	32.72 ± 1.36	0.014	- S
Photopic 3.0 Flicker a	18.38 ± 2.07	18.70 ± 2.04	0.586	- NS
Photopic 3.0 Flicker b	30.69 ± 2.57	31.37 ± 2.18	0.324	- NS
Oscillatory potentials N2	21.91 ± 0.85	22.45 ± 1.02	0.044	- S
Oscillatory potentials P2	25.49 ± 1.01	26.13 ± 0.94	0.027	- S
Oscillatory potentials N3	29.01 ± 1.37	29.64 ± 1.04	0.081	- NS
Oscillatory potentials P3	32.72 ± 1.78	33.32 ± 1.26	0.189	- NS

The significantly delayed responses, between diabetic patients and healthy subjects older than 50 years, were the dark-adapted oscillatory potentials N2 (21.91±0.85ms versus 22.45±1.02ms, p=0.044, Figure 2) and P2 (25.49±1.01ms versus 26.13±0.94ms, p=0.027, Figure 3), dark-adapted, scotopic 3 "b" wave (42.89±2.74ms versus 44.99±3.16, p=0.015, Figure 4) and light adapted, photopic 3 "b" wave (31.62±1.62ms versus 32.72±1.36ms, p=0.014, Figure 5). Other ERG parameters showed a delay for DM+ patients, but the statistical significance was slightly above 0.05, so further studies on larger patients group should be conducted.

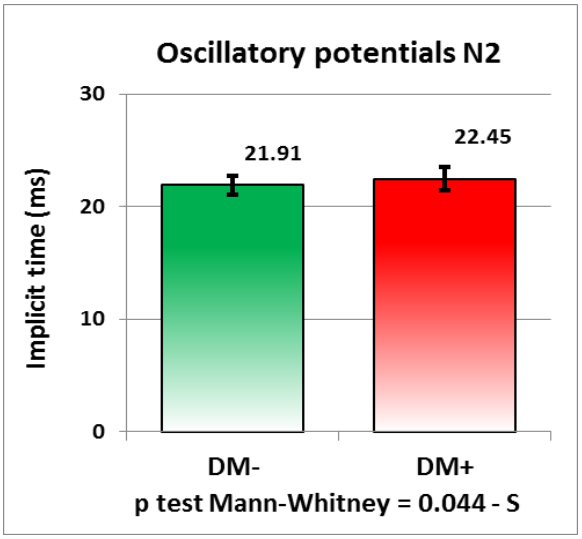

Fig. 2. Comparison of mean values of implicit times for dark-adapted oscillatory potentials N2 wave between DM- and DM+ subjects

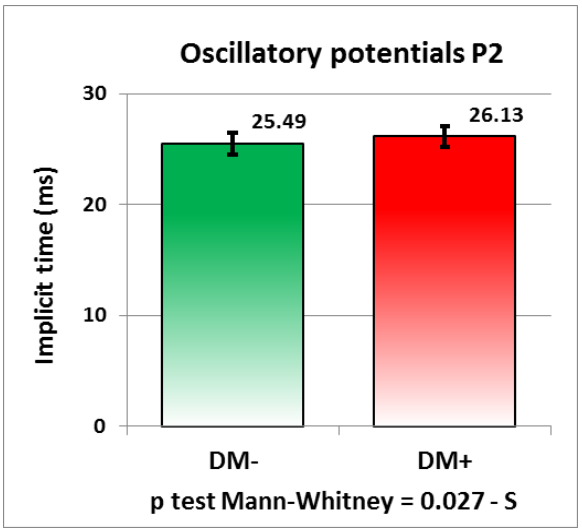

Fig. 3. Comparison of mean values of implicit times for dark-adapted oscillatory potentials P2 wave between DM- and DM+ subjects

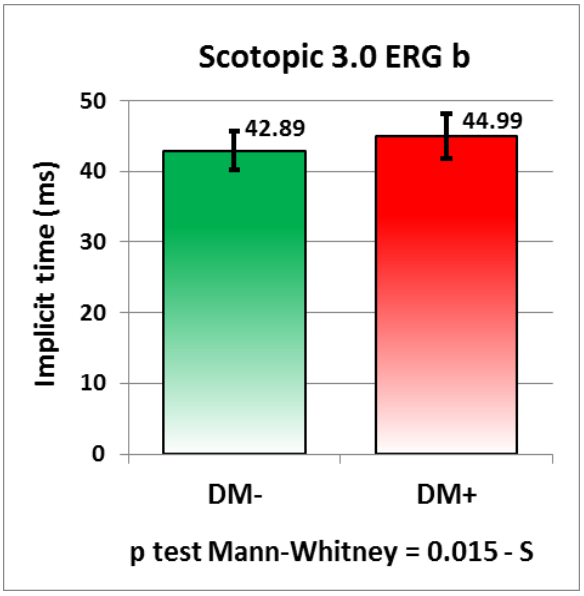

Fig. 4. Comparison of mean values of implicit times for dark-adapted (scotopic) 3 "b" wave between DM- and DM+ subjects

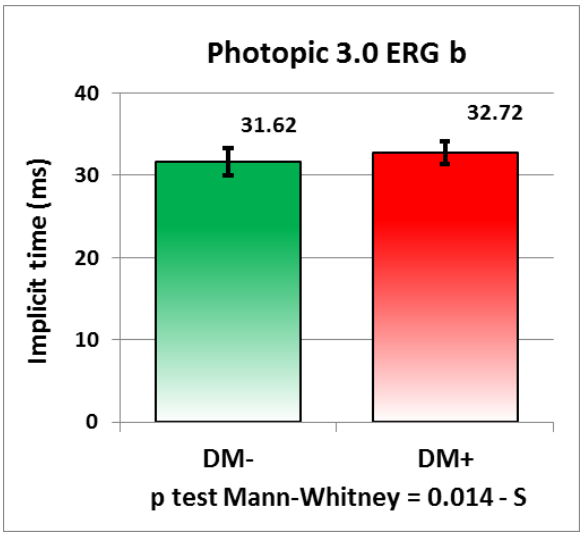

Fig. 5. Comparison of mean values of implicit times for light-adapted (photopic) 3 "b" wave between DM- and DM+ subjects

DISCUSSIONS

As we stated before, diabetes is a disease with both neurologic and vascular components. Recent studies showed that neurodegeneration is an early event in the pathophysiology of diabetic retinopathy and is already present before any vascular abnormalities can be seen in ophthalmoscopic examination [7]. The results of the present study strengthen these findings, showing ERG abnormalities in patients with diabetes and normal ophthalmologic examination.

The full-field ERG is an objective method which reflects the electrical answer from the whole retina [5] and thus a specific tool for assessing the functional response of this tissue. As symptoms do not appear in the first stages of diabetic retinopathy, early diagnosis is a challenging step in the prevention of complications.

Oscillatory potentials deterioration was one of the earliest functional retinal abnormalities reported in the literature [1, 9] in patients with diabetes but without diabetic retinopathy. Our study is in consent with these reports, showing a delayed OP2, which probably indicates early damage of the neuronal synaptic activity of the amacrine and horizontal cells.

The "a" wave of both rod and cone systems had a slightly higher, but statistically insignificant implicit time ($p>0.05$), compared to age-matched controls. This finding is not in consent with a previous study that showed a delayed "a" wave [2], reflecting that the photoreceptors are more affected by age than diabetes, at least at the onset of the disease as yet there are no changes of diabetic retinopathy.

In our study, the averaged "b" wave implicit time was delayed for both rod and cone systems in diabetic patients compared to the aged-matched controls. These changes in the "b" wave implicit time are consistent with damage in the inner retinal layers in the early stages of disease. Holopigian et al showed in their study [10], that apart from oscillatory potentials, "b" wave is also a sensitive indicator of retinal damage in patients with diabetes and no visible diabetic retinopathy.

CONCLUSIONS

By eliminating age influence on ERG responses, because the most affected parameters in the diabetic patients without diabetic retinopathy are the scotopic oscillatory potentials, "b" wave in scotopic 3.0 and photopic 3.0 ERG, we can conclude that the retinal dysfunction in Diabetes Mellitus appears first in the inner retinal layers. Therefore, the aforementioned parameters could be used to assess the evolution in the initial stages of DM, before clinical ophthalmological symptoms appear.

The electrophysiological findings from the present study showed that, apart from oscillatory potentials changes, reported in previous studies, there was a significant delay in the cone system b wave, which indicates that the functional integrity of the inner retina is compromised before the appearance of clinical changes.

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ASPECTE TEMPORALE ALE ERG FULL-FIELD LA PACIENTII CU DIABET FĂRĂ RETINOPATIE DIABETICA

REZUMAT

Scop: Evaluarea modificărilor latentei undelor ERG full-field la pacienții cu diabet zaharat tip 2, fără retinopatie diabetica. Material și metodă: Studiul prospectiv a inclus 11 pacienți cu diabet zaharat, cu vârste cuprinse între 50 și 80 de ani, fără retinopatie diabetica si 14 subiecți cu vârste similar, folosiți ca lot martor. Tuturor participanților li s-a efectuat ERG full-field și examinare oftalmologică pentru a exclude orice patologie oftalmologică asociată. Electroretinogramele au fost înregistrate cu sistemul Metrovision MonPackOne, care are parametrii de stimulare in conformitate cu standardul ISCEV. A fost analizata latenta pentru undele "a" și "b", pentru ERG 0,01 cu adaptare la întuneric, ERG 3 cu adaptare la întuneric, potențialele oscilatorii cu adaptare la întuneric, și ERG 3.0, 30Hz flicker cu adaptare la lumină, care au fost comparaţi între pacienții diabetici și subiecții sănătoși. Rezultate: Răspunsurile întârziate semnificativ, la compararea lotului de pacienți diabetici cu lotulde subiecți sănătoși cu vârstă peste 50 de ani, au fost potențiale oscilatorii cu adaptare la întuneric N2 (21.91 ± 0.85ms versus 22.45 ± 1.02ms, p = 0,044) și P2 (25.49 ± 1.01ms față 26.13 ± 0.94ms, p = 0,027), unda "b" pentru ERG 3, cu adaptare la întuneric (42.89 ± 2.74ms versus 44.99 ± 3.16, p = 0,015) și unda "b" pentru ERG 3, cu adaptare la lumina (31,62 ± 1.62ms versus 32.72 ± 1.36ms, p = 0,014). Concluzie: Rezultatele electrofiziologice din prezentul studiu au arătat că, în afară de modificările potențialelor oscilatorii, raportate în studiile anterioare, a existat o întârziere semnificativă pentru unda "b", ceea ce indică faptul că integritatea funcțională a starturilor interne ale retinei este compromisă înainte apariția modificărilor clinice.

Cuvinte cheie: ERG, diabet zaharat, latenta

ANTIBIOTIC RESISTANCE OF *ENTEROCOCCUS SPP*. STRAINS ISOLATED FROM OUTPATIENTS WITH URINARY TRACT INFECTIONS

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ABSTRACT
Objective: The main aim of our study was to isolate and identify enterococci from cases of urinary tract infection, to know antibiotic susceptibility pattern of the isolates and also to establish the resistance phenotypes.
Material and Methods: The present study, was conducted in the Microbiology Department of Intermed Laboratory, Cluj-Napoca, from january 2010 to december 2012. Identification, isolation and testing of the germs were done by the standard conventional methods.The testing of antibiotic resistance was performed through Kirby-Bauer disk-diffusion method, according to CLSI standards.
Results: A total of 62 strains of enterococci were isolated from a total of 5261 urine samples.Out of 62 strains, 58 (94%) were *Enterococcus faecalis*, 4(6%) were *E. faecium*. The antimicrobial resistance profile for *Enterococcus faecalis* was: 24.13% to ampicillin, penicillin, streptomicin, gentamicin, 15.51% to nitrofurantoin, 13.79% to ciprofloxacin, levofloxacin, ciprofloxacin, 6.89% to tetracycline. Most of the four *Enterococcus faecium* strains were sensitive to antibiotics, only one from these strains presents resistance to fluoroquinolones
Conclusion: The role of the microbiology laboratory, essential for a correct anti-infective therapeutic approach, consists of performing an antibiogram, of establishing and interpreting the resistance phenotypes, thus providing information about the resistance development mechanisms and the way of spreading of these phenotypes.
Keywords: *Enterococcus; Enterococcus faecalis; Enterococcus faecium;* urinary tract infections; urine culture; antibiotics

INTRODUCTION

It is a known fact that enterococci cause a large variety of infections. Most frequently, they infect the urinary tract, the abdomen, the blood flow, the endocardium, the biliary tract, lesions caused by burns, and permanent catheters [1]. 80 to 90% of these infections are caused by *E.faecalis* and just 10% by *E.faecium* [2].
The data provided by the literature indicate that enterococci rank 4th among the causes for infections that require hospitalization, and 3rd among the causes for bacteremia in the United States [3]. The statistics for these cases/the degree of fatality for enterococcal bacteremia show values ranging from 12 to 68%, with deaths caused by septicaemia in 4 to 50% of cases [4].
Urinary tract infections are among the most frequent human infections [5].
The aetiology of urinary infections is varied: the species isolated most frequently is *E.coli,* which produces approximately 90% of these infections, but lately, *Enterococcus* species have also been frequently isolated and have become important etiological agents of these infections [6].
The ideal treatment for urinary infections requires knowing the bacteria involved in the aetiology of these infections and their sensitivity to antibiotics. The increase in the number of antibiotics as well as their empirical and abusive use favoured the occurrence of certain strains that are multiresistant to antibiotics. Antibiotic resistance represents one of the difficult issues that medical practice is facing, but also one of the priority research themes in microbiology [7].

MATERIAL AND METHODS

A total of 5261 urine sample recovered from outpacients, were processed. Samples from outpatients from whom *Enterococcus* species were isolated were collected during the period from january 2010 to december 2012.
Midstream urine sample in early morning was collected in wide mouth sterile container. The urine samles were collected in sterile condition and all of them were cultured on sheep blood agar and lactose medium for enterobacteria and incubated at 37°C.
After 48 hours, each of the enterococcal positive cultures over than 10^5 CFU/ml was identified as UTI [8].

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Identification of the isolates to the genus level was performed according to: reaction on Gram staining; cellular morphology; growth and blackening of the bile-esculine agar; growth on CPS medium (bioMérieux) and Trypticase soy agar - 5% sheep blood (bioMérieux); absence of catalase.
Antibiotic susceptibility studies were performed by disc diffusion method (Kirby-Bauer) according to the Clinical and Laboratory Standard Institute (CLSI 2007) [9].
High level aminoglycoside resistance was determined by using discs with 120µg/mL of gentamicin and 300µg/mL of streptomycin. Isolates were interpreted as susceptible or resistant according to the sensitivity zones of the particular antimicrobial as recommended by CLSI.
Comparing antibiotic susceptibility was performed for the following antibiotics: Ampicillin (AM), Penicillin (P), Gentamicin (GM120), Streptomycin (STR 300), Tetracycline (TE), Linezolid (LZD), Ciprofloxacin (CIP), Levofloxacin (LEV), Rifampicin (RIF),Norfloxacin (NOR), Nitrofurantoin (FT), Vancomycin (VA).
To confirm the secretion of beta lacatamase we realized nitrocefin test (Nitrocef Matchbook stick-Hardy Diagnostics).

RESULTS

A total of 5261 urine samples were studied from patients with suspected signs and symptoms of urinary tract infection, out of which 976 specimens were positive in culture.

Table I. Frequency distribution of microbial pathogens in midstream urine of outpatients studied

Microbial Isolates / Strains	No. of strains	Frequency%
E.coli	703	72%
Proteus mirabilis	43	4%
Klebsiella pneumoniae	49	5%
Enterobacter cloacae	1	
Enterococcus faecium	4	1%
Enterococcus faecalis	58	5%
Streptococcus agalactiae	55	6%
Staphylococcus saprophyticus	24	3%
Staphylococcus epidermidis	22	2%
Staphylococcus aureus	9	1%
Serratia odorifera	2	
Serratia marcescens	3	
Pseudomonas fluorescens	1	
Pseudomonas aeruginosa	2	
TOTAL	976	100 %

The frequency of occurrence of the isolates and their strains therefore, were as follows: *E.Coli* (72%), *Streptococcus agalactiae* (6%), *Enterococcus faecalis* (5%), *Klebsiella pneumoniae* (5%), *Staphylococcus saprophyticus* (3%), *Staphylococcus epidermidis* (2%), *Enterococcus faecium* (1%), *Staphylococcus aureus* (1%) in that descending order (Table I). Enterococci were isolated in pure cultures in clinically significant numbers in 62 (6%) specimens.

Table II. Different *Enterococcus spp*. isolated

Enterococccus spp.	No. of isolates	Percentage
Enterococcus faecalis	58	94
Enterococcus faecium	4	6
TOTAL	62	100

Only two species of enterococci were isolated. Out of these two, *E. faecalis* (94%) was the predominant species followed by *E. faecium* (6%)(Table II).

Table III. Susceptibility of the *Enterococcus faecalis* isolates to different antimicrobials

Antimicrobial disc	Susceptible		Intermediate		Resistant	
	Number	Percentage	Number	Percentage	Number	Percentage
Penicillin	44	75.86	-	-	14	24.13
Ampicillin	44	75.86	-	-	14	24.13
Levofloxacin	50	86.2	-	-	8	13.79
Ciprofloxacin	48	82.75	2	3.44	8	13.79
Norfloxacin	48	82.75	2	3.44	8	13.79
Gentamicin	44	75.86	-	-	14	24.13
Streptomicin	44	75.86	-	-	14	24.13
Rifampicin	58	100	-	-	-	-
Tetracyclin	54	93.1	-	-	4	6.89
Linezolid	58	100	-	-	-	-
Vancomycin	58	100	-	-	-	-
Nitrofurantoin	49	84.48	-	-	9	15.51

E. faecalis isolates were resistant to ampicillin, penicillin, streptomicin, gentamicin (24.13%) and sensitive to vancomicin, linezolid and rifampicin (100%).
Most of the four *Enterococcus faecium* strains were sensitive to antibiotics, only one from these strains presents resistance to fluoroquinolones.

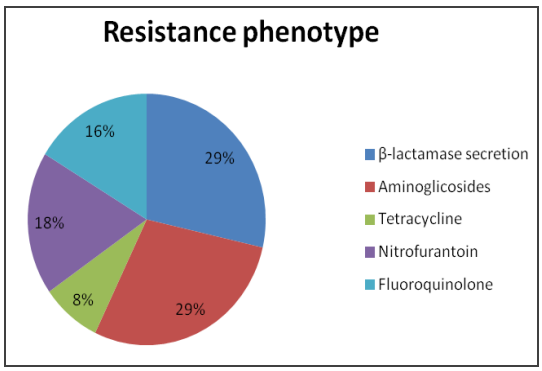

Fig. 1. The distribution of resistance phenotypes of *E. faecalis* strains. Antibiotic resistance of *Enterococcus spp*. strains isolated from outpatients with urinary tract infections

DISCUSSIONS

Over the past years, there has been an increasing interest in enterococci, not only because of their capacity to induce severe infections, but also due to their increased resistance to a number of antimicrobial agents [10].

In the present study, *E. coli* was the bacterium predominantly involved in urinary tract infections (72%), which is in accordance with the data obtained in other studies [11].

Miskeen *et al.* [12] detected enterococci in 7.4% of patients with urinary tract infections.

Cornia *et al.* [13] identified enterococci as etiological agents of bacteriuria among elderly male inpatients and outpatients in 22.5% of the cases.

In our study, from 976 positive urocultures, 58 *E. faecalis* strains (94%) and 4 *E. faecium* strains (6%) were isolated.

These 2 identified species are known to be significantly associated with severe clinical infections, their isolation being a serious reason for concern [14].

These results are comparable to those obtained by other authors [15], who mention the fact that *E. faecalis* has been isolated in the majority of the urinary tract infection cases (63.89%), being followed by *E. faecium* (11.37%).

From the point of view of resistance, enterococci have an intrinsic resistance to oxacillin and cephalosporins. *E. faecalis* is naturally resistant to clindamycin.

For enterococci isolated from urinary tract infections, the CLSI excludes erythromycin and clindamycin from the test panel, so that no data on macrolide and lincosamide resistance are available.

Our study showed that the isolated enterococci strains had a 24.13% resistance to penicillin, ampicillin, gentamicin and streptomycin, and only a 6.89% resistance to tetracycline.

In a study including 27 European countries performed by Schouten *et al.* [16], the resistance of enterococci to gentamicin varies between 1%-49%.

Recent literature data indicate a dramatic increase in the resistance levels of enterococci to penicillin of up to 100% [17], and to ampicillin of up to 62% [18].

Data obtained in our study evidence a low resistance (15.51%) of enterococci strains to nitrofurantoin, similarly to results obtained in other studies [19].

Doctors should be encouraged to use nitrofurantoin for the treatment of urinary tract infections due to the high efficiency and low cost of this antibiotic.

All enterococci strains identified in our study had a 100% sensitivity to vancomycin, which is in agreement with data published in other recent studies [20].

Antimicrobial resistance surveillance in Europe 2012 [21] shows a vancomycin resistance lower than 1% for Romania.

Resistance to glycopeptides (vancomycin and teicoplanin) is variable, ranging between 1%- 39% for countries using glycopeptide derivatives (avoparcin) as growth factors in animals [13].

CONCLUSIONS

1. The aim of this paper was to study the antibiotic resistance of *Enterococcus spp.* strains isolated from outpatients, as well as to establish the resistance phenotypes of these strains in order to select the most efficient antibiotic and to prevent the selection of multiresistant bacterial strains.
2. The study was performed on 62 *Enterococcus spp.* strains isolated from 5261 outpatient urocultures.
3. Two species of enterococci were identified: *E. faecalis* (94%) and *E. faecium* (6%).
4. From the point of view of resistance, our study showed that only one *E. faecium* strain had an isolated resistance to fluoroquinolones, while the isolated *E. faecalis* strains had a 24.13% resistance to ampicillin, penicillin, gentamicin, streptomycin and a 100% sensitivity to vancomycin.
5. The monitoring of antibiotic prescription is required, because empirical treatment in the absence of an antibiogram induces an increase in the resistance of *Enterococcus spp*. strains.
6. The role of the microbiology laboratory, essential for a correct anti-infective therapeutic approach, consists of performing an antibiogram, of establishing and interpreting the resistance phenotypes, thus providing information about the resistance development mechanisms and the way of spreading of these phenotypes.

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21. Antimicrobial resistance suveillance in Europe 2012

REZISTENTA LA ANTIBIOTICE A TULPINILOR DE ENTEROCOCCUS SPP. IZOLATE DIN INFECTII URINARE LA PACIENTII DIN AMBULATOR

REZUMAT

Scop: Studiul urmareste izolarea, identificarea si rezistenta la antibiotice a tulpinilor de *Enterococcus spp*. izolate din infectii urinare la pacientii din ambulator, precum si stabilirea fenotipurilor de rezistenta in care se incadreaza aceste tulpini.

Material si metoda: In perioada ianuarie 2010 - decembrie 2012 s-au luat in studiu 5261de uroculturi provenite de la pacientii laboratorului de analize medicale Intermed Cluj-Napoca. Izolarea, identificarea si testarea sensibilitatii germenilor izolati s-a realizat in laboratorul mai sus mentionat. Testarea rezistentei la antibiotice s-a realizat prin metoda difuzimetrica conform standardelor CLSI.

Rezultate: Din cele 5261 uroculturi efectuate la pacientii laboratorului Intermed, 976 au fost pozitive, izolandu-se 58 de tulpini de *E. faecalis* si 4 de *E.faecium*. Tulpinile de *E. faecalis* izolate au prezentat o rezistenta de: 24,13% la ampicilina, penicilina, gentamicina si streptomicina, 15,51% la nitrofurantoin, si de doar 6,89% la tetraciclina. Din cele 4 tulpini de *E. faecium* numai una a prezentat rezistenta izolata la fluoroquinolone.

Concluzii: In elaborarea unei terapii antiinfectioase corecte, rolul laboratorului de microbiologie consta in efectuarea antibiogramei, precum si stabilirea si interpretarea fenotipurilor de rezistenta, oferind astfel informatii asupra mecanismului de instalare a rezistentei si asupra modalitatilor de raspandire a acestor fenotipuri.

Cuvinte cheie: *Enterococcus; Enterococcus faecalis; Enterococcus faecium;* infectiile tractului urinar; urocultura; antibiotice

AUTOFLUORESCENCE IMAGING VIDEO ENDOSCOPY SYSTEM: PRINCIPLES, TECHNIQUE, COLOUR PATTERNS OF EARLY LARYNGEAL CANCER

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ABSTRACT

We conducted a prospective study to evaluate visualization of laryngeal lesions using AFE videoendoscopy, which is a new illumination system that allows for real-time WLE, but also makes it possible to switch to AFE endoscopy by the press of a single button. In total, 78 patients (53 men and 25 women) with signs or symptoms suggesting laryngeal dysfunction were included in this analysis. Histopathology revealed 30 cases (24%) of benign lesions such as granullomas, polyps, chronic laryngitis and papillomas. Mild dysplasia occurred in 35 cases (28%), moderate dysplasia in 18 cases (14.4%), severe dysplasia or carcinoma in situ in 32 cases (25.6%) and early invasive carcinoma in 10 cases (8%). Our results similar to other studies, have demonstrated a sensitivity by white light laryngoscopy of 88% and specificity of 77%. The sensitivity by autofluorescence laryngoscopy was 94% and specificity to 87%. The diagnosis accuracy by white light laryngoscopy was 73% (n=57) and that of autofluorescence 84% (n=66). Interest in fluorescence diagnostics in vivo has increased considerably in the last few years, as a result of several factors: the availability, decreasing cost and clinical suitability of the required technologies, development of new exogenous fluorophores and more detailed understanding of autofluorescence.

Key words: photodiagnosis, autofluorescence endoscopy, colour laryngeal patterns

INTRODUCTION

1. Fluorescence imaging: historical background

Early detection and proper treatment monitoring of neoplastic disease with the main function of preserving organs are some of the most challenging problems in the field of ENT oncology [1, 2]. In recent years there has been a great interest in fluorescence-based technique applied to clinical oncology especially in the ENT field. These techniques such as: laser-induced fluorescence (LIF), fluorescence correlation spectroscopy (FCS), photodynamic diagnosis (PDD) by autofluorescence (AF) or by exogenous fluorescence (induced fluorescence or IF) have a significant impact on the development of photodynamic therapy (PDT) of solid tumors.

The first study in cancer photodetection or tissue characterization using AFE (autofluorescence endoscopy) was reported by Policard in 1924 concerning the fluorescence of tumours under illumination with UV/violet light [3]. In 1942 Auler and Banzer [4] and Figge [5] observed red fluorescence of animal tumours after administration of exogenous porphyrins. The first use of fluorescein, to improve the detection and identification of brain tumours in vivo, was reported by Moore et al. in 1948 [6]. The groups of Profio [7], Alfonso [8], Lohmann [9] and Yang [10] did pioneering in vitro and in vivo studies of human and animal tumour autofluorescence. Recently, Zhang et al. [11] reported differences in fluorescence excitation and emission spectra between slowly and rapidly growing cells.

After exposing of instruments and tissue fluorescence principles we will discuss the clinical applications of in vivo fluorescence endoscopy for early cancer detection.

Secondly we will classify the colour patterns of early laryngeal lesions in AFE (autofluorescence endoscopy) images and then we will investigate the correlation between the patterns and histopathological findings.

2. Physical Principles and Instrumentation

When the mucosal surface is illuminated by light, the light can be reflected, back-scattered, absorbed or induce cellular autofluorescence. During malignant transformation morphological alterations of premalignant and normal tissues produce AF spectral changes.

Autofluorescence endoscopy is based on the excitation of tissue-specific fluorescence of the mucosa by

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short-wave blue light of the visible spectrum (375-440nm). The appearance and degree of autofluorescence depend especially on tissue structure's fluorophores content.
Fluorophores involved in cellular metabolism include reduced nicotinamide adenine dinucleotide (NADH), flavins, aromatic amino-acids (e.g. tryptophan, tyrosine, phenylalanine), carious porphyrins and lipopigments (e.g. ceroids, lipofuscin) that are the end-products of lipid metabolism. In addition, red porphyrin fluorescence due to bacteria may be significant in certain body sites and/or lesions.
Characteristics that are important to consider in optimizing and interpreting clinical studies include the following: a) each florophore has a distinct excitation and emission spectrum. b) the fluorophores are not uniformly distributed in tissue. c) any given tissue contains a mixture of many fluorophores of different concentration.
First AF endoscopy proved to be of great value in the detection of early lung cancer using the LIFE (Laser Induced Fluorescence Endoscopy) System (Xillix Technology Vancouver) [12, 13]. Then multiple publications were reported on its clinical applicability in the diagnosis of head and neck malignancies, mainly in laryngeal localizations [14, 15].
Recently the Storz Company, using the xenon light source with a band of excitation wavelength from 375 to 440 nm and producing AF imaging in a green spectral range, has developed a less expensive device, the D-light AF System [16].

3. Clinical applications

In urology, the detection of early-stage urothelial lesions with fluorescence illumination has pushed forwards the development of PDD technique [17]. Also in gynaecology PDD is used to diagnose precursors of malignant tissue that are not visible under white light [18]. Fluorescence bronhoscopy seems to be successful in the detection of early lung cancer [19] and in otolaryngology, fluorescence imaging of PP IX seems to be helpful in the detection of neoplastic lesions in the oral cavity [20]. In dermatology, efforts have been made to diagnose and demarcate basal cell carcinoma, for example [21]. Neurosurgeons se PP IX fluorescence for intraoperative detection of tumours and tumour rests after resection of malignant gliomas [22].

METHODS

AFE videoendoscope system

We conducted a prospective study to evaluate visualization of laryngeal lesions using AFE videoendoscopy, which is a new illumination system that allows for real-time WLE, but also makes it possible to switch to AFE endoscopy by the press of a single button. The blue light source was provided by a xenon short arc lamp with an excitation wavelength ranging from 375 to 440nm (D-Light C/AF Light System, 20133620), while an integrated optic filter block out the remitted excitation light up to 440nm. The camera system was a target - integrating high-resolution colour charge device (CCD-camera, Tricam PDD, 20221037; Karl Storz Co.). This camera was connected to a 70° rigid-angled endoscope (Karl Storz, Tuttlingen, Germany).

Patients: In total, 78 patients (53 men and 25 women) with signs or symptoms suggesting laryngeal dysfunction were included in this analysis. Main symptoms were: hoarseness, foreign body sensation in the throat, throat pain and fatigue of voice. The ages of the patients ranged from 34 to 79 years (mean 55 years).

Endoscopic examinations (procedure): First, routine endoscopic examinations were carried out using the WLE mode of the AFE videoendoscope system to identify abnormal mucosal areas in the larynx. We then examined the larynx by switching to the AFE mode with a press of a foot switch. Pictures made of abnormal mucosal areas were then assembled of both WLE and AFE and examined. In each case tissue sample were taken from the areas that were suspected. According to the size and extension of a laryngeal lesion microlaryngoscopy with excisional biopsy or laser CO2 cordectomy was performed in all cases to confirm the diagnosis histologically. Diagnostic accuracy, sensitivity and specificity were evaluated by correlating histopathologic diagnosis with visual impression.

RESULTS

A total of 78 patients with a total of 125 benign and malignant lesions of the vocal folds were examined. Histopathology revealed 30 cases (24%) of benign lesions such as granullomas, polyps, chronic laryngitis and papillomas. Mild dysplasia occurred in 35 cases (28%), moderate dysplasia in 18 cases (14.4%), severe dysplasia or carcinoma in situ in 32 cases (25.6%) and early invasive carcinoma in 10 cases (8%).
In the larynx all lesions were localized in the glottis region. Under AF videoendoscopy, the healthy mucosal areas of the head and neck regions displayed a typical bright green colour. The AF appearance of the premalignant lesions was found to be variable. If marked hyperkeratosis was present, it appeared as a light green or white colour, depending on its thickness.
The bright green colour in marked keratosis can be explained by the intensive fluorescence of keratin in the superficial epithelial layer. However, the intensity of the superficial layer may hide a subjacent precancerous lesion rendering impossible a complete evaluation. Therefore, the result should be regarded critically in strongly keratinizing lesions. Mucosal alteration, histologically defined as moderate dysplasia, showed a clearly recognizable altered green fluorescence with a

shift to reddish-blue, violet fluorescence. In situ and infiltrative squamous cell carcinomas all displayed a markedly altered green fluorescence, presenting reddish-violet colour. The AF image of the cancer was less variable, in most of the cases being recognized as a violet or reddish-blue colour (Table I).

Table I. The recorded AFE images and the colour patterns were classified into the following types depending on the epithelial thickness, inflammation or vascularization grade

Laryngeal lesion	AFE (LAF)	Remarks
Hyperkeratosis	Green to white(0)	Depending on the thickness
Normal mucosa, benign lesions	Green(0)	Better contrast of blood vessels
Simple hyperplasia, mild dysplasia	Green to violet(0-1)	Depending on inflammation grade
Chronic laryngitis, moderate dysplasia	Violet to red-violet(1-2)	Depending on inflammation grade
Teleangiectatic polyp, granulomas	Violet to red-violet(1-2)	Depending on vascularization grade
Papillomas	Red-violet(2)	Depending on vascularization grade
Severe dysplasia	Red-violet(2)	No differentiation from CIS/ICA
Carcinoma in situ	Red-violet(2)	No differentiation from DIII/ICA
Invasive cancer	Red-violet(2)	Yellow-orange in ulcerated tumors

Abbreviations: AFE autofluorescence endoscopy; LAF loss of autofluorescence(0=none, 1=mild,2= marked); CIS carcinoma in situ; ICA invasive cancer; DIII severe dysplasia.

Moreover, smaller, flat superficial epithelial lesions were found to be better outlined by AF photodiagnosis then large, ulcero-infiltrative tumours. The demarcation of these lesions provided a sharp, good image quality, but often showed a greater size than we expected from the white light examination, indicating the structural changes in the dysplastic mucosa around the infiltrating tumour. Some benign lesions, such as granulation tissue, papilloma and vocal fold polyp, also displayed a loss of green fluorescence. This may be the results that the hem molecule in dilated subepithelial vessels absorbs the incident light and therefore contributes to a further loss of autofluorescence. On the ulcerated necrotic surface of the tumours bacterial colonization was observed, reflecting as an orange or yellow bright fluorescence under the excitation light.

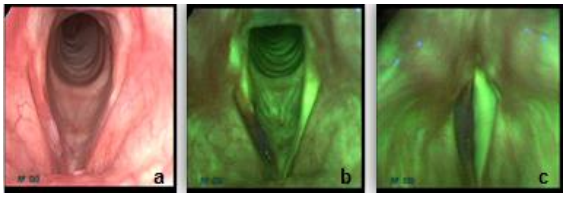

Fig. 1. Invasive Carcinoma (pT1a). White light endoscopy (a) demonstrates an irregular thickening of the right vocal fold; Autofluorescence endoscopy (b, c) shows a marked loss of autofluorescence, whereas induced fluorescence endoscopy.

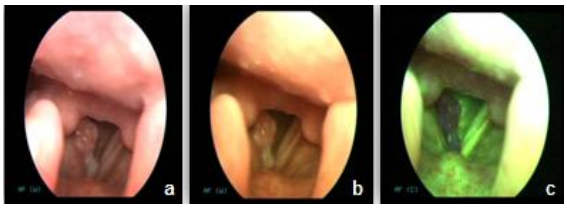

Fig. 2. Moderate differentiated squamous cell carcinoma of the right vocal fold. White light endoscopy (a); Yellow light endoscopy (b); Autofluorescence endoscopy (c) shows a marked loss of autofluorescence, whereas induced fluorescence endoscopy.

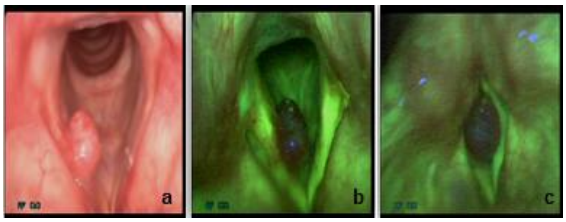

Fig. 3. Pyogenic granuloma on the right vocal fold: White light endoscopy (a); Autofluorescence endoscopy (b, c) shows a marked loss of autofluorescence, whereas induced fluorescence endoscopy.

Our results similar to other studies, have demonstrated sensitivity by white light laryngoscopy of 88% and specificity of 77%. The sensitivity by autofluorescence laryngoscopy was 94% and specificity to 87%. The diagnosis accuracy by white light laryngoscopy was 73% (n=57) and that of autofluorescence 84% (n=66).

DISCUSSION AND FUTURE PERSPECTIVES

Interest in fluorescence diagnostics in vivo has increased considerably in the last few years, as a result of several factors: the availability, decreasing cost and clinical suitability of the required technologies, development of new exogenous fluorophores and more detailed understanding of autofluorescence.

The detection of premalignant lesions or early cancer using autofluorescence depends on changes in one or more of:

a) the fluorophore concentration or spatial distribution;
b) the tissue architecture, such as mucosal thickening or loss of layered structure;

c) the biochemical/biophysical microenvironment of the tissue;
d) the metabolic status: NADH is fluorescent only in its reduced form;
e) the wavelength-dependent light attenuation due to the concentration and distribution of (non-fluorescent) chromophores, particularly haemoglobin.

But, the large number of false positive results with a consequent low positive predictive value implies a potential benefit from adjunct methods such as microendoscopy-narrow band imaging (ME-NBI), optical coherence tomography (OCT) or confocal laser endomicroscopy (CLE) [23] which would provide greater detection specificity. The use of trimodal imaging endoscopy that includes WLE, AFI and ME-NBI incorporated in one endoscopy system might improve diagnostic accuracy for high-grade dysplasia and early cancer [24]. ME-NBI is currently considered the technique of choice for improvement of diagnostic accuracy because it reduces the high rate of false-positive results associated with WLE and AFI. Consequently, the development of infrared technique may provide greater tissue penetration, obtaining images with greater contrast between lesions and their surrounding regions, and allowing the visualization of vascularization in deeper lesions, including the submucosa. Another possible strategy could be the supplementation of the AF technique with contact endoscopy (CE) [16, 25] or the combination of the AF method with protoporphyrin IX induced fluorescence endoscopy [26]. Although our series includes only a small number of patients treated by endoscopic laser surgery, this clinical application of the D-Light AF System in indirect laryngoscopy seems to be a very promising method in the control of the surgical margins.

If the goal of early detection of primary or recurrent tumours can be achieved, this may increase the likelihood of successful radical treatment and reduce complications. Additional potential applications of these techniques are to provide guidance in locationg the optimum sites for biopsy [27] to define the surgical margins for tumour resection [28, 29] and to optimize and monitor PDT treatments [30].

CONCLUSIONS

The potential clinical advantages of fluorescence techniques include: the high sensitivity; flexibility in the anatomical sites that can be investigated, especially using small-diameter optical fiber probes; reduction in the use of random tissue biopsies and the ease of use by the clinician. The potential for reduced health-care costs as a consequence for the minimally invasive nature and speed of the techniques and the improved patient outcome may be significant.

ACKNOWLEDGMENT

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SISTEMUL VIDEO DE ENDOSCOPIE CU AUTOFLUORESCENTA: PRINCIPII, TEHNICA, MODELE DE CULOARE PENTRU CANCERUL LARINGIAN INCIPIENT

REZUMAT

Am efectuat un studiu prospectiv pentru a evalua modelele de culoare ale leziunile laringiene utilizand videoendoscopia cu autofluorescenta, un sistem nou de iluminare ce permite in timp real vizualizarea atat cu lumina alba, cat si posibilitatea de a schimba in modul autofluorescent numai prin apasarea unui singur buton. In studiu au fost inclusi un numar de 78 pacienti (53 barbati si 25 femei) cu semne si simptome sugestive pentru o disfunctie laringiana. Rezultatele histopatologice au evidentiat 30 cazuri (24%) de leziuni benigne ce includ granuloame, polipi, laringita cronica si papiloame. Displazia slaba a putut fi decelata in 35 de cazuri (28%), displazia moderata in 18 cazuri (14,4%), displazia severa sau carcinomul in situ in 32 de cazuri (25,6%) si carcinomul invaziv incipient in 10 cazuri (8%). Rezultatele noastre, similare cu alte studii, au demonstrat o sensibilitate pentru endoscopia cu lumina alba de 88% si o specificitate de 77%. Sensibilitatea in cazul endoscopiei cu autofluorescenta a fost de 94% si specificitatea de 87%. Acuratetea diagnostica pentru endoscopia cu lumina alba a fost de 73% (n=57), iar pentru endoscopia cu autofluorescenta de 84% (n=66). Interesul pentru diagnosticul fluorescent *in vivo* a crescut considerabil in ultimii ani ca si rezultat al catorva factori: disponibilitatea, costurile mai scazute, adaptarea clinica a noilor tehnologii cerute, dezvoltarea unor noi fluorofori exogeni si nevoia de intelegere mai amanuntita a autofluorescentei.

Cuvinte cheie: fotodiagnosticul, endoscopia cu autofluorescenta, modele de culoare laringiana.

PLACENTAL STEM CELLS MODULATE TUMOR FORMATION IN MOUSE MODEL OF UTERINE SARCOMA

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ABSTRACT

Background: The adult mesenchymal stem cells (MSCs) are one of the most studied populations of adult multipotent stem cells, being isolated from bone marrow, peripheral blood, or other solid tissues. The fetal MSCs are isolated from placenta, amniotic fluid, umbilical cord and umbilical cord blood. Placental stem cells (PSC) are at the crossroads of adult stem cells and embryonic stem cells (ESCs). Although they do not have the same proliferative and differentiation potentials of ESCs, the placenta-derived multipotent cells may be superior to adult stem cells, so that their increased potential should be explored in more detail for further therapeutic strategies.

Materials and methods: Placental stem cells (PSCs) were isolated from 20 term-delivered placentas using enzymatic digestion (*Collagenase I A*). Further cultivation and expansion of single-cell suspension was performed in standard medium containing *alpha*-MEM, 10% FCS, and antibiotic solution, placed in 6-well culture dishes at 37° C and 5% CO_2. Morphological characterization showed fibroblast-shape cellular appearance, adherent to plastic surface, and reaching confluence in spindle-like colonies. For mouse model (CD1 Nu/Nu) of uterine sarcoma we used an immortalized cell line commercially available (MES-SA), admixed with Matrigel scaffold and we injected $1x10^6$ tumor cells in the experimental animal group (n = 10). Tumor formation was completed in 4 weeks. PSCs were transfected with GFP by electroporation method, and $1x10^6$ GFP-expressing PSCs were injected at tumor site for every mouse. For comparative analysis we used bone marrow-derived MSCs stably expressing GFP, and we visualized cellular migration, homing and effects by serial pictures taken every 3 days for 2 weeks using *in vivo* imaging system Hamamatsu Aequoria.

Results: PSCs obtained using enzymatic digestion were similar to bone marrow-derived mesenchymal stem cells in morphological and functional characteristics. Tracking of *in vivo* migration of GFP-PSCs comparative with MSCs-GFP expressing showed an increased ability of PSCs to decrease the size of initial tumor, evidenced by measurement of tumor diameter every 3 days for 2 weeks.

Conclusion: The present study shows that PSCs have an increased ability to modulate tumor growth compared to their counterparts, the bone marrow-derived MSCs, which makes them more suitable for therapeutic approaches in cancer treatment.

Key words: mesenchymal stem cells, placental stem cells, tumor growth

INTRODUCTION

The adult mesenchymal stem cells (MSCs) are one of the most studied populations of adult multipotent stem cells, being isolated from bone marrow, peripheral blood, or other solid tissues. The fetal MSCs are isolated from placenta, amniotic fluid, umbilical cord and umbilical cord blood. Placental stem cells (PSCs) are at the crossroads of adult stem cells and embryonic stem cells (ESCs). Although they do not have the same proliferative and differentiation potentials of ESCs, the placenta-derived multipotent cells may be superior to adult stem cells, so that their increased potential should be explored in more detail for further therapeutic strategies.

Despite recent advances in understanding of the molecular abnormalities associated with uterine cancer, these results have not been translated into better therapy for patients [1-3]. Uterine cancer is the most common cancer of the female genital tract. These cancers include carcinomas, sarcomas, and mixed epithelial-mesenchymal tumors. For all subtypes, the only available treatment is hysterectomy. There is no effective therapy once these tumors have spread outside the uterus; adjuvant therapy for metastatic disease does not enhance survival. Thus, further study is

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needed, not only to identify chemotherapeutic agents to treat uterine cancer after it has developed, but also to discover new strategies to prevent or delay disease progression.

Mesenchymal stem cells can be broadly grouped into two different subgroups adult MSCs and fetal MSCs. Adult MSCs are isolated from bone marrow, peripheral blood. Fetal MSCs are isolated from Placenta, amniotic fluid, umbilical cord and umbilical cord blood [4]. Placenta provides one of the most reliable and abundant source of MSCs [5]. Term placental tissues are discarded after birth, hence these tissues can be effectively utilized for research as well as clinical application without much ethical concern.

The aim of the present study was to establish the role of the PSCs in modulating tumor development. For this reason we comparatively analyze the bone marrow-derived MSCs and PSCs from morphological and phenotypical point of view. Further we induced tumor formation in mouse model and infused both MSCs and PSCs in order to establish their role in tumor development.

MATERIALS AND METHODS

Cell culture and expansion

Placental stem cells (PSCs) were isolated from 20 term-delivered placentas using enzymatic digestion (*Collagenase I A*) or explants method. Further cultivation and expansion of single-cell suspension was performed in standard medium containing *alpha*-MEM, 10% FCS, and antibiotic solution, placed in 6-well culture dishes at 37° C and 5% CO_2. Morphological characterization showed fibroblast-shape cellular appearance, adherent to plastic surface, and reaching confluence in spindle-like colonies. At passage 2, cells were submitted to differentiation assays towards osteoblasts, adipocytes, and chondrocytes, and were analyzed based on flowcytometric markers (CD34, CD45, CD90, CD73, and CD105) (data not shown).

For mouse model (CD1 Nu/Nu) of uterine sarcoma we used an immortalized cell line commercially available (MES-SA, ATCC® CRL-1976™). This cell line was was established from a surgical tumor specimen obtained at the time of hysterectomy. Initially, the cells were grown in soft agar, and later they were transferred to multiwell plates. Using the MES-SA cell line, tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with 10^7 cells. Literature data provides evidence that the tumor is a poorly differentiated uterine sarcoma. The nonepithelial origin of the cells was supported by ultrastructural studies and the absence of staining for mucin. The cells are sensitive to a number of chemotherapeutic agents including doxorubicin, dactinomycin, mitomycin C, taxol and bleomycin. They are resistant to vinblastine, dacarbazine, cisplatin, melphalan, vincristine, methotrexate and etoposide.

The base medium for this cell line is ATCC-formulated McCoy's 5a Medium Modified (ATCC®, Manassas, Virginia, USA) supplemented with fetal calf serum (FCS, ATCC®) to a final concentration of 10%.

Transfection procedure

The PSCs used in tumor models were transfected with Green Fluorescent Protein (GFP) using Amaxa™ Nucleofector™ II Device (Amaxa, Lonza Group Ltd, Basel, Switzerland). GFP is derived from the copepod Pontellina and can be used as a positive control to monitor transfection efficiency in cells of interest (Figure 1). The cells were passaged 2-3 days before nucleofection and cells were nucleofected after reaching 70 - 85% confluency (higher cell densities may cause lower nucleofection efficiencies). We used plasmid pmaxGFP (Amaxa) conc 0.5 ug/ul - 4 uL/reaction, and the procedure was as follows: nucleofection with Amaxa Nucleofector II, program U-23, for maximum eficiency. The kit used for transfection was Lonza (Amaxa) MSC Nucleofector kit. We used 5 x 10^5-10^6 cells/reaction. After trypsinization, the cells were centrifuged and then resuspended in 100 ul Nucleofector MSC solution and 2 ug GFP-containing plasmid. The cells are further electroporated, transfered in a new culture flask and expanded in mesenchymal stem cells medium supplemented with 20% FCS.

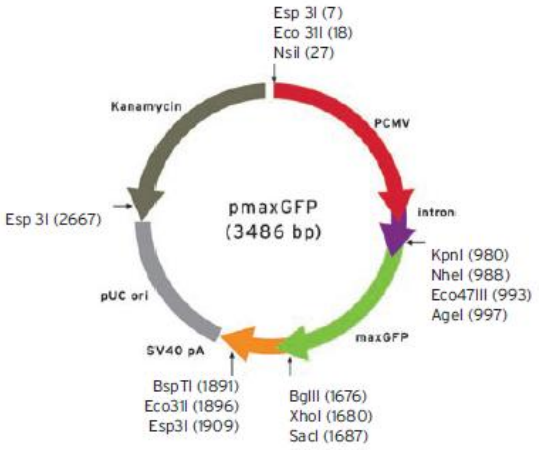

Fig. 1. GFP containing plasmid

Tumor formation in mouse models

MES-SA cells admixed with Matrigel scaffold (BD *Matrigel*™ Basement Membrane Matrix, BD Bioscience) and we injected $1x10^6$ tumor cells in the experimental animal group (n = 10). Tumor formation was completed in 4 weeks. PSCs were transfected with GFP by nucleofection method, and $1x10^6$ GFP-expressing PSCs were injected at tumor site for every mouse. For comparative analysis we used bone marrow-derived MSCs stably expressing GFP, and we visualized cellular

migration, homing and effects by serial pictures taken every 3 days for 2 weeks using *in vivo* imaging system Hamamatsu Aequoria (Hamamatsu Photonics Deutschland GmbH, Germany).
The mice were anesthetized with Sevofluran – induction and maintenance, while the dark box in which the animals were placed was maintained at 37° C. Cellular migration was tracked for 2 weeks, taking serial photographs, which were interpreted with the dedicated software of the *in vivo* imaging system. All animal experiments described herein comply with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Directive 86/609, Strasbourg, 1986) and the experimental protocol was reviewed and approved by the University of Medicine and Pharmacy Timisoara Board for Animal Experiments.

RESULTS AND DISCUSSION

Following infusion of $1x10^6$ tumor cells (human uterine sarcoma cell line MES-SA), subcutaneous tumor formation occurred within 4 weeks (Figure 2, Panel A). Peri-tumoral injection of $1x10^6$ GFP-expressing MSCs induced a decrease in size of mouse tumors after 1 week and 2 weeks (Figure 2, Panel B). Prior to injection at tumor site, $2x10^6$ PSCs were transfected with GFP with a transfection rate of 50%. Then, $1x10^6$ GFP-expressing PSCs were injected in tumor mouse model and we noticed a progressive decrease in tumor diameter at 1 week and 2 weeks (Figure 2, Panel C). Compared to MSCs-treated tumors, the ratio between initial tumor size and final tumor size was favorable to tumors treated with PSCs.

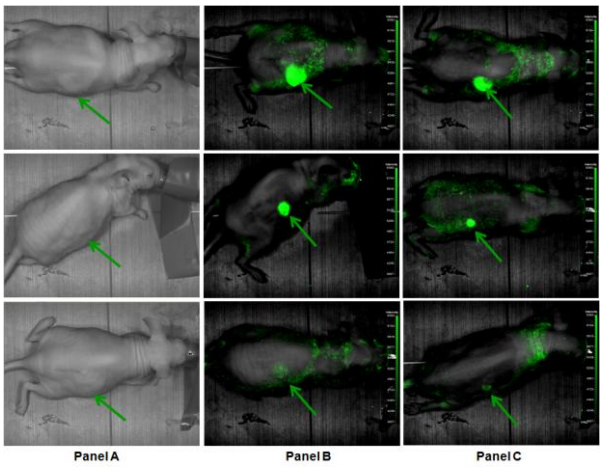

Fig. 2. Timeline of tumor development induced by MES-SA uterine sarcoma cells in CD1 nu/nu mice, injected with GFP-expressing MSCs or GFP-transfected PSCs

Mesenchymal stem cells (MSCs) are multipotent stem cells with the ability to differentiate into a variety of cell types, including chondrocytes, osteoblasts, adipocytes, muscles, neurons, stromal cells, and other cell types [6]. Several studies have indicated that human placenta-derived mesenchymal stem cells (PSCs) are similar to stem cells from bone marrow with respect to cell characteristics and their potential for multilineage differentiation [7-9]. As placental tissues originate during the first stages of embryological development, these tissues might contain cells that have retained the prosperities of early embryonic cells from which they derive. Furthermore, the placenta is fundamental for maintaining feto-maternal tolerance during pregnancy, suggesting that cells present in placenta tissue may have immonomodulatory characteristics. Meanwhile, recent studies showed that mesenchymal isolated from placenta tissue have the ability to specifically homing to multiple tumor site.
These three key aspects make cell from placenta extremely attractive candidate for possible use in cell therapy approaches in direct cancer therapy [10]. Some studies have engineered MSCs to express interferon b (IFNb) in gliomas [11], metastatic melanoma [12], and breast cancer models [13]. These studies showed that MSCs can travel to the same homing destination as the migrating cancer stem cell with unusual abilities to migrate to oncogenetic site. Meanwhile, PSCs have been shown to be more advantageous in cell procurement, storage, and transplantation than bone marrow-derived MSCs. Moreover, mesenchymal stromal cells from bone marrow have a risk of viral infection [14] and their differentiation capacity decreases significantly with donor age [15]. Furthermore, the placenta is generally discarded after birth; as such, this tissue is available in large supply, and the isolation of stem cell from this tissue does not involve any invasive procedures for the donor and avoids ethical controversy. These key aspects make cells isolated from placenta good candidates for possible use in cell therapy. The primary interest of this study has been focused on whether PSCs could specifically migrate to the tumor site and overcome tumor progression in a human uterine cancer model.

CONCLUSION

The present study shows that PSCs have an increased ability to modulate tumor growth compared to their counterparts, the bone marrow-derived MSCs, which makes them more suitable for therapeutic approaches in cancer treatment.

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CELULELE STEM DERIVATE DIN PLACENTA MODULEAZA FORMAREA TUMORILOR IN MODELUL ANIMAL DE SARCOM UTERIN

REZUMAT

Introducere: Celulele stem mezenchimale adulte (MSCs) sunt dintre cele mai studiate populatii de celule stem multipotente adulte, fiind izolate din maduva osoasa hematogena, sange periferic sau alte tesuturi. Celulele stem mezenchimale fetale sunt izolate din placenta, lichid amniotic, cordon ombilical si sange din cordonul ombilical. Celulele stem izolate din placenta (PSCs) se afla la limita dintre celule stem adulte si celule stem embrionare (ESCs). Cu toate ca nu au acelasi potential proliferativ si de diferentiere ca al ESCs, celulele stem multipotente izolate din placenta sunt superioare celulelor stem adulte, astfel incat potentialul lor crescut ar trebui explorat in detaliu in vederea utilizarii acestora pentru diverse strategii terapeutice.

Materiale si Metode: Celulele stem placentare (PSCs) au fost izolate din 20 de placente la termen folosind digestia enzimatica (*Coagenaza I A*). Cultivarea ulterioara si expandarea celulara au fost efectuate in placi de cultura cu 6 godeuri, la 37^{o} C si 5% CO_2, in mediul de cultura standard, care contine *alpha*-MEM, 10% ser fetal bovin (FCS) si solutie de antibiotic. Caracterizarea morfologica a aratat celule alungite, fibroblast-like, aderente la suprafata de plastic, care ajung la confluenta prin formarea de colonii in vartejuri. Pentru modelul animal (CD1 Nu/Nu) de sarcom uterin am folosit o linie celulara imortalizata disponibila comercial (MES-SA), care a fost amestecata cu matrice Matrigel si au fost injectate 1x10^{6} celule tumorale/animal in grupul animalelor folosite pentru acest experiment (n=10). Formarea tumorilor a fost completa in decurs de 4 saptamani de la injectare. PSCs au fost transfectare cu GFP prin metoda electroporarii si au fost injectate 1x10^{6} PSCs fluorescente/animal la nivelul tumorilor formate. Pentru analiza comparativa am folosit celule

stem mezenchimale izolate din maduva osoasa care exprima stabil GFP (MSC/GFP) si am urmarit migrarea celulara, localizarea si efectul acestor celule prin imagini seriale efectuate la fiecare 3 zile timp de 2 saptamani cu ajutorul sistemului de imagistica *in vivo* Hamamatsu Aequoria.

Rezultate: PSCs obtinute prin digestie enzimatica au fost similare celulelor stem mezenchimale derivate din maduva osoasa in ceea ce priveste caracteristicile morfologice si functionale. Urmarirea comparativa a migrarii in vivo a PSCs/GFP si MSCs/GFP a aratat ca celulele stem placentare prezinta o capacitate mai mare de a induce regresia dimensiunii initiale a tumorilor, evidentiata prin masurarea diametrului tumoral timp de 2 saptamani, la fiecare 3 zile.

Concluzie: Studiul prezent arata ca PSCs au o capacitate crescuta de a modula dezvoltarea tumorala comparativ cu celulele stem mezenchimale izolate din maduva osoasa, fiind mai potrivite pentru abordari terapeutice in tratamentul cancerului.

Cuvinte cheie: celule stem mezenchimale, celule stem placentare, dezvoltare tumorala

EPITHELIAL MARKERS EXPRESSED BY HUMAN MESENCHYMAL STEM CELLS UPON *IN VITRO* INDUCTION

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ABSTRACT
Although the property of adult stem cells to regenerate and repair damaged tissues may be controversial, bone marrow derived cells are reported to have potential contribution to tissue repair in both physiological and pathological conditions, including various types of epithelia. In this study we verify the effect of some chemical inductors to differentiate *in vitro* bone marrow-derived adult mesenchymal stem cells (MSCs) into epithelial-like cells. After isolation and expansion, induction of MSCs towards the epithelial lineage was made using various cytokines and growth factors (epidermal growth factor – EGF, hepatocyte growth factors – HGF, fibroblast growth factor - FGF), added in the culture alone or in combination. Differentiated cells were analyzed using immunofluorescence for vimentin, cytokeratin and E-cadherin markers. In all differentiated MSCs, some modifications of the cell morphology were noticed, as they become more polygonal and had a tendency to form multilayered culture. Expression of vimentin was weaker in the epithelial-like cells, compared with MSCs, whereas expression of cytokeratin was stronger in epithelial-differentiated cells. The results of RT-PCR showed increased expression of cytokeratin 19 and E-cadherin in epithelial cells, especially in cells cultured in media without FGF, suggesting a possible role FGF in inhibition of mesenchymal to epithelial transition. The experiments showed that MSCs differentiation toward the cells expressing epithelial markers is relatively easy to obtain using a certain combination of inductors, without genetic manipulation of the cells.
Key words: mesenchymal stem cells, epithelial-like cells, differentiation media, immunocytochemistry

INTRODUCTION

The epithelium is a type of tissue that lines all the body surfaces and cavities including skin, ocular surface, gastrointestinal tract or uro-genital system, providing a protective barrier [1,2]. Cell loss is continuously counter-balanced by cell production via the paracrine effects of various cytokines and growth factors including Epidermal Growth Factor (EGF), Hepatocyte Growth factor (HGF) or Fibroblast Growth Factor (FGF) maintaining thus epithelial homeostasis [2]. The source of cells involved in tissue regeneration is poorly defined and remains unknown. A possible source can be stem-like progenitor cells, found mainly in neural [5], vascular [6], hepatic [7], pancreatic [8] and epidermal tissue [9], or bone marrow derived stem cells, including hematopoietic stem cells (HSCs), involved in blood cell production and mesenchymal stem cells (MSCs) [2]. Various approaches have been followed in the field of stem cell engineering, in order to exploit the differentiation ability of *in vitro* MSCs to not only the well-known osteogenic, adipogenic and chondrogenic lineages [3], but also towards neural and epithelial lineages [1,2].

The aim of our study was to verify the effect of some chemical inductors to differentiate *in vitro* MSCs into epithelial-like cells, without genetically manipulate the cells.

MATERIALS AND METHODS

MSCs isolation and expansion

Normal human mesenchymal stem cells (MSCs) were obtained from bone marrow of 10 healthy Orthopedics patients undergoing hip replacement surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages and used in our experiments. The MSCs were further cultured and expanded in alpha-minimum essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37°C in 5% CO_2 atmosphere. Medium replacement was performed every third day and when reaching 80-90% confluence, the

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cells were passed using 0.25% Trypsin-EDTA solution (Sigma-Aldrich Company, Ayrshire, UK) followed by centrifugation (10 minutes, 300g) and replated in T75 culture flasks at a density of 10,000 cells/cm^2. Starting with passage two, part of the cells were used for further phonotypical analyses and differentiation assays, while MSCs expanded to passages 2-5 were used in the subsequent experiments.
All tissue samples were obtained after signing the informed consent elaborated under an approved protocol, according to the World Medical Association Declaration of Helsinki.

Epithelial differentiation of human MSCs
Human MSCs at passage three, obtained as described above, were plated on adherent uncoated culture flasks, and the differentiation process was induced using a protocol previously described [2]. Shortly, the induction medium was constituted of basal DMEM low glucose (1 g/l, Gibco BRL, Invitrogen, Carlsbad, CA, USA) with 10% FCS (PromoCell, Heidelberg, Germany), supplemented gradually with 10 ng/ml keratinocytes growth factor (KGF, Peprotech Inc., Rocky Hill, NJ), 20 ng/ml epidermal growth factor (EGF, Peprotech Inc., Rocky Hill, NJ), 10 ng/ml hepatocyte growth factor (HGF, Peprotech Inc., Rocky Hill, NJ) and 60 ng/ml insulin-like growth factor-2 (IGF-2; Human Recombinant, Peprotech Inc., Rocky Hill, NJ).

Differentiation analysis
After 24 days of differentiation, cells morphology was assessed using light and fluorescence microscopy. Specific cell surface antigens, including stem markers: CD29, CD44, CD54, CD73, CD90, CD95, CD105, CD106, CD166, and epithelial markers - E-cadherin, Ep-CAM and HER2, were measured by flow cytometry (FACScalibur, BD Biosciences, San Jose, CA, USA). Immunofluorescence staining was also performed for cytokeratin, vimetin, and E-cadherin. All the analysis was performed for the differentiated MSCs and for normal undifferentiated MSCs as control.

RESULTS AND DISCUSSION

In all differentiated MSCs, some modifications of the cell morphology were noticed, as they become more polygonal and had a tendency to form multilayered culture. Expression of vimentin was weaker in the epithelial-like cells, compared with MSCs, whereas expression of cytokeratin was stronger in epithelial-differentiated cells (Figure 1).

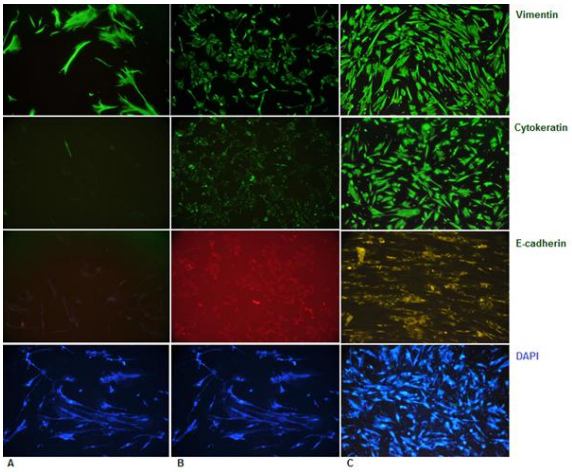

Fig. 1. Immunofluorescence staining for Vimentin, Cytokeratin, E-cadherin, and DAPI. Cell were analyzed by fluorescence microscopy at 20x magnification, for undifferentiated hMSCs (A), HK2 epithelial line (B) and differentiated hMSCs (C).

Expression of MSCs characteristic markers assessed by flowcytometry confirmed their definition criteria: positive for CD90, CD73 and CD105, these cells being negative for CD34 and CD45 (Figure 2).

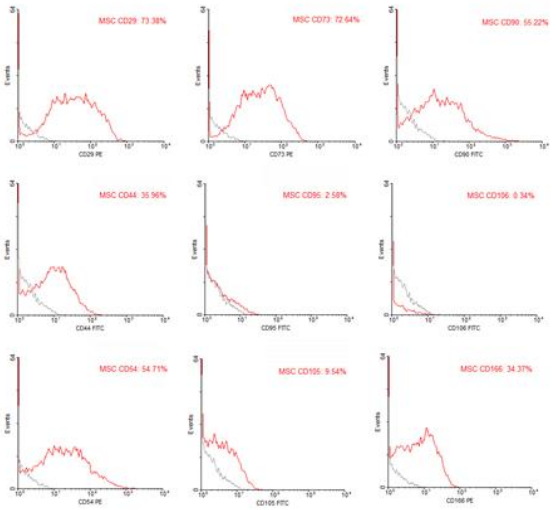

Fig. 2. Undifferentiated MSCs expressing characteristic surface markers

When the MSCs were induced towards epithelial lineage, the cells were induced towards a different expression pattern of surface markers (Figure 3).

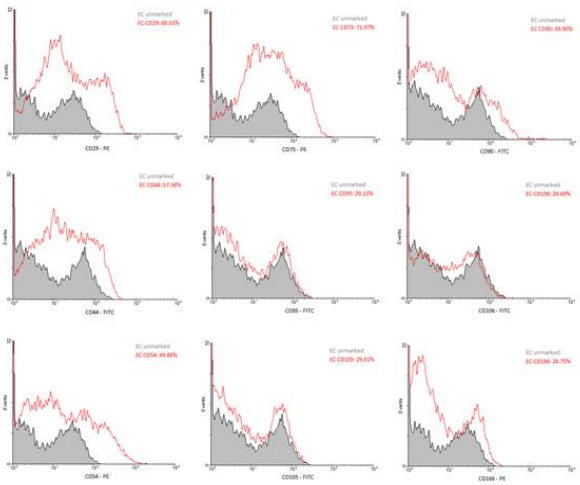

Fig. 3. Epithelial-differentiated MSCs showing different patterns of expression for surface markers

Regarding expression of epithelial characteristic markers, we demonstrated an increase of Ep-CAM, E-cadherin and Her2 in epithelial-differentiated cells, compared to undifferentiated MSCs (Figure 4).

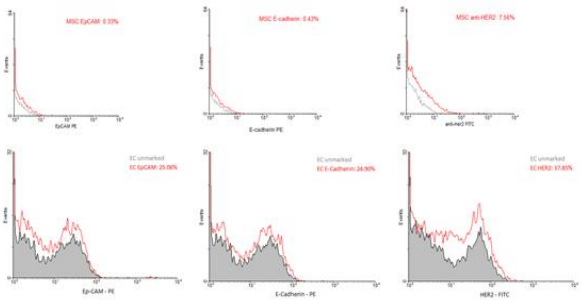

Fig. 4. Flowcytometric analysis of surface markers for undifferentiated MSCs (upper panel) and epithelial-like cells (lower panel)

There are few data regarding MSCs in vitro differentiation towards epithelial-like cells, so that MSCs ability in this case is an unrevealed subject. This phenomenon is interesting for evaluation of epithelial-mesenchymal (EMT) and mesenchymal epithelial transition (MET), mechanisms which are involved in tumor development [10, 11]. Although the data from literature provides information regarding the culture condition containing *trans retinoic acid* for induction of differentiation, our protocol suggested the use of growth factors, such as KGF (keratinocyte growth factor), EGF (epidermal growth factor), HGF (hepatocyte growth factor), and IGF-2 (insulin-like growth factor-2).

Several *in vitro* studies supported the idea of pluripotent ability of mesenchymal stem cells, which could be used for regeneration and repair of damaged tissues. Even though there are controversies regarding stem cells regeneration ability, there are solid evidence that stem cells can induce tissue repair in both physiological and pathological conditions, in different non-hematopoietic tissues, including here diverse epithelial types.

MSC homing and differentiation *in vivo* into type I pneumocytes was demonstrated [12], as well as acquirement of phenotypical characteristics of all main lung cells [13], using an animal model of lung injury. Moreover, MSCs were differentiated into pigmented retinian cells [14,15], skin [16], ductal pithelial cells [17], and epithelial renal tubular cells [18,19]. In this study, we demonstrated that MSCs can differentiate into epithelial-like cells with keratinocyte phenotype, which can be further used in cellular therapies.

CONCLUSION

The experiments showed that MSCs differentiation toward the cells expressing epithelial markers is relatively easy to obtain using a certain combination of inductors, without genetic manipulation of the cells.

ACKNOWLEDGEMENT

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MARKERI EPITELIALI EXPRIMATI DE CELULELE STEM MEZENCHIMALE UMANE IN CONDITII DE DIFERENTIERE *IN VITRO*

REZUMAT

Cu toate că abilitatea celulelor stem adulte de a regenera și repara țesuturile lezate rămâne controversată, celulele derivate din măduva hematogenă sunt raportate ca având o contribuție însemnată proceselor de reparare tisulară, atât în condiții fiziologice cât și patologice, incluzând de asemenea și numeroase tipuri de epitelii. În acest studiu, verificăm efectul anumitor substanțe biochimice inductoare pentru diferențierea *in vitro* a celulelor stem mezenchimale adulte (MSCs) derivate din măduva hematogenă, către celule epithelial-like. După izolare și expandare, inducția MSCs spre linie epitelială s-a realizat utilizând diferite citokine și factori de creștere (EGF – Factorul de creștere epidermală; HGF – Factorul de creștere al hepatocitelor; FGF – Factorul de creștere al fibroblastelor), adăugati individual în cultură sau în combinație. Celulele diferențiate au fost analizate prin imunohistochimie pentru vimentină și citokeratină, iar expresia genică pentru citokeratină 19 și E-caderină, prin RT-PCR. În toate MSC-urile diferențiate, s-au observat modificări morfologice, ele devenind mai poligonale cu tendință de a forma culturi pluristratificate. Expresia de vimentină a fost scăzută în celulele epithelial-like, în comparație cu MSC, pe când expresia de citokeratină a fost mai intensă în celulele diferențiate. Experimentele au demonstrat că diferențierea MSC spre celule care exprimă markeri epiteliali este relativ ușoară, prin combinația diferiților factori inductori, fără modificări genetice ale celulelor.

Cuvinte cheie: celule stem mezenchimale, celule epithelial-like, mediu de diferențiere epitelială, RT-PCR, imunohistochimie

EVALUATION OF LARGE AND GIANT INCIZIONAL HERNIA REPAIR: A 5-YEARS RETROSPECTIVE OBSERVATIONAL STUDY

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ABSTRACT

Large and giant incisional hernia defect is challenging in order to improve thepostoperative results. We conducted a 5 years retrospective observational study on large and giant incisional hernia repair in our Clinics in order to evaluate the surgical outcomes in relation with the patient's demographic characteristics, comorbidities, and the local surgical conditions. We have established the target group of 124 patients with large and giant ventral herniaout of a total number of 409, using inclusion and exclusion criteria. We collected data about demographics, comorbidities, hernia characteristics, operative details, and follow-up data (length of hospital stay, incidence of early complications). Data are presented as mean and range.Sex distribution shown a ratio of F: M= 3.34: 1. The peaks of incidence for female patients was the decade 60-69 y and 50-59 y, and for males the decade 70-79 y and 80-89 y. Prior hernia onset gynecology surgical procedures (39.5%) and classic gallbladder surgery (33.8%), have been noticed. 19% of the patients were admitted for a recurrence or multi recurrence. High blood pressure, obesity, diabetes, cardiac and respiratory diseases were the most obvious comorbidities. Alloplastic substitution with polypropylene, polyester and Dacron mesh has been used in all cases. The most preferred mesh position was intraperitoneal (78%), then retromuscular (15%) and preperitoneal (7%). Immediate postoperative complications occurred in 10% (12 cases) of the patients. 58.3% of them have been postoperative wound infection, then hemorrhagic shock, acute renal insufficiency, parietal abscess. Length of hospital stay varied widely according to the presence of complications, between 7 days and maximum 62 days. In large and giant incisional hernia defect size, location, depth of involvement, contamination, and comorbidity influence the repair outcomes. Pre, intra and postoperative management focused on patient's status and characteristics has to be done in a multidisciplinary team of clinicians, anesthesiologist, general surgeons and plastic surgeons.

Key words: large hernia, giant hernia, comorbidities, alloplastic substitution, mesh position, hospital stay

INTRODUCTION

Incisional hernias whose real incidence is difficult to determine are considered one of the most common complications after abdominal surgery with high morbidity and important socioeconomic costs. Sanders si Kingsnorth in a review on scientific papers from 1970-2012 have found big differences on repored incidence values [1]. The reported incidence after a midline laparotomy ranges from 3% to 20% and is doubled if the index operation is complicated by wound infection [2]. About 50% of incisional hernias are detected within one year of surgery, but they can occur several years afterwards, with a subsequent risk of 2% a year [2, 3]. Apparently incisional hernias can occur after any type of laparotomy incision but the most common are after midline (especially upper midline) and transverse incisions [3].

Since now risk factors such as patient's multiple comorbid conditions (age >65, male, obesity, diabetus, renal failure, smoking, others) have been reported in many studies, but the relative importance of many of the proposed risk factors is poorly understood yet [1, 4, 5]. Surgical factors that can influence the surgical outcomes have been mentioned in several studies. Type of technique as well as type of suture are to be mentioned. Reported recurrence rates remain high. For instance, the recurrence rate after open suture repair can be up to 54%, and up to 36% for open mesh repair. In general,

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recurrence rates are slightly lower, with a mean of about 15% [6]. A systematic review found that in the case of hernia repair without prosthetic mesh the recurrence rates is 12-54%, whereas in the case of hernia repair with mesh the recurrence rates range between 2-36% [6, 7].
In the case of large incisional hernia defect size, location, depth of involvement, contamination, and comorbidity influence the management of abdominal wall defects.
We conducted a 5 years retrospective observational study on large and giant incizional hernia repair in our Clinics in order to evaluate the patients demographic characteristics, comorbidities, and the surgical conditons (defect size, infection, bowel resection, mesh type, drain use, length of hospital) and surgical outcomes.

MATERIAL AND METHODS

We have conducted a retrospective observational study on big and large hernia repair with alloplastic substitution performed in the Surgery Clinics II of Emergency County Hospital Timisoara. Electronic medical of all 409 consecutive patients who had a ventral hernia repair from 2008 until 2012 have been revised. We have establish the target group of 124 patients with large and giant ventral hernia for whom we have revised also the operative reports, clinic notes, imaging reports. Hernia size of 10-15 cm was defined as large and more than 15 cm as giant. Data were collected using a standard Microsoft excel worksheet. We included in the study primary ventral hernia repairs and recurrent hernia repairs. Every recurrent hernia repair during the time period of the study was counted as a separately. We excluded ventral hernia repairs that were performed as a part of other procedures from our study.
The data that was obtained included demographics, comorbidities, and hernia characteristics such as status of natural history (reduced versus incarcerated). Operative details included the hernia management: defect size, mesh type, bowel resection type (colon vs small bowel), mesh positioning. Followup data consisted of length of hospital stay, the incidence of early complications. Data are presented as mean and range.

RESULTS

During 2008-2012, 409 patients have been operated in our Clinics for ventral hernia. Out of them 124 patients (23%) presented large and giant hernia. Most of them (87%) had a hernia defect between 10-15cm and the rest (13%) presented a giant hernia.
Distribution according to sex has shown a ratio of F:M = 3.34 : 1.
The age limits of the group were between 30-89 y. The peaks of incidence for female patients was the decade 60-69 years and 50-59 years, and for males the decade 70-79 years and 40-49 years (Figure 1).

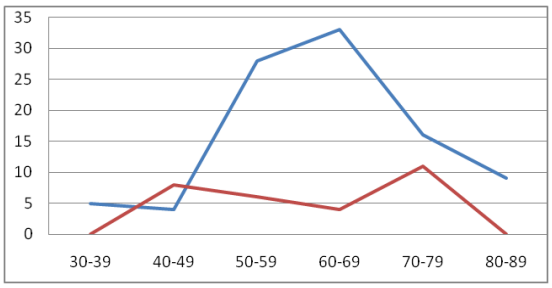

Fig. 1. Age groups and sex distribution of the cases. Legend: blue - females; red - males.

According to the type of laparotomy incision prior hernia onset we noticed: 39.5% gynecology surgical procedures (49 cases), 33.8% clasic gallbladder surgery (42 cases), 11.3%colon cancer surgery (14 cases), followed by other pathology conditions: gastric perforated ulcer, umbilical hernia, hidatic cyst, intestinal infarction, apendicectomy, pancreas cancer, ilio-femural bypass (Figure 2).

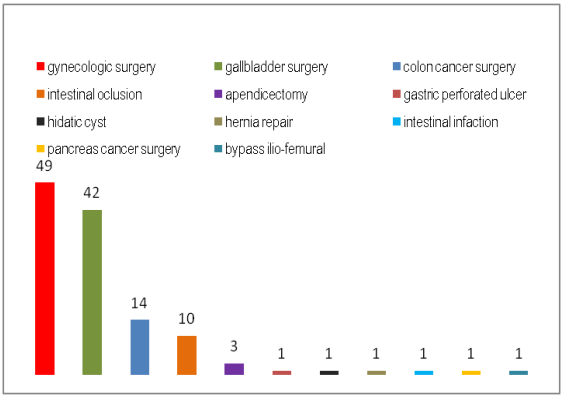

Fig. 2. Distribution of type of laparotomy incision prior hernia onset

In the group of 124 patients, 81% presented no recurrence. The rest of 19% have been distributed as following: 15% (18 patients) have been recorded with recurrence afer direct suture of the initial defect and 45 (5 patients) with multi recurrence (Figure 3).

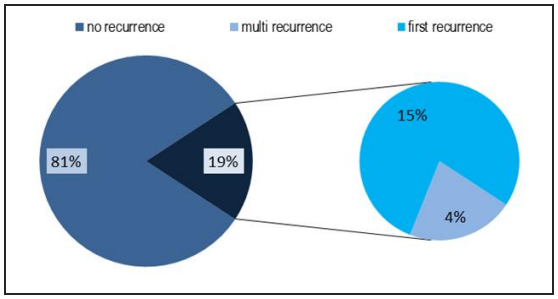

Fig. 3. Percentage distribution of recurrences.

Comorbidities associated with our group consisted in order in high blood pressure, obesity, diabetes, cardiac and respiratory diseases.

Considering the localisation of hernia the most frequant was at midline (78%), then xifo-umbilical, sub-umbilical and supra-umbilical. Lateral hernia heve been encoutered in 17% of cases, and transversal hernia in 5% (Table I).

Table I. Localisation of hernia

Median		Lateral		Transvers	
xifo-ombilical	39	subcostal	10	sub-ombilical	6
supra-ombilical	28	paramedian	8		
sub-ombilical	30	Right iliac fosse	3		
TOTAL	97(78%)	TOTAL	21(17%)	TOTAL	6(5%)

In 17 cases out of 124, the intervention was an emergency (irreducible or incarcerated ventral hernia and strangulated ventral hernia). All the patients were operated on under general anesthesia. For alloplastic substitution the most used were from polypropilene, polyester and Dacron. As for mesh position the most preferred was intraperitoneal (97 cases), retromuscular (19 cases) and preperitoneal (8 cases). Figure 4 is illustrating the precentage distribution of mesh positioning.

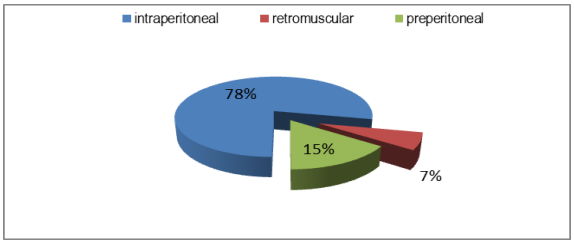

Fig. 4. Distribution of mesh positioning

Immediate postoperative complications have been noted in 10% (12 cases) of the patients: wound infection (7 cases), hemorrhagic shock, acute renal insufficiency, parietal abscess.
Length of hospital stay varied widly according to the presense of complications, between 7 days and maximum 62 days.

DISCUSSION

The correction of abdominal hernias remains one of the most common surgical procedures since it occurs in about 11% of laparotomies [8].
In our study we noticed as types of laparotomy incision prior hernia onset gynecology surgical procedures (39.5%), clasic gallbladder surgery (33.8%), colon cancer surgery (11.3%), and other pathology conditions. 19% of the group have been prezented for a recurrence or multi recurrence.
In 17 cases out of 124, the intervention was an emergency for irreducible or incarcerated ventral hernia and strangulated ventral hernia. Considering the localisation of hernia the most frequant was at midline (78%).
Better appreciation of patient characteristics may help to support which type of procedure or mesh use will likely succeed. Risk factors for the development of abdominal wall incisional hernias may include the following: overweight, smoking, age greater than 60 years, wound infection, re-laparotomy, postoperative wound dehiscence, postoperative wound infection chronic medical conditions (such as cirrhosis or cardiopulmonary disease, diabetes), and chronic steroid use [9, 10, 11].
In our study the risk factors were age greater than 60 years, female sex, and associated comorbidities consisted in order in high blood pressure, obesity, diabetes, cardiac and respiratory diseases.
In terms of hernia repair there are three options with respect to the location of the prosthesis: pre-muscleaponeurotic (onlay), retro-muscleaponeurotic (underlay or sublayer) or retro-muscular, and intraperitoneal (inlay) [8]. It seems to be no statistically significant the difference regarding recurrence between prostheses placed in retro-muscular or pre-muscleaponeurotic position [12]. The placement of the prosthesis intraperitoneally in the open operation, is an indication to the large incisional hernias, to the multi-recurrent, when associated with intra-abdominal injury, in obese patients and when the laparoscopic approach is contraindicated [8].
In our study, alloplastic substitution with polypropilene, polyester and Dacron mesh have been used in all cases. As for mesh position the most preferred was intraperitoneal (78%), retromuscular (15%) and preperitoneal (7%).
Suture repair is likely to produce results twice as bad as mesh repair and the current techniques that surgeons are using to repair incisional hernias with prosthetic mesh continue to yield recurrence rates of greater than 20% [13].
Major complications which can occur in repair of large incisional hernias include mesh infection and enterocutaneous fistula which may result in prolonged morbidity and require re-operation [14].
Our followup data have shown immediate postoperative complications in 10% (12 cases) of the patients. Most of them (58.3%) confrunted with postoperative wound infection (7 cases), then hemorrhagic shock, acute renal insufficiency, parietal abscess. Lenght of hospital stay varied widly according to the presense of complications, between 7 days and maximum 62 days.
Before performing a surgical approach for large or giant hernia repair it is important that the patient is advised about the possibility that their expectations regarding the outcome, both aesthetic and functional, could notto be always achieved.
Langer and colleagues in a comparative, retrospective study of over 400 incisional hernia operations over a 25-year period, estimated that the most important prognostic factor is the surgeon's experience [15]. Actually pre, intra and postoperative management of hernia repair focused on patient's status and charateristics should be done in a multidisciplinairy team of clinicians, anethesiologits, general surgeons and plastic surgeons.

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EVALUAREA REPARĂRII HERNIILOR INCIZIONALE MARI ȘI GIGANTE: STUDIU RETROSPECTIV OBSERVAȚIONAL PE 5 ANI

REZUMAT

Repararea defectului cauzat de hernia incizională mare și gigantă este o provocare pentru îmbunătățirea rezultatului postoperator. Am efectuat unstudiu retrospectiv observațional pe 5 ani privind repararea herniei mari și gigante în Clinica noastră, pentru a evalua rezultatele chirurgicalein relație cu caracteristicile demografice si comorbiditațile pacientului si condițiile chirurgicale locale. A fost selectat un grup țintă de 124 pacienți cu hernia incizională mare și gigantă din cei 409, folosind criterii de acceptare și respingere. Au fost colectate date demografice, de comorbiditate, caracteristici ale decfectului parietal, detalii de intervenție chirurgicală si date de supraveghere (durata de spitalizare, incidenta complicatiilor imediate). Datele sunt prezentate ca medii si distributii.Repartiția pe sexe a arătat o rată F:M= 3,34: 1. Vârfurile de incidență pentru femei au fost decadele 60-69 ani și 50-59 de ani, iar pentru bărbați decadele 70-79 si 80-89 de ani. Intervențiile chirurgicale din sfera ginecologică (39,5%) și chirurgia vezicii biliare (33,8%) au fost cele mai frecvente laparotomii înainte de apariția herniei. 19% dintre pacienți s-au internat pentru recidivă sau multi recidivă.Cele mai frecvente comorbidități semnalate au fost hipertensiunea arterială, obezitatea, diabetul zaharat, afecțiuni cardiace și respiratorii. In toate cazurile s-a folosit substituția alloplastică cu meșe din polipropilenă, poliester sau Dacron. Poziționarea meșei s-a făcut cel mai des intraperitoneal (78%), apoi retromuscular (15%) și preperitoneal (7%). Complicațiile postoperatorii imediate au apărut la 10% dintre pacienți (12 cazuri). 58,3% dintre ele au fost reprezentate de infectarea plăgii postoperatorii, urmate de șoc hemoragic, insuficiență renală acută, abces parietal. Durata de spitalizare a fost in funcție de prezența complicatiilorposoperatorii, respectiv între 7 zile și maxim 62 zile. In cazul defectelor parietale mari și gigante, mărimea defectului, localizarea, structurile implicate, prezența contaminarii și comorbiditățile pot influența rezultatele postoperatorii. Managementul pre, intra și postoperator focusat pe statusul și caracteristicile pacientului este de preferat să se facă în echipă multididciplinară de medici internişti, anestezişti, chirugi generaliţti si chirurgi plasticieni.

Cuvinte cheie: hernie mare, hernie gigantă, comorbidități, substituție alloplastică, poziționarea meșei, durata de spitalizare

POLYURETHANE NANOSTRUCTURES INCORPORATING URSOLIC AND OLEANOLIC ACIDS: *IN VITRO* ANTIPROLIFERATIVE EVALUATION

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ABSTRACT

In the last years, the frequency of melanoma is increasing while melanoma death-related causes currently represent more than 80% of all skin cancers. Oleanolic and ursolic acids are pentacyclic triterpenes that were proven as efficient antitumor agents in several cancers such as colon, liver, breast and prostate. The aim of this study was synthesis of a new delivery system for ursolic and oleanolic acids, as a solution for their poor water solubility that presumably causes low bioavailability. Using the interfacial polycondensation technique combined with spontaneous emulsification, nanoparticles with diameter between 30 and 68 nm were obtained.*In vitro*antriproliferative assay was conducted on several melanoma cell lines using the free pure compounds as blank A significant antiproliferative effect of ursolic acid on human and murine melanoma cells was observed, while the same doses of oleanolic acid exhibited only low potency on all tested cell lines. When incorporated into polyurethane nanoparticles, the active compounds lack their antiproliferative effect, probably due to a weak release from the final nanostructures.

Keywords: antiproliferative, melanoma, oleanolic acid, polyurethane nanoparticles, ursolic acid

INTRODUCTION

In the last years, the frequency of melanoma is increasing [1] while melanoma death-related causes currently represent more than 80% of all skin cancers [2]. The highest incidence occurs between age 20 and 45, with a mortality rate higher in men than in women; however, the incidence is higher among women, due to the fact that,usually, melanoma occurs on areas hard to be noticed on men, while on women it occurs on lower legs [3, reviewed in 4].

New treatment strategies are generated byincreasedresearch in the field, natural compounds being a significant part of the currently studied compounds. Oleanolic and ursolic acids are pentacyclic triterpenes that were proven as efficient antitumor agents in several cancers such as colon, liver, breast and prostate [5-7]. Also, several studies have reported their efficacy against melanoma [8, 9].

The aim of this study was synthesis of a new delivery system for ursolic and oleanolic acids, as a solution for their poor water solubility thatpresumably causes low bioavailability. The delivery system is based on polyurethane nanoparticles which were physicochemically analyzed in terms of particle size; in order to evaluate their efficacy, an *in vitro* antriproliferative assay was conducted on several melanoma cell lines using the free pure compounds as blank.

MATERIALS AND METHODS

Substances

Ursolic and oleanolic acids of analytical purity were purchased from Fluka (Sigma Aldrich, Steinheim, Germany). The reagents were purchased as follows: Merck (Germany) provided isophoronediisocyanate (IPDI), Polyethylene glycol M = 200 (PEG), acetone, Span®85 and Tween®20. 1,4-butanediol (BD) was purchased from Carl Roth GmbH (Germany) and ethylene glycol (EG) from Lach-Ners.r.o. (Czech R.).

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Synthesis of polyurethane drug delivery system
The method described in literature by Bouchemal*et al*. (10) was used for the polyurethane particles synthesis. The method consists in a multi-step procedure: interfacial polycondensation technique and spontaneous emulsification. An organic phase (mixture of 1.5 ml IPDI, 1.5 ml Span®85, and 15 ml acetone heated at 30°C) was injected into an aqueous phase (0.8 ml EG, 0.8 ml BD, 0.3 ml PEG, and 1.5 ml Tween®20 mixed with 15 ml distilled water and heated at 30° C) under magnetic stirring (500 rpm). The final mixture was heated at 40°C for four hours in order to ensure complete chemical reactions. The nanoparticles obtained from the procedure described above were maintained as layers of 3 mm thickness in Petri dishes at 80° C for 12 hours, for water and acetone removal, and then the products were washed with a mixture (water-acetone 1:1 v/v). The two triterpenic acids were added in concentration of 57 μM in two experiments and empty polymeric nanoparticles were used as a control.

Nanoparticles' size measurements
A particle size analyzer (Vasco from Cordouan Tech., France) was used in order to measure the size of the obtained polyurethane nanoparticles. Solutions of 1:100 (w/w) in ethanol were prepared. The parameters used were: 190 channels, 25° C temperature, 80% laser power, 18 μs time interval, continuous acquisition mode and Log-normal dispersion. The average value of three consecutive measurements was considered.

Alamar Blue in Vitro Analysis
A375 human melanoma cells (Sigma-Aldrich, Bucharest, Romania) were cultured in DMEM (Sigma-Aldrich, Bucharest, Romania) containing 15% FCS (fetal calf serum, PromoCell, Heidelberg, Germany) and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell). SK-mel 2 human melanoma cells (ATCC, Germany) were cultured in EMEM (ATCC, Germany) containing 10 % FBS (fetal bovine serum, ATCC, Germany) and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell). B16 4A5 murine melanoma cells were cultured in DMEM (Sigma-Aldrich, Bucharest, Romania) containing 10% FCS (fetal calf serum, PromoCell, Heidelberg, Germany),1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell) and 1% glutamine (PromoCell). Cells were maintained at an atmosphere of 5% CO_2 at 37°C.
The cell lines were seeded onto a 96-well microplate (5,000 cells/plate) and attached to the bottom of the well for 24 h. The next day, 150μL of new medium containing the tested substances were added and cells were incubated for 48 h.ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acids (UA_nano), oleanolic acid (OA) and polyurethane nanoparticles incorporating oleanolic acid (OA_nano) were added in the concentrations of 25, 50, 75 and 100 μM. The two highest concentrations (75 and 100 μM) of theempty polyurethane nanoparticles were tested. After the exposure time, 15 μL of the Alamar Blue solution was added and the cells were incubated for at least 4 h at 37 °C. A microplate reader was used to spectrophotometrically analyze the samples at 570 nm and 600 nm respectively. Untreated cells were used as controls. Since the tested substances were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Ayrshire, UK) and stored as stock solutions, the highest concentration of DMSO in the final medium (0.1%) was tested and did not have any significant effect on cell proliferation. For all experiments, final concentrations of the tested compounds were prepared by serial dilutions of the stock solution (10 mM) with the DMEM and EMEM medium.
Cell viability was calculated using the formula:

$$\{[(\varepsilon_{OX})\lambda_2 A\lambda_1 - (\varepsilon_{OX})\lambda_1 A\lambda_2 \text{ of test agent dilution}]/[(\varepsilon_{OX})\lambda_2 A°\lambda_1 - (\varepsilon_{OX})\lambda_1 A°\lambda_2 \text{ of untreated positive growth control}]\} \times 100$$

Where,
ε_{OX}= molar extinction coefficient of alamar Blue oxidized form (BLUE);
A = absorbance of test wells;
A° = absorbance of positive growth control well (cells without tested compounds);
λ_1= 570 nm and λ_2 = 600 nm

All *in vitro* experiments were performed on microplates with at least four parallel wells. The results are are presented as mean ± standard deviation. One way Anova test was used to determine the statistical difference between various experimental groups;*, ** and *** indicate $p<0.05$, $p<0.01$ and $p<0.001$.

RESULTS

Nanoparticle's diameter
Polyurethane nanoparticles with the two tested acids were prepared. Figure 1 exhibits the results of particle measurements for the polyurethane nanostructures with oleanolic acid (a), polyurethane nanoparticles with ursolic acid (b) and empty polyurethane nanoparticles (c). One can notice that nanoparticles with diameter between 30 and 68 nm were obtained, as follows: 30.91 nm for polyurethane nanoparticles with oleanolic acid, 58.90 nm for polyurethane nanoparticles containing ursolic acid and 67.63 nm for empty polyurethane samples. Only one population was obtained for all the samples, thus indicating their homogeneous nature.

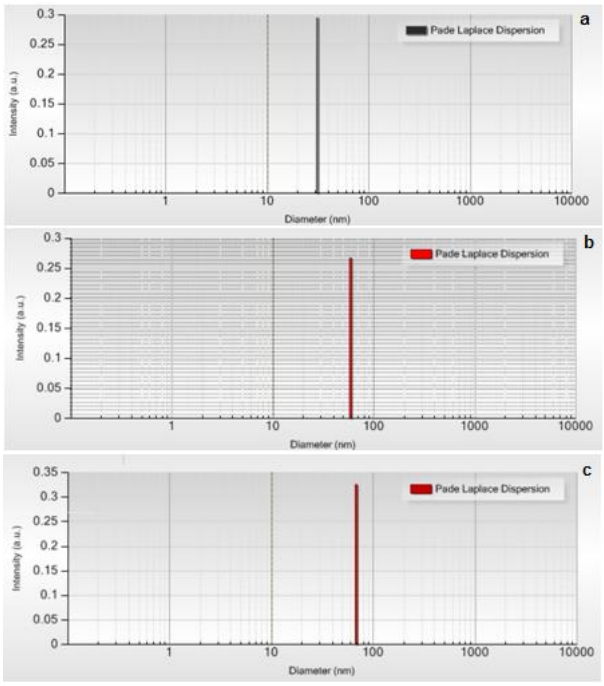

Fig. 1. Diameter of polyurethane structures for: (a) polyurethane nanoparticles with oleanolic acid, (b) polyurethane nanoparticles with ursolic acid and (c) empty polyurethane nanoparticles. Parameters used were: temperature (25° C), 190 channels, laser power (80%), time interval (18 μs), acquisition mode (continuous) and analysis mode (Pade-Laplace).

Cell proliferation assay

Figure 2 present the *in vitro*antiproliferative effects of ursolic and oleanolic acids compared to the encapsulated compounds on A375 human melanoma cell line. Exposure to ursolic acid for 48 h caused thedecrease of A375 cells' viability in a dose-dependent manner. The viability values were as follows: 96.60 ± 1.63 %, 76.25 ± 4.60 %, 52.61 ± 9.74 % and 33.23 ± 3.20 % for 25, 50, 75 and 100 μM, respectively. Encapsulation of ursolic acid inside polyurethane nanoparticles led to the following results: 97.98 ± 2.46 %, 85.74 ± 8.15 %, 100.44 ± 7.37 %, and 79.49 ± 4.54 %, for the same concentrations, respectively. In case of oleanolic acid, the viability values were: 77.94 ± 7.92 %, 75.70 ± 2.88 %, 75.15 ± 6.90 %, 82.54 ± 0.20 % for 25, 50, 75 and 100 μM, respectively. The same concentrations of the polyurethane nanoparticles incorporating ursolic acid led to the results: 86.68 ± 2.48 %, 88.03 ± 7.83 %, 88.59 ± 6.37 %, 85.58 ± 5.15 %, respectively. Also, the highest concentrations (75 and 100 μM) of empty polyurethane nanoparticles were tested and the viability values after 48 h of exposure were: 94.72 ± 6.69 % and 86.33 ± 2.20 %, respectively.

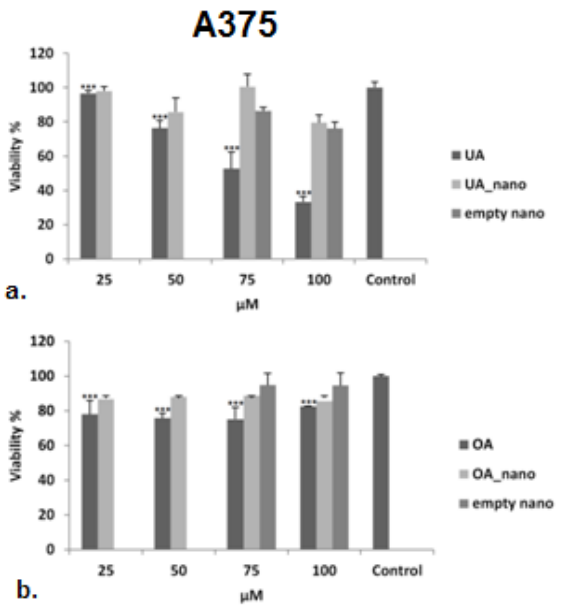

Fig. 2. *In vitro* viability of A375 human melanoma cells after 48 h of exposure to: (a) ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acid (UA_nano), empty polyurethane nanoparticle (empty_nano) and (b) oleanolic acid (OA), polyurethane nanoparticles incorporating oleanolic acid (OA_nano), empty polyurethane nanoparticle (empty_nano) as compared to the untreated cells (control).

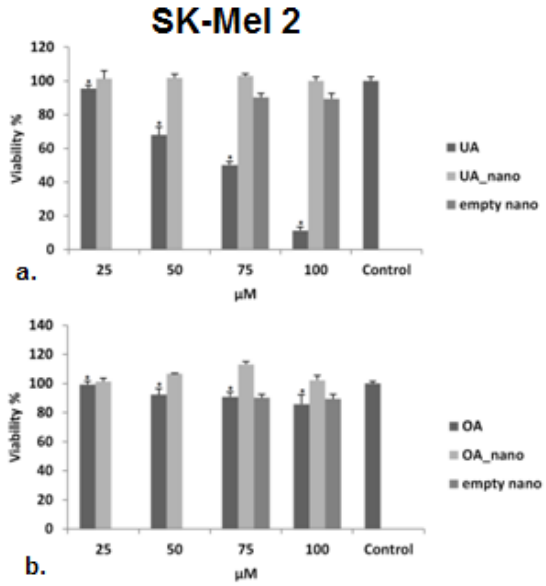

Fig. 3. *In vitro* viability of SK-Mel 2 human melanoma cells after 48 h of exposure to: (a) ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acid (UA_nano), empty polyurethane nanoparticle (empty_nano) and (b) oleanolic acid (OA), polyurethane nanoparticles incorporating oleanolic acid (OA_nano), empty polyurethane nanoparticle (empty_nano) as compared to the untreated cells (control).

As figure 3 shows, the viability values for SK-Mel 2 cells treated with ursolic and oleanolic acids as well as their nanoparticles are: 95.40 ± 1.89 %, 67.90 ± 4.77 %, 50.12 ± 2.16 %, 11.24 ± 2.19 % for 25, 50, 75 and 100 µM of ursolic acid, respectively. Treatment with nanoparticles incorporating ursolic acid resulted in the following values: 101.34 ± 4.77 %, 101.76 ± 2.22 %, 102.91 ± 1.35 % and 100.00 ± 2.41 % for 25, 50, 75 and 100 µM, respectively. Oleanolic acid's treatment led to the following results: 99.25 ± 2.19 %, 92.30 ± 4.23 %, 90.83 ± 3.16 % and 85.73 ± 4.38 % for 25, 50, 75 and 100 µM, respectively. For the same concentration of polyurethane nanoparticles incorporationgoleanolic acid the viability values were: 101.23 ± 2.31 %, 106.51 ± 0.48 %, 113.07 ± 2.00 % and 102.21 ± 3.28 %, respectively. The empty nanoparticles led to 90.20± 2.37 % for 75 µMand 89.34 ± 3.26 % for 100 µM.

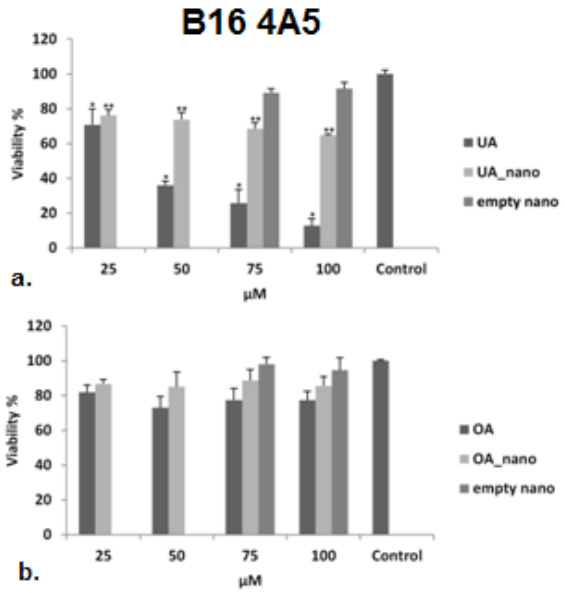

Fig. 4. *In vitro* viability of B16 4A5 murine melanoma cells after 48 h of exposure to: (a) ursolic acid (UA), polyurethane nanoparticles incorporating ursolic acid (UA_nano), empty polyurethane nanoparticle (empty_nano) and (b) oleanolic acid (OA), polyurethane nanoparticles incorporating oleanolic acid (OA_nano), empty polyurethane nanoparticle (empty_nano) as compared to the untreated cells (control).

B16 4A6 murine melanoma cells viability values after treatment with the tested compounds are presented in Figure 4. Ursolic acid's treatment produced decrease in cell viability as follows: 70.35 ± 9.23 %, 36.04 ± 2.32 %, 25.85 ± 7.80 % and 12.70 ± 4.11 % for 25, 50, 75 and 100 µM, respectively. For the same concentrations of nanoparticles with ursolic acid the results were: 76.08 ± 3.31 %, 73.68 ± 4.10 %, 68.43 ± 3.53 % and 64.77 ± 0.68 %, respectively. Treatment with oleanolic acid resulted in the following values: 81.23 ± 4.23 %, 73.00 ± 6.49 %, 77.41 ± 6.57 % and 77.31 ± 5.18 % for 25, 50, 75 and 100 µM, respectively. At the same concentrations, the results for nanoparticles with oleanolic acid were: 86.68 ± 2.48 %, 85.04 ± 8.53 %, 88.59 ± 6.37 % and 85.58 ± 5.15 %, respectively.

DISCUSSION

The role of pentacyclictritepenes in cancer treatment is the subject of many reports. Ursolic and oleanolic acid extracted from various plants were proven to act as antiproliferative agents in many cancer types. Meng*et al*. [11] reported the ursolic acid's activity on DU 145, PC-3 and LNCap prostate cancer lines with IC_{50}values ranging between 22 and 36 µM. Also, they reported ursolic acid's antitumor effect on HeLa (cervix) and RPMI 8226 (myeloma) cells [11]. Shan *et al*. [12] reported the *in vitro* antitumor activity of ursolic acid on HL60 and K562 (leukemia) cells as well on HL60 and H562 – Adriamycin resistant leukemia cells. Other studies also reported the*in vivo* anticancer activity of ursolic acid in HCT11 colon cancer xenograft [13] as well as in DU145 prostate cancer cells implanted in athymicBalb/c nude male mice [14].

In terms of oleanolic acid's anticancer activity, *in vitro* effects were reported for BGC-823 (gastric) and A549 (lung) cancer cells [15]. Kartini*et al*. reported positive results of oleanolic acid against SiHa (cervix) cells [16]. Cheng *et al*. reported the oleanolic acid antitumor activity on MCF-7 (breast) cancer cells; Cheng's results are contradictory to Hsu *et al*. that reported a weak anticancer activity of oleanolic acid on MCF-7 and MDA-MB-231 cells [17].

Our data revealed the *in vitro*antiproliferative activity of ursolic acid on all tested melanoma cell lines. The viability decrease manifested in a dose-dependent manner for all three cell lines, however with different potencies. The IC_{50}values ranged between 75 and 100 µM for A375 (human) melanoma cells, between 25 and 50 µM for B16 4A5 (murine) melanoma cells and 75 µM for SK-Mel 2 (human) melanoma cells. The same concentration of oleanolic acid seems to produce a weak response as antiproliferativeagent on the tested cells. For all cell lines, the IC_{50} values were not found in the tested range of concentration, the lower viability being recorded for B16 4A5 (73 %). SK-Mel 2 proved to be the most resistant to oleanolic acid. Our results are consistent with previous data, suggesting that ursolic acid is more effective than the oleanolic acid [18, 19] in terms of antiproliferative activity.

In recent years, nano- and micro-particles have raised medical interest, being used as drug delivery systems [20]. Some of the new strategies of drug delivery in nanotechnology are polymeric nanoparticles, microemulsions, liquid cristals systems, solid lipid nanoparticles and liposomes [21]. Along with other advantages, it was reported that nanostructures could improve the solubility and stability of the active substances

and alsoassociate compounds with different hydrophilicity/lipophilicity degrees [22, 23]. These advantages could lead to the reduction of the therapeutic dose and the improvement of pharmacological activity [21].
The present synthesis of polyurethane nanoparticles was based on the method previously described by Bouchemal*et al*. [10], who obtained nanocapsules as drug carriers for α-tocopherol, a good antioxidant with a high sensitivity on light, heat and oxygen. Based on this procedure, Borcan*et al*. also obtained microparticles using isophorondiisocyanate, with nontoxic *in vitro* activity on mesenchymal stem cells and reduced*in vivo* noxiousness on CD1Nu/Nu mice [20].
As the present study revealed, the encapsulation of ursolic and oleanolic acid into poly(ether) urethane nanoparticles did not improve the *in vitro* activity of the active compounds, leading to a high viability of the three cancer cell lines, as compared to the antiproliferative activity of the free substances. A possible explanation may consist in the strong entrapment of the active agent inside the polymeric nanostructures which presumably causes an impossibility to exert its pharmacological activity. Further research should be focused on the *in vivo* study of the polyurethane nanoparticles loaded with the active compounds.

CONCLUSIONS

The present study reportsa significantantiproliferative effect of ursolic acid on human and murine melanoma cells while the same doses of oleanolic acid exhibited only low potency on all tested cell lines. When incorporated into polyurethane nanoparticles, the active compounds lack their antiproliferative effect, probably due to a weak release from the final nanostructures.

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NANOSTRUCTURI POLIURETANICE INCORPORÂND ACIZII URSOLIC ȘI OLEANOLIC: EVALUAREA EFECTELOR ANTIPROLIFERATIVE *IN VITRO*

REZUMAT
În ultimii ani, frecvența melanomului este în creștere, iar mortalitatea produsă de melanom reprezintă mai mult de 80% din cancerele pielii. Acizii oleanolic și ursolic sunt triterpene pentaciclice care s-au dovedit a fi agenți antitumorali eficienți în câteva tipuri de cancere precum colon, ficat, sân și prostată. Scopul studiului a fost sinteza unui noi sistem de livrare a acidului ursolic și oleanolic, ca o soluție pentru slaba solubilitate în apă, care posibil să fie cauza unei biodisponibilități scăzute. Utilizând tehnica de policondensare interfacială combinată cu emulsificarea spontană, au fost obținute nanoparticule cu diametru intre 30 și 68 nm. S-au realizat teste antiproliferative *in vitro*, pe câteva linii celulare de melanom, utilizând compuși puri liberi ca martori. A fost observat un efect antiproliferativ semnificativ al acidul ursolic pe liniile celulare melanomice umane și murinice, în timp ce aceleași concentrații de acid oleanolic au avut o potență redusă pe toate liniile celulare testare. După incorporarea în nanoparticule poliuretanice, compușii activi și-au pierdut efectul antiproliferativ, probabil datorită unei eliberări scăzute din nanostructurile finale.
Cuvinte cheie: antiproliferativ, melanom, acid oleanolic, nanoparticule poliuretanice, acid ursolic