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CONTENTS

CUPRINS

TESTING THE DISCRIMINANT POWER OF ECG SIGNAL SONIFICATION PROCEDURES

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ABSTRACT

The purpose of the paper is to develop a protocol for testing the potential improvement in ECG investigations by adding sound to visual display of ECG signal. As there are numerous procedures for sonification of biological signals, which can be tested on both normal and pathological signals, this protocol allows a systematic approach, associated with a quantitative estimation of any of the sonic representations: acoustic continuous or quasicontinuous display, as well as real time, magnified or compressed display. The analysis of the potential use of various types of tempolenses is also discussed. An application of such a protocol will bring important data for further studies on potential use of sonifcation tools in the analysis of various biological signals.
Keywords: ECG, sonification, tempolens, discriminant power.

INTRODUCTION

Sonification is a method using acoustic non verbal convey for data representation (1). Its potential use for cardiac signals representation was revealed by the pioneering work of Ballora (2). However, sonification of biological signals is still in its infancy, studied by just a few research teams. This is due not only to the absence of appropriate equipment, but also to the lack of a proper semiology, to allow the potential user to retrieve, recognize and classify a signal - the essential step to bring this method within the arsenal of cardiac investigations. This paper is the first in a set of articles which intend to present a set up of a methodology for a classification of some cardiac signals, sonified by various methods, starting with a short presentation of these sonification methods.

SONIFICATION METHODS

A short presentation of sonification methods, as described by Mihalas et al. (3) is necessary for a better understanding of the proposed methodology.

1.1. Sonification levels

There are several ways to transform a parameter of a signal into a parameter of the corresponding sound. It should be mentioned that most usual techniques record and/or store the signal in a digital form; hence it has usually a discrete original representation. One can distinguish three major levels for sonification, briefly presented bellow.

a) Acoustic Transform

This level assumes a direct correspondence between the frequency of the sound and the amplitude of the signal (linear of exponential). There are two ways to display the sound:

i - with a continuous change of frequency between two neighbor points, called "Level A"

ii - with a quasicontinuous representation, which preserve the discrete form of the signal, producing a sound of a constant frequency for a duration equal to sampling frequency. (In fact we can set an arbitrary value for the duration this sound). This is called "Level Q".

b) Sonic Transform

Let us mention here that the frequencies produced by the acoustic transform belong to a continuous spectrum. If we apply the procedure of level Q, but associate only frequencies from a discrete spectrum (ex the musical notes), the representation obtained is called "Level S". For level S we can add algorithms for obtaining various durations of the sounds.

c) Musical Transform

An extension of level S, by introducing rhythm and harmony (and timber), would yield a representation which sounds like music - this is the "Level M". This level will not be analyzed in this paper.

1.2. Tempolenses

Various tools developed for enhancing the performances of a visualization procedure can be transposed also for sonification. Such a useful tool is the "lens" used for magnifying certain areas of an image. The equivalent transposition which can enhance the temporal resolution of a sonic representation was called "temporal lens" or "tempolens" (4). The main parameter which characterizes a tempolens is the "magnification" - m, defined as the ratio between the duration of the display and the original duration of the segment. For m>1 we have a "dilation" of the signal, while for m<1 the signal is "compressed" (a useful procedure for shortening the display duration of some signals).

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The tempolenses can be classified into:

tempolenses with constant magnification

tempolenses with variable magnification (5), which have different magnifications for different segments of the signal. The preliminary results show a good resolution, without inutile prolongation of other segments.

1.3. Signals

For this phase of the study we worked with cardiac signals for humans downloaded from PhysioBank (6), from both healthy subjects and from patients presenting sleep apnea, congestive heart failure and arrhythmia. Only one ECG lead was used; with sampling rate of 100 Hz. When it was originally recorded with a different sampling rate, it is resampled. This standardization will bring a standard processing procedure for all signals. The ECG signal is imported in MATLAB and filtered (20 Hz low pass). The sonic representation was generated in MATLAB R2011b.

METHODOLOGICAL SETUP

In order to build a methodology to cover all aspects necessary for introducing a new procedure as an investigation tool, it is worth to note that it comprises several phases:

a) understanding the procedure- in our case it will be represented by collection of demo signals, sonified in various ways. Our previous work (3) showed that using visualization in parallel with sonification raises the quality of understanding.

b) testing the discriminant power of various sonification procedures – the work in this phase is very difficult, taking into account the very large number of tests and the degree of subjectivity of listeners in ranking the procedures; however, this phase is essential before using the procedures for classification.

c) learning phase – similar to any other method, once established the procedure, step by step, a large collection of signals will be displayed, both video and audio; the "supervised learning" procedure will be applied, having for each signal all additional information about diagnosis, age gender etc.

d) classification assessment – the last phase, when the users will be presented the now signals which have to be classified.

e) the classical analysis (sensitivity/specificity, predictive values, accuracy and error rate etc.) will be performed, separately for each class of signal and sonification procedure.

In this paper only the phase about testing of discriminant power will be presented in detail, without describing the learning phase and classification assessment. However, the demo phase will be described, since it precedes the testing phase.

METHODOLOGY FOR UNDERSTANDING THE SONIFICATION PROCEDURE

This phase has two major purposes:

a) to understand the sonification levels A and Q (levels S and M have not been yet introduced in our studies).

b) to understand tempolenses

3.1. Understanding sonification levels

A couple of demo signals have been built (7) to have both quick and slow variations.

The A representation is unique; however, for Q representation one can choose various temporal steps (multiplying the sampling period with an integer, q). The same signal can be reproduced several times, starting with a very short steps (0.02 s for q=2) when it sounds like A and increasing gradually up to 0.05 s (q=5) and 0.2 s (q=20), when each step is heard separately. It is worth to note here that, due to the very short duration of some events (for instance the R wave of the QRS complex in ECG), an increase of the step (q) might yield a "jump" over such short events (no longer observing Shannon's sampling theorem). Hence, we have chosen the maximal value from each set of q samples. For the preliminary trial we selected q=5.

For S representation, the distinction from level Q with the same temporal step is less perceivable at quick variations, but might be perceived at slow variations. We can mention here that the distinction will be clearer when the sonification is performed on more channels simultaneously.

3.2. Understanding tempolenses

A. Tempolenses with constant magnification

Let us first remind that for A level we have only one way of magnification, which can be easily understood and perceived if, after a display with normal speed, we can dilate it 2 times (and/or 4 times), then compress it 2x (and/or 4x). Let us now observe that for Q and S levels, the tempolens can be applied in two ways:

a) method N (no interpolation) each temporal step is magnified "n" times (here "magnified" can be dilated or compressed); there is no interpolation(or skip) for frequencies

b) method I (interpolation) each time step is split into "n" subintervals (n>1 for dilation and n < 1 for compression) and the corresponding interpolated frequencies are computed (for compression this is equivalent to skipping some values).

The procedure for A and Q levels will start with the original sound, followed by "4xN" and "4xI" (dilated 4 times, no interpolation, then interpolation).

Note: for the rhythm analysis (RR signals), instead of dilation the signal will be compressed, and the "Observation sheet" was adjusted accordingly.

B. Tempolenses with variable magnification

As we showed in a previous paper (4) the interest paid to various regions of the sound is different. The tempolens with variable magnification used by us, in both demo and real signals, dilates the signal 2x for the first quarter of the duration, preserves unchanged the next quarter and compresses 2: the rest half.

For understanding this lens the original signal will be first displayed at A level without lens and then with lens, followed by Q level, both N and I method. For demo signals the S level sounds similar to Q and will not be displayed separately.

METHODOLOGY FOR TESTING THE DISCRIMINANT POWER OF VARIOUS SONIFICATION PROCEDURES

The major interest of the study is to test the capacity of users to distinguish the difference between signals originating

from patients with different diseases. Of course, these differences might be better or less perceivable depending on the sonification procedure chosen the sonification procedure chosen for representation.

a) Working groups

The study will be performed on three groups of persons: a group of cardiologists, a group of medical students and a group of musicians (students). Each person will have a User ID, and a file comprising users relevant data will be built (age, gender, profession etc)

b) Display procedures

Individual setup is prepared for the setups users with two versions of display: head phones or loud speakers. The length of an audio sample will be 10 seconds.

The working protocol comprises more phases:

accommodation phase – the user will be presented successively the demo signals for each of the displaying scheme to be used

several testing phases, each one dedicated to a pair of two signals. At the beginning, each pair will have a normal signal displayed first; however, it can be changed, when the differential diagnosis comes to be tested.

The screen displayed to a user looks like in figures 1 and 2. In Figure 1 the signals were displayed with normal rate, while in fig.2, the same signals were displayed with a tempolens with magnification 4x.

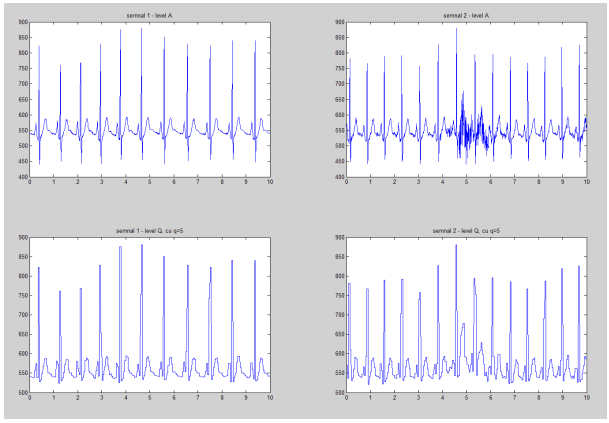
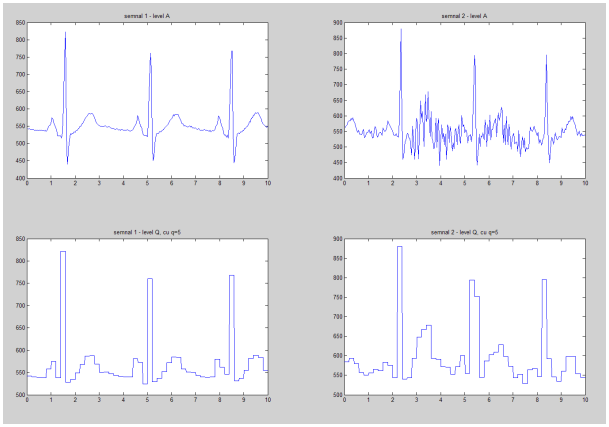


Fig.1. Visual display of two signals at normal rate (no tempolens). Left side - reference signal (normal ECG), right side - tested signal (ECG in sleep apnea). Upper side - level A, bottom - level Q

The quadrants are allocated standard conditions: the top two correspond to the A level and the two at the bottom correspond to the Q level (for q=5); the value of q can be changed, if necessary. On the vertical axes, the left side comprises the visual display of the signal 1 (the normal ECG in the first set of trials), while the right side displays signal 2.

When the program starts, the first quadrant (up left) is displayed and the corresponding sound is heard (standard duration is 10 seconds); then, after a pause of 0.5 seconds, the second quadrant is visualized and heard. The program stops and the user can now estimate the score of his perception. With a click the program starts the display of the third, then the fourth quadrant, corresponding to the Q level; the programs stops again and restarts with a new click.

Fig.2. Visual display of the same two signals with tempolens with magnification 4x. Starting point of magnification for ECG in sleep apnea was t=4



The next test phase displays the same two signals, but with different processing parameters: magnification 4 times without interpolation, then magnification 4 times with interpolation and finally applying a tempolens with variable magnification (2x : 1x : 1/2x).

The sounds can be heard by accessing the library of sounds, at the address: <www.medinfo.umft.ro/dim/sonification/test-andor>

The user can repeat any of the phases. Moreover, when applying a tempolens, he can choose the starting point (by specifying the starting time for either reference signal 1, or tested signal 2, or both). This point will be preserved for all magnifications in that tour. A full round for comparing two signals takes 10-15 minutes, depending on the number of repetitions required by the user.

c) Perception scale

The users will be asked to rank the quality of perception of the differences between the two signals to be compared. As the signals will be displayed both visual and acoustic, the users will rank also the differences perceived visually.

The following scale for ranking the differences is proposed (Table I):

Table I. Ranking scale for differences

Class	Score	Description
Not relevant	0	No differences
	1	Small differences, difficult to describe
Relevant	2	Not large, but clearly distinguishable
	3	Large differences
Other	9	No resemblance/seem to be different signals
	X	Could not hear/see the signal, technical problems

A demo with examples for each score is presented before starting the testing.

d) Observation file

The results of tests will be recorded on an "Observation file" presented in Annex 1.

Such a file will be filled in for each pair of signals to be compared.AS it can be seen, the users can ask a repetition of the signals for deciding upon the differences; the number of displays is also recorded (#); a value #>1 represents repetitions.

CONCLUSIONS

The procedures described above allow a solid approach for estimating the real potential of using sonification as a complementary tool in ECG investigations. Introducing such a tool in clinical practice must pass first the full set of tests mentioned above. This paper refer to the first protocol of studies to be performed before introducing more sophisticated transformations, corresponding to levels S, or even M, where the number of mappings between the signal parameters and sonic parameters can increase dramatically.

Let us mention here that such a study is also very useful for comparing sonification with visualization (8). There are high expectations from an integrative representation, combining both visualization and sonification, not only for a higher refinement, but also bringing in supplementary data about the overall physiological condition of a patient (9).

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ANNEX 1. OBSERVATION FILE

I. ESTIMATION OF CLARITY OF AUDIO/VIDEO DISPLAY

User ID .....
Date .....
Time .....

Table AI. Accommodation

Accommodation phase		
full	short	no

II. COMPARISON OF TWO SIGNALS

Signal 1 ID: ..... Signal 2 ID: .....

Table AII. Discriminant power between 2 signals, by A and Q levels

Type processing		Normal		4X - N		4X - I		Var	
Method display		A	Q	A	Q	A	Q	A	Q
Sound	S								
Visual	V								
No of displays	#								

Table AII is repeated in the Observation Sheet several times; one such table is filled in for each pair of signals.

III. REMARKS

Table AIII. Was sonification helpful in perceiving the differences between these two signals?

Not at all	Not very much	A little	Clearly yes	I don't know

3.b. If yes, which was the most relevant procedure? (you can choose more than one)
3.c. Did you like any of the sounds? Which one?
3.d. Other comments: .....

TESTAREA PUTERII DE DISCRIMIARE A SEMANALELOR ECG PRIN PROCEDURI DE SONORIZARE

REZUMAT

Scopul acestui studiu a fost dezvoltarea unui protocol de testare a imbunatatirii investigatiilor ECG prin adaugarea sunetelor la imaginea grafica a semnalelor ECG. Deoarece exista numeroase proceduri pentru sonorizarea semnalelor biologice, care pot fi testate atat in cazul semnalelor normale, cat si in cazul celor patologice, acest protocol permite abordarea sistematica, asociata cu estimarea cantitativa a oricarui tip de reprezentare sonora: prezentarea acustica continua sau cvasicontinua, precum si cea in timp real, amplificata sau comprimata. Este discutata de asemenea si analiza potentialelor aplicatii ale tipurilor variate de tempolene. Aplicarea unui astfel de protocol va aduce date importante in studii viitoare legate de utilizarea instrumentelor de sonorizare in analiza diverselor semnale biologice.

Cuvinte cheie: ECG, sonorizare, tempolene, putere discriminanta

PREGNANCY AND BIRTH OUTCOME AFTER MATERNAL EXPOSURE TO CEFTRIAXONE

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ABSTRACT

Objective. To examine the risk of congenital abnormalities, preterm birth, low birth weight and impaired liver function after exposure to ceftriaxone *in utero*.

Patients and Methods. 55 pregnant patients (20 to 35 years), who redeemed a prescription for ceftriaxone during their pregnancy, were randomly allocated in three study groups, accordingly to their pregnancy stages: first trimester (n = 23), second trimester (n = 22), and respectively third trimester (n = 10). The control group consisted of 55 women who did not redeem any prescriptions until the end of pregnancy. All adverse drug events were monitored.

Results. In the group of women exposed to ceftriaxone at any time during pregnancy, average birth weight was 3202 g, 3.90% had a low birth weight, 4.94% had a preterm delivery, and 3.54% gave birth to a child with a minor congenital malformation. The corresponding data in the control group were similar, respectively: 3328 g, 5.27%, 7.32%, and 4.13 %. 7 patients (12.72%) from the first and second study group required two series of intravenous ceftriaxone administrations for recurrent severe pyelonephritis. Biliary pseudolithiasis has been reported in 8.1% of these cases and reversible cholelithiasis was revealed in 2 cases of newborns from women who received ceftriaxone therapy in the third trimester of pregnancy

Conclusion. Ceftriaxone is a well tolerated third generation cephalosporine, which can be safely used during pregnancy. Biliary pseudolithiasis and newborn cholestasis are possible adverse effects due to extensively drug use.

Key words: ceftriaxone, pregnancy, safety, biliary pseudolithiasis, reversible cholestasis.

INTRODUCTION

Cephalosporins are the most widely used class of antibiotics. Based on their spectrum of activity against gram-negative bacteria, they are classified into four generations. Many of the first and generation cephalosporins have been studied extensively in pregnant patients and are considered safe in any stage of pregnancy.

We recently reviewed the current status of antibiotic therapy for pre-term, pre-labor rupture of membranes (pPROM) cesarean delivery, newly evolving strategies to enhance the effectiveness in reducing post-natal infection, and to monitor the adverse effects on the embryo and fetus. We conducted a full PubMed (January 1976-August 2011) search using the key words "pregnancy", "infections", "antibiotics" and "transplacental transmission." The purpose of this analytic review of selected studies was to reveal the safety of antibiotic use in pregnancy. A total of 355 articles were identified and supplemented by a bibliographic search (1).

Second and third generation cephalosporins are prescribed today to pregnant women, although there are no appropriate data on their safe use during pregnancy, and particularly during embryogenesis.

Ceftriaxone is a second generation cephalosporin (Figure 1):

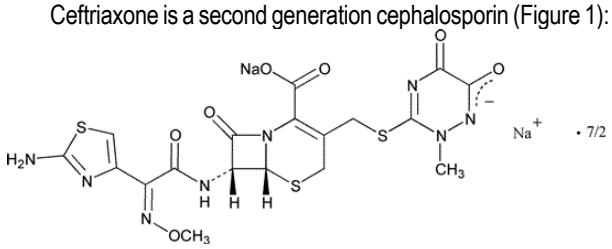

Fig. 1. Chemical structure of ceftriaxone sodium

Ceftriaxone is prescribed as an antibiotic of first choice in pyelonephritis in pregnancy, pneumonia, otitis, sinusitis and meningitis. It has bactericidal activity against *Streptococcus pneumoniae, Klebsiella pneumoniae, Staphylococcus aureus,* Group A beta haemolytic *Streptococcus,* and Gram negative bacteria (2,3).

It is a cephalosporin for parenteral administration. In adults, the usual dose is 1 to 2 g of ceftriaxone, administered once a day (every 24 hours). In cases of serious infections the dose can be raised-up to 4g, administered once a day intravenously. Completely absorbed following parenteral administration, the peak plasma concentrations (about 80 mg/l) occurs between 2

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and 3 hours after dosing. The pharmacokinetics of ceftriaxone is non-linear with respect to the dose. The decrease of binding to plasma proteins explains the non-linearity and leads to an increase in distribution and elimination of the drug.

Hepato-biliary complication due to use of ceftriaxone were described in the last ten years considering children and adults, but not pregnant women (5).

Previous researches have revealed that ceftriaxone has no effect on reproduction or mutagenic activity; however, only few studies pointed out that this third generation cephalosporin crosses the placenta and is excreted in human milk at low concentrations (4).

The aim of the present study was to prospectively evaluate the safety of ceftriaxone during pregnancy and to evaluate the potential risks of this third generation cephalosporin on pregnancy outcome, fetus and newborn.

PARTICIPANTS, MATERIALS AND METHODS

Study design and eligibility criteria

Fifty-five eligible pregnant women participated in this randomized, prospective and comparative survey (study groups). The patients, ranging in age from 20 to 35 years, who redeemed a prescription for ceftriaxone during their pregnancy, were classified in three subgroups, accordingly with their pregnancy stages: first trimester (n = 23), second trimester (n = 22), and respectively third trimester (n = 10).

The control group consisted of 55 women who did not redeem any prescriptions from 2 months before pregnancy until the end of pregnancy (Figure 2).

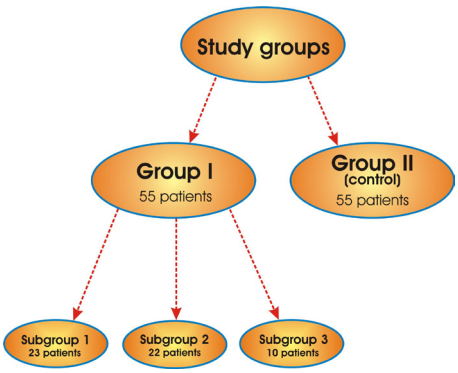

Fig. 2. Study groups

Exclusion criteria: pregnant women with co-morbidities, hypersensitivity to ceftriaxone, use of different antibiotics during pregnancy within the past three months. Patients with risk factors for billiary stasis/sludge or history of reno-urinary lithiasis were also excluded from the study. This study was performed between February 2011- March 2012, in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the declaration of Helsinki, as revised in 2000. All the participants gave informed consent and were recruited from the patient list of a family healthcare provider from Timisoara, Romania.

MATERIAL AND METHODS

Exposed women from study and control groups were paired for age, with references being exposed to non-teratogenic antibiotics administered for the same indications. Full clinical details of the studied patients were obtained from their electronic case notes.

The electronic ICMed system, used by family healthcare providers, includes the type and amount of the prescribed drug according to the Anatomical Therapeutical Classification Code (ATC), the defined daily dose, the date of issuing the prescription, and the personal register number of the patient (CPR number) which is assigned to all patients. This electronic registry contains information on all births; the main variables include maternal age, birth order, gestational age, length and weight at birth, and CPR number of both mother and child.

The patients received intravenous ceftriaxone therapy, for different bacterial infections, for five to ten days. All adverse drug events were monitored.

Data on prescriptions for ceftriaxone and outcome were obtained from population-based registries from the family healthcare unit were the pregnancies were followed-up. Using a case–control design, we compared pregnancy outcomes between women who had been prescribed ceftriaxone during pregnancy and those who had not, adjusting for available potentially confounding factors.

Statistical analysis

Logistic regression analyses was used in order to estimate the prevalence of congenital abnormalities, preterm birth (gestational age <37 weeks), and low birth weight (<2500 g) associated with ceftriaxone exposure, adjusted for maternal age, birth order and smoking. All statistical analyses were performed using the SPSS software package (version 6.0 for Windows, SPSS Inc, Chicago, IL.).

RESULTS

We identified 55 women who redeemed a prescription for ceftriaxone during pregnancy, and 23 of them redeemed their prescription during the first trimester. The control group consisted of 55 women who did not redeem any prescriptions before conception until delivery.

In the group of women exposed to ceftriaxone at any time during pregnancy, average birth weight was 3202 g, 3.90% had a low birth weight (restricted to full term deliveries > 37 weeks), 4.94% had a preterm delivery, and 3.54% gave birth to a child with a congenital malformation. The corresponding numbers in the control group were 3328 g, 5.27%, 7.32%, and 4.13 %. No cases of perinatal death in the study and control group were revealed.

7 patients (12.72%) from the first and second study groups required two series of intravenous ceftriaxone administrations for recurrent severe pyelonephritis. Biliary pseudolithiasis has been reported in 8.1% of these cases and reversible cholelithiasis was revealed in 2 cases of newborns from women who received ceftriaxone therapy in the third trimester of pregnancy (Table I).

Table I. Characteristics of participants and outcomes in study and control group

Characteristics	Exposed to Ceftriaxone First trimester (n=23)	Exposed to Ceftriaxone Second trimester (n=22)	Exposed to Ceftriaxone Third trimester (n=10)	Con-trols (n=23)
Mean age	26.5 (20-35)	26.0 (22-31)	26.3 (23-30)	26.7 (20-35)
No of Prescriptions	25	22	12	–
Smokers (%)	32.28	31.63	33.52	32.83
Birth Weight (mean)	3176 g	3245 g	3185 g	3328 g
No of Low Birth Weight (%)	5.22	3.38	3.11	5.27
No of Preterm Deliveries (%)	6.85	4.22	3.76	7.32
No of malformations (%)	5.42	2.84	2.36	4.13
Ceftriaxone induced biliary sludge/calculi cases)	–	2	3	–
No of Ceftriaxone induced cholestasis in the neonate(cases)	–	–	2	–

DISCUSSION

Although antibiotics are commonly prescribed to pregnant women, details relating to the effects of many of these drugs remains poorly understood. If an antibiotic must be prescribed, it is important to be aware of the effects such drugs can have on pregnancies, in order to prescribe the most suitable treatment with the least risk to mother and fetus.

Ceftriaxone is a third generation cephalosporin, commonly used in general practice for various infections in all age groups, because of its antimicrobial activity against many gram-positive and gram-negative organisms. There are controversies regarding "contraindication in pregnancy" induced by ceftriaxone (6).

Pharmacokinetic changes occurring during pregnancy, as well as fetal pharmacokinetics and passage of the drugs to the unborn are correlated with reproductive and developmental pharmacology. The passage of cephalosporins is different in the three stages of pregnancy. Most studies of drug transfer across the maternal and embryonic/fetal barrier are concerned with the end of pregnancy; but little is known about the transport of substances in the early phases of pregnancy, in which, morphologically and functionally, both the yolk sac and the placenta develop and change in performance (7). Because of these considerations, our study involved pregnant women of all three trimesters of pregnancy, who received treatment with ceftriaxone.

In the third month of pregnancy, the fetal liver is already capable of activating or inactivating chemical substances through oxidation (8). It is very important that, in the fetal compartment, the detoxification of drugs and their metabolites takes place at a low level, certainly in the first half of pregnancy. The excretion in the amniotic fluid explains the accumulation of biological active substances might take place in the fetal compartment.

Although fetal treatment is still an exception, it is of great interest that in the case of prevention of vertical infections, at the time of functioning circulation and kidney excretion, antibiotics *(penicillins, cephalosporins)* concentrate in the fetal compartment (9).

Our prospective and comparative study did not reveal statistical significant differences among study groups (Table I). In accordance with other researches we concluded that ceftriaxone is a safe antibiotic (10). Considering all these, some adverse effects during ceftriaxone therapy are important to be revealed. In previous studies we revealed the importance of drug-induced lithiasis and the composition of ceftriaxone calculi (11). In the present study we revealed reversible precipitates in the gallbladders of 2 pregnant women who received ceftriaxone in the second trimester and 3 in the third trimester; the duration of therapy was ten days, as reported in the literature (12).

Both ceftriaxone exposure cases and their references had only minor malformations in the newborn, revealed within the expected baseline risk for the general population. The five minor malformations in the ceftriaxone study group were as follows: one cases of undescended testis, one case of metatarsus adductus, one haemangioma, one minor syndactyly, and one case of left *hallux valgus* associated with left preauricular trags (Figures 3 and 4):

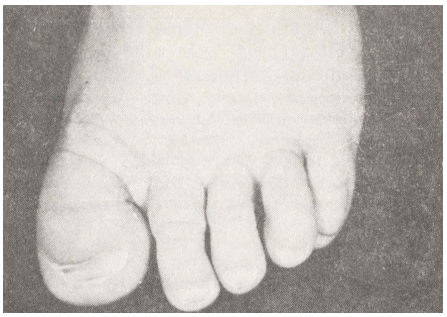

Fig. 3. Left halux valgus in a neonate after maternal second trimester exposure to ceftriaxone

The presence of preauricular trags requires an attentive follow-up of the patient for hearing impairment and especially for hidden abnormalities of the urinary tract.

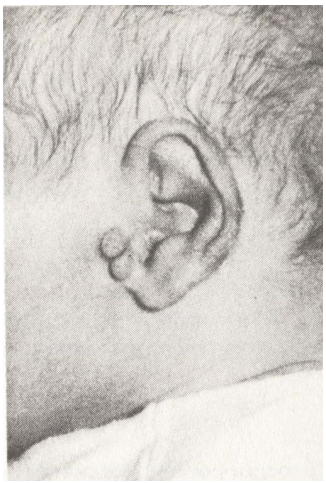

Fig. 4. Left preauricular traggs in a neonate after maternal third trimester exposure to ceftriaxone

In three cases of pregnancies associated with severe pyelonephritis requiring 3g ceftriaxone/day, administered prior to delivery, potentially dangerous complications like reversible cholestasis occured in neonates. All three neonates were born at term and weighted over 3000 grams. They were icteric, with the liver palpable 3 cm below the right costal margin; no splenomegaly was associated. Aspartate aminotransferase ranged between 88 and 120 units, prothrombine time was prolonged to 36-42 seconds, total bilirubin was 38-44 mg/dl with direct component of 33-36 mg/dl. After 3 days the icterus subsided and the blood testes normalized. The reversible cholestatic syndrome in the newborn has been widely discussed in the literature (13-15). Frequently, a reversible higher level of liver enzymes may prove the previously action of ceftriaxone (16,17).

The presented study has some limitations. One limitation is the small sample size and the broad confidence limits. The precise mechanisms of ceftriaxone action during pregnancy will be clarified by further studies.

CONCLUSION

Ceftriaxone is a well tolerated third generation cephalosporine, which can be safely used during pregnancy. Biliary pseudolithiasis and newborn cholestasis are possible adverse effects due to extensively drug use.

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EFECTELE EXPUNERII MATERNE LA CEFTRIAXONA ASUPRA SARCINII SI NASTERII

REZUMAT

Obiectiv. Examinarea riscului de aparitie a anomaliilor congenitale, nasterilor premature, greutate mica la nastere si afectarea functiei hepatice dupa expunerea in utero la ceftriaxona.

Pacienti si metode. 55 paciente gravide (cu varsta intre 20 si 35 ani), care au primit prescriptie medicala pentru ceftriaxona in timpul sarcinii, au fost distribuite in trei grupuri de studiu, in functie de stadiul sarcinii: primul trimestru (n = 23), al doilea trimestru (n = 22), respectiv trimestrul trei (n = 10). Grupul de control a fost format din 55 de femei care nu au avut prescriptie medicala pentru nici un fel de medicament pana la sfarsitul sarcinii. Au fost monitorizate toate efectele adverse.

Rezultate. In grupul femeilor expuse la ceftriaxona pe parcursul sarcinii, greutatea medie la nastere a fost 3202 g, 3,90% au avut greutate mica la nastere, 4,94% au avut nasteri premature si 3,54% au dat nastere unui copil cu malformatii congenitale minore. In grupul de control, datele au fost similare, respectiv: 3328 g, 5,27%, 7,32% si 4,13 %. 7 paciente (12,72%) din primul si al doilea grup de studiu au necesitat administrarea intravenoasa a ceftriaxonei pentru tratamentul pielonefritei severe recurente. In 8,1% dintre cazuri a fost identificata pseudolitiaza biliara iar in 2 cazuri de nou-nascuti a fost relevata colelitiaza, in cazul femeilor carora li s-a administrat ceftriaxona in timpul trimestrului trei de sarcina.

Concluzie. Ceftriaxona este o cefalosporina de generatia a treia care este bine tolerata si poate fi administrata in siguranta in timpul sarcinii. Pseudolitiaza biliara a nou-nascutului si colestaza sunt efecte adverse posibile, datorate utilizarii abuzive a acestui medicament.

Cuvinte cheie: ceftriaxona, sarcina, siguranta, pseudolitiaza biliara, colestaza reversibila

OPTIMIZATION OF PROTOCOLS FOR COMPLETELY DECELLULARIZE EQUINE CORNEA

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ABSTRACT

Cornea can be damaged by various diseases, in which the only treatment is corneal transplantation. A severe shortage of donor corneas exists worldwide, so alternative solutions are needed. As so, researchers tried to tissue-engineer corneas using autologous cells cultured on biological scaffolds like decellularized xenograft tissue. The aim of present study was to obtain complete decellularized equine cornea.

Corneas were obtained from 20 normal healthy horses, sacrificed under strict rules and regulation in slaughter houses. Several protocols that include chemical and mechanical methods were carried out for completely decellularize cornea: hypertonic solutions, detergents, buffers, and alcohol, combined with mechanical agitation or direct stromal perfusion.

DNA extraction and measurement, RT-PCR, DAPI immunofluorescence and HE histochemistry were performed for evaluating effectiveness of our protocols. DNA measurements revealed 91% decellularization in corneas treated with Triton-X, SDS and Tris on continuous agitation (757 ng/mg), and 96% decellularization in corneas cannulated and perfused with the same chemicals (321 ng/mg), compared to control (8060 ng/mg). The results were confirmed by DAPI nuclear staining, while HE staining revealed integrity of stromal fibers. RT-PCR showed fragmented DNA and expression of GAPDH in all samples.

We succeed 91% decellularization in corneas treated by classical methods which involve cornea placed in detergents-buffer combination on continuous agitation, and 96% in cannulated corneas, data suggesting that our new method could be studied further to see if the integrity of stromal fibers together with basement membranes maintain as much as possible to physiological conditions.

Key words: equine cornea, decellularization, cannulate**,** DNA extraction, HE staining

INTRODUCTION

The cornea can be damaged by various diseases that can cause visual impairment and even blindness (1,2). So far, the only treatment for visually impairing corneal damage or scarring is corneal transplantation. Since the first human corneal transplant in 1905, corneal transplantation is one of the most successful forms for tissue transplantation (3-5). Although corneal transplantation is very useful, a severe shortage of donor corneas exist worldwide (6). In addition, complications such as infection, graft failure and immune reactions are possible, and allograft reaction has been reported in patients because of penetrating keratoplasty. Hence, alternative solutions for corneal transplantation are needed to overcome the shortage of corneal donor organs and the complications resulting from the procedure, especially rejection and graft failure of the transplanted cornea (7,8). To solve this problem, several researchers have tried to tissue-engineer corneas using autologous cells cultured in various extracellular 3D matrices and synthetic polymers (9,10). Both synthetic and biological matrices have previously been tested for corneal tissue engineering (11-13), but another new strategy for preparing a scaffold is the use of decellularized xenograft tissue, in which the donor cells and antigen molecules are completely removed to eliminate the host immune reaction (14,15).

The aim of the present study was to obtain complete decellularized equine cornea, including all corneal layers: epithelial layer, stroma, and endothelial cells. Considering that both outer layers can be mechanically removed, the main challenge in obtaining decellularized cornea is represented by the stromal compartment, populated by independently functioning specialized keratinocytes. We optimized different protocols largely used for decellularization procedures in order to completely remove the possible antigenic components from the stromal layer.

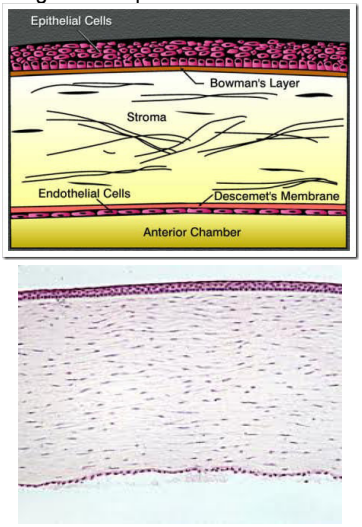

Fig. 1. Schematic representation of corneal layers (epithelial cells, Bowman membrane, stroma, Descemet membrane, and endothelial cells) (upper panel) and histological image of hematoxylin and eosin staining, depicting the same corneal structure (lower panel)

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MATERIALS AND METHODS

Equine corneas were obtained from 20 normal bread healthy horses, sacrificed under strict rules and regulation in slaughter houses. The eyeballs were harvested under sterile conditions and transported to the laboratory on ice. The surrounding ocular muscles and conjunctivae were removed aseptically and the globes were washed in sterile medium containing PBS (Dulbecco's Phosphate Buffer Saline, Sigma-Aldrich Company, Ayshire, UK) and 2% Pen/Strep (10,000 IU/mL, PromoCell, Heidelberg, Germany). All animals experiments on comply with European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, France, 1986). The University of Medicine and Pharmacy Timisoara Ethical Committee approved the experimental protocol.

1. Equine corneas processing methods

Three methods were used for equine cornea processing before the decellularization protocols were applied. The first method consisted of harvesting the cornea with a fine sclera limbus for maintaining the shape and physical properties of corneal tissue (Figure 2b.). The second method considered obtaining smaller fragments of corneal tissue. For this purpose, the equine eyeballs were trephined in the middle of corneal tissue using 6 mm disposable biopsy punch (Kai Medical, Lohdorfer, Germany), and the resulting corneal fragments were further submitted to decellularization protocols. This method was used for achieving better results in penetration of substances which are required for cellular removal from all corneal layers (Figure 2c.).

The third method used in processing the cornea was adapted from vascularized organs decellularization protocols, in which the organs are perfused with detergent substances. Large sclera limbus and cornea were harvested from the equine eyeball; the whole fragment was stretched and bound to a 5 cm diameter Petri dish, placed in a vertical position, and cannulated in the upper part, while a small incision was made in the lower part of the cornea (Figure 2d.). The whole construct was perfused with 1% SDS with a flow of 1 ml/hour for 24 hours. After detergent wash, the cellular residues were removed using continuous perfusion with PBS for 12 hours. Corneal deswelling was performed in 100% glycerol for 6 hours.

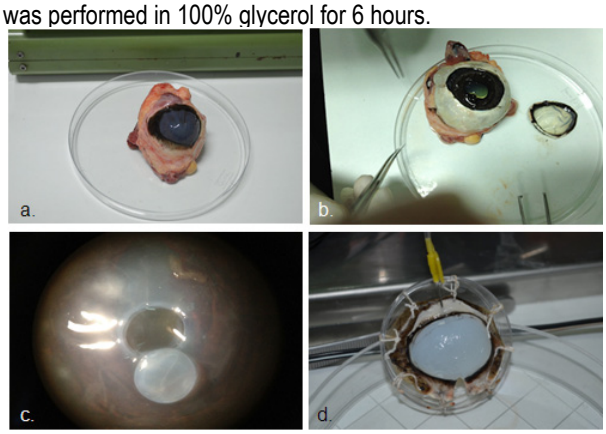

Fig.2. Methods for equine corneas processing. a. entire equine eyeball; b. equine cornea harvested with fine sclera limbus; c. trephined cornea; d. equine cornea harvested with large sclera tissue, cannulated and vertically perfused

2. Decellularization of equine corneas

Protocol 1. Detergent decellularization protocol. Corneas were placed in 50 ml centrifuge tubes (Falcon, BD) with 1% sodium dodecyl sulphate (SDS; Sigma-Aldrich Company) at room temperature on the orbital shaker and continuous agitation for 12 hours. The ratio between SDS and corneal volume was 25:1. In order to remove the detergents, the corneas were washed three-five times with PBS, for 2 hours each, at room temperature. Another washing step included 12 hours placement in 75% ethanol, at room temperature, followed by 3-5 times wash in PBS, for 1 hour, at room temperature.

Protocol 2. Ethanol decellularization protocol. Corneas were placed in 50 ml tubes, and 75% ethanol (20:1) was added at 20°C, for 72 hours, on orbital shaker. Solvent replacement was performed at 1, 3, 6, 12, 24, 48, and 72 hours. Distilled water was used for washing, for 12 hours, at room temperature, followed by Trypsin-EDTA 0.05% (Sigma-Aldrich Company) (15:1) incubation, for 96 hours, at room temperature.

Protocol 3. DNAse and RNAse decellularization protocol. Corneas were subjected to non-surfactant treatment involving incubation in 1.5 M NaCl, for 48 hours, at room temperature (change of medium was performed every 24h). This method was modified in the second step by treating the corneas with DNAse 5 U/ml + RNAse 5U/ml (Sigma-Aldrich Company), for 48 hour, at room temperature (dilution buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 50 µg/ml BSA). The third step consisted of PBS wash, for 72 hours, at room temperature (medium was changed every 24 hours).

Protocol 4. Aprotinin decellularization protocol. In this protocol, corneas were treated with 10 mM Tris buffer + EDTA 0,1% + aprotinin 10 KIU, for 12 hours, at 4° C; 0.3% SDS in TBS, EDTA 0.1%, aprotinin 10 KIU/ml were added for 20 hours, at room temperature. Next step wash 3 times wash in TBS, followed by treatment with DNAse I 50 U/ml + 1 U/ml RNAse A (dilution buffer: 50 mM Tris-HCl, 10 mM $MgCl_2$, 50 µg/ml BSA), for 3 hours, at 37° C with agitation. The final washing step was 3 times in TBS and the corneas were deswelled for 6 hours in 100% glycerol (Figure 3). All chemical substances and reagents were purchased from Sigma-Aldrich Company, unless otherwise specified.

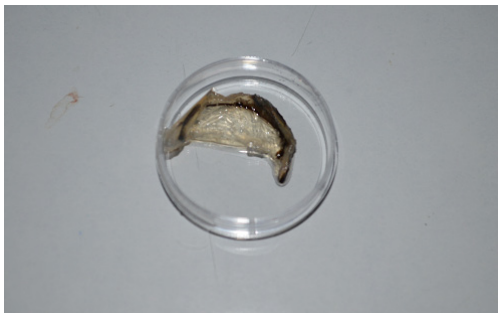

Fig.3. Aspect of deswelled sectioned equine cornea. After 6h placement in 100% glycerol, the thickness of corneas was reduced to less than 1/3, compared to swelled corneas, but it was significantly thicker than normal, freshly isolated ones

Protocol 5. Osmotic gradient plus detergents. Corneas were subjected to a stepwise treatment in which we used combination of hypotonic and hypertonic buffers, together with detergents (SDS or Triton X-100). This protocol was adapted from Roy et al. (16). In the first step, corneas were treated with hypotonic buffer – 10 mM Tris (pH 8) for 24 hours, at 4 °C, on orbital shaker. After 24 hours, the buffer was replaced with hypertonic solution containing 1% Triton X-100, 1.5 M KCl, and 10 mM Tris (pH 8). In the last decellularization step, the hypertonic buffer was replaced with detergent solution containing 1% SDS and 10 mM Tris (pH 9) for 24 hours, under the same temperature conditions. In order to remove the detergent, corneas were incubated at 37 °C for 30 minutes in 1% Triton X-100, and then washed with PBS for 72 hours (change every 24 hours).

Protocol 6. Cannulated corneas, osmotic gradient and detergents. Our newly developed protocol was a three step protocol, in which we used the previous described method (protocol 5) perfused into the cannulated cornea with a flow rate of 1 ml/hour, for 24 hours. The last step consisted in extensive wash of cellular remains using PBS, with the same flow rate, for 12 hours. The entire procedure was performed at room temperature and all chemical substances and reagents were purchased from Sigma-Aldrich Company. Deswelling procedure followed the same protocol described before, using 100% glycerol for 6 hours at room temperature.

3. DNA extraction and PCR evaluation

For DNA extraction we used Fast Tissue-to-PCR Kit (Fermentas, Life Sciences), and followed the manufacturer instructions. Shortly, DNA was extracted from 5 mg of equine decellularized cornea, which was mixed by vortexing initially with 100 µl of Tissue Lysis Solution and 10µl of Proteinase K Solution. The mixture was incubated at room temperature for 10 minutes, and then additional 3 minutes at 95 °C. At the end of the incubation, not all the tissue was completely digested, but this fact did not affect the further PCR performance. In a next step, 100 µl of Neutralization Solution T was added to the sample and mix by vortexing. The neutralized tissue extract was either stored at 4 °C or used immediately in PCR.

The PCR analysis was performed with Tissue Green PCR Master Mix, which includes a specialized formulation of the efficient DreamTaq DNA polymerase, dNTPs and buffer, optimized for use with the reagents used for DNA extraction. Loading dyes and density reagent are also included in the master mix for direct loading of the PCR product onto an agarose gel. The primers used for the PCR reaction were the following: G3PDH F: GA-AGG-TCG-GAG-TCA-ACG-GAT-TTG-GT; G3PDH R: AT-GTG-GGC-CAT-GAG-GTC-CAC-CAC, with an amplicon size of 1000 bp. The program followed for DNA amplification consisted of subsequent temperature cycles: 50 °C - 30 minutes, 95 °C - 15 minutes, 35x (94 °C - 1 minute, 60 °C - 1 minute, 72 °C - 1 minute), 72 °C -10 minutes, 4 °C - ∞. The amplification products were migrated in electrophoresis 2% agarose gel, and visualized using UV Fluor-S MultiImager (Bio-Rad) and Quantity One Basic software.

4. Histochemical and fluorescence evaluation

Corneas decellularized using different protocols were cut in small fragments (10 mm^3) each and embedded in Killik – cryostat embedding medium (Bio-Optica, Milano, Italy), snap-frozen in liquid nitrogen, and preserved at -80° C until further processing. 3 µm thick sections were performed using cryotome, and the slides were stained using haematoxylin-eosin (DakoCytomation Hematoxylin Mayer's, Lillie's modification, Dako) classical labeling, for revealing collagen fibers organization after decellularization procedures. For showing the degree of decellularization, we used the fluorescent nuclear staining – 4,6-diamidino-2-phenylindole (DAPI), counted the remaining nuclei on five different microscopic fields, and performed comparative statistic analysis between all protocols using the average value. Microscopy analysis was achieved on a Nikon Eclipse E800 microscope equipped with adequate fluorescence filters.

5. Statistic analysis

Statistic analysis was performed using Excell Microsoft Office 2007 (Microsoft Corporation) software. The central tendencies of the variables were expressed as a mean (M), and the dispersion ones as standard deviation (sd). In order to perform the statistic comparisons, „t"-Student test and the variance analysis (ANOVA) were used for continuous variables. Differences were considered significant for $p < 0.05$.

RESULTS

DNA content and integrity

The DNA content analysis revealed different concentrations of DNA remaining in the decellularized samples, correlated with the complexity and duration of the protocols used. All DNA samples were expressed as ng of DNA / mg of dried cornea, and calculated in respect with suspension volume. We used Nanodrop ND-1000 spectrophotometer for quantification of DNA, and the 260/280 ratio was between 1.2-1.8 in all samples measured (Figure 4). The following were the average values of DNA amount obtained using different decellularization protocols (± sd): Protocol 1 – 3466.6 ± 124 ng/mg; Protocol 2 – 4808 ± 203 ng/mg; Protocol 3 – 3111 ± 98 ng/mg; Protocol 4 – 2923 ± 87 ng/mg; Protocol 5 – 757 ± 58 ng/mg; Protocol 6 – 321 ± 35 ng/mg. Control integral cornea had a significant increased content – 8060.4 ± 109 ng/mg DNA (Figure 5). Based on DNA content, protocols 5 and 6 had the best decellularization rate, estimated to 91% and 96%, respectively.

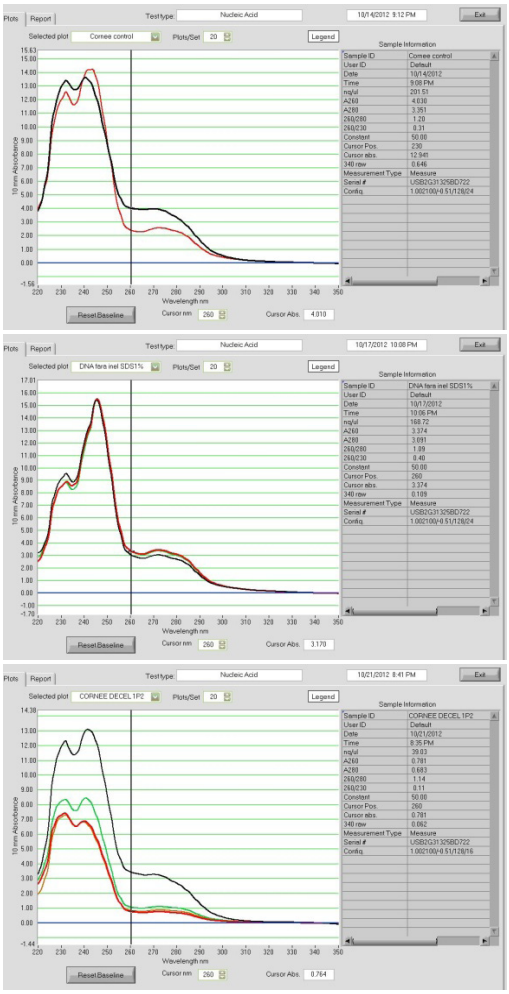

Fig. 4. DNA content measured using Nanodrop ND-1000 spectrophotometer in samples extracted after decellularization of equine cornea using different protocols. Highly impure DNA content, reflected by the 260/280 ratio which was lower than 2 in all samples, except for control

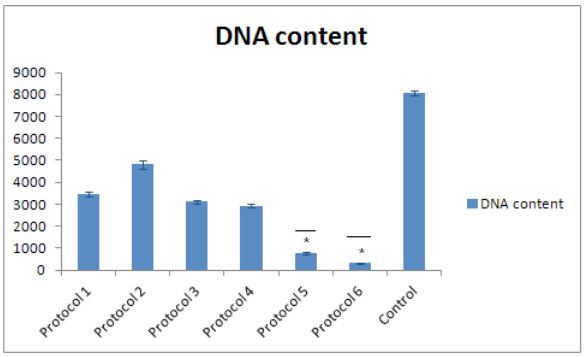

Fig. 5. Statistic analysis of DNA content in samples obtained after using all 6 decellularization protocols. Significant decrease is found in all samples, compared with control ($p < 0.05$), but highly significant decrease in DNA content was found in cornea samples decellularized using Protocols 5 and 6 ($p < 0.001$)

Control DNA semi-quantitative expression showed integral pattern of molecular aspect (Figure 6a). RT-PCR showed fragmented DNA and expression of GAPDH in samples extracted after decellularization with protocols 1 and 2 (Figure 6b). Samples extracted after using more complex decellularization protocols (protocols 3 and 4) showed a clear 1000 bp band when migrated in agarose gel, with no fragmentation, while decellularization protocols 5 and 6 showed a faded band when amplified for GAPDH expression. The results are clearly indicating that the decreased DNA amount is relevant for a good decellularization protocol, and protocols 5 and 6 seem do have the best outcomes.

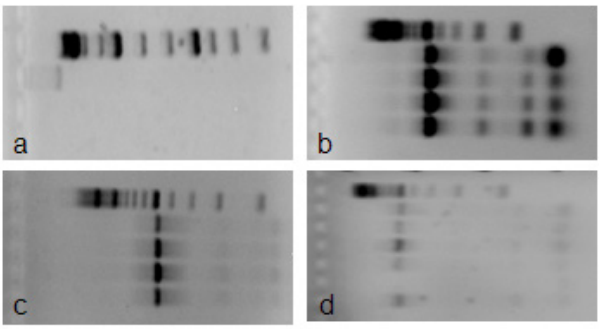

Fig.6. Agarose gel electrophoresis migration of DNA. a. control DNA, no amplification; b. protocols 1 and 2 obtained DNA expression of GAPDH (1000 bp ladder); c. protocols 3 and 4 obtained DNA qualitative expression of GAPDH; d. protocols 5 and 6 obtained DNA amplified for specific expression of GAPDH

Structural integrity of corneal stromal fibers and cell removal

HE staining was used for quantification of cell number, but also for revealing the disposition of stromal fibers within the equine cornea. The fibers had a parallel arrangement, both with respect of each other, and the two basal enclosing membranes (Figure 7a). DAPI staining showed cellular nuclei in the equine cornea. In normal integral cornea, DAPI staining showed abundant cellular population within stroma, as well as the endothelial (mono)layer and epithelial cells placed on basal membranes (Figure 7b).

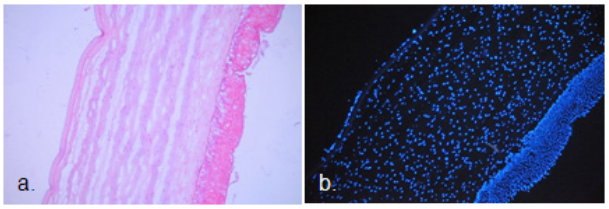

Fig.7. Normal integral equine cornea. a. HE staining (magnification 200x); nuclear DAPI staining (magnification 100x)

Decellularization protocols used in this study induced changes in morphological and structural aspects of equine cornea, in different degrees, depending on the methods used. Protocols 1-4 provoked a markedly disarrangement in fibers structure, while the cellular content was less influenced, the number of nuclei presented a small decrease, compared to control (Figures 8 and 9).

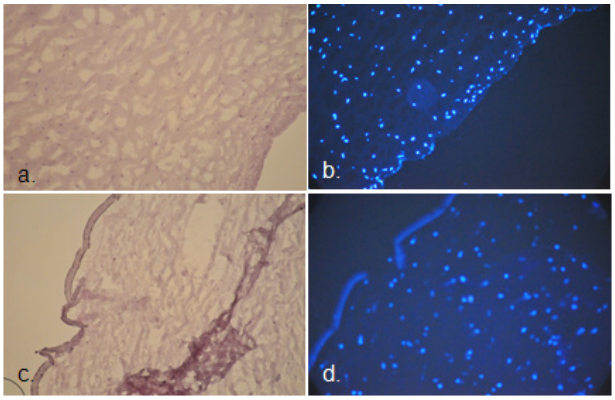

Fig. 8. Detergent and ethanol methods for decellularization of equine cornea. a. Protocol 1, HE staining; b. Protocol 1, DAPI staining; Protocol 2 (c. HE; d. DAPI) (magnification 200x)

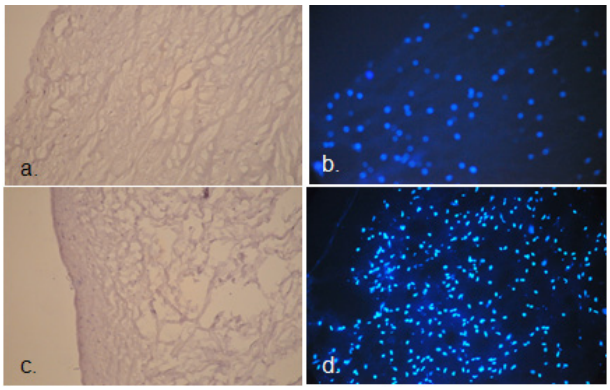

Fig.9. Nuclease and aprotinin decellularization methods. Protocol 3 (a. HE; b. DAPI). Protocol 4 (c. HE; d. DAPI) (magnification 200x)

When protocols 5 and 6 for decellularization were used, the number of nuclei had a spectacular decrease, thus showing that the cells were removed in a greater extent. Regarding the fibers arrangement, protocol 5 seemed to be more aggressive on overall morphological structure of equine cornea (Figure 10 a and b), while using protocol 6 we were able to maintain fibers structure in a close to normal disposition (Figure 10 c and d).

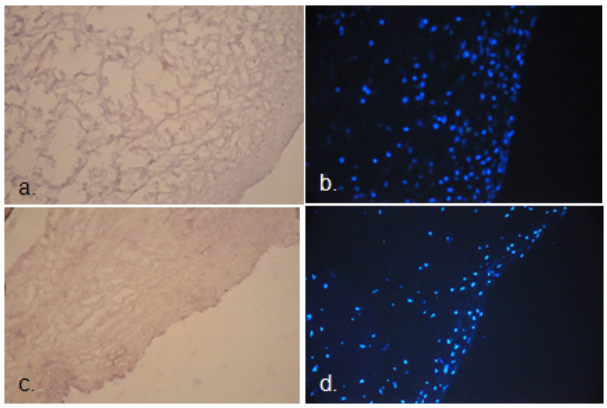

Fig. 10. Canulated equine cornea, perfused with SDS for 24h. Dehydrated cornea right after SDS perfusion and stained with HE (a) and DAPI (b). Additional PBS wash for 12 hours, followed by deswelling in 100% glycerol (c. HE; d. DAPI) (magnification 200x)

DISCUSSION

We were able to develop and optimize a new protocol for efficient decellularization of equine cornea, consisting in using methods and reagents already described in literature (16), but introducing a revolutionary concept of stretched and cannulated cornea, which proved to remove 96% of the stromal cells, while maintaining integrity and fibers arrangement. This protocol could be applied in cases of bioengineered constructs, which will be further used in *in vivo* transplants.

Successful tissue engineering depends on the provision of a scaffold during the initial stage of reconstruction. A suitable scaffold is readily fabricated and results in a 3D porous structure, which affects the structure and function of the generated tissue. Moreover, the proper scaffold not only provides an environment in which the cells proliferate, integrate and form ECM, but is important for metabolic support and waste removal (17,18). Until now, there have been no synthetic scaffolds that can mimic the structure of the lamellar stroma, and synthetic and natural biological materials used as scaffolds for tissue engineering lack a lot of natural properties, which limit their use for clinical implantation (19). Without a proper 3D assembly, cells lack the proper clues for growing into the variety of cells that make up a particular tissue.

We demonstrated that this tissue can be processed using a variety of techniques that can maintain the unique ECM structure. In this study, treatments were selected to be compatible with corneal tissue. We may speculate that these decellularized corneas should also provide the specific microenvironment for the stromal and epithelial cells to migrate and repopulate the tissue graft, both *in vitro* and *in vivo*.

Acknowledgements

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OPTIMIZAREA PROTOCOALELOR PENTRU DECELULARIZAREA COMPLETA A CORNEEI ECVINE

REZUMAT

Corneea poate fi alterata in diferite afectiuni oftalmologice, in care singurul tratament este transplantul de cornee. In lumea intreaga exista un deficit al donatorilor de cornee, astfel incat este nevoie de solutii alternative. Cercetarile actuale sunt indreptate spre obtinerea corneei prin inginerie tisulara, folosind celule autologe, cultivate pe suporturi biologice, cum ar fi xenografoane decelularizate. Scopul acestui studiu a fost acela de a obtine decelularizarea complete a corneei ecvine.

Au fost obtinute 20 de cornee de la cai de rasa comuna, sanatosi, care au fost sacrificati in conditii reglementate de legislatia Romaniei. Au fost utilizate diverse protocoale care folosesc metode chimice si mecanice pentru decelularizarea completa a corneei: solutii hipertone, detergenti, solutii tampon, alcool, in combinatie cu agitare mecanica sau perfuzie stromala directa.

Pentru evaluarea eficientei de decelularizare a protocoalelor utilizate, a fost efectuata extractia ADN, urmata de RT-PCR si au fost utilizate metode de imunofluorescenta (DAPI) si histochimie (HE). Masurarea continutului de ADN a aratat o decelularizare de 91% la corneele tratate cu Triton-X, SDS si Tris, cu agitare continua (757 ng/mg) si o decelularizare in proportie de 96% in cazul corneelor canulate si perfuzate cu aceleasi substante chimice (321 ng/mg), comparativ cu corneea control (continut ADN - 8060 ng/mg). Rezultatele au fost confirmate prin marcarea nucleara cu DAPI, iar colorarea histochimica HE a aratat integritatea fibrelor stromale. Prin RT-PCR s-a aratat fragmentarea ADN si expresia GAPDH in toate probele analizate.

Am reusit decelularizarea corneei ecvine in proportie de 91% folosind metode clasice, care utilizeaza o combinatie de detergenti si solutii tampon si agitatie continua, in timp ce prin canularea corneei am obtinut o decelularizare in proportie de 96%, aceste date sugerand ca noua metoda dezvoltata prin acest studiu ar trebui investigata in viitor pentru a arata daca integritatea fibrelor stromale si membranelor bazale este suficienta pentru mentinerea conditiilor fiziologice ale micromediului celular.

Cuvinte cheie: cornee ecvina, decelularizare, canulare, extractie ADN, marcare HE

ANALYSIS OF SYNTHETISED STEROID DIMERS BASED ON THE STRUCTURE OF PENTACYCLIC TRITERPENES

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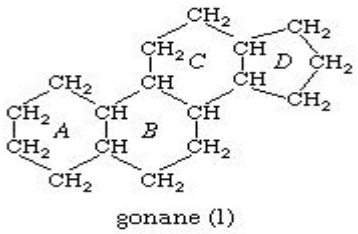
ABSTRACT

Steroid dimers can serve as architectural components in the chemistry of molecular recognition and may be used as chiral building blocks to construct artificial receptors. The synthesis of steroid dimers based on pentacyclic triterpenes such as betulin or betulinic acid, was attempted. A simple esterification reaction between betulin and betulinic acid was not satisfactory. The research not shows better results even in the case of betulinic acid activation with thionyl chloride. 1,4-butanediol was used to create a spacer group and to avoid the steric hindrance and the final product was topical applied on hairless mice skin.

Keywords: steroid, betulin, betulinic acid, esterification, HPLC-MS

INTRODUCTION

The steroids are organic compounds which contain a specific arrangement of four cycloalkane rings which are connected to each other (1). These substances form a class of biologically active lipophilic molecules which include steroid hormones, plant sterols (known as phytosterols), cholesterol, and bile acids. The parent structure, named gonane (also known as the steroid nucleus), a tetracyclic seventeen carbon steroidal ring system is known as 1,2-cyclopentano-perhydro-phenanthrene or simply cyclopentaphenanthrene (Figure 1) (2).



gonane (1)

Fig. 1. Gonane skeleton

The four rings of gonane skeleton are lettered A, B, C, and D, and the carbon atoms are numbered beginning in the A ring. There is well-known that B, C, and D rings of steroids are always trans-fused, and in most natural steroids, the first two rings are also trans-fused.

The steroid dimers are representatives of an important family of chemical compounds which are produced by various marine organisms, and there are also synthesized in laboratories. These organic compounds possess different pharmacological and biological properties, and can also be used to create "molecular umbrellas" for drug delivery (2). Steroid dimers were first observed as synthetic byproducts and then discovered in nature (3, 4). The steroid dimers possess interesting micellular, detergent, and liquid crystal properties (5).

The steroid dimers are linear or cyclic compounds, connected via rings in the steroid nucleus (A–A, B–B, etc.) or through the side chains, the latter being commonly used to form bile acids dimers. There are many kinds of acyclic structures based on bile acids, among which the most interesting are the following: ionophores (6), molecular umbrellas (7), cleft type structures (8), molecular tweezers (9), dendrons (10), gelling agents (11), and inclusion compounds (clathrates) (12).

The steroid dimers represent a class of compounds that have attracted attention for their rigid and inherently asymmetric architecture (13).

A synthesis of 6E-hydroximino steroid homodimers was reported in the literature (Figure 2) (14). These unusual 6E-hydroximino-3-oxo-4-ene steroids were discovered in the sponge *Cinachyrella alloclada*. A rapid and efficient synthesis of homodimers of these cytotoxic compounds was studied. To 6-ketones (X = O) three or four carbon atom linkers with terminal double bonds were attached in the position 3β. The allylic ether (n = 1) afforded E-homodimer in 82% with small amounts of the sterically less favoured Z-isomer. In the case of homoallylic ether (yield 80%) the ratio E/Z was 1.5:1. After chromatographic purification, the dimers were converted to the 6E-oximes. How-

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ever, homodimers were proved to be not very cytotoxic when compared with monomer counterparts.

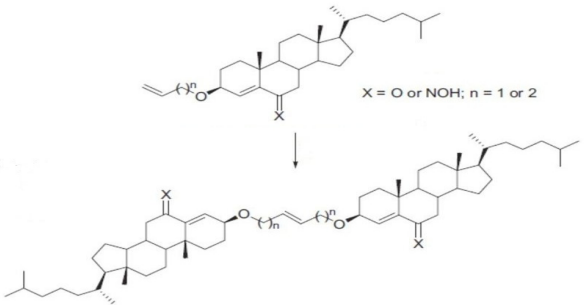

Fig. 2. Synthesis of homodimers

L.P. Valverde and his collaborators have reported a method (15) that involves the modification of the hydroxyl group (C-3 from A-ring of pregnenolone) in order to synthesize pregnenolone dimers with and without spacer groups that can be used as chiral building blocks to construct artificial receptors and as architectural components in biomimetic or molecular recognition chemistry (Figure 3). They reported an easy procedure to synthesize pregnenolone dimer via connection between the two A rings. The purpose to use of long spacer arm in the pregnenolone-dimer was to avoid the steric hindrance that the support could have when interacting with other biological molecules.

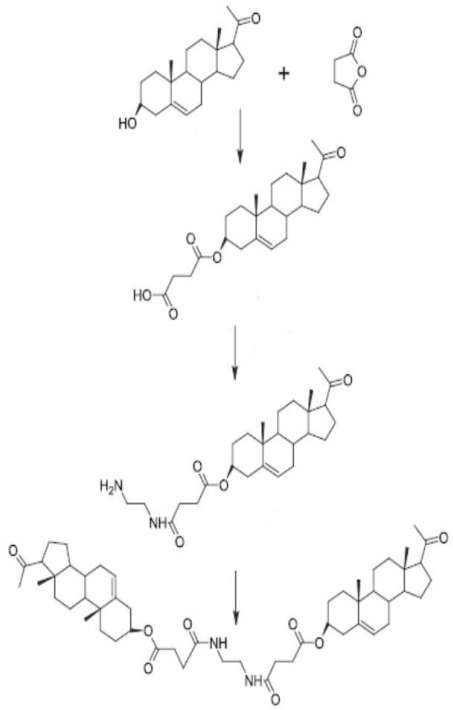

Fig. 3. Three steps synthesis of pregnenolone-pregnenolone dimer via Ring A-Ring A connection
(1) pyridine/toluene; (2) ethylenediamine dihydrochloride, acetonitrile/water; (3) 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride, acetonitrile/water

H. Kuhl and H.D. Taubert have reported the synthesis of androgene dimers coupled at C3-C3 and C17-C3 and of an androgen-progestogen combination as long-acting hormonal steroids (16).

Cholest-5-en-3-spiro-[6' α,5'-oxa]-5' α-cholest-3'-one (2), cholest-5-en-7-spiro-[4' α,5'-oxa]-5' α-cholest-7'-one and 3β-substitutedcholest-5-en-7-spiro-[4' α,5'-oxa]-3' β-substituted-5' α-cholestan-7'-ones were synthesized starting from cholest-5-en-3-one, cholest-5-en-7-one and 3β-substituted-cholest-5-en-7-ones respectively (17).

The need of new antifungal drugs with high efficacy, low toxicity and new action mode is well-known. Synthesis and antifungal activity of bile acid derived new steroidal dimers such as N1,N3-Diethylenetriamine-bis-[cholic acid amide] (Figure 4a) and N1,N3-Diethylenetriamine-bis-[deoxycholic acid amide] (Figure 4b) having novel amphiphile topology was described in a US Patent (18).

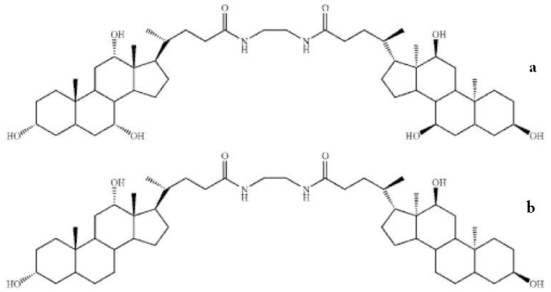

Fig. 4. Structures of bile acid derived new steroidal dimers

In this study, betulin and betulinic acid were used as raw materials in an esterification reaction in order to obtain steroid dimers compounds. 1,4-butanediol was used to create a spacer group because there were not obtained good results even after the betulinic acid activation with thionyl chloride.

MATERIALS AND METHODS

Betulin and betulinic acid (Figure 5) were isolated with dry column chromatography from external birch bark (*Betula Pendula Roth*) as was described in the literature (19, 20) and its IR spectra were compared with the reference standards. Betulinic acid was crystallized from chloroform-methanol as shining needles with a melting point 294-296 °C (21). Solvents, thionyl chloride and all the others substances at analytical reagent grade, were purchased from Fluka (Germany) and were used without any previous purification.

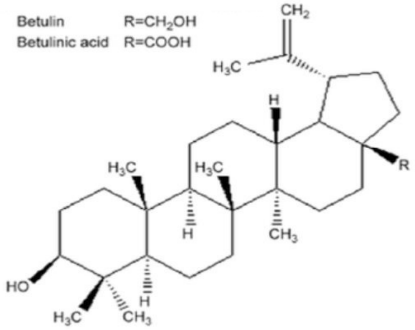

Fig. 5. Structures of betulin and betulinic acid

Dimerization procedure

As it was already described in the literature in the case of betulinic acid acyl glucuronide synthesis (22), betulinic acid was coupled with betulin in the presence of potassium carbonate (K_2CO_3) in a heterogeneous solution of CH_2Cl_2 / H_2O.

HPLC-MS

Effective analysis was performed by two complementary methods. It was first performed the screening analysis, looking for the three compounds that may exist in the sample (betulinic acid, betulin and corresponding dimer). It was run a gradient of 0.4% formic acid in water/methanol and it was record the chromatogram.

Betulinic acid activation

The reaction apparatus consists of a one-neck flask with magnetic stir bar and reflux condenser. For draining of the evolving gases (HCl and SO_2) the reflux condenser is connected to a wash bottle filled with an aqueous solution of sodium hydroxide (20%). The reaction flask is charged with 10 ml carbon tetrachloride and 14.50 µl (0.2 mmol) freshly distilled thionyl chloride. Whilst stirring, 45.67 mg (0.1 mmol) betulinic acid is added in the flask. The reaction mixture is initially slowly heated whilst stirring (as far as possible because of strong formation of gas) up to 50 °C, afterwards stirring is continued for 2 additional hours at 80 °C.

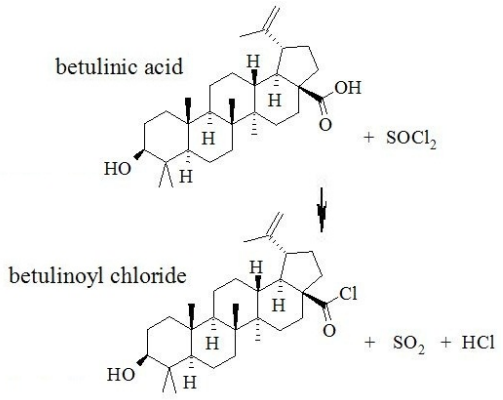

Fig. 6. The reaction of betulinic acid activation

After the cooling down of the reaction mixture, the reflux condenser is replaced by a distillation bridge and the excess of thionyl chloride is removed by distillation (23). The crude acid chloride is for most uses pure enough, so that one can do without distillation. The product is a colourless, strong refractive liquid, which crystallizes whilst cooling as a colourless solid product.

The dimerization procedure was repeated using activated betulinic acid in reaction with betulin and the products were analyzed by FT-IR spectra using a Jasco FT-IR/410 spectrometer. In the last step, it was used 1,4-butanediol in order to avoid the steric hindrance.

Ten weeks old females of hairless mouse SKH1 were used in this experiment. Creams with and without the final product were applied on mice skin for 9 weeks (0.3 mL / application). There were used 4 mice: group FP (2 mice, treated with cream with the final product), group 0 (2 mice, treated with blank cream). The applications were done twice a week and the measurements were done only at the begging and the end of the experiment period. The measurements on the mice skin were carried out with a Multiprobe Adapter System (MPA5) from Courage-Khazaka, Germany.

RESULTS

The obtained chromatogram in the screening analysis is presented in Figure 7.

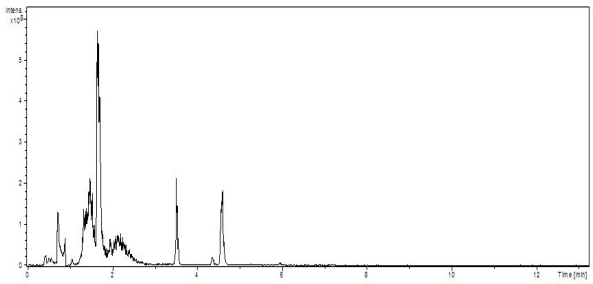

Fig.7. The chromatogram of the first reaction products

The molecular mass was the principle of betulinic acid and betulin identification, after about 1.8 minutes. The two signals at 3.6 and 4.5 minutes correspond to plasticizers from solvent (not from analyzed sample). The corresponding mass spectra are shown in Figure 8. There were not identified any compound with expected molecular weight for a dimer (M = 898, the expected m / z = 899 or 881 for dehydrated molecule).

The raw materials were quantitative analyzed in order to see their ratio because in the first stage of analysis it was not revealed any dimer.

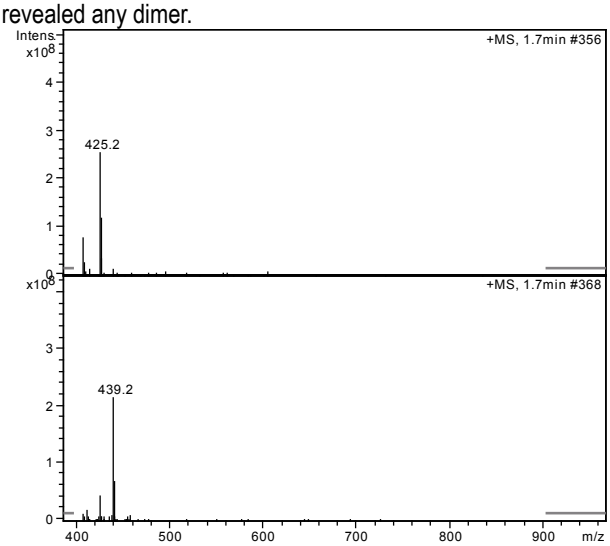

Fig. 8. The mass spectra of first reaction products

The absence of any important change in mice skin parameters may be observed on Figure 9.

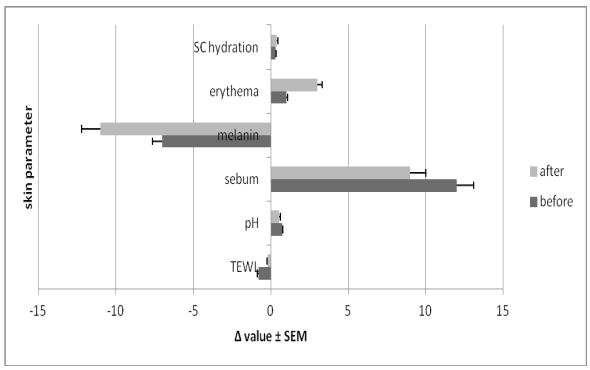

Fig. 9. Differences of mice skin parameters between cream with and without final product before and after experiement

DISCUSSION

After the first reaction was done, it was weighed a quantity of 6.98 mg product sample, dissolved in acetone with ultrasound and analyzed the 1:1000 solution with HPLC-MS. The test results showed that the powder contains about 70% betulin (± 3-4%) and 32% betulinic acid (± 3-4%). It is unlikely that dimer exist in this sample given the reactants ratio in the sample provided.

There were used the calibration curves from Figure 10.

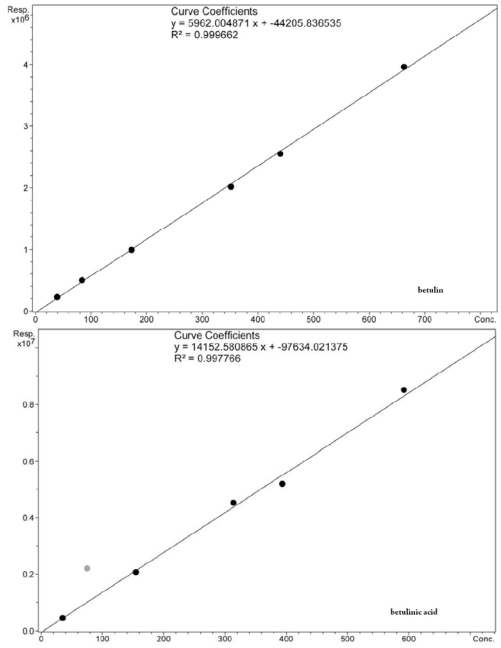

Fig. 10. Calibration curves of betulin and betulinic acid

The betulinic acid activation was the second step in order to obtain the steroid dimer. It was used the procedure described in the literature (23). After the betulinoyl chloride was purified and checked by FT-IR spectra, the dimerization procedure was repeated.

The reaction products were checked by FT-IR spectra. It is well-known that organic acids presents a strong absorption between 1700-1725 cm^{-1} for C=O vibration, a strong and very broad signal at 2500-3300 cm^{-1} characteristic for O-H vibration, and a strong band between 1210-1320 cm^{-1} for C-O bond. The O-H bond from alcohols presents signals between 3200-3700 cm^{-1}, depend on the presence of hydrogen bonds. A strong band between 1735-1750 cm^{-1} (C=O) and two or more bands between 1000-1300 cm^{-1} (C-O) are specific for esters (24).

The IR spectra of the products sample did not reveal the presence of the ester group. Then, 1,4-butanediol was used in the synthesis in order to avoid the steric hindrance. In this case the FT-IR spectrum contains the signals specific for esters. The new product was topical applied on the back skin of hairless mice for its toxicological characterization. There were not observed important changes of skin parameters (Figure 9).

CONCLUSIONS

The synthesis of symmetric molecules derived from joining two identical parts has gained importance in last decade. Steroid dimers may be linear or cyclic compounds which are connected via rings or through the side chains, the latter being commonly used to form bile acids dimers. In this experimental study it was attempted the synthesis of a steroid dimer using pentacyclic triterpenes such as betulin and betulinic acid as raw materials. It was done a simple esterification reaction and the products were analyzed by HPLC-MS; the analysis results show that this procedure was not satisfactory. The procedure was then repeated after the betulinic acid activation with thionyl chloride, but FT-IR spectra did also not show the expected results. It seems that the steric hindrance prevents the chemical reaction which was done to obtain the steroid dimer. It was synthesized an ester with 1,4-butanediol which not shown any important changes of mice skin parameters.

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ANALIZA DIMERILOR STEROIDICI SINTETIZATI PE BAZA STRUCTURII TRITERPENELOR PENTACICLICE

REZUMAT
Dimerii steroidici pot servi in calitate de componente arhitecturale in chimia recunoasterii moleculare si pot fi folositi ca blocuri chirale de constructie a receptorilor artificiali. A fost incercata sinteza dimerilor steroidici pe baza triterpenelor pentaciclice, cum ar fi betulina sau acidul betulinic. O reactie de esterificare simpla intre betulina si acidul betulinic nu a fost satisfacatoare. Cercetarea nu prezinta rezultate mai bune chiar si in cazul activarii acidului betulinic cu clorura de tionil. 1,4-butandiol a fost folosit pentru a crea o grupare spatioasa și pentru a evita impiedicarea sterica, iar produsul final a fost aplicat topic pe piele de soarece fara par.
Cuvinte cheie: steroid, betulina, acid betulinic, esterificare, HPLC-MS

SERUM SOLUBLE INTERLEUKIN-2 RECEPTOR LEVELS IN CHRONIC HEPATITIS C BEFORE INTERFERON THERAPY

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ABSTRACT

Background: The release of soluble interleukin-2 receptor (sIL-2R) appears to be a characteristic marker of T lymphocyte activation and might be related to immunoregulatory function. **Objectives**: To determine serum sIL-2R levels in hepatitis C virus (HCV) infection as compared to normal controls and its association with histological inflammatory indicators. **Materials and methods**: Fifteen patients with chronic HCV infection (6 male, 9 female, mean age 47.35±10.78 years) and 14 healthy subjects (6 male, 8 female; mean age 35.00±15.45 years) were included in this study. The diagnosis of the patients with chronic HCV infection was established on the basis of clinical, laboratory, ultrasonographis and histopathologic findings. The healthy subjects had negative hepatitis serology, normal liver function tests and normal ultrasonographic findings. Serum sIL-2R was measured before interferon (IFN) alpha treatment. Serum levels of sIL-2R were measured by ELISA. Statistical significance was assumed for $p<0.05$. **Results**: Serum sIL-2R levels were significantly higher 809±43.6 pg/mL than those of healthy controls 634.5±32.7 pg/mL, $p<0.02$ by Student t test. There were some correlations between serum sIL-2R levels and histological activity score ($r=0.482$, $p<0.01$) and serum alanine aminotransferase levels ($r=0.363$, $p<0.01$). Serum sIL-2R was significantly higher in HCV genotype 1 as compared to genotype 3 (889±54.8 vs. 756.7±37.9; $p<0.02$). **Conclusion**: Serum sIL-2R levels increase due to HCV infection, and the amount of increase corresponds to the degree of inflammation.

Key words: soluble interleukin-2 receptor, hepatitis C virus infection, pathophysiology

BACKGROUND

The pathogenic mechanisms of chronic HCV infection remain unclear. Objectives of this study were to assess the potential role of sIL-2R in chronic HCV infection and to clarify whether there is a difference in the activation of T lymphocytes among HCV genotypes.

MATERIAL AND METHODS

Fifteen patients with chronic HCV infection (6 male, 9 female, mean age 47.35±10.78 years) and 14 healthy subjects (6 male, 8 female; mean age 35.00±15.45 years) were included in this study. The diagnosis of the patients with chronic HCV infection was established on the basis of clinical, laboratory, ultrasonographis and histopathologic findings. The healthy subjects had negative hepatitis serology, normal liver function tests and normal ultrasonographic findings. Serum levels of sIL-2R were measured by ELISA. The individual samples were done in duplicate. The Mann–Whitney test was used for group comparisons and Fisher's exact test was used to calculate the differences in categorized data. For testing associations, Spearman rank correlation coefficients were calculated. For normally distributed paired samples, paired t-tests were used. Bonferroni's correction was used where appropriate, and corrected $p<0.05$ were considered significant. Values are given as mean±SD. Correlation between serum sIL-2R level and the immunohistochemical reading score was analyzed by a simple curve fit test. Survival curves were plotted with method of Kaplan-Meier. The statistical difference between groups was compared by the log-rank test. χ^2 analysis showed that the serum sIL-2R levels correlated well with histological inflammatory indicators.

RESULTS

Serum sIL-2R was measured in 15 patients with chronic HCV infection before interferon (IFN) alpha treatment. Serum sIL-2R levels were significantly higher 809±43.6 pg/mL than those of healthy controls 634.5±32.7 pg/mL, $p<0.02$ by Student t test. There was some correlation between serum sIL-2R levels and histological activity index scores ($r=0.482$, $p<0.01$) and serum alanine aminotransferase levels ($r=0.363$, $p<0.01$). Serum sIL-2R was significantly higher in HCV genotype 1 as compared to genotype 3 (889±54.8 vs. 756.7±37.9; $p<0.02$).

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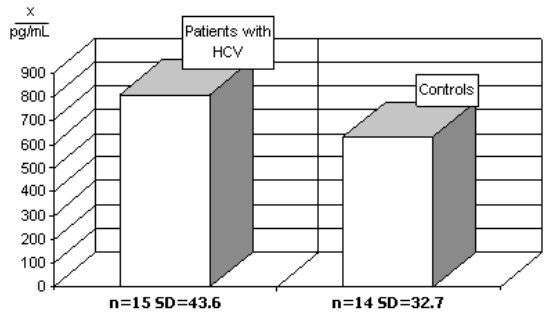
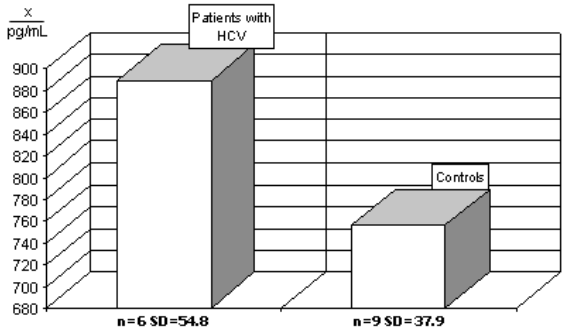


p<0.02

Fig.1. Mean value of the sIL-2R levels in patients with chronic HCV infection as compared to controls



p<0.02

Fig. 2. Mean value of the sIL-2R levels in HCV genotype 1 as compared to genotype 3

DISCUSSION

In this study we measured sIL-2R levels in serum of patients with chronic HCV infection. SIL-2R in serum of HCV patients were significantly increased compared to those of normal controls. We also examined, retrospectively, the relationship between the severity of chronic liver disease, assessed histologically, and sIL-2R levels in selected patients. Clinically it has been reported that the level of sIL-2R in serum is a useful marker to follow disease activity. In addition to activated lymphocytes, increased IL-2R gene expression can be found in chronic HCV infection. Activated T cells can be an important source of elevated sIL-2R in HCV patients. This elevated sIL-2R level can in turn act as a prognostic serum biomarker to assess the aggressiveness of HCV infection (1-4). Further studies are needed to elucidate the mechanism by which expression of sIL-2R may be regulated pathophysiologically.

CONCLUSION

Increased concentrations of sIL-2R, a marker of T lymphocyte activation have been found in chronic HCV infection. Higher sIL-2R levels were related to disease progression and genotype.

Conflict of Interest

The authors have declared no conflicts of interest.

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NIVELURILE RECEPTORULUI SOLUBIL AL INTERLEUKINEI-2 IN HEPATITA CRONICA C INAINTE DE TERAPIA CU INTERFERON

REZUMAT

Introducere: Se consideră că producerea receptorului solubil al interleukinei 2 (sIL-2R) este un marker caracteristic al activării limfocitelor T şi ar putea să fie în legătură cu funcţia de imunoreglare. **Obiective**: Determinarea nivelurilor de sIL-2R în ser în infecţia cu virusul hepatitei C (HCV) comparativ cu martori sănătoşi şi asocierea cu indicatorii histologici ai inflamaţiei. **Material şi metode**: Cincisprezece pacienţi cu infecţie cronică cu HCV (6 bărbaţi, 9 femei, vârsta medie 47,35±10,78 ani) and 14 subiecţi sănătoşi (6 bărbaţi, 8 femei; vârsta medie 35,00±15,45 ani) au fost incluşi în studiu. Diagnosticul pacienţilor cu infecţie cu HCV a fost stabilit pe baza examenului clinic, datelor de laborator, de ultrasonografie şi histopatologice. Subiecţii sănătoşi au prezentat serologie negativă pentru hepatită, testele funcţiei hepatice normale şi datele de ultrasonografie normale. Nivelurile serice de sIL-2R au fost măsurate folosind ELISA. Semnificaţia statistică a fost stabilită pentru p<0,05. **Rezultate:** sIL-2R în ser a fost măsurat la 15 pacienţi cu infecţie cronică cu HCV înaintea tratamentului cu interferon (IFN) alfa. Nivelurile serice de sIL-2R au fost semnificativ mai crescute 809±43,6 pg/ml decât cele ale subiecţilor sănătoşi 634±32,7 pg/ml, p<0,02 după testul Student t. Au existat corelaţii între nivelurile serice de sIL-2R şi scorul activităţii histologice (r=0,482, p<0,01) şi nivelurile serice de alanin aminotransferază (r=0,363, p<0,01). sIL-2R în ser a fost semnificativ mai crescut la pacienţi cu HCV genotip 1 comparativ cu genotip 3 (889±44,8 vs. 756,7±37,9; p<0,02). **Concluzie**: Nivelurile serice de sIL-2R cresc din cauza infecţiei cu HCV, iar nivelul corespunde gradului de inflamaţie.
Cuvinte cheie: receptorul solubil al interleukinei 2, infecţia cu virusul hepatitei C, fiziopatologie

EFFICACY AND TOXICOLOGICAL CHARACTER OF CORIANDER AND CHAMOMILE EXTRACTS

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ABSTRACT

Plants have the ability to synthesize a wide variety of chemical substances that are used to perform important biological functions, and to defend against attack from predators such as insects, fungi and herbivorous mammals. In this study, coriander and chamomile extracts were obtained using two different pathways (Soxhlet and maceration). The extracts were characterized by density, pH, and dried substance content measurements, by Franz diffusion cell and applications on the mice skin. Generally the results indicate the obtaining of extracts good enough to be used in cosmetics.

Keywords: diffusion cell, TEWL, melanin content, erythema, Stratum corneum

INTRODUCTION

A new stage of plant chemistry (phytochemistry) started in 1806, when Belgian pharmacist Sertűrner isolated for the first time morphine from poppy latex. In his experimental study, it was established for the first time a procedure to obtain the plants' active principles, so-called the "quintessential" advocated by his predecessors. In the following centuries, the research is characterized by large-scale studies on phytochemicals, and their principles (1, 2). These chemicals as essential oils, tannins, vitamins, alkaloids, were studied in laboratories and clinics, establishing their therapeutic properties. Scientific studies have also shown that active principles have well-defined action, and medicinal plants contain a number of substances that complement and enhance their action. The active principles are sometimes scattered throughout the plant, and in this case it is used together with the root, sometimes they are located in large amounts only in leaves, flowers, roots, seeds, fruits and so on; it is harvested and uses only those parts of the plant (3).

Coriander, *Coriandrum sativum L.* (*Apiaceae* family), is an annual plant with thin, and less branched roots. It has a cylindrical long stem (about 30-60 cm), finely furrowed, and branched at the top. The flowers are white, reddish or purplish. It blooms in June and July (4). Coriander is a culture plant that grows on brown forest soils. The fruits (*Fructus coriander*) are collected in order to be used in August and September (5).

Fig. 1. Coriander (6)

The main component of coriander is the volatile oil with concentrations between 0.2-1.7% and it depend on the climatic conditions in which the plant grew. Smaller fruits are rich in volatile oil. Coriander fruits contain 13-20% fatty oil. There were also determined protein substances, different acids, mannitol, tocopherol, coumarins, a mixture of flavonoids, and resins (7).

Coriander oil is a colorless or pale yellow liquid with aromatic odor, characteristic and pleasant, like the rose, and with spicy taste. It is less dense than water (d = 0.780 to 0.885) and is optically active. It is soluble in alcohol, ether, chloroform, petroleum ether, fatty oils and is insoluble in water, but it communicates its flavor. Coriander oil is constituted mostly of linalool and other terpenes (pinene, geraniol, geranyl acetate, borneol, citronellol, camphor). Coriander fruits possess spasmolytic and bactericidal actions. The volatile oil is used mainly as a flavoring in perfumery (8).

Chamomile, *Chamomilla Matricaria recutita* (*Asteraceae* family) is an annual herbaceous plant which has a branched surface root in soil. The stem is often branched, each branch terminated by an inflorescence. It blooms from May to August (9). Chamomile is a common plant throughout the country, found in uncultivated places, green land, beside roads, meadows, healthy soils. The flowers (*Flores Chamomillae*) are used for their content: bitter sesquiterpene substances such as matricine.

Fig. 2. Chamomile (10)

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Other substances isolated from chamomile are flavonoids, represented by flavones and flavonols as aglycones, but mostly as glycosides. There have been highlighted two coumarins, phenolic acids, and high fatty acids. Chamomile flowers also contain mucilage, a phytosterol, a tricyclic alcohol ($C_{15}H_{26}O$), a tricyclic hydrocarbon ($C_{15}H_{24}$), and a keto-alcohol ($C_{15}H_{24}O_2$). The most important component is the volatile oil which is the most active ingredient of chamomile. It is a blue, dense liquid, with aromatic odor and bitter taste. In contact with air and light its color changes to green, watery brown (11).

In this study, coriander and chamomile extracts were obtained through two different pathways and there were characterized by density, pH, and dried substance content measurements; the concentrated extracts were tested by the mice skin model.

MATERIALS AND METHODS

The coriander and chamomile extracts were obtained through two different pathways: (i) extraction with Soxhlet apparatus at 78 °C for 2 hours, and (ii) maceration at room temperature for 7 days.

Table I. Samples codes and descriptions

Code	Raw materials *Technique*	M.U.	Quantity
CoS	Coriander Ethanol (96%) Distilled water *Soxhlet*	g ml ml	5 40 10
ChS	Chamomile Ethanol (96%) Distilled water *Soxhlet*	g ml ml	5 40 10
CoM	Coriander Ethanol (96%) Distilled water *Maceration*	g ml ml	5 40 10
ChM	Chamomile Ethanol (96%) Distilled water *Maceration*	g ml ml	5 40 10

The obtained extracts were finally concentrated in order to obtain suspensions which can adhere to the mice skin. These suspensions efficacy was evaluated by Franz diffusion cell in the following conditions: 7 mL phosphate buffer (pH = 7.4) as acceptor phase; effective diffusion area was 1.767 cm^2; rotation speed was 450 rpm; experiments were done at a temperature of 37±0.5 °C for 6 hours. Acceptor phase was extracted with an automatically system and replaced with fresh medium. Quantitative measurements were made with a UV-Vis spectrophotometer Hewlett Packard model 8453. A synthetic membrane and samples of rat skin were used for the study.

Ten weeks old females of hairless mouse SKH1 were maintained during the experiments in National Institute of Animal Health (NIAH) recommended conditions: 12 hours light-dark cycles, food and water *ad libidum*, temperature 24±2 ºC, humidity above 55%. The suspensions were applied on mice skin for 9 weeks (0.3 mL / application). There were used 15 mice: group CoS (3 mice, treated with suspension based on CoS extract), group ChS (3 mice, treated with suspension based on ChS extract), group CoM (3 mice, treated with suspension based on CoM extract), group ChM (3 mice, treated with suspension based on ChM extract) and group 0 (3 mice as blank treated with ethanol). The applications and measurements were done twice a week.

After applications, each determination was performed within 30 minutes. All the measurements on the mice skin were carried out with a Multiprobe Adapter System (MPA5) from Courage-Khazaka, Germany.

RESULTS

The coriander and chamomile extracts present different pH, density, and dried substance content values depending on the chosen pathway (Table II).

Table II. Samples characterization

Code	Density [g/cm^3]	pH	Dried substance content [%]
CoS	0.94	5.66	3.8
ChS	0.98	6.20	5.6
CoM	0.92	5.72	4.9
ChM	0.91	6.08	6.1

Suspensions with different concentrations were used to draw the calibration curves. Calibration curves were done in order to evaluate the suspensions efficacy with Franz diffusion cell (Figure 3).

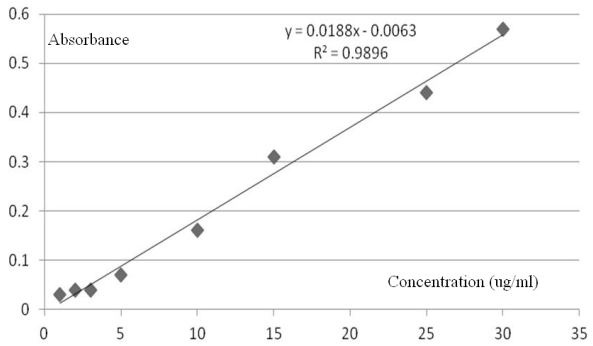


Fig. 3. Calibration curve used in the case of CoS sample

It was draw the release profile of every obtained suspension. An example of these is Figure 4.

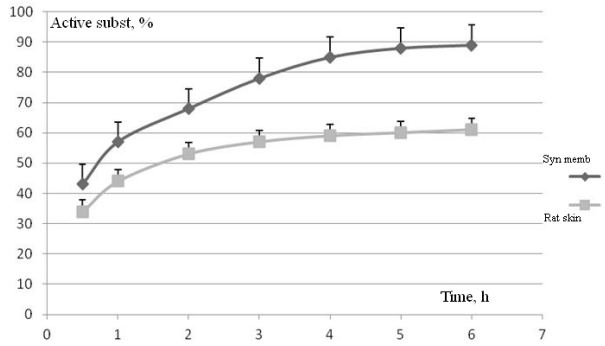


Fig. 4. Release profile of CoS sample

There were not observed any differences about the mice skin macroscopic aspect between the first and the last week of the experiment (Figure 5). The evolutions of the skin parameters are presented in Figure 6.

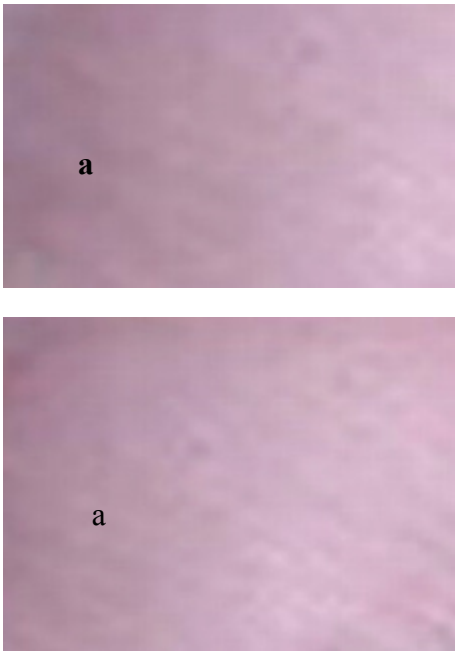
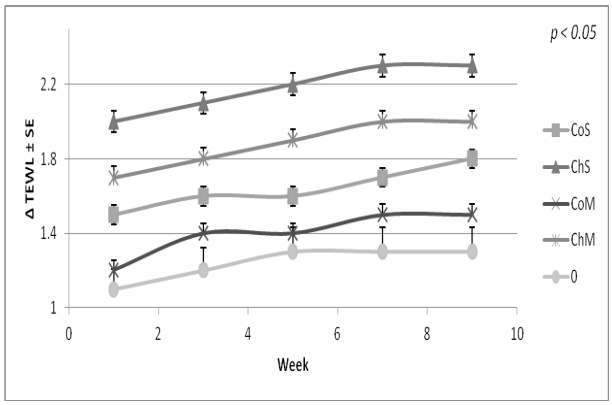
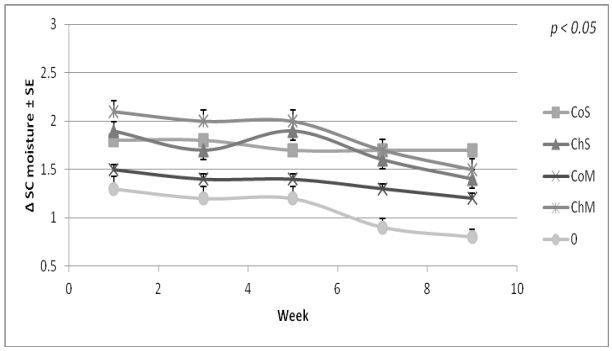
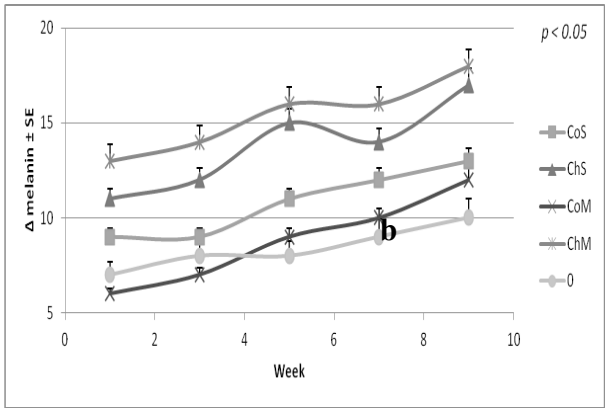


Fig. 5. Macroscopic aspect of back skin (magnification: 3x) for one mouse from CoS group in
(a) first week; (b) last week of experiment







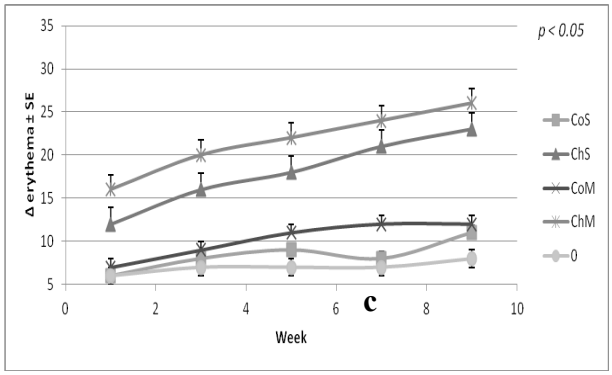


Fig. 5. Evolution of skin parameters: (a) transepidermal water loss; (b) hydration of Stratum corneum;
(c) melanin content; (d) erythema

DISCUSSION

The diffusion capacity of the active principle from the obtained suspensions was studied on synthetic membrane (PORAFIL Membranfilter) and on rat skin (*Spraque Dawley*). In all cases, the amount of active principle which diffuses through the membrane is high (over 40%), regardless of membrane used. The best results were obtained from samples obtained by maceration extraction process.

TEWL measurements showed increases in transdermal water loss less pronounced, which indicates low skin alterations or substances with low harmful effect. The melanin content

evolutions correspond to an upward trend, which could not be correlated with the type of suspension. In many studies there is a slight linear increase melanin content in the skin over skin testing period. Suspensions based on coriander extracts present lower erythema differences and this trend is probably due to the chamomile allergic reaction (12). Level of hydration of the *Stratum corneum* had a downward trend in all cases.

CONCLUSIONS

Medicinal plants are used for their medicinal properties. Only few plants or their phytochemical constituents have been proven to have medicinal effects by rigorous science or have been approved by regulatory agencies. In this research there were obtained coriander and chamomile extracts using two different pathways. The extracts were concentrated and their efficacy was evaluated by Franz diffusion cell and applications on the mice skin. There were recorded good results of diffusion through membranes and good results of TEWL and melanin content. It was observed the well known chamomile allergic reaction by erythema evolution study.

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EFICACITATEA SI CARACTERUL TOXICOLOGIC AL UNOR EXTRACTE DE CORIANDRU SI MUSETEL

REZUMAT

Plantele au capacitatea de a sintetiza o mare varietate de substante chimice, care sunt utilizate pentru a efectua diverse functii biologice importante, si pentru a apara impotriva atacurilor unor pradatori, cum ar fi insectele, ciupercile si mamiferele erbivore. In acest studiu, extracte de musetel si coriandru au fost obtinute folosind doua cai diferite (Soxhlet si macerare). Extractele au fost caracterizate prin masuratori de densitate, pH si continut de substanta uscata, cu ajutorul celulei de difuzie Franz si prin aplicatii pe piele de soarece. In general rezultatele indica obtinerea unor extracte destul de bune pentru a fi folosite pentru produse cosmetice.

Cuvinte cheie: celula de difuzie, pierdere de apa transdermica, continut de melanina, eritem, Stratum corneum

OLEANOLIC AND URSOLIC ACID IN HUMAN SKIN CANCER – A PRELIMINARY IN VITRO COMPARATIVE STUDY

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ABSTRACT

Triterpenic compounds are the subject of many studies because of their multiple activities including anticancer activity. The aim of this study is to compare the activity of ursolic acid (UA) and oleanolic acid (OA) on A375 human melanoma cells, using the MTT *in vitro* cell viability assay. A375 cells were treated with different concentration of ursolic and oleanoic acid (10, 20, 30, 50, 75 μM) for 24 and 48 hours. UA acted as a cytotoxic compound at concentration of 50 and 75 μM, cell viability decreasing slightly after 24 h (80% and 97%), and more pronounced after 48 h (47% and 46% respectively) leading to the conclusion that ursolic acid could be an in vivo anti-proliferative agent on A375 human melanoma cells in a dose- and time-dependent manner. For cells treated with OA, at 48 h, we observed a very slightly decreasing in cell viability (93-102%) for all the concentration, showing a poor anti-proliferative effect at the same concentration.

Key words: ursolic acid (UA), oleanolic acid (OA), anti-proliferative, MTT assay

INTRODUCTION

Importance of research skin cancer increases with its incidence, statistics showing that the number of skin cancers is increasing every year. In this direction, triterpenic compounds are the subject of many studies because of their multiple activities including anticancer activity (1). Oleanoic acid (OA) and its isomer, ursolic acid (UA) are pentacyclic triterpene compounds, with 30 carbon atoms (2) which exist in nature both in free form and as aglycones for saponins triperpenice: oleanane and ursane (3, 4, 5). Their existence is widespread in nature, oleanolic acid were isolated from over 120 species of plants of which the best known are rosemary, oregan, lavender, *Olea Europeae L.*, *Swertia mileensis T.*, *Ligustrum lucidum it.*, and *Actostaphylos uva-urs*, *Eriobotrya japonica*, *Rosmarinus officinalis*, and *Glechoma hederaceae* (2, 4,5).

The pharmacological activity of oleanolic acid and ursolic is multiple, related, totaling antitumor activity, antiviral, anti-inflammatory, antimicrobial, hepatoprotective, antidiabetic, gastroprotective and hemolytic (3, 4). Antitumor effects of ursolic and oleanolic acid were observed in various types of cancers such as breast cancer, melanoma, hepatoma, prostate carcinoma and leukemia and lymphomas (3, 6). Ursolic acid has demonstrated cytotoxic effects on P-388 and L-1210 leukemia cells, A-549 human lung carcinoma cell, KB human epidermoid carcinoma (2, 4). Feng's study results achieved by MTT method, show that ursolic acid causes inhibition of B16 cell proliferation with IC50 value at 10 μM (2), which strengthens the hypothesis that supports anti-proliferative effects of ursolic acid.

Moreover, Es-Saady et al. showed ursolic acid's effect on G1 cell cicle on B16 melanoma cells (7). Baconasoni et al. examined the effect of ursolic acid on the proliferation and differentiation of A375 human melanoma cells and and showed that UA inhibits tumor cells proliferation in a dose- and time- dependent maner (8). However, the mechanisms by which these triterpenes act as tumor inhibitors in the skin may be multiple and complex, some of which are still under study (9).

The aim of this study is to compare the activity of ursolic acid and oleanolic acid on A 375 human melanoma cells, using the MTT cell viability test.

MATERIALS AND METHODS

Preparation of ursolic and oleanolic acid solution

Ursolic and oleanolic acid (Sigma Aldrich Germany) were dissolved in Dimethyl sulfoxide (DMSO; Sigma-Aldrich, Ayrshire, UK) and stored at 2-8 °C. For all experiments, final concentration of the tested compounds was prepared by diluting the stock solution with DMEM.

Cell culture

A375 human melanoma (ATCC, USA) was cultured in DMEM containing 10% FCS (fetal bovine serum, PromoCell, Heidelberg, Germany), 1% glutamine, and 1% penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany). Cells were cultured in 5 % CO_2 atmosphere at 37 ° C.

MTT in Vitro Analysys

The A375 cell line (ATCC, USA) was seeded onto a 96-well microplate and attached to the bottom of the well overnight. After 24 h, 150 μL of new medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL,

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Invitrogen, Carsbad, Ca, USA) and the tested substances were added and incubated for 24 h and 48 h respectively; the medium was supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 1% penicillin/streptomycin mixture (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany). Melanoma cells were passaged at confluence after treatment with 5 mM EDTA; the living cells were then assayed by the addition of 15 µL of 5mg/mL MTT solution. The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 3-h contact period. The medium was then removed, and the precipitated crystals were dissolved in 150 µL dimethyl sulfoxide (DMSO; Sigma-Aldrich, Ayrshire, UK). Finally, the reduced MTT was spectrophotometrically analyzed at 570 nm using a microplate reader; wells with untreated cells were used as controls. All *in vitro* experiments were performed on two microplates with at least four parallel wells. DMSO was used to prepare stock solutions of the tested substances, and we used DMSO solution as a negative control. The dilution rate was 1:400 and the concentration of stock solution was 10 mM.

RESULTS

Cell morphology

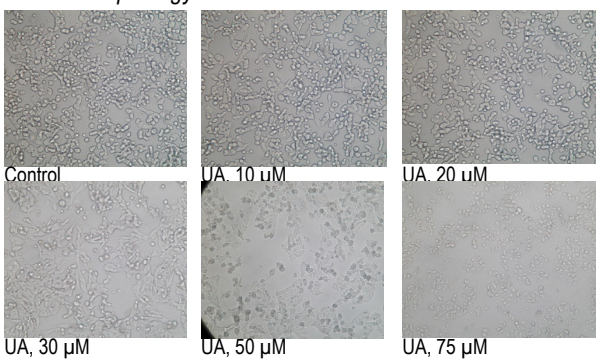

Fig.1. A375 cell morphology: control, cells treated with different concentration of ursolic acid (10, 20, 30, 50, 75 µM)

Figure 1 show the morphology of A375 cell treated with ursolic acid at different concentration and non-treated cells (control). The morphology of cells treated with oleanolic acid looked like the morphology of cells treated with ursolic acid.

Cell proliferation assay

Cell proliferation assay was performed using the MTT assay. MTT is a tetrazolium salt that is cleaved to formazan by the succinate dehydrogenase system which belongs to the mitochondrial respiratory chain and is only active in viable cells. The mitochondrial succinate dehydrogenase reduces the yellow tetrazolium salt into water-insoluble purple formazan. A375 cells were treated with different concentration of ursolic and oleanoic acid (10, 20, 30, 50, 75 µM) for 24 and 48 hours.

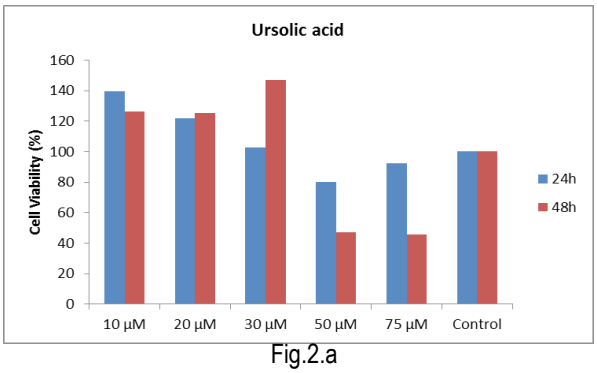

Fig.2.a

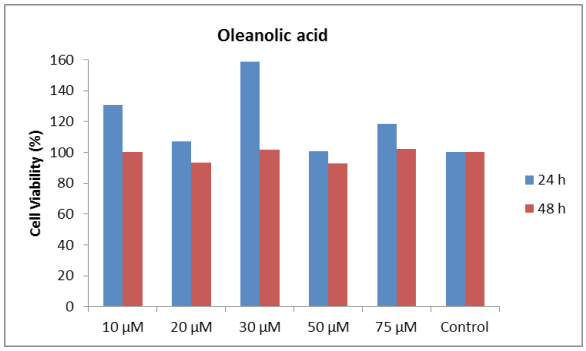

Fig.2. Effect of UA (a) and OA (b) on A375 melanoma cells viability. Cell were treated with different concentration and cell viability was assesed by MTT assay at 24 h and 48 h.

After 24 h in cells treated with UA, we observed an increase of proliferarion to 139%, 121%, 102 % (10, 20, 30 µM) compared to control. For the higher concentration, 50 and 75 µM we observed a decrease of cell viability percentage to 78%, 98 % respectively. For cells treated with OA, at 24 h there all the concentration (10-75 Mm) showed an increase of proliferation (131%, 107%, 158%, 101%, and 119% respectively). At 48 h, for cells treated with UA, MTT assay did not show a difference in cell proliferation between 10-30 µM, but for 50, 75 µM, it showed a decrease of cell viability from 78% to 47% and from 97% to 46% respectively (Figure 2.a). For cells treated with OA, at 48 h, we observed a slightly decreasing in cell viability (93-102%) for all the concentration (Figure 2.b).

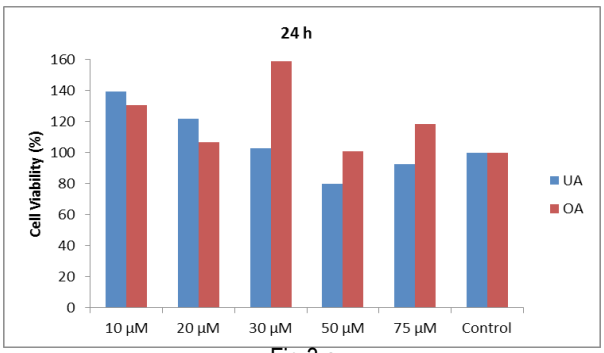

Fig.3.a

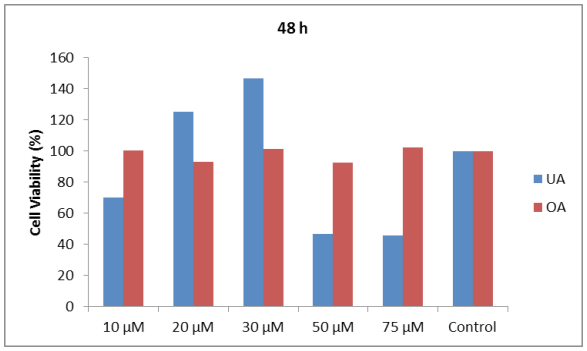

Fig.3. Comparative effect of ursolic and oleanolic acid at 24 h (a) and 48 h (b), assesed by MTT assay.

DISCUSSION

Due to continuous increase in skin cancer frequency, in the past 10 years the number of melanoma cases augmented more than any type of cancers (10), researcher's attention heads to obtain more effective and less toxic anticancer compounds (11). In this prospect, some of the compounds studied for their anticancer potential are ursolic and oleanolic acid. Ursolic acid inhibits growth of tumor cells through multiple functions including cytotoxicity, induction of apoptosis and prevention of angiogenesis (12). Our study attempted to discover the activity of ursolic and oleanolic acid and to make a comparison between their activities on A375 cell line. A375 is a human melanoma cell line, amelanotic, derived from a 54 year old female with malignant melanoma.

Using the MTT assay we demonstrated that UA has an anti-proliferative effect on A375 melanoma cells line in a dose and time-dependent manner. UA acted as a cytotoxic compound at concentration of 50 and 75 µM, cell viability decreasing slightly after 24 h (80% and 97%), and more pronounced after 48 h (47% and 46% respectively). Lower concentrations did not induce any cytotoxic activity neither after 24 h, nor after 48 h. Furthermore concentrations between 10 and 30 µM showed an increase of MTT reduction, which could lead to the conclusion that ursolic acid induce the proliferation of A375 melanoma cells. Other authors reported that MTT could underestimate the anti-proliferative effect of substances demonstrating through other methods such as Trypan blue exclusion method (10) or counting with hemocytometer (7) that the true number of viable cells could not correspond with the values obtained by MTT assay. This is the case of M4Beu melanoma (10), B16 mouse melanoma (7), MCF7 human breast cancer and HaCaT keratinocyte-derived cells (10, 13). As we did not use another method to evaluate the effects of UA on A375 cells, we cannot decide if the MTT values are the true values or not.

Other results on ursolic acid's activity on melanoma cancer cells, such as melanoma B16 murine melanoma and M4Beu human melanoma report that depending on exposure time, anti-proliferative activity of ursolic acid occurs at concentrations IC = 10-50 µM (10, 14, 15). However, Manu et al, reported that after 48 hours of exposure, concentration between 10-50 µM, showed a non-toxic activity on B16 F10 murine melanoma cells, but it found to be 76 % and 100% cytotoxic at concentration of 75 µM and 100 µM (16).

Comparison to the ursolic acid, oleanolic acid did not show a good anti-proliferative activity at the same concentration neither after 24 h of exposure, nor after 48 h. George et al, showed using a MTT assay on HaCaT cells, after exposure of 200 µM for 72 hrs, only 26.2% cytotoxicity was observed (11). Oleanolic acid also was observed to reduce metastatis of B16 melanoma cells (17). All taken together could lead to the conclusion that oleanolic acid's antiproliferative activity is at higher concentration than ursolic acid.

CONCLUSION

In conclusion, we may say that ursolic acid could be an *in vivo* anti-proliferative agent on A375 human melanoma cells in a dose- and time-dependent manner. Oleanolic acid show a poor anti-proliferative effect at the same concentration and further investigation with higher concentration are needed.

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ACIDUL OLEANOLIC SI URSOLIC IN CANCERELE CUTANATE UMANE – STUDIU COMPARATIV PRELIMINAR IN VITRO

REZUMAT

Compusii triterpenici sunt investigati in multiple studii actuale datorita actiunii acestora in diverse afectiuni, inclusiv activitatea antitumorala. Scopul acestui studiu a fost acela de a compara activitatea acidului ursolic (UA) si oleanolic (OA) asupra celulelor liniei de melanom uman A375, folosind testul *in vitro* de viabilitate MTT. Celulele liniei A375 au fost tratate cu diferite concentratii de acid ursolic si oleanolic (10, 20, 30, 50, 75 μM) timp de 24 de ore, respectiv 48 de ore. UA a avut activitate citotoxica la concentratii de 50 si 75 μM, viabilitatea celulara prezentand o scadere usoara dupa 24 de ore (80% si 97%), dar mult mai pronuntata dupa 48 de ore de actiune (47%, respectiv 46%), ceea ce a dus la concluzia ca acidul ursolic ar putea fi un agent anti-proliferativ in cazul celulelor de melanom uman A375, actiunea acestui compus fiind dependenta de doza si de timpul de actiune. Pentru celulele tratate cu OA, la 48 de ore se observa o scadere usoara a viabilitatii celulare (93-102%), pentru toate concentratiile utilizate, ceea ce sugereaza un efect anti-proliferativ scazut al acestui compus comparativ cu UA, pentru concentratii similare.

Cuvinte cheie: acid ursolic (UA), acid oleanolic (OA), anti-proliferativ, test MTT

THE RELATIONSHIP BETWEEN DIABETIC RETINOPATHY AND CORONARY ARTERY DISEASE IN BANAT COUNTY

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ABSTRACT

We conducted an observational study to assess the relationship between diabetic retinopathy and coronary artery disease in a population from Banat County, Romania. Our study included 231 patients (121 men and 110 women) with type 2 diabetes aged 40-65 years who were examined for retinal microvascular changes by retinal photographs in the Ophthalmology Clinic Timisoara between May 2008 and September 2012. The patients involved in our study were selected from a larger group of 3,400 patients. The mean follow-up period was 28 ± 12 months. The initial ophthalmologic examination revealed that 109 subjects had some degree of DR (prevalence rate of ~ 40%), including 87 patients with non-proliferative (NPDR) (prevalence rate of 32%), and 22 patients with proliferative diabetic retinopathy (PDR), (prevalence rate of 8%). After adjustments for risk factors, diabetic retinopathy was associated with two times higher incidence of unstable and stable angina - odds ratio =1.95 (95% CI 1.13 - 3.35), and higher incidence of myocardial infarction-odds ratio = 2.36 (95% CI 0.78 - 7.14). The risks of CAD were higher in individuals with proliferative retinopathy (PDR) - for unstable and stable angina odds ratio =2.86 (95% CI 1.13 - 7.22), for myocardial infarction odds ratio = 4.29 (95% CI 1.1-16.7). Also, in patients with non-proliferative retinopathy (NPDR) was a slightly higher incidence of unstable and stable angina odds ratio=1.76 (95% CI 0.99 - 3.14), and myocardial infarction - odds ratio=1.43 (95% CI 0.44 - 4.59). Our data show that the presence of retinopathy in individuals with type 2 diabetes was associated with a twofold higher risk of incident CAD (unstable and stable angina, myocardial infarction), independent of glycemic levels and cardiovascular risk factors. This association appears to be graded with retinopathy severity, as in patients with proliferative retinopathy was associated with four time higher incidence of myocardial infarction and three time higher incidence of stable and unstable angina.

Key words: diabetic retinopathy, coronary artery disease, myocardial infarction, angina, incidence

PURPOSE

We conducted an observational study to asses the relationship between diabetic retinopathy and coronary artery disease in a population from Banat County, Romania.

INTRODUCTION

Diabetic retinopathy (DR) is a common and devastating microvascular complication in type 1 and 2 diabetes patients. The leading cause of mortality in patients with diabetes is coronary arteries disease (CAD) (1,2). CADs are listed as the cause of death in ≈ 65% of persons with diabetes (3). In diabetic patients myocardial ischemia due to coronary atherosclerosis commonly occurs without symptoms. As a result, multi-vessel atherosclerosis often is present before ischemic symptoms occur and before treatment is instituted. The risk of a myocardial infarction in diabetic patients with no evidence of CAD matches that in patients without diabetes who have had a previous myocardial infarction, supporting the idea of diabetes as a coronary equivalent (4). A delayed recognition of various forms of CAD undoubtedly worsens the prognosis for survival for many diabetic patients (5). A number of recent studies showed that diabetes is an independent risk factor for CAD in both men and women (6,7). However, it is unclear if the presence of DR, a microvascular complication, is an increased risk of CAD.

METHODS

Study population

Our study included 231 patients (121 men and 110 women), aged 40-65 years who were examined for retinal microvascular changes by retinal photographs in Ophthalmology Clinic Timisoara between May 2008 and September 2012. The patients were selected from a larger group of 3,400 patients who visited the Ophthalmology Clinic Timisoara for eye examination between May 2008 and September 2012. We selected for inclusion in our study only patients with fasting plasma glucose of ≥ 7.0 mmol/l (126 mg/dl) or more, using diabetic medications, or a physician's diagnosis of diabetes. Participants diagnosed with diabetes were categorized as newly diagnosed diabetes (NDM) patients. Those with self-reported diabetes and either on current treatment (insulin or oral hypoglycemic medication) or with diabetic glucose values were categorized as having known diabetes (KDM). In these patients we assessed hypertension on the basis of systolic and diastolic blood pressure at three consecutive visits (more than 140 systolic or/and 90 diastolic mmHg, measure in a rest condition with a mercury sphygmomanometer). The diagnosis of hypertension was confirmed by a cardiologist.

We excluded from our study patients with history of stroke

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or transient ischemic attack; patients with known prior coronary arteries diseases (history of angina, previous myocardial infarction), significant peripheral vascular disease (previous surgery or symptoms of intermittent claudication); aortic aneurysm; thyroid disease; nephrotic syndrome and patients with type 1 diabetes. In all patients with known or recent diabetes a complete physical examination, ECG, and blood pressure was performed, then the patients were referred for a complete eye examination. Required data were collected, including: sex, age, disease duration, type of diabetes, dyslipidemia (based on laboratory findings or use of any lipid-lowering drugs).

Participants were asked to go to a specified laboratory for collection of blood samples, following a 12-hour fast to identify the undiagnosed patients. Glycosylated hemoglobin (HbA1c) level was measured and values less than 7% were considered as indicators of good glycemic control. Body mass index (BMI) (weight in Kg, divided by height in meters squared) was calculated. The WHO (1977, 1979) classification for BMI was used to estimate the degree of obesity. Complete eye examinations were performed. Fundus photography performed with a digital camera and full retinal and macular examination conducted by two experimented operators were carried out. Diabetic retinopathy is primarily classified into non proliferative DR (NPDR), formerly termed simple, or background retinopathy (mild, moderate and severe), and proliferative DR (PDR). We use for classification of diabetic retinopathy an adaptation of "The International Clinical Diabetic Retinopathy Disease Severity Scale" (8). DR was defined to include the presence of any of the above lesions.

The presence of coronary arteries diseases was defined as presence of pectoris angina (with one of the following: specific ischemic changes on ECG, positive effort test, positive angiocoronarography) and acute coronary syndrome (ST elevation myocardial infarction -typical chest pain, specific rise and fall of cardiac enzyme and ST elevation on ECG, unstable angina-typical chest pain, ischemic modification on ECG, without rise of cardiac enzyme). The diagnosis of coronary arteries disease was made by a cardiologist from Cardiovascular Disease Institute Timisoara. We performed angiocoronarography in all patients with CAD.

Statistical analysis

We used the MedCalc Software for calculating the odds ratio, confidence interval, prevalence proportion. The results for continuous variables were given as mean ± SD and for categorical variables as percentage. The limit of P value of statistical significance was considered 0.05.

RESULTS

We included in our study 231 patients (121 men and 110 women) with type 2 diabetes, aged 40-65 years. The mean follow-up period was 28 ± 12 months. The initial ophthalmologic examination revealed that 109 subjects had some degree of DR (prevalence rate of ~ 40%), including 87 patients with non-proliferative (NPDR) (prevalence rate of 32%), and 22 patients with proliferative diabetic retinopathy (PDR), (prevalence rate of 8%). We detected clinically significant macular edema (CSME) in 17 patients (6.25%). The clinical characteristics of patients are shown in Table I. Patients with DR (109 patients) were included in group A. Patients without DR (122 patients) were included in group B. The characteristics of patients with DR (group A) and without DR (group B) are shown in Table II. The patients with DR had a significantly longer duration of known diabetes ($P < 0.001$), a higher ratio of being on insulin therapy ($P < 0.001$), higher serum creatinine levels ($P = 0.026$). There were no significant differences with regard to patient age, sex, risk factors for CAD, and other biochemical parameters (Table II).

Table I. Characteristics of total patients with type 2 diabetes

	Total (n = 231)
Clinical characteristics	
Age (years)	51 ± 12
Sex (Men/Women)	121/110
BMI (kg/m ^{2})	27.2 ± 6.1
Diabetes	
Duration (years)	7.4 ± 4
HbA_{1c} (%)	8.5 ± 2.2
Retinopathy (%)	109 (40%)
Proliferative retinopathy (%)	22 (8.0)
Macular edema	17(6.25%)
Nephropathy (%)	74(32%)
Peripheral neuropathy (%)	47 (22%)
Cardiovascular risk factors	
Hypertension (%)	162 (70%)
Dyslipidemia (%)	150 (65%)
Smoking (%)	99 (43%)
≥2 Cardiovascular risk factors (%)	143 (62%)

Table II. Characteristics of patients in group A and B

Characteristics	**Group A (With DR) 109 pts**	**Group B (Without DR) 122 pts**	***P* value**
Age (years)	52±10 y	53±9 y	P=NS
Sex (Men/Women)	59/56	62/54	P=NS
BMI (kg/m ^{2})	26.5±5	27.3±6	P=NS
Duration of diabetes (years)	10.4±6	6.2±4	P < 0.001
HbA_{1c} (%)	7.9±1.3	8.1±1.7	P=NS
Creatinine (mg/dL)	1.13±0.47	0.94±0.20	P=0.026
No. of patients taking insulin	33(30%)	17 (14%)	P<0.001
Total cholesterol (mg/dL)	201.4±50.2	213±48.6	P=NS
HDL (mg/dL)	42.3±9.5	40.1±8.7	P=NS
LDL (mg/dL)	126.5±33.8	128±40.2	P=NS
≥2 Cardiovascular risk factors (%)	69 (63.3%)	74 (61%)	P=NS
Smoking (%)	47 (43%)	52 (42.6%)	P=NS
Hypertension	75 (69%)	87 (71%)	P=NS

Over the follow-up period, 101 patients (43.7%) were diagnosed with CAD, 85 patients (36.7%) with stable and unstable angina, and 16 patients (~7%) with myocardial infarction. In group A there were 59 patients (54%) with CAD, 49 patients (45%) with stable and unstable angina, and 10 patients (9.17%) with myocardial infarction. The incidence of CAD was significantly higher than in group B: 42 patients (34.4%) with CAD, 36 patients (29.5%) with stable and unstable angina, and 6 patients (5%) with myocardial infarction ($p < 0.001$) (Table III).

Table III. Incidence of coronary arteries disease

	Total patients 231 pts	**Group A with DR 109 pts**	**Group B without DR 122 pts**
CAD	101 pts-43.7%	59 pts-54%	42 pts- 34.4%
Unstable angina, stable angina	85 pts-36.7%	49 pts-45%	36 pts-29.5%
Myocardial infarction	16 pts-7%	10 pts-17%	6 pts-5%

After adjustments for risk factors, diabetic retinopathy was associated with two times higher incidence for unstable and stable angina - odds ratio = 1.95 (95% CI 1.13 - 3.35), higher incidence of myocardial infarction - odds ratio = 2.36 (95% CI 0.78 - 7.14). The risk of CAD was higher in individuals with proliferative retinopathy (PDR) - for unstable and stable angina - odds ratio = 2.86 (95% CI 1.13 - 7.22), for myocardial infarction - odds ratio = 4.29 (95% CI 1.1 - 16.7). Also, in patients with non-proliferative retinopathy (NPDR) was a slightly higher incidence of unstable and stable angina - odds ratio = 1.76 (95% CI 0.99 - 3.14), and myocardial infarction - odds ratio = 1.43 (95% CI 0.44 - 4.59).

DISCUSSION

Our study conducted on a sample of patients with type 2 diabetes showed that the prevalence of CAD is significantly higher in patients with DR compared with those without DR. It is well know from observational studies that patients with advanced diabetic retinopathy have a poor life expectancy. A retrospective review of 128 diabetics followed-up at the Radcliff Infirmary, Oxford, England found the 5-year mortality was 45% for those with PDR, 8% for those with only microaneurysms, and 8% for those without any retinopathy (9). Davis and colleagues (10) reported in a prospective study of 709 patients with type 2 diabetes taking insulin and followed-up for up to 13 years a 5-year mortality of 44% for those with PDR, 19% for those with moderate NPDR, and 1% for those with no or minimal retinopathy at baseline.

In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) based on an 8.5-year follow-up of 1,370 persons with older-onset diabetes mellitus, the age-adjusted and sex-adjusted hazard ratios for CAD mortality were 1.50 (95% confidence interval, 1.22 - 1.85) in patients with mild NPDR, 1.93 (95% confidence interval, 1.43 - 2.61) in those with moderate NPDR, and 2.07 (95% confidence interval, 1.48 - 2.91) in those with PDR (11). This study involved patients with type 1 diabetes, while we included in our study only patients with type 2 diabetes. In the Atherosclerosis Risk in Communities Study was demonstrated that among patients with type 2 diabetes, the presence of diabetic retinopathy is associated with a twofold higher risk of CAD events, and a threefold higher risk of CAD death, independent of cardiovascular risk factors, diabetes duration and control, and large-vessel atherosclerosis (12). In a study conducted by Mittinen and colleagues (13) that included 1,059 patients during the 7-year follow-up has been shown that 255 (24%) patients developed serious CAD events. In patients with proliferative retinopathy at baseline, the risk of CAD events during the follow-up was statistically significantly higher compared with patients without retinopathy changes (odds ratio [OR] 2.31, 95% CI 1.21 - 4.40). The association between proliferative retinopathy and CAD events remained significant when other cardiovascular risk factors were controlled for (13).

In a study of 2,329 type 1 diabetic patients without prior CAD, the 7-year incidence rate of CAD was 8.0 (per 1,000 person-years) in men and 10.2 in women. Multivariate standardized Cox proportional hazards models showed that age (hazard ratio 1.5), albumin excretion rate (1.3 in men and 1.6 in women), waist-to-hip ratio (1.3 in men), smoking (1.5 in men), fasting triglycerides (1.3 in women) or HDL cholesterol (0.74 in women), and systolic BP (1.3 in women) were predictors of CAD (14). In the pooled analysis of the 17 included studies, the OR for all-cause mortality and/or CV events of the presence of DR was 2.34 (95% CI 1.96 - 2.80) compared with patients without DR. All but three studies reported an increased risk for CV events. However, a significant heterogeneity among the individual estimates was evident when the magnitude of the association was evaluated (I^2 = 62.7%, P < 0.001). When advanced DR was evaluated (10 reports), the OR for all-cause mortality and/or CV events was 4.22 (95% CI 2.81 - 6.33) compared with patients without retinopathy (I^2 = 63.0%, P = 0.004). The overall sensitivity of advanced DR for the combined outcome was 19% (13 - 28), and the specificity was 94% (91 - 96). The positive likelihood ratio was 3.64 (2.52 - 5.26) and the negative likelihood ratio was 0.84 (0.77 - 0.92) (15).

These meta-analyses and cohort studies showed that the presence of any degree of DR or advanced DR was associated with an increased risk for all-cause mortality and CV events (fatal and nonfatal) in both type 2 and type 1 diabetic patient. We found similar data in our study. After adjustments for risk factors, diabetic retinopathy was associated with two time higher incidence for unstable and stable angina -odds ratio =1.95 (95% CI 1.13 - 3.35), higher incidence of myocardial infarction-odds ratio = 2.36 (95% CI 0.78 - 7.14). The risks of CAD were higher in individuals with proliferative retinopathy (PDR) - for unstable and stable angina odds ratio =2.86 (95% CI 1.13 - 7.22), for myocardial infarction odds ratio = 4.29 (95% CI 1.1 - 16.7). Also in patients with non-proliferative retinopathy (NPDR) was a slightly higher incidence of unstable and stable angina odds ratio = 1.76 (95% CI 0.99 - 3.14), myocardial infarction- odds ratio = 1.43 (95% CI 0.44 - 4.59). A recent study of 824 type 2 diabetic patients, using fundoscopic examinations to evaluate retinopathy, showed a gender difference in the association of background diabetic retinopathy with CAD death, raising the possibility that microvascular disease might be more important in the development of CAD in women than in men (16). We didn't follow

the gender difference regarding incidence of CAD in our study.

A number of important limitations of this study should be mentioned. The people in our study were aged between 40-65 years, white Caucasian. Thus, caution should be taken when extending these findings to other segments of the population - older and younger age groups, other ethnic groups, etc. We didn't use in our study methods to diagnose silent cardiac ischemia (stress echocardiography, scintigraphy, PET etc.).

CONCLUSION

Our data show that the presence of retinopathy in individuals with type 2 diabetes was associated with a twofold higher risk of incident CAD (unstable and stable angina, myocardial infarction) independent of glycemic levels and cardiovascular risk factors. This association appears to be graded with retinopathy severity: in patients with proliferative retinopathy was associated with four time higher incident of myocardial infarction and three time higher incidence of stable and unstable angina.

Our study showed that in diabetic patients evaluation of diabetic retinopathy is important because the presence of any modification is associated with risk of developing coronary arteries disease. Further studies are necessary to asses the correlation with cardiovascular risk factor, diabetic retinopathy and cardiovascular mortality.

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RELATIA DINTRE RETINOPATIA DIABETICA SI BOALA CORONARIANA IN REGIUNEA DE VEST A ROMANIEI

REZUMAT

Am efectuat un studiu observational pentru a evalua relatia dintre retinopatia diabetica si boala coronariana la nivelul populatiei din Regiunea de Vest a Romaniei. Studiul a inclus 231 de pacienti (121 barbati si 110 femei) cu diabet tip 2, cu varsta cuprinsa intre 40-65 ani, care au fost examinati pentru modificari microvasculare retiniene prin tehnici de imagistica retiniana in Clinica de Oftalmologie, in perioada mai 2008 – septembrie 2012. Pacientii implicati in acest studiu au fost selectati dintr-un grup mai mare, care a cuprins 3400 de pacienti. Perioada medie de urmarire a pacientilor a fost de 28 ± 12 luni. Examinarea oftalmologica initiala a evidentiat 109 pacienti cu un grad de retinopatie diabetica (DR) (prevalenta ~ 40%), dintre care 87 de pacienti cu retinopatie diabetica non-proliferativa (NPDR) (prevalenta 32%) si 22 de pacienti cu retinopatie diabetica proliferativa (PDR) (prevalenta 8%). Dupa modificarea factorilor de risc, retinopatia diabetica a fost asociata cu incidenta de 2x mai crescuta a anginei instabile si stabile - odds ratio = 1,95 (95% CI 1,13 - 3,35), precum si cu incidenta mai crescuta a infarctului miocardic - odds ratio = 2,36 (95% CI 0,78 - 7,14). Riscurile de boala coronariana (CAD) au fost mai crescute la pacientii cu retinopatie proliferativa (PDR) – pentru angina instabila si stabila, odds ratio = 2,86 (95% CI 1,13 - 7,22), iar pentru infarctul micocardic, odds ratio = 4.29 (95% CI 1.1-16.7). De asemena, la pacientii cu retinopatie non-proliferativa (NPDR) a fost observata o incidenta usor mai crescuta a anginei instabile si stabile, odds ratio = 1,76 (95% CI 0,99 - 3,14), in timp ce pentru infarctul miocardic - odds ratio = 1,43 (95% CI 0,44 - 4,59). Datele prezentate releva faptul ca prezenta retinopatiei la indivizii cu diabet zaharat tip 2 a fost asociata cu un risc de doua ori mai crescut de aparitie a CAD (angina instabila si stabila, infarct miocardic), independent de nivelurile glicemice si prezenta factorilor de risc cardiovascular. Aceasta asociere pare sa fie corelata cu severitatea retinopatiei, deoarece la pacientii cu retinopatie proliferativa incidenta infarctului miocardic a fost de 4 ori mai crescuta, in timp ce incidenta anginei instabile si stabile a fost crescuta de 3 ori.

Cuvinte cheie: retinopatie diabetica, boala coronariana, infarct miocardic, angina, incidenta

THE PATHOLOGIC SPECTRUM OF TONSIL MALIGNANT TUMORS IN TIMISOARA - STATISTIC STUDY

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ABSTRACT

More than 90% of the oropharynx tumors are squamocellular carcinomas. The rest are malignant melanomas, minor salivary glands tumors, sarcomas, plasma cell tumors, lymphomas and other rare tumors. Metastases in orophayinx have been also identified.

The retrospective study was performed on a group of 134 patients with tonsil tumors admitted in the ENT Clinic of the Clinical City Hospital Timişoara between 2006 and 2010. The variables analyzed were the histopathologic type of the tumor, local extension and lymph node invasion as well as distant metastases. The specimens were fixed in buffered formalin, sectioned at 5 μm and stained with haematoxyllin and eosin for the histopatologic diagnosis. The majority of the tumors studied were squamous cell carcinomas, which was expected, given the increased general incidence of this type of malignant tumors from this region. The lymphomas were present in 2 female patients, one aged 16, and the other aged 61. The last type of malignant tumor that we have noticed was lymphoepithelial carcinoma, present in a 35 years old patent.

Keywords: squamocellular carcinomas, tonsil carcinoma, pathology, radiotherapy

INTRODUCTION

More than 90% of the oropharynx tumors are squamocellular carcinomas. The rest are malignant melanomas, minor salivary glands tumors, sarcomas, plasma cell tumors, lymphomas and other rare tumors. Metastases in orophayinx have been also identified (1-4). Lymphoepithelial carcinoma is more frequent in the tonsil region and in the base of the tongue. The differential diagnosis between lymphoepithelial carcinoma and squamous cell carcinoma is important, because the first is radiosensitive. Non–Hodgkin lymphoma is encountered in 5% of the malignant tumors of the tonsil and is a rare occurence in the base of the tongue.

Squamous cell carcinoma is an epithelial malignant tumor with squamous differentiations such as intercellular bridges and keratin formation. Its origin is in the cornified squamous epithelium of the oropharynx mucosa or in the respiratory epithelium with squamous metaplasia (5). In the oropharynx, the most common localizations include the base of the tongue and the tonsils.

Microscopically, squamous cell carcinomas are characterized by invasive growth and squamous differentiations demonstrated by the formation of intercellular bridges, with or without cornification; and by the formation of keratin pearls. From the immunohistochemical point of view, squamous cell carcinomas express epithelial markers, such as cytokeratins and epithelial membrane antigen (EMA). The cytokeratin expression pattern is determined by the differentiation grade of the tumor and the keratinization degree (6).

The basaloid carcinoma is a poorly differentiated squamous cell carcinoma type composed by basaloid and squamous cells, with a clinically agressive behaviour. It was first described by Wain et al. in 1986 (7). It has a predilection for the aerodigestive tract, but it was described also in the uterine cervix (8), esophagus (9), lungs (10) and the anal canal (11). The preferential localization is the hypopharynx (the piriform sinus), but it was described in the oropharynx as well (12,13) and in the oral cavity (14-16). The precursor of this tumor type is considered a totipotent primitive cell localized in the basal layer of the surface epithelium or in seromucous glands (7,16). The lymphoepithelial carcinoma is a poorly differentiated form of the squamous cell carcinomas or an undifferentiated carcinoma associated with a dense lymphocytic infiltrate in the stroma. From the morphologic point of view it can not be distinguished from type III WHO nasopharynx carcinoma (17). It was initially described in the rhynopharynx in 1921 by Regaud and Reverchon (18), and independentently by Schmincke (19). The squamous cell papillary carcinoma is a rare variant of squamous cell carcinoma initially described by Crissman et al. in 1988 (20). Its main features are a papillary growth pattern and a good prognosis. In the head and neck region, it is preferentially localized in the oropharynx, hypopharynx, larynx and sinonazal tract (20-25). It was found in the skin (26), uterine cervix (27), conjunctiva (28), and thymus. The verucous carcinoma (Ackerman tumor) is a well differentiated variant of squamous cell carcinoma initially described by Ackerman in 1948 (29). It is characterized by an exophytic, verucous growth, with slow local invasion which produces extensive destructive lesions if untreated. It rarely metastasizes. The majority of these tumors (75%) are localized in the oral cavity and 15% in the larynx. In

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the oral cavity, the most frequently affected are the oral mucosa and the gum, and in the larynx the vocal folds. It is rare in other zones of the head and neck, such as nasal cavity, the sinonasal tract or the rhynopharynx. They were described in the skin, anus, external genital organs, urinary bladder and esophagus (30).

MATERIAL AND METHODS

The retrospective study was performed on a group of 134 patients with tonsil tumors admitted in the ENT Clinic of the Clinical City Hospital Timişoara between 2006 and 2010. The variables analyzed were the histopathologic type of the tumor, local extension and lymph node invasion as well as distant metastases. The specimens were fixed in buffered formalin, sectioned at 5 µm and stained with haematoxyllin and eosin for the histopatologic diagnosis.

RESULTS

The histopatologic diagnosis of tonsil tumors

The majority of the tumors studied were squamous cell carcinomas, which was expected, given the increased general incidence of this type of malignant tumors from this region. Squamous cell carcinomas were characterized by the presence of neoplastic squamouas cells, usually with euchromatic, but monstruous nuclei, with prominent nucleoli, with marked nuclear anaplasia and anisonucleolosis. The cytoplasm of the neoplastic cells was abundant, predominantly eosinophyllic, sometimes with a granular aspect. In some rare cases, a basophyllic material, with basal membrane aspect accumulated around neoplastic cells. In these cases, the tumors were classified as squamous cells carcinomas, because other features (the type of tumor cells and the general microscopic architecture of the tumor) of basal cells carcinomas were absent. Out of the 134 tumor specimens, 123 were squamous cells carcinomas, mostly moderately differentiated. The histopatologic types of the lesions studied are represented in Figure 1.

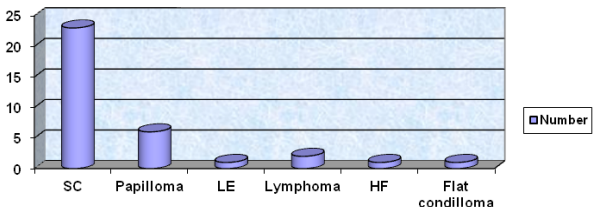

Fig.1. Histopatologic types of the lesions studied

Squamous cells carcinoma

The squamous cells carcinoma was majoritar not only among the malignant tumors from the study, but from the total of the specimens. The majority of the tumors were nonkeratinized squamous cells carcinomas, moderately differentiated (G_2). The distribution of the squamous cells carcinomas is represented in Figure 2.

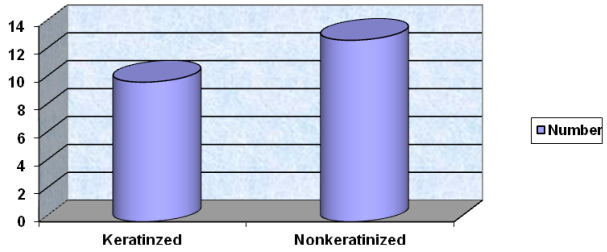

Fig. 2. Squamous cells carcinomas subtypes studied

The differentiation grade of the squamous cell carcinomas was in the majority of the cases moderate (G_2), with tumors with keratinization and keratozic pearls formation, with squamous cells with euchromatic nuclei with prominent nucleoli and with abundant eosinophyllic cytoplasm. The mitotic count on 200x field was moderate, and no tumor necrosis was noticed.

The well differentiated squamous cell carcinoma was characterized by abundant keratinization.

As mentioned, the moderatly differentiated squamous cell carcinoma is characterized by moderate formation of keratozic pearls (keratinocite maturation), and the cells are uniform, some o them with basaloid features. The mitotic count on 200x field is low, and the tumor cells are grouped.

The poorly differentiated squamous cell carcinoma is characterized by marked cellular and nuclear atypia, without keratinocite maturation and without keratinization, a high mitotic count and often atypic mitoses. We have noticed this type of squamous cell carcinoma in 3 cases.

The nonkeratinized squamous cell carcinoma was present in more variants, most frequently with cells with indistinct limits, atypic, with a high mitotic count and sometimes with local necrosis, predominantly comedonecrosis. Often the tumors were exulcerated and inflamed, with local suprainfection, which modify the microscopic aspect. The lymph node metastases of nonkeratinized carcinomas had a particular morphologic pattern, with local necrosis and cystic degeneration.

The mitotic count in this tumor type (squamous cell nonkeratinized carcinomas) was high, with easily visibile mitoses even at low magnification (medium power field), many of them being atypic.

Comedonecrosis was frequent in poorly differentiated nonkeratinized squamous cell carcinomas, probably by alteration of the tumor vascular supply produced by the rapid tumor growth with insufficient angiogenesis. The tumor growth with high mitotic index doesn't seem to be the main mechanism of necrosis, because this phenomenon is noticed also in poorly differentiated nonkeratinized squamous cell carcinomas with low mitotic index.

The rarer variants of nonkeratinized squamous cell carcinoma were those with foci of umor cell maturation and keratinization and those involving the deep part of the tonsil crypts. In his later situation, the tumor cells had indistinct limits, cellular and nuclear anaplasia; euchromatic nuclei with prominent

nucleoli and anisonucleolosis, and the tumor cells islands were infiltrated by lymphocytes, which are normal in this area even in normal patients.

The nonkeratinized squamous cell carcinomul arising from the tonsil crypt epithelium was noticed in a single case.

Another rare variant of nonkeratinized squamous cell carcinoma is that with maturation of the cells from the periphery. The peripheral cells maturation is proven y their better differentiated aspect and by keratin production, which accumulates around the islands of tumor cells. We have noticed this type of tumor in 2 cases.

Almost all of the lymph node metastases had a solid pattern, resembling the primary tumor, with islands of squamous tumor cells mostly without keratin deposition. In one case we hav noticed a lymph node metastasis with cystic appearance, without necrosis or degeneration.

The nondifferentiated squamous cell carcinoma was present in 2 cases, both patients male, one aged 35 and the other aged 53. It was characterized by large groups of big tumor cells, with indistinct cellular limits, with abundant eosinophyllic cytoplasm and monstruous nuclei, with marked nuclear anaplasia, prominent nucleoli and anisonucleolosis, with high mitotic count and atypical mitoses. The tumor microvessel density was also increased, and the tumor stroma tumorală was massively infiltrated with lymphocytes.

The last rare variant of squamous cell carcinoma was that with papillary pattern, noticed in a 59 years old male patient, which presented with a large friabile exophytic tumor, without necrosis or suprainfection. Microscopically, the tumor is formed by fine loose connective tissue axes, sometimes infiltrate with inflammatory cells, without massive fibrosis or keratin deposits, covered by a relatively well differentiated neoplastic epithelium, with medium sized tumor cells, with eosinophyllic cytoplasm and euchromatic nuclei with prominent nucleoli and anisonucleolosis, arranged in layers. In the described case, the squamous cell carcinoma with papillary pattern was associated in variable proportions with tonsilar epithelium displasia. The displasia was moderate, with cellular and nuclear atypia and with disorganized maturation of epiteliale cells.

The lymphomas were present in 2 female patients, one aged 16, and the other aged 61. B cells lymphoma is a medium sized tumor, of 3/3/1.2 cm, ulcerated. Microscopically, the tumor was formed of neoplastic lymphocytes, with surface epithelium ulceration.

The last type of malignant tumor that we have noticed was lymphoepithelial carcinoma, present in a 35 years old patent. It was a small tumor, with a maximum dimension of 0.6 cm, with elastic consistency and formed of large cells, with abundant eosinophyllic cytoplasm, with euchromatic nuclei with nucleoli with atypia and sometimes of multinucleated cells. The epithelial cells described are intermixed with small lymphocytes, with phenomena of emperipolesis and peripolesis.

The majority of the malignant tumors studied were squamous cells carcinomas, keratinized or nonkeratinized, of different grades, moderately differentiated, followed by far by lymphomas and by lymphoepithelial carcinoma, as illustrated in Figure 3.

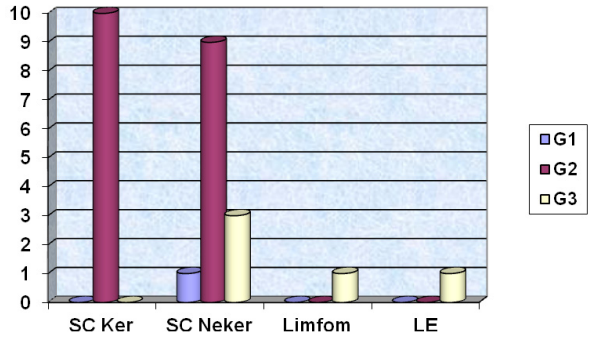

Fig. 3. The malignant tumors studied. The high frequency of squamous cell carcinomas is easily noticeable.

DISCUSSION

The1 34 patients with tonsil lesions group is significant for the analyzed period and for the size of the diagnosis and treatment center where the study was carried on; the majority of the studies published in the literature are on tens of patients series, the only large groups studies being on periods longer than 10 years as archival epidemiologic studies. The biggest populational study published so far is the one of Mehta et al (31), which consideres all of the oropharynx and tonsil squamous cell carcinoma from SEER database of the National Cancer Institute from 1976 to 2006. The central conclusion of this study is the fact that the tumor differentiation grade has evolved from poorly differentiated tumors in the '70s, to well differentiate tumors with longer survival intervals, including an increase of the disease-free interval. In our study, the majority of the patients with squamous cell carcinomas had moderatly differentiated tumors, with G_2 grade; a few patients had poorly differentiated or anaplasic tumors. The well differentiated tumors were even rarer. The age was also considered as a risk factor in a study on a large number of patients (5538), establishing the age of 50 as a threshold (32). In this study, the same as in ours, the age was not correlated with the increased incidence of the tonsil carcinoma, even if combined with human papillomavirus seemed to have a slightly higher role. As for the lymph node metastases, a remarkale fact is that the tonsil tumors may present with clinically occult metastases, which can evolve in residual tumors and may compromize the efficiency of the therapy. Most patients from our study had lymph node metastases, because most had locally advanced tonsil squamous cell carcinomas and thus cervical lymph node dissection was necessary due to the extent of the local lesions. In the case of locally early stage tumors, the selective dissection of the regional lymph nodes is important, because it can identify occult lymph node lesions, which may diminish the efficacy of the treatment, and which have usually a bad prognosis, both from the disease-free interval and the survival rate (33).

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SPECTRUL PATOLOGIC AL TUMORILOR AMIGDALIENE MALIGNE IN TIMISOARA – STUDIU STATISTIC

REZUMAT

Mai mult de 90% dintre tumorile orofaringiene sunt carcinoame scuamocelulare, restul fiind melanoame maligne, tumori ale glandelor salivare mici, sarcoame, tumori plasmocitare, limfoame sau alte tumori rare. Au fost identificate de asemenea metastaze la nivelul orofaringelui.

Studiul retrospectiv a fost efectuat pe un grup de 134 de pacienti cu tumori amigdaliene, care au fost internati in Clinica ORL a Spitalului Municipal Timisoara intre anii 2006-2010. Variabilele analizate in acest studiu au fost: tipul histologic al tumorilor, gradul de extindere locala si invazia nodulilor limfatici, precum si metastazele la distanta. Specimenele au fost fixate in formalina tamponata, sectionate la o grosime de 5 µm si colorate cu hematoxilina si eozina pentru stabilirea diagnosticului histopatologic. Majoritatea tumorilor studiate au fost carcinoame cu celule scuamoase, ceea ce era de asteptat data fiind incidenta crescuta a acestui tip de tumori maligne in aceasta regiune. Limfoamele au fost intalnite doar la 2 paciente cu varsta de 16, respectiv 61 de ani. Un ultim tip de tumora maligna intalnit a fost carcinomul limfoepitelial, care a fost prezent la un pacient in varsta de 35 de ani.

Cuvinte cheie: carcinom scuamocelular, carcinom amigdalian, patologie, radioterapie