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Profesorul universitar doctor Vlad ARTENIE la 75 de ani

Este o tradiţie în Facultatea de Biologie a Universităţii „Alexandru Ioan Cuza” din Iaşi ca la debut de an academic să omagieze activitatea didactică, ştiinţifică şi organizatorică a marilor formatori de şcoală. În acest debut de nou an universitar avem onoarea de a omagia activitatea celui care a pus bazele şcolii de biochimie din facultatea noastră şi a contribuit în mod hotărâtor la dezvoltarea acestei ştiinţe – profesor universitar doctor Vlad Artenie – cu ocazia împlinirii frumoasei vârste de 75 de ani

Profesorul universitar Emeritus dr. Vlad Gr. Artenie este unul dintre oamenii de ştiinţă care au contribuit substanţial la dezvoltarea biochimiei şi enzimologiei în ţara noastră, atât prin studii teoretice, fundamentale, cât şi aplicate, ca rezultat al unei activităţi îndelungate şi deosebit de bogate în învăţământul superior şi cercetarea ştiinţifică, bucurându-se de o largă recunoaştere pe plan naţional şi internaţional.

Născut la data de 16 septembrie 1936 în satul Valea Glodului, comuna Vultureşti din Judeţul Suceava, Vlad Artenie şi-a petrecut copilăria la ţară, însuşindu-şi de mic respectul faţă de muncă, datorie şi adevăr, aşa cum a văzut la părinţii săi, oameni cu puţină carte, dar cu mult bun simţ, modeşti, oneşti şi muncitori. Absolvind clasele primare şi gimnaziale (1944-1951) în satul natal, Vlad Artenie şi-a continuat studiile la Liceul „Nicu Gane” din Fălticeni, unde a avut profesori remarcabili, fiind impresionat de profesorul de Chimie şi Fizică, Vasile Bogdăneţ, care i-a transmis pasiunea şi dragostea pentru studiul chimiei. După absolvirea liceului în anul 1954, a dat examen de admitere la Facultatea de Chimie a Universităţii „Alexandru Ioan Cuza” din Iaşi, unde a urmat specialitatea de Chimie Organică, avându-i profesori pe Radu Cernătescu, Margareta Poni, Magda Petrovanu, Ioan Zugrăvescu, Eugen Papafil, C. H. Budeanu, Boris Arventiev, Vasile Ababii şi asistenţi pe Iulian Zaharia, Eugenia Rucinschi, Domnica Furnică, Natalia Hurduc, Angela Popa şi alţi. În iunie 1959 a susţinut examenul de stat, prezentând lucrarea de diplomă „*Preparate chimicoterapice pe baza de arsenic, stibiu şi bismut*”, având conducător ştiinţific pe prof. dr. docent Vasile Ababii. La cele trei probe ale examenului de stat, desfăşurat sub preşedinţia prof. dr. doc. Ioan Zugrăvescu (1910-1989), Membru Corespondent al Academiei Române, Vlad Artenie a obţinut note de zece şi „Diploma de merit”.

Ca urmare a rezultatelor excepţionale obţinute în timpul studenţiei, absolventul Vlad Artenie a fost numit, începând cu 1 octombrie 1959, ca preparator la disciplina de Chimie Biologică din Catedra de Chimie Fizică şi Chimie Generală a Facultăţii de Chimie (Şef de Catedră – prof. dr. Eugen Papafil) de la Universitatea „Alexandru Ioan Cuza” din Iaşi. La 1 octombrie 1960 a ocupat prin concurs postul de asistent la disciplina de Chimie Biologică din catedra menţionată, titularul disciplinei fiind prof. univ. dr. Elisabeta Văscăuţeanu (1897-1989). În calitate de asistent a condus lucrări practice de laborator la disciplina de Chimie Biologică cu studenţii biologi.

În perioada 3 ianuarie 1963 – 30 iunie 1966, Vlad Artenie a efectuat un *stagiu de doctorat cu frecvenţă* la Universitatea de Stat „M. V. Lomonosov” din Moscova (Rusia), sub conducerea ştiinţifică a academicianului profesor Serghei E. SEVERIN, biochimist de renume mondial. În mai 1966 Vlad Artenie a obţinut titlul ştiinţific de *doctor în ştiinţe biologice*, specialitatea *Biochimie animală*, cu teza „*Izolarea, purificarea şi proprietăţile colin-acetilazei*” – enzimă implicată în biosinteza acetilcolinei, important mediator al impulsurilor nervoase.

La terminarea stagiului de pregătire prin doctorat, Vlad Artenie a revenit pe postul de asistent la disciplina de Chimie Biologică de la Universitatea „Alexandru Ioan Cuza” din Iaşi. În anul universitar 1966-1967 a funcţionat ca asistent cu delegaţie de lector la disciplina de Chimie

Biologică din Catedra de Chimie Generală a Facultăţii de Chimie. La 1 octombrie 1967, a ocupat, prin concurs, postul de lector la aceeaşi disciplină de Chimie Biologică. De la 1 octombrie 1969 a onorat postul de conferenţiar la disciplina de Biochimie, aparţinând Catedrei de Chimie Organică şi Chimie Biologică de la Facultatea de Chimie, până la 15 septembrie 1974 când a trecut cu postul respectiv în cadrul Facultăţii de Biologie-Geografie-Geologie. În urma concursului susţinut în prima jumătate a anului 1990, Vlad Artenie a dobândit, începând cu 15 septembrie 1990, titlul de profesor universitar la disciplina de Biochimie în cadrul Facultăţii de Biologie a Universităţii „Alexandru Ioan Cuza", unde a desfăşurat, cu pasiune şi conştiinciozitate, o vastă activitatea didactică şi ştiinţifică până la 30 septembrie 2005, când devine profesor consultant, iar de la 1 octombrie 2006 profesor asociat.

În calitate de lector (1967 – 1969), conferenţiar (1969-1990), profesor titular (1990-2005), profesor consultant (2005-2006) şi profesor asociat (2006 şi în prezent), Vlad Artenie a fost titularul disciplinelor de *Biochimie* de la Facultatea de Educaţie Fizică şi Sport, de la specializările de Biologie, Chimie, Biofizică şi Fizică Medicală, *Metabolismul glucidelor şi lipidelor, Biochimie Ecologică şi Biochimie clinică* (specializarea Biochimie, Facultatea de Biologie) şi *Biochimia Metabolismului* (specializarea Biochimie Tehnologică, Facultatea de Chimie) din **Ciclul de licenţă** – cursurile sale fiind extrem de apreciate de studenţi şi de colegi prin claritatea şi noutatea informaţiei, remarcându-se printr-un talent didactic de excepţie. La **Ciclul de master**, profesorul Vlad Artenie a predat cursurile *Capitole Speciale de Metabolism, Biochimie Ecologică şi Toxicologie* (Masterul de Enzimologie şi Biotehnologie, Facultatea de Chimie), *Biochimia Macromoleculelor Informaţionale, Biochimia Metaboliţilor la Microorganisme, Proteom* şi *Transformări Metabolice în Materiile Prime Agroalimentare* (Mastere de la Facultatea de Biologie). Pe baza amplei experienţe didactice, profesorul Vlad Artenie a publicat lucrări de sinteză bogate în conţinut şi moderne în ceea ce priveşte concepţia de structurare: două cărţi, patru cursuri, trei prelegeri post-universitare şi trei îndrumătoare de laborator. În toate aceste lucrări se reflectă excepţionala pregătire profesională şi vasta cultură de specialitate a autorului. Ca şi cadru didactic, profesorul Artenie era o persoană foarte bine apreciată de studenţi, nu numai profesional, ci şi ca om.

Între anii 1970 şi 2007, profesorul Vlad Artenie a condus *6 lucrări* metodico-ştiinţifice pentru obţinerea gradului didactic I în învăţământ, specialitatea Biologie şi a participat ca preşedinte în Comisiile de Inspecţie Specială pentru acordarea gradului didactic I în învăţământ unui număr de 52 de profesori de biologie şi unui număr de 3 profesori de chimie. În aceeaşi perioada, a condus **259 teze de licenţă** la specializările de Biologie, Ştiinţe Naturale şi Agricole şi Biochimie de la Facultatea de Biologie şi specializările de Chimie şi Biochimie Tehnologică de la Facultatea de Chimie, precum şi **43 de lucrări de disertaţie** la diferite mastere de la Facultatea de Biologie, respectiv Facultatea de Chimie. Astfel a contribuit din plin la instruirea şi educarea a numeroase serii de biologi, biochimişti, biofizicieni, chimişti şi absolvenţi de educaţie fizică şi sport.

În perioada 1970 – 2007 a făcut parte din **87 de comisii de concurs** pentru ocuparea a 23 posturi de profesor în diferite centre universitare din ţară, a 30 posturi de conferenţiar, a 17 posturi de şef de lucrări, a 11 posturi de asistent şi a 6 posturi de preparator. La acestea se adaugă un număr de 7 posturi de cercetător ştiinţific principal gradul I, 4 posturi de cercetător ştiinţific principal gradul II şi 2 posturi de cercetător ştiinţific principal.

Ca recunoaştere a calităţii activităţii sale didactice, domnului Vlad Artenie i s-a acordat în anul 1988 de către Ministerul Învăţământului titlul de **Conferenţiar universitar evidenţiat.**

În paralel cu ampla si fructuoasa activitate didactică, profesorul Vlad Artenie a desfăşurat o meritorie activitate pe linie administrativă. Astfel, a fost *membru al Consiliului Profesoral* de la Facultatea de Chimie (10 februarie 1970 – 15 septembrie 1974), de la Facultatea de Biologie-Geografie-Geologie (1 octombrie 1981 - 30 septembrie 1988, iar în perioada 1 noiembrie 1984 - 30 septembrie 1988 a îndeplinit funcţia de *secretar ştiinţific al Biroului* acestui *Consiliu Profesoral*) şi de la Facultatea de Biologie (9 martie 1990 - 30 septembrie 2005) de la Universitatea „Alexandru Ioan Cuza” din Iaşi.

În perioadele 1 octombrie 1991 - 25 decembrie 1995, 1 noiembrie 1998 – 25 decembrie 1999, 1 ianuarie 2000 – 27 noiembrie 2003 şi 1 decembrie 2003 - 30 septembrie 2005, profesorul Vlad Artenie a fost şef al Catedrei de Biochimie-Genetică-Microbiologie de la Facultatea de Biologie. În activitatea de conducere a catedrei a militat pentru modernizarea planurilor de învăţământ şi dotarea laboratoarelor de biochimie, genetică şi microbiologie cu aparatură şi echipamente adecvate, la nivelul standardelor europene. Ca şef de catedră a impus un stil de muncă propriu, la baza căruia a stat puterea exemplului personal. Totodată a căutat să propage şi să întreţină o atmosferă de colegialitate şi respect reciproc între membrii catedrei şi în special între membrii colectivului de biochimie.

În anii 1990 – 1991, profesorul Vlad Artenie a contribuit substanţial la fundamentarea planurilor de învăţământ corespunzătoare şi organizarea specializării de **Biochimie** din cadrul Facultăţii de Biologie, precum şi a specializării de **Biochimie Tehnologică** de la Facultatea de Chimie. De asemenea, a avut o contribuţie importantă la înfiinţarea modulului franco-român al masterului de **Enzimologie şi Biotehnologie** din cadrul Facultăţii de Chimie, respectiv o contribuţie hotărâtoare la fondarea şi implementarea masterului franco-român de **Bioprocedee în domeniul agroalimentar** la Facultatea de Biologie, care a început să funcţioneze din anul universitar 2003-2004.

În perioada 2000 – 2002, profesorul Artenie a coordonat acţiunile legate de mutarea Colectivului de Biochimie din laboratoarele ocupate în corpul A al Universităţii „Alexandru Ioan Cuza”, în spaţiul Facultăţii de Biologie. Pe această linie, beneficiind de înţelegere şi sprijin din partea Rectorului, prof. dr. Dumitru I. Oprea şi a decanului, prof. dr. Gheorghe Mustaţă, profesorul Vlad Artenie a reuşit organizarea a două laboratoare pentru lucrările practice la disciplina de Chimie generală şi la disciplinele de biochimice, precum şi a două laboratoare de cercetare în domeniul biochimiei.

Pe lângă activitatea didactică bogată, profesorul Vlad Artenie a desfăşurat şi o remarcabilă activitate de **cercetare ştiinţifică**. În urma cercetărilor ştiinţifice efectuate, singur sau în colaborare cu alţi colegi, a publicat **364** de lucrări ştiinţifice originale, dintre care:

- **94** (28 în extenso şi 66 în rezumat) în reviste din străinătate şi
- **270** (212 în extenso şi 58 în rezumat) în reviste de specialitate din ţară.

Multe din lucrările elaborate ca singur autor sau în colaborare sunt citate de autori din ţară şi străinătate în articole şi cărţi de specialitate.

La cele 364 de lucrări ştiinţifice originale, se adaugă:

- 9 brevete de invenţie;
- 10 lucrări de popularizare ştiinţifică (biografii, istoria biologiei şi chimiei, probleme de actualitate în biochimie);
- 26 de recenzii la cărţi de specialitate, din care 23 de autori străini, ceea ce i-a permis raportarea la cercetările internaţionale;
- referent ştiinţific la 26 de cărţi de specialitate publicate de autori români.

Diversele probleme abordate în cercetările ştiinţifice efectuate de profesorul Vlad Artenie, singur sau în colaborare cu alţi colegi şi cercetători ştiinţifici, pot fi înscrise în domeniile de enzimologie, biochimie animală, biochimie clinică umană, biochimia microorganismelor, biochimie vegetală, biochimie ecologică / xenobiochimie şi biologie moleculară.

Un domeniu de cercetare pe care a căutat să-l introducă şi să-l dezvolte în cadrul Laboratorului de Biochimie al Universităţii „ Alexandru Ioan Cuza ” din Iaşi este domeniul de **enzimologie** privind izolarea, purificarea şi caracterizarea unor enzime sintetizate de animale, microorganisme şi plante. Astfel, putem menţiona purificarea şi caracterizarea colin-acetiltransferazei din creierul de iepure, catalazei sintetizate de *Penicillium chrysogenum* sau a proteazelor din frunzele de *Plantago*. Unele din rezultatele privind purificarea de enzime microbiene şi vegetale au constituit obiectul a *două* brevete de invenţie, care pot sta la baza unor biotehnologii cu implicaţii în industria de medicamente. O altă direcţie din domeniul enzimologiei a urmărit obţinerea de biocatalizatori heterogeni prin imobilizarea de enzime pe diferite suporturi organice sau anorganice, având drept scop creşterea stabilităţii şi perioadei de viaţă a enzimelor studiate. În urma colaborărilor începute din anul 1983 cu cercetători din cadrul Institutului de Cercetări Biologice din Iaşi (dr. Valeriu Rugină, biochimist Dumitru Cojocaru), de asemenea cu profesorii inginer dr. Severian Dumitriu şi inginer dr. Marcel Popa din grupul condus de renumitul academician profesor inginer dr. Cristofor I. Simionescu (1920 – 2007) de la Facultatea de Tehnologie Chimică a Institutului Politehnic „Gheorghe Asachi” din Iaşi, profesorul Vlad Artenie a reuşit imobilizarea pepsinei şi tripsinei pe Biozan R, a ureazei pe carboximetilceluloză, a catalazei pe xanthan sau fibre de celuloză etc., ceea ce a lărgit gama de utilizare a enzimelor respective în diferite domenii de interes practic. Cercetările având drept scop imobilizarea pepsinei, tripsinei, catalazei etc. pe diferiţi polimeri organici au constituit un punct de plecare în stabilirea Acordului de cooperare ştiinţifică cu specialiştii din Laboratorul de Tehnologia Substanţelor Naturale (actualmente Laboratorul ProBioGEM) de la Universitatea de Ştiinţe şi Tehnologii din Lille, Franţa, al cărui director a fost prof. dr. Didier Guillochon. În urma acestei colaborări au fost susţinute două teze de doctorat în cotutelă, având ca subiecte studiul imobilizării pepsinei pe oxid de aluminiu în scopul obţinerii de peptide biologic active prin scindarea hemoglobinei.

În general, cercetările de **biochimie animală** vizează nivelul principalilor componenţi biochimici şi dinamica activităţii unor enzime din sângele şi ţesuturile păsărilor domestice (găini, porumbei), animalelor de laborator (şobolani, cobai), peştilor şi ovinelor, în dependenţă de condiţiile de viaţă şi furajare sau în anumite stări fiziopatologice.

Un număr mai mare de lucrări (23), realizate în colaborare cu cercetătorii de la Staţiunea de Cercetări Ştiinţifice „Stejarul”- Pângăraţi din Judeţul Neamţ (dr. Klaus Battes, dr. Maria Apetroaei), Staţiunea de Cercetare şi Producţie Salmonicolă Potoci - Bicaz din Judeţul Neamţ (dr. Ionel Miron, dr. Costică Misăilă, dr. Elena Rada Misăilă) şi Staţiunea de Cercetări pentru Acvacultură şi Ecologie Acvatică Iaşi (dr. Costică Misăilă, dr. Elena Rada Misăilă), reprezintă contribuţii valoroase la cunoaşterea mecanismelor moleculare care stau la baza creşterii şi dezvoltării salmonidelor în condiţii de acvacultură intensivă în viviere flotabile în apa lacurilor de acumulare sau în ape termostatate etc. În aceste studii, întreprinse pe bază de contracte, s-au obţinut date originale despre profilul metabolic sanguin şi bioritmul activităţii enzimelor digestive la păstrăvul curcubeu, păstrăvul de lac şi lostriţă. Valoarea acestor rezultatele a fost atestată între altele, prin elaborarea a **cinci** brevete de invenţie cuprinzând reţete de hrană concentrată granulată pentru furajarea păstrăvului curcubeu de diferite vârste, crescut în condiţii de acvacultură intensivă.

O direcţie principală de cercetare, abordată cu specialiştii de la Staţiunea de Cercetare şi Dezvoltare pentru Ovine Popăuţi, Judeţul Botoşani, sub conducerea cercetătorului ştiinţific principal gradul I dr. Gheorghe Hrincă, se axează pe probleme privind particularităţile sistemelor genetico-biochimice ale rasei de ovine Karakul de Botoşani în corelaţie cu potenţialul ei morfo-productiv.

Merită să fie menţionate şi lucrările în care se studiază unele laturi ale stresului oxidativ în procesele de învăţare şi memorare la şobolanii trataţi cu antagonişti şi agonişti specifici sistemelor neurotransmiţătoare colinergic şi catecolaminergic.

În lucrările de **biochimie clinică umană** se urmăresc aspecte vizând studiul electroforetic al proteinelor membranei eritrocitului uman, activitatea catalazei în unele forme de cancer uman, catabolismul acizilor sialici în eritrocitele umane normale şi în hematiile aflate în curs de îmbătrânire, dinamica activităţii unor enzime antioxidante la bolnavii cu afecţiuni neuropsihice, starea metabolismului lipidelor serice la bolnavii cu sindrom nefrotic, determinarea parametrilor biochimici care exprimă disfuncţiile ficatului în hepatitele virale la pacienţii cu hepatită cronică. Tot aici trebuie menţionat studiul mutaţiilor, secvenţelor necunoscute şi polimorfismelor, specifice genelor BRCA1 şi BRCA2 răspunzătoare de predispoziţia ereditară la cancerul mamar şi ovarian în populaţia din Nord-Estul României.

Cercetările referitoare la **biochimia microorganismelor** au fost realizate în colaborare cu membri ai Colectivului de Microbiologie condus de regretatul profesor univ. dr. Napoleon D. Topală (1928 - 1988) de la Facultatea de Biologie-Geografie-Geologie a Universităţii „Alexandru Ioan Cuza” din Iaşi, pe baze de contracte cu diferiţi beneficiari, în special, Întreprinderea de Antibiotice din Iaşi. Aceste cercetări, urmărind dinamica unor enzime implicate în metabolismul glucidelor şi proteinelor (amilază, catalază, dehidrogenaze, fosfomonoesteraze, proteaze etc.) la producătorii de substanţe biologic active (antibiotice, vitamine, aminoacizi etc.), au relevat o corelaţie directă între activitatea enzimelor respective şi biosinteza diferitelor antibiotice ca penicilina, tetraciclina, streptomicina. Rezultatele obţinute oferă o cale de control al procesului biotehnologic de obţinere a substanţelor antibiotice. Pentru ciclul de lucrări „*Studiul unor microorganisme producătoare de substanţe biologic active*” profesorul Vlad Artenie a primit, împreună cu profesorul Napoleon D. Topală, premiul „Emanoil Teodorescu” al Academiei Române în anul 1977.

În cercetările de **biochimie vegetală** se studiază particularităţile biochimice ale unor soiuri de soia, ale unor populaţii locale, soiuri şi hibrizi de porumb, ale unor varietăţi de nuc, ale unor populaţii de fasole, ale unor plante medicinale (dracila, vinca) şi ale unor soiuri de viţă de vie, evidenţiindu-se calităţile plantelor respective şi importanţa lor practică. În unele lucrări se cercetează proporţia diferitelor fracţiuni proteinice din cariopsele principalelor populaţii de porumb din Moldova, comportarea cromatografică a proteinelor solubile din cariopsele de porumb pe Sephadex G-100 şi pe DEAE-celuloză. De asemenea, s-a urmărit variaţia sezonală a nivelului de clorofile şi carotenoide la diverse specii de plante, evidenţierea cu ajutorul electroforezei în gel de poliacrilamidă în condiţii denaturante a deosebirilor cantitative şi calitative ale proteinelor din seva viţei de vie în dependenţă de genotip, dinamica activităţii unor enzime şi a conţinutului de pigmenţi fotosintetici la unele specii de plante tratate cu radiaţii gamma sau microunde, apoi dinamica activităţii unor enzime implicate în scindarea amidonului pe parcursul germinaţiei seminţelor de la diferite specii de graminee spontane şi cultivate, precum şi studiul proteinelor prin electroforeză în gel de poliacrilamidă şi a unor enzime în germinaţia seminţelor de glădiţă şi salcâm etc.

Cercetările de **biochimie ecologică / xenobiochimie** urmăresc influenţa diferitelor erbicide(derivaţi sulfonamidaţi ai acizilor clor-fenoxi-alcanoici şi beta-naftoxiacetic, metilclor, Gramoxone, 2,4-D) asupra activităţii unor enzime implicate în procesele anabolice şi catabolice la specii de plante de cultură sau din flora spontană, de asemenea, efectul radiaţiilor gamma, radiaţiilor ultraviolete şi câmpului electromagnetic asupra unor procese biochimice la diferite specii de animale şi plante.

Cercetările de **biologie moleculară** constituie o direcţie mai nouă pe care profesorul Vlad Artenie a putut să o dezvolte după anul 2000, datorită colaborărilor internaţionale desfăşurate pe parcursul mai multor ani cu membrii Laboratorului condus de profesorul dr. Stéphane Bouquelet de la Universitatea de Ştiinţe şi Tehnologii din Lille, precum şi cu grupul de specialişti condus de profesorul dr. Roderich Brandsch din Institutul de Biochimie şi Biologie Moleculară al Universităţii „Albert-Ludwig" din Freiburg, Germania.

Cercetările realizate în colaborarea cu Laboratorul din Lille au constat în caracterizarea prin metode moderne de biochimie, biologie moleculară şi bioinformatică a fructozokinazei la bifidobacterii, bacterii Gram pozitive, capabile să metabolizeze fructo-oligozaharidele.

Colaborarea cu grupul profesorului Roderich Brandsch, Profesor de Onoare al Universităţii „Alexandru Ioan Cuza" din Iaşi, s-a axat pe abordarea la nivel molecular a căilor şi enzimelor corespunzătoare implicate în catabolizarea nicotinei de către specia *Athrobacter nicotinovorans* care conţine megaplasmidul pAO1. Prin tehnicile de biologie moleculară s-a reuşit identificarea genelor ce controlează biosinteza unui număr de cinci enzime cantonate pe megaplasmidul pAO1, precum şi caracterizarea moleculară şi cinetică a acestor enzime implicate în metabolismul nicotinei, respectiv în metabolizarea glucidelor de către *Athrobacter nicotinovorans*.

Pe linia cercetării ştiinţifice, trebuie să remarcăm şi susţinuta activitate depusă de profesorul Vlad Artenie în calitate de conducător de doctorat la specialitatea biochimie, drept care i-a fost acordat în anul 1982 şi reconfirmat în anul 1990. Până în prezent a condus activitatea de pregătire prin doctorat a **64** de doctoranzi care au susţinut public teza de doctorat şi au primit titlul ştiinţific de doctor în biologie. Din cei 64 de doctori, trei sunt cetăţeni străini (Grecia, Israel şi Ucraina), iar şase doctoranzi români au realizat teza de doctorat pe bază de cotutelă cu Universitatea de Ştiinţe şi Tehnologii din Lille şi unul cu Universitatea Paris VII din Franţa. Din cei 9 membri ai actualului Colectiv de Biochimie din cadrul Laboratorului Profesional de Biochimie şi Biologie Moleculară de la Facultatea de Biologie a Universităţii „Alexandru Ioan Cuza", şapte şi-au trecut doctoratul sub conducerea ştiinţifică a profesorului Vlad Artenie, iar unul a finalizat teza în cotutelă cu Laboratorul de Fiziologia Plantelor al Academiei de Ştiinţe din Republica Moldova.

Din 1970 şi până în 2010 profesorul Artenie a fost **membru-referent in 104 Comisii de Doctorat** pentru conferirea titlului de doctor în ştiinţe la Universitatea Bucureşti, Universitatea Babeş-Bolyai din Cluj-Napoca, Universitatea „Dunărea de Jos" din Galaţi, Universitatea de Stat din Chişinău (Republica Moldova), Universitatea de Medicină şi Farmacie „Gr. T. Popa" din Iaşi, Universitatea de Ştiinţe Agricole şi Medicină Veterinară „Ion Ionescu de la Brad" din Iaşi, Universitatea Tehnică „Gheorghe Asachi" din Iaşi şi bineînţeles, la Universitatea „Alexandru Ioan Cuza" din Iaşi.

Profesorul Vlad Artenie , în calitate de şef al Colectivului de Biochimie şi al Catedrei de Biochimie-Genetică-Microbiolgie de la Facultatea de Biologie a promovat cu perseverenţă imaginea Universităţii „Alexandru Ioan Cuza" din Iaşi în lumea universitară europeană, prin

stabilirea de colaborări ştiinţifice şi didactice cu universităţi din Franţa şi Germania. Pe această linie menţionăm:

1) Profesorul Vlad Artenie a fost responsabil din partea Universităţii,, Alexandru Ioan Cuza” din Iaşi pentru Programul TEMPUS-PEC nr.4398/1992-1995, pentru Proiectele de Măsuri Complementare PMC TEMPUS-PHARE No.1173/1995 şi No.02034/1996-1997, coordonate de Universitatea Paris-Sud (Paris XI) şi conduse de doamna Directeur de Recherche II dr. Rita BAROT, Membru de Onoare al Senatului Universităţii Ieşene.

2) Profesorul Vlad Artenie a organizat, începând din anul 1995 şi până în anul 2004, în fiecare an, în cadrul Universităţii „Alexandru Ioan Cuza” din Iaşi Şcoala de Vară Franco-Română de Biochimie, intitulată „*Biologie et Pathologie Moléculares. Biotechnologies*”, condusă de profesorul emerit Jean Montreuil (1920-2010) de la Universitatea de Ştiinţe şi Tehnologii I din Lille. La cele 10 ediţii (1995 – 2004) ale Şcolii de Vară Franco-Română de Biochimie organizată la Iaşi au participat peste 1000 de cursanţi (studenţi, masteranzi, doctoranzi, cadre didactice din învăţământul superior şi liceal, cercetători şi specialişti din domeniul medical, farmaceutic, alimentar etc.).

3) Profesorul Vlad Artenie a perfectat şi a condus **Acordul de cooperare ştiinţifică** (2000 – 2004) între Laboratorul de Biochimie al Catedrei de Biochimie-Genetică-Microbiologie din cadrul Facultăţii de Biologie a Universităţii „Alexandru Ioan Cuza” din Iaşi şi Laboratorul de Tehnologia Substanţelor Naturale (actualmente Laboratorul ProBioGEM), având ca director pe profesorul dr. Didier Guillochon din Universitatea de Ştiinţe şi Tehnologii I din Lille.

4) Profesorul Vlad Artenie s-a implicat în înfiinţarea Programului de master franco-român *Bioprocedee în domeniul agroalimentar* la Facultatea de Biologie din Iaşi, modul în cadrul căruia s–a concretizat şi o colaborare pe linie didactică între Laboratorul Ieşean de Biochimie şi Laboratorul condus de profesorul Didier Guillochon, Doctor Honoris Causa al Universităţii „Alexandru Ioan Cuza”.

5) Profesorul Vlad Artenie a coordonat finalizarea a şase teze de doctorat la specialitatea de Biochimie pe bază de cotutelă cu Universitatea de Ştiinţe şi Tehnologii I din Lille.

În acest context, trebuie să arătăm că profesorul Vlad Artenie a fost profesor invitat la:

- Universitatea „Albert Ludwig”din Freiburg, Germania, în 2000 (22 iunie - 1 iulie), 2003 (18 - 24 mai), 2004 (5 - 11 septembrie) şi 2006 (30 iulie - 9 august), respectiv

- Universitatea de Ştiinţe şi Tehnologii din Lille, Franţa, în anul 2002 (1 - 30 mai) şi 2006 (1 – 15 martie).

Meritele ştiinţifice şi didactice ale profesorului Vlad Artenie au fost recunoscute prin alegerea sa timp de aproape 7 ani (20 aprilie 1994 – 9 octombrie 2000) ca membru în Consiliul Naţional de Evaluare Academică şi Acreditare Bucureşti.

Profesorul Vlad Artenie a făcut parte din comitetul de redacţie/referent ştiinţific al revistelor *Studii şi Cercetări de Biochimie* şi *Revue Roumaine de Biochimie* (1987 – 1991), *Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza“ din Iaşi (Serie Nouă), Secţiunea IIa. Biologie Vegetală* (1992 – 2000), *Romanian Journal of Physiology[Physiological Science]*, Editura Academiei Române (2006 – 2010), apoi a fost redactor-adjunct (2000 – 2007), editor executiv (2007-2009) şi din 2010 este redactor-şef al revistei *Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza“ din Iaşi (Serie Nouă), Secţiunea IIa. Genetica şi Biologie Moleculară.* De asemenea, a fost membru al Comisiei de Cercetare Consultativă şi de Recomandare Ştiinţifică a Institutului Biografic American (American Biographical Institute – ABI), **SUA**, 2002 - 2005.

Datorită aleselor calităţi profesionale şi morale, profesorul Vlad Artenie a fost ales vicepreşedinte al Subcomisiei de Biochimie a Filialei Iaşi a Academiei Române, preşedinte al Filialei Iaşi a Societăţii de Biochimie şi Biologie Moleculară şi membru în Consiliul de conducere al acesteia, de asemenea a fost membru al Societăţii de Ştiinţe Biologice din România, membru al Fundaţiei MEDBIOCHIM Bucureşti, membru al Fundaţiei Internaţionale „Ştefan Lupaşcu“ pentru Ştiinţă şi Cultură, Iaşi, membru al Asociaţiei Oamenilor de Ştiinţa, Filiala Iaşi, transformată după anul 1990 în Academia Oamenilor de Ştiinţă din România.

Din cele expuse mai sus se desprinde imaginea unui mare profesor – creator de şcoală, om de ştiinţă complet, cu alese trăsături morale, plin de dinamism social, admirat de studenţi, masteranzi, doctoranzi, colaboratori şi colegi.

Pentru tot ce a făcut în cei peste 50 de ani de activitate în învăţământul superior de biochimie, pentru Catedra de Biochimie şi Biologie Moleculară, pentru Facultatea de Biologie şi pentru Universitatea „Alexandru Ioan Cuza” din Iaşi, profesorului Vlad Artenie i s-a acordat de Senatul Universităţii Ieşene la 18 octombrie 2007 titlul de **Profesor Emeritus**, iar Ministerul Educaţiei, Cercetării, Tineretului şi Sportului şi CNCSIS i-au conferit la 13 mai 2010 premiul **Opera Omnia**.

Fire extrem de generoasă, gata oricând să ajute, să acorde sprijin celor ce-l solicită, cu dragoste de muncă şi pasiune pentru Biochimie, însoţite de deschiderea şi apropierea faţă de studenţii cu care a lucrat, faţă de doctoranzi şi tinerele cadre didactice din Catedra de Biochimie şi Biologie Moleculară a Facultăţii de Biologie de la Universitatea „Alexandru Ioan Cuza” din Iaşi, profesorul Vlad Artenie constituie un exemplu elocvent pentru ceea ce trebuie să fie un adevărat profesor universitar, un simbol al profesiei.

În aceste zile când domnul profesor doctor Vlad Artenie împlineşte frumoasa vârstă de 75 de ani, membrii Colectivului de Biochimie, ai Laboratorului profesional de Biochimie şi Biologie Moleculară şi ai Facultăţii de Biologie îi transmit un sincer „La mulţi ani!”, sănătate deplină şi noi realizări ştiinţifice. Să ne trăiţi mulţi ani stimate domnule PROFESOR!

Prof. univ. dr. Dumitru Cojocaru
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GENETICS ASPECTS OF DIABETIC NEPHROPATHY

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Keywords: diabetes mellitus, diabetic nephropathy, genetics, susceptibility genes
Abstract: Diabetic nephropathy is a clinical syndrome characterized by persistent albuminuria, a relentless decline in GFR, raised arterial blood pressure, and increased relative mortality for cardiovascular diseases. The pathogenesis of diabetic nephropathy is multifactorial, with contributions from metabolic abnormalities, hemodynamic alteration, and various growth and genetic factors.

The identification of the main genes would allow the detection of those individuals at high risk for diabetic nephropathy and better understanding of its pathophysiologyas well.The present review discusses the main information available in literature regarding some genetic variants (involved in the renin-angiotensin system, glucose and lipid metabolism and some cytoskeleton proteins) that reaffirms the importance of genetic factors in diabetic nephropathy.

INTRODUCTION

Diabetes mellitus (DM) is a set of metabolic disorders with different etiologies characterized by hyperglycemia resulting from defects in insulin secretion and/or action. In 2000, 171 million cases of DM worldwide were estimated, and that number is expected to increase to 366 million cases in 2030 (Wild *et al*., 2004).Diabetes mellitus is associated with severe complications including nephropathy, neuropathy, retinopathy and accelerated cardiovascular disease.

Diabetic nephropathy is the leading cause of end-stage renal disease (ESRD) in developed countries (The World Health Report, 2006). Evidence for a genetic component to diabetic nephropathy comes from family studies displaying familial aggregation of diabetic nephropathy both in type 1 and in type 2 diabetes mellitus (Seaquist *et al*.,1989; Pettitt *et al*.,1990; Quinn *et al*.,1996), as well as differences in the prevalence of diabetic nephropathy between ethnic groups (Nelson *et al*. 1988;Chandie *et al*., 2006).

Numerous metabolic pathways and associated groups of genes have been proposed as candidates to play a role in the genetic susceptibility to diabetic nephropathy.

STRATEGIES FOR IDENTIFICATION OF GENES ASSOCIATED WITH DIABETIC NEPHROPATHY

Genes that confer susceptibility to DN can be sought in different ways. In broad terms, there are three strategies for identifying susceptibility genes for DN: linkage analysis, population association (case-control) and the new technology Genome-Wide Association Scans (GWAS).

Linkage analysis by use of affected pedigrees is generally preferred to case-control studies but there are difficulties in establishing such a resource for DN.

Candidate genes are often analyzed in case-control studies by comparing the frequency of polymorphisms/ mutations in candidate genes among patients with and without the disease. This is an appropriate study for investigating complex genetic transmission, and it is especially useful in situations where the genetic influence is relatively low and disease-related alleles are common in a population (Adler *et al*.,2000).

Technological advances permit the genotyping of large numbers of SNPs in thousands of individualswithin the scope of a large project grant. For example, high-throughput commercial genotyping platforms such as Affymetrix (http://www.affymetrix.com) and Illumina (http://www.illumina.com) are now able to genotypeup to one million SNPs in several thousand individuals at a cost of less than GBP 1,000,000. Thus a GWAS is now a practical option for identifying nephropathy susceptibility variants, but given the still significant costs of such a scan, careful consideration must be given to the design of the study in order to maximize the chance of success (Wang *et al*., 2005).

Discovery of genetic variants that underpin susceptibility to nephropathy would permit identification of patients at risk of nephropathy shortly after diagnosis of diabetes rather than much later when persistent microalbuminuria develops, by which time there is already histological evidence of renal injury (Conway and Maxwell, 2009).

STUDIES OF CANDIDATE GENES

One approach to identify genes associated to DN is the study of candidate genes. There are many studies of candidate genes for DN but the results are inconsistent. The choice of the gene to be studied dependson knowledge concerning its actions in DN pathophysiology such as those involving blood pressure control, severity of proteinuria, insulin resistance, lipid metabolism or other pathways involved in the progression of DN. The present review discusses the main information available in literature regarding some genetic variants (involved in the RAS, glucose and lipid metabolism and some cytoskeleton proteins) that reaffirms the importance of genetic factors in diabetic nephropathy (Table 1.).

Angiotensin converting enzyme

The angiotensin-converting enzyme (ACE), a potent vasoconstrictor, catalyzes the conversion of angiotensin I to angiotensin II. It also inactivates bradykinin, a vasodilator, by bringing about its proteolysis (Crisan and Carr, 2000).

The angiotensin-converting enzyme gene (ACE) on chromosome 17q23 has repeatedly been evaluated for a role in DN. Polymorphisms in this gene are clearly associated with circulating ACE levels (Rigat *et al*., 1990) and reports suggested association between the ACE DD allele and type 1 DN (Hadjadj *et al*., 2001; Boright *et al*., 2005).

A meta-analysis including 47 studies (14 727 subjects) showed that subjects with the II genotype had a 22% lower risk of DN than carriers of the D allele [OR = 0.78, CI95% (0.69–0.88)]. Also this study support a genetic association of the ACE I/D polymorphism with diabetic nephropathy. These findings may have implications for the management of diabetic nephropathy using ACE inhibitors especially among type 2 diabetic Asians (Ng *et al*., 2005). Although a large meta-analysis failed to confirm the DN association in white individuals (Kunz *et al*., 1998) a recent report (Hadjadj *et al*., 2007) from the European Rational Approach for the Genetics of Diabetic Complications (EURAGEDIC) Study Group detected evidence for association of several ACE polymorphisms (including the "D" deletion allele) in a large case-control study, with somewhat consistent findings in a family-based transmission disequilibrium testing analysis.

In summary, it appears that polymorphisms in the ACE gene may have a role in the progression of DN, rather than in the susceptibility to it. Thus, evaluating the ACE I/D polymorphism is by no means a reliable and cost-effective tool to identify patients at risk and those who may benefit the most of renoprotective therapy with ACE inhibitors or angiotensin II antagonists and, possibly, with other inhibitors of the RAS, such as renin and aldosterone antagonists.

Angiotensinogen and angiotensin II receptor type 1

Other variants in the renin–angiotensin system that were also widely studied and reproduced, such as the rs699 variant of AGT and the rs5186 polymorphism of AGTR1, were not associated with diabetic nephropathy in a meta-analysis (Mooyaart *et al*., 2011).

Aldose reductase

Aldose reductase is an important enzyme in the polyol pathway, and is suggested as contributing to diabetic microangiopathic complications. Several mechanisms have been proposed to explain how AKR1B1 activity leads to hyperglycaemia-induced lesions in different tissues (Chung and Chung , 2003)

Ko *et al.*were the first to identify seven alleles at the locus of the (AC)n dinucleotide repeat sequence upstream of AKR1B1. The most common allele contains 24 (AC) repeats and was named Z (Ko *et al.,* 1995). Several studies have demonstrated a correlation between the Z-2 allele (23 repeats) and susceptibility to an increased risk of DN in both T1DM and T2DM (Shah *et al*., 1998; Olmos *et al*., 1999; Moczulski *et al*., 2000; Neamat-Allah *et al*., 2001; Liu *et al*., 2002)

A second AKR1B1 polymorphism has been observed at position−106 of its promoter region. This C106T polymorphism was identified in both Caucasian and Asian subjects with T1DM or T2DM, and association with DN has been observed. Sivenius *et al.* (2004) and Gosek *et al.* (2005) suggested that this polymorphism could be involved in the early development of microalbuminuria in Finnish T2DM patients and is a risk factor for development of DN in T2DM patients with poor glycaemic control, respectively. All these results suggest that AKR1B1 polymorphisms play a role in DN development.

Glucose transporter 1

The glucose transporter 1 (SLC2A1, also know as GLUT1) is the major representative of the family of facilitative glucose transporters that are expressed in glomerular, mesangial, endothelial cells and podocytes. SLC2A1 is likely to be pivotal in raising intracellular glucose levels by activating pathogenic pathways (Koya and King, 1998; Larkins and Dunlop, 1992; Vlassara,1997).

Several works have tried to determine whether SLC2A1 might be a candidate gene conferring susceptibility to DN. In a study Ng *et al*., (2002) confirms that SNPs at the GLUT1 (XbaI -intron 2 and HaeIII SNPs -exon 2) are associated with susceptibility to diabetic nephropathy in type 1 diabetes. Although these SNPs confer a considerable

personal risk for diabetic nephropathy, they account for a limited proportion of cases among type 1 diabetic patients. A meta-analysis concluded that there is, indeed, a significant association between the SLC2A1 *XbaI* polymorphic site and DN, but larger studies are needed (Zintzaras and Stefanidis, 2005)

Apolipoprotein E

The apolipoprotein E gene (APOE) on chromosome 19q has also been associated with susceptibility to type 1 DN (Araki *et al*., 2000) and type 2 DN (Hsu *et al*., 2005). APOE is a polymorphic protein that consists of three isoforms, E2, E3, and E4, encoded by the alleles ε2, ε3, and ε4, which are defined by a single amino acid substitution at two sites (reviewed by Mahley and Rall, 2000). The E2 allele is thought to lead to an increased risk of diabetic nephropathy and the E4 allele is thought to have a protective effect. Both the E2 and the E4 allele were associated with diabetic nephropathy in a meta-analysis (Mooyaart *et al*., 2011)

Adionectin

Adiponectin is an adipocytokine that is produced by fat cells (Guzik *et al*., 2006). Circulating levels of adiponectin are reduced in individuals who are obese and have diabetes, and levels rise after weight loss. Positive correlations have been reported between circulating high molecular weight adiponectin level and HDL cholesterol, with negative correlations among circulating inflammatory markers, triglycerides, and homeostasis model assessment of insulin resistance. Adiponectin gene (ADIPOQ) polymorphisms reportedly play a protective role in susceptibility to coronary heart disease (Qi *et al*., 2006). With clear relationships between atherosclerosis and DN, ADIPOQ is likely a gene that may play a role in both vascular processes. In a recent analysis, rs17300539 of ADIPOQ, which is believed to mitigate vascular damage, was not associated with diabetic nephropathy (Mooyaart *et al.,* 2011)

Peroxisome proliferator activated receptor gamma 2

Peroxisome proliferator activated receptor gamma 2 (PPARγ2) is the predominant adipose isoform of this receptor and is expressed selectively in the adipose tissue where it modulates the expression of target genes implicated in adipocyte differentiation and glucose homeostasis. PPARγ2 is considered, therefore, a major candidate gene for T2DM and/or obesity and, recently, for type 2 DN. Three studies have evaluated its association with type 2 DN. In the study by Herrmann *et al*. (2002) the Pro12Ala polymorphism was associated with lower albumin excretion rates among Ala12 carriers with type 2 DN, which may indicate a protective effect of this allele. These findings were confirmed by Caramori *et al*. (2003). More recently, Pollex *et al*. (2007) showed that the Ala12 allele carriers have reduced occurrence of microalbuminuria (1.5-fold reduction of the albumin/creatinine ratio). All these results indicate a protective correlation between the Ala12 polymorphism and the albumin excretion rate. The mechanism underlying the protective effect of the Ala12 allele is yet unknown.

Adducins

Adducin (ADD) is a heterodimeric cytoskeleton protein composed of α, β and γ-subunits. These proteins are encoded by three genes (ADD1, ADD2 and ADD3) that map to different chromosomes. ADD genes show a similar gene structure, suggesting their deviation from a single gene that has undergone duplications and rearrangements during evolution. In humans, the ADD1 is widely expressed, while the ADD2 is especially expressed in neuronal, renal and erythropoetic tissue (Matsuoka *et al*., 2000).

The α-subunit regulates the activity of transmembrane ion pumps and is encoded by the adducing 1 (ADD1) gene, located in chromosome 4q21.

In a large study that investigate the role of the α-adducin gene in genetic susceptibility to diabetic nephropathy, Conway *et al*., (2004) have found no evidence of association between variation in the α-adducin gene and the development of nephropathy in the Irish population. While they cannot exclude the possibility of a minor gene effect, it is unlikely that common variation within the α-adducin gene plays a major role in the genetic predisposition to diabetic nephropathy in Irish population. Another study of the same group investigated the ADD2 gene and their results suggest that common polymorphisms and putatively functional variants in the ADD2 gene do not strongly influence genetic susceptibility to diabetic nephropathy in the White population studied with type 1 diabetes (Currie *et al*., 2008). Most studies on the role of adducins were performed on hypertensive patients and less on diabetic nephropathy. Recently Lanzani *et al*., described an epistatic interaction between the adducing gene (ADD1 and ADD3) in a large cohort of never treated hypertensive individuals. Patients who carried both the mutated ADD1 Trp allele and ADD3 G/G had the higher systolic and diastolic blood pressure values (p=0.002). (Lanzani *et al*., 2005). So far there are a small number of studies on the effect of adducins in the patology and progression of diabetic nephropathy.

Table 1. Candidate genes associated with type 1 diabetic nephropathy

Class	Gene name	Symbol	Location
Renin-angiotensin system	Angiotensin-converting enzyme 1	ACE 1	17q23
	Angiotensinogen	AGT	1q42-43
	Angiotensin II receptor	AGT1	3q21-25
Glucose metabolism	Aldose reductase	AKR1B1	7q35
	Glucose transporter-1	SCL2A1	1p35
Lipid metabolism	Apolipoprotein E	APOE	19q13.2
	Adiponectin	ADIPOQ	3q27
	Peroxisome proliferator activated receptor gamma 2	PPARγ 2	3p25
Cytoskeletal genes	Alfa adducin	ADD1	4p16.3
	Beta adducin	ADD2	2p13.3
	Gamma adducin	ADD3	10q25.2

CONCLUSION AND PERSPECTIVES

Due to the growing burden of the management of diabetes and its complications, it is important to identify DN predictors, in order to facilitate its diagnosis and treatment. Identification of risk genes could provide a powerful tool for identifying the subset of patients who have diabetes and will progress to nephropathy and ESRD. Early identification will facilitate earliar intervention, ultimately delaying and reducing the impact of DN.

Most genetic studies have been performed in selected populations but they are heterogeneous between them. It should also be pointed out that an isolated candidate gene is sought when various genes are probably involved and possibly interlinked (Carpena *et al*., 2010). Joint efforts are essential to achieve robust findings in the study of genetics of DN. In the light of remarkable advances in this area of study, we hope that in the near future patients at high risk for developing DN could be identified and benefited with earlier specific therapies. Hence, by combining the expertise of geneticists and clinicians, there is real hope that the genetic basis of diabetic nephropathy and other complex renal diseases may be unravelled offering new opportunities for screening and therapeutic intervention.

In addition, new pharmacogenomic developments will contribute to better treatment choices for DN and, more importantly, will help preventing it based on an individual's genetic characteristics.

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ANGIOTENSIN-CONVERING ENZYME INSERTION/DELETION POLYMORPHISM IN TYPE I DIABETIC NEPHROPATHY

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Keywords: angiotensin converting enzyme gene, insertion/deletion polymorphism, diabetic nephropathy
Abstract: Angiotensin converting enzyme gene has been described with an insertion/ deletion polymorphism (I/D) of a 287-basepair sequence of DNA in intron 16 leading to three genotypes, DD and II homozygotes and ID heterozygote.

We examined the frequency of ACE I/D polymorphism in 217 patients, of which 59 with diabetes mellitus type I (controls), 37 with incipient diabetic nephropathy and 121 with end-stage renal disease (cases). The ACE I/D polymorphism was detected by PCR using three oligonucleotide primers in a single reaction. This study has found no evidence that the insertion/deletion polymorphism in the ACE gene plays a major role in the progression of diabetic nephropathy. In particular, the DD genotype, which has previously been implicated in diabetic nephropathy both type I and II, in our study, is not associated with an increased risk of developing type I diabetic nephropathy. Although this study found no association between ACE I/D polymorphism and diabetic nephropathy, we found that DN and ESRD patients have higher prevalence of dyslipidemia and blood pressure ($p < 0.001$), this parameters being major determinants of progression.

INTRODUCTION

Diabetic nephropathy is the most serious complication of diabetes mellitus and affects approximately a third of diabetic patients. Importantly, it is the leading cause of end-stage renal disease requiring dialysis or transplantation in developed countries (National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, 2003) as well as in rapidly developing countries in Asia (Singapore Renal Registry, 1997).

Genetic studies have revealed the the genes of renin-angiotensin system (RAS) are highly polymorphic, raising the possibility that in addition to environmental factors, the genetic make up of RAS affects the status of RAS in individuals. One of such is the insertion/deletion polymorphism of ACE gene. Studies of familial clustering have consistently demonstrated that genetic susceptibility plays an important role in diabetic nephropathy (Krolewski *et al*., 2001) and the gene encoding angiotensin-I converting enzyme (ACE) is a potential candidate gene in its etiology.

ACE, a potent vasoconstrictor, catalyzes the conversion of angiotensin I to angiotensin II. It also inactivates bradykinin, a vasodilator, by bringing about its proteolysis (Crisan and Carr, 2000). ACE gene has been described with an insertion/ deletion polymorphism (I/D) of a 287bp sequence of DNA in intron 16 (Rigat *et al*., 1992) leading to three genotypes, DD and II homozygotes and ID heterozygote. The mean plasma/serum ACE level in the DD subjects is reported to be approximately double that of II subjects, with ID subjects having intermediate values (Rigat *et al*., 1990).

In a pioneering study, Marre *et al*. (1994) proposed a protective effect of the II genotype against the development of diabetic nephropathy in insulin-dependent diabetes mellitus. Thereafter, a sizeable number of association studies have investigated the possible role of ACE I/D polymorphism in the pathophysiology of diabetic nephropathy and most of them have recorded association of the D allele as a risk factor (Ng *et al.*, 2005).

The goal of this study is to test the role of ACE gene, especially the insertion/deletion polymorphism, as a potentially reliable candidate gene for the progression rate in type I diabetic nephropathy. The relevance of this question has not only the clinical usefulness of identifying new prognostic indicators, but should help in dissecting some of the pathogenetic mechanism of diabetic renal diseases.

MATERIAL AND METHODS

Subjects and clinical data. A total of 449 patients with T1DM were recruited from the Diabetic and Nephrology Out-patients Clinics and from the Division of Transplantation at San Raffaele Hospital. Inclusion criteria were: a) established T1DM, 2) patient who underwent a kidney or simultaneous kidney-pancreas transplantation in the past 15 years, 3) absence of secondary nephropathy, 4) absence of chronic comorbidities other than diabetes mellitus. No restriction was adopted based on age, sex, BMI, blood pressure values, HbA1c levels, renal failure stage, disease duration, antihypertensive treatment and its duration.

Genotype was available for 217 patients. Informed consent was obtained.

Genotyping. DNA was extracted from venous whole blood by standard methods.

DNA was amplified by the PCR using three oligonucleotide primers in a single reaction. Two primers flanked the insertion-deletion site (5'-CTGGAGACCACTCCCATCCTTTCT-3'and 5'-GATGTGGCCATCACATTCGTCAGAT-3') and generated a product of 190bp from D alleles, and a product of 490 bp from I alleles. Due to preferential amplification of D allele, the 490bp band is not always visible in heterozygous samples. To avoid the resulting misidentification of ID heterozygotes as DD homozygotes, a third primer complementary to sequence within the insertion was included in all reaction (5'-TGGGATTACAGGCGTGATACAG-3') (Morgan *et al*., 1999).

This consistently generated a fragment of 159bp from I alleles only. The 159 and 190bp fragments were used for genotyping.

Amplification reactions were carried out in a total volume of 20 µl, using 100 ng genomic DNA (2 µl), 0.32 µl $MgCl_2$ (50 mM) (Bioline), 2 µl NH_4 (Bioline), 0.4 µl dNTPs (10 mM) (Promega), 0.8 µl (100 ng/µl) of each primer and 0.2 µl of 5U/µl Taq polymerase (Bioline). Samples were denatured at 96ºC for 5 min, followed by 34 cycles of denaturation at 94ºC for 30s, primer annealing at 58ºC for 1 min and DNA extension at 72ºC for 1 min, with a final 10 min extension stage at 72ºC.

Detection of ACE I/D polymorphism by electrophoresis. The reaction products were separated by electrophoresis in 2% agarose gels (SeaKem LE Lonza) and stained with ethidium bromide. Under ultraviolet light two bands, insertion (I) and deletion (D) were visible (Figure 1).

RESULTS AND DISCUSSIONS

Diabetic patients without nephropathy (DM) were 221, the genotype was determined in 59 of them, diabetic patients with incipient nephropathy (DN) were 94, 37 were genotyped, and diabetic patients with ESRD were 131, in 121 of them information about genotype was available.

Clinical and biological characteristics of each group are summarized in Table 1.

The mean age of the patients was 31.5 ± 0.8 years for DM, 40.8 ± 1.5 years for DN and 40.0 ± 0.7 years for ESRD group. Mean systolic and diastolic blood pressure were 120.0 ± 1.0 and 74.5 ± 0.7 mmHg for diabetic patients, 134.9 ± 2.1 and 81.3 ± 1.0 mmHg for DN group and 137.1 ± 1.7 mmHg for ESRD group respectively. Mean total cholesterol, triglycerides and HbA1c levels were: for cholesterol 171.9 ± 2.7 mg/dl (DM), 192.0 ± 5.4 mg/dl (DN), 214.0 ± 15 mg/dl (ESRD), triglycerides 79.5 ± 3.5 mg/dl (DM), 115.9 ± 7.7 mg/dl (DN), 214.0 ±15 mg/dl (ESRD) and HbA1c 8.3 ± 0.1 % (DM), 8.6 ± 0.2 % (DN) and 8.1 ± 0.6 % (ESRD) respectively.

As expected, patients with nephropathy and ESRD were older, with high levels of BP (both systolic and diastolic), and more severe metabolism alterations (cholesterol and triglycerides significantly elevated). Creatinine clearance values were estimated based on Cockcroft-Gault formula (Cockcroft and Gault,1976).

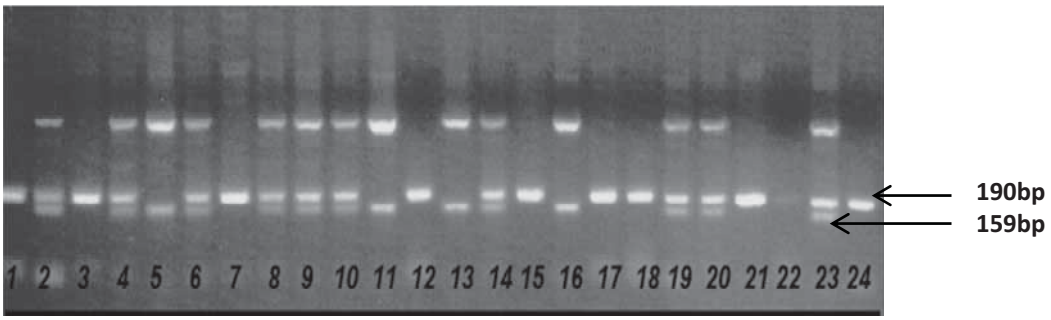

Figure 1. Agarose gel electrophoresis of PCR products of ACE gene. Lines 5, 11, 13, 16 homozygous II cases, 2, 4, 6, 8-10, 14, 19-20, 23 heterozygous ID and lines 1, 3, 7, 12, 15, 17, 18, 21, 24 homozygous DD cases

Patient characteristics (n = 217) according to ACE I/D genotypes are shown in Table 2. The distribution of patients as per genotyping was II (DM-19, DN-11, ESRD-45), ID (DM-27, DN-21, ESRD-63) and DD (DM-13, DN-5, ESRD-13). In each study group, the genotype frequency distributions of this polymorphism were in Hardy-Weinberg equilibrium.

In our study ID genotype was the most frequent, present in 45.8% (DM), 56.8% (DN) and 52.1% (ESRD), followed by II in 32.2 % (DM), 29.7% (DN) and 37.2% (ESRD) and DD was found in only 22% (DM), 13.5% (DN) and 10.7% (ESRD) (Table 2). There is a significant difference between DD genotype in cases (DN and ESRD) and controls

(DM). The DD genotype frequency decreases from 22% in diabetic patients without nephropathy to 13.5% and 10.7% in diabetics with nephropathy and ESRD patients, but there is no association between the D allele and diabetic nephropathy ($p > 0.05$).

Although this study found no association between ACE I/D polymorphism and diabetic nephropathy, we found that DN and ESRD patients have higher prevalence of dyslipidemia and blood pressure ($p < 0.001$), this parameters being major determinants of progression. Paradoxically, no correlation exists between HbA1c levels and BMI. This finding suggestthat the etiology of diabetes, dyslipidemia, hypertension and nephropathy may have a common factor(s), and it also provides clues for the high incidence of micro- or macrovascular complications in T1DM patients.

Table 1. Clinical features of patients with T1DM

	Diabetes (DM)	Diabetic Nephropathy (DN)	ESRD	p
Age	31.5 ± 0.8	40.8 ± 1.5	40.0 ± 0.7	< 0.001 DN & ESRD vs DM
Diabetes duration (years)	20.5 ± 0.6	22.6 ± 1.0	26.3 ± 0.7	< 0.05 vs all
BMI (kg/m^2)	22.9 ± 0.2	23.6 ± 0.4	23.2 ± 0.3	ns
SBP (mmHg)	120.0 ± 1.0	134.9 ± 2.1	137.1 ± 1.7	< 0.001 DN & ESRD vs DM
DBP (mmHg)	74.5 ± 0.7	81.3 ± 1.0	81.9 ± 1.0	< 0.001 DN & ESRD vs DM
Cholesterol (mg/dl)	171.9 ± 2.7	192.0 ± 5.4	214.0 ±15	< 0.001 DN & ESRD vs DM
Triglycerides (mg/dl)	79.5 ± 3.5	115.9 ± 7.7	165.5 ± 15	< 0.001 DN & ESRD vs DM
HbA1c (%)	8.3 ± 0.1	8.6 ± 0.2	8.1 ± 0.6	ns
Creatinine clearance	113 ± 2.4	114 ± 12	54.4 ± 1.3	-------

Table 2. Distribution of ACE genotype and allele frequencies in the three groups

	DM n=59 (%)	DN n=37(%)	ESRD n=121 (%)	p
Genotype frequency				
II	19 (32.2)	11 (29.7)	45 (37.2)	ns
ID	27 (45.8)	21 (56.8)	63 (52.1)	ns
DD	13 (22.0)	5 (13.5)	13 (10.7)	ns
Allele frequency				
I	46 (53.5)	32 (58.2)	108 (58.7)	ns
D	40 (46.5)	23 (41.8)	76 (41.3)	ns

Despite the huge amount of studies looking for candidate genes, the ACE gene remains the unique, well-characterized locus clearly associated with pathogenesis and progression of chronic kidney disease.

We examined insertion/deletion (I/D) polymorphism of theACE gene, one of the important genes in RAS, in T1DM patients with (DN and ESRD) and without nephropathy (DM). The primary objective of the study was to find the pattern of distribution of ACE I/D polymorphism in T1DM, in T1DM with incipient nephropathy and ESRD patients and to study the relation between DD gene polymorphism and DN. Although the data from Caucasian studies failed to confirm an increased risk for development of DN in T1DM and T2DM being associated with D-allele, a role of this genetic marker in Asian patients with T2DM cannot be ruled out (Kunz *et al*., 1998). A meta-analysis of 8663 type I and type II diabetics with incipient or overt nephropathy (defined, respectively, by the presence of microalbuminuria or macroalbuminuria/proteinuria, with or without renal insufficiency) and 6064 diabetic controls with no evidence of renal disease (defined as a urinary albumin excretion below the threshold for microalbuminuria) included in 47 studies published from 1994 to 2004, showed that those with the II ACE polymorphism had a 22% lower risk for nephropathy than homozygous or heterozygous carriers of the D allele. (Ng *et al*., 2005).

This study has found no evidence that the insertion/deletion polymorphism in the ACE gene plays a major role in the progression of diabetic nephropathy. In particular, the DD genotype, which has previously been implicated in diabetic nephropathy both type I (Ng *et al*., 2005) and II (Lee and Tsai, 2002), is not associated with an increased risk of developing diabetic nephropathy.

Thus, evaluating the ACE I/D polymorphism is by no means a reliable and cost-effective tool to identify patients at risk and those who may benefit the most of renoprotective therapy with ACE inhibitors or angiotensin II antagonists and, possibly, with other inhibitors of the RAS, such as renin and aldosterone antagonists. Several research groups have used control groups consisting solely of diabetic patients with normoalbuminuria despite a long duration of diabetes (Grzeszczak *et al*., 1998; Azar *et al*., 2001). This approach could yield clearer evidence for a true association between ACEI/D polymorphism and diabetic nephropathy, since the use of these controls can help reduce case misclassification.

Abreviation

ACE= angiotensin converting enzyme
BMI= body mass index
D=deletion
DN=diabetic nephropathy
DM= diabetes mellitus
ESRD= end-stage renal disease
I=insertion
PCR= polymerase chain reaction
RAS= renin-angiotensin system
T1DM= type 1 diabetes mellitus
T2DM= type 2 diabetes mellitus

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CLONING AND PURIFICATION OF A REPRESSOR PROTEIN FROM *ARTHROBACTER NICOTINOVORANS* PAO1

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Keywords: *Arthrobacter,* repressor, carbohydrates

Abstract: The pAO1 megaplasmid of *A. nicotinovorans* consists of 165 ORF's related mainly to nicotine degradation, uptake and utilization of carbohydrates, amino acids and sarcosine. The putative sugar catabolic pathway consists of 11 ORF's organized as a single operon and coding for an ABC-type sugar-transport system and several putative oxidoreductases and dehydrogenases. The current work is focused on orf32, a putative PdhR related protein, most probably involved in the control of the whole operon. The approx. 700 kb *orf32* gene was cloned in the pH6EX3 plasmid vector and the gene product purified to homogeneity as a 29 kDa His-tagged recombinant protein. As indicated by GPC, it consists of a monomeric protein with a native molecular weight of 32 kDa. The specific UV/Vis spectra showed only a single peak at 280 nm common for all proteins and did not indicated the presence of any colored cofactors. This is in good agreement with the fact that PdhR-family proteins contain a winged helix-turn-helix (wHTH) domain responsible for DNA binding, and not a Zn-finger or any other metal containing domains.

INTRODUCTION

Plasmids are simple genetic elements, independent from the bacterial chromosome, involved both in vertical and horizontal-gene transfer. Most of the time, the plasmids encode different properties (resistance to antibiotics, to highly toxic compounds) which give the host cell an evolutionary advantage. The ability to use less common compounds as carbon and nitrogen sources is such an advantage, allowing the bacteria to be present in many environments as natural autochthonous microflora with a high potential for bioremediation of pollutants. Several plasmid-encoded pathways were described (ex: for metabolism of phthalate (Eaton A., 2001) or naphthalene (Rosselló-Mora, Lalucat & García-Valdés, 1994)) but only few are completely elucidated.

The presence of the 165- kb pAO1 megaplasmid inside the cells of the gram positive soil bacteria *Arthrobacter nicotinovorans* allows this microorganism to use nicotine as sole carbon and nitrogen sources. The complete sequence of this plasmid was determined and two putative pathways could be described (Igloi & Brandsch, 2003): on one hand the nicotine-degrading pathway, fully characterized by Brandsch (Brandsch Roderich.,2006) and on the other hand an yet unknown putative sugar-catabolic pathway. The overall GC content of the pAO1 plasmid indicates that nicotine-catabolism gene clusters are a new acquisition, being attached during the evolution to an older plasmid, containing the sugar-catabolic pathway. Recently shown analogies of the pAO1 encoded pathway for nicotine metabolism and the chromosome encoded one from *Nocardioides* sp. strain js614 (Ganas *et al.*, 2008) would suggest a horizontal gene transfer.

The sugar-catabolic pathway is comprised of several genes, among which a putative cellulase, an ABC-transporter system gene cluster and a cluster of several dehydrogenases and oxidoreductases. This last cluster probably encodes the last steps of the pathway, connecting it to the general metabolism of the cell. All of these genes are clearly organized as a single operon, with *orf32,* a putative DNA binding -protein having an opposite orientation and thereby being the perfect candidate for the repressor involved in the control of the whole operon (figure 1).

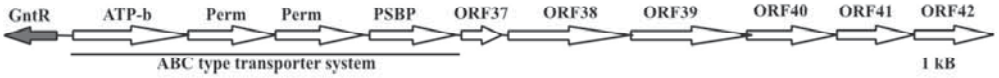

Figure 1. General organization of the putative carbohydrate utilization gene cluster from pAO1

Our current study is focused further characterization the ORF32 protein and elucidating its possible role in the cell. By cloning the gene in the expression vector pH_6EX_3 (Berthold *et al.*, 1992), we were able to express it as a recombinant His-tagged protein, to easily purify it to homogeneity and, using antibodies developed in rats, to indicate a possible inducer by Western-blot.

MATERIAL AND METHODS

Strains and growth conditions. For all recombinant DNA-techniques and protein purifications, *E. coli* XL1 Blue grown on Luria-Bertani (LB) medium was used. Arthrobacter nicotinovorans pAO1+ and pAO1- were a kind gift from prof. Dr. Brandsch R, and were grown an citrate medium supplemented with carbohydrates at 10 mM final concentration.
Isloation and cloning of *orf32*. The *orf32* was isolated by PCR using the primers in table 1 and a suspension of *Arthrobacter nicotinovorans* cells as template. Directional cloning (Sambrook J, Fritsch EF, Maniatis T,1989) of the fragment containing the *orf42* in the pH_6EX_3 vector was achieved by using *BamHI* şi *XhaI* (NEB, U.K) enzymes and Rapid DNA ligation Kit, Roche). Transformed *E. coli XL1* Blue competent cells were selected on plates containing ampiciline (50 microg/ml) and the recombinant plasmid was checked for the presence of insert by restriction enzyme digestion.
Table 1. Oligo-nucleotides used for isolation of *orf32*

	Sequence
Orf32forw	5′-GGCCGAGGATCCATGGACG-3′
Orf32rev	5′-CGCTACCACTCGAGGCTGACC-3′

Protein expresion was done using auto-inducible medium as described elsewhere. (Mihasan, Ungureanu & Artenie,2007) **Protein purification** was achieved using standard IMAC techniques (Ausubel et. Al., 2002) on Fast-Flow Ni-chelating Sepharose (Amersham Biosciences, Sweden). **Native molecular weight determination** was done using gel permeation chromatography on an HiLoad 16/60 Superdex 200 column connected to an AKTA Basic FPLC system. **Protein concentration** was assayed using the dye-binding method of Bradford (Bradford,1976). **SDS-PAGE** was performed using the discontinuous system of Laemlli fallowing the procedure described by Sambrook, 1989(Sambrook J, Fritsch EF, Maniatis T,1989). **Antibodies** against the purified protein were developed in rats and used as primary antibodies in Western-Blots. **Carbohydrate metabolism** assay was performed with the API 50CHL (Biomerieux, France) per producer's indications.

RESULTS AND DISCUSSIONS

***ORF32* encodes a monomeric protein.** The recombinant protein obtained by cloning orf39 in pH6EX3 has the N-terminal sequence as follows: HHHHHLVPRGSEA**L**, where the leucine in bold is the native start codon. This allowed for a one step purification process of the protein from the *E.coli* cell lysate using mobilized metal affinity chromatography. The purified enzyme had a relative molecular weight of 47 kDa, in good accordance with the theoretical mass. The purity of our preparations was very high (over 95% on SDS-PAGE, fig. 2)

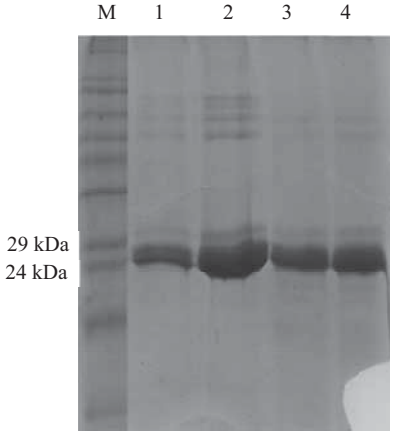

Figure 2. Orf32 encoded protein was purified to homogenity.
M –Molecular Weight Marker Sigma Wide Range
1,2- Purified protein, 200 mM imidasol elution
3,4 – Purified protein, 500 mM imidasol elution

Repressor proteins are usually tetramers or dimers in solution. In order to establish native state of this protein a

gel permeation chromatography was performed. Approximately 1.6 mg purified ORF40 were injected on a HiLoad 16/60 Superdex 200 column. The chromatogram is presented in figure 3. The protein eluted as a single peak, corresponding to a molecular weight of 32 kDa. This indicates that surprisingly, ORF32 is monomer in solution.

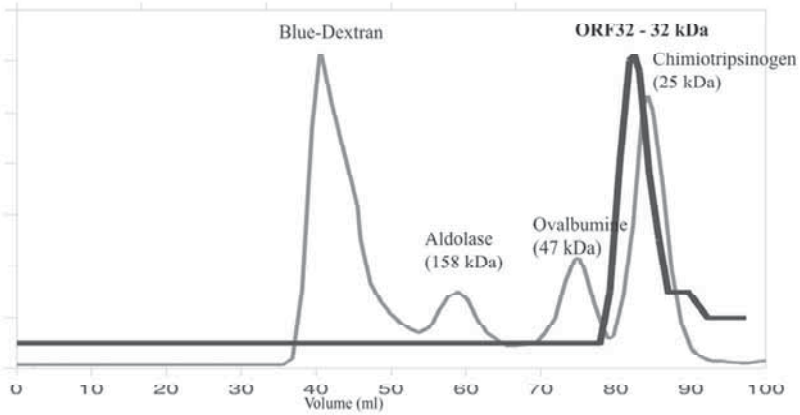

Figure 3. Native molecular mass determination of ORF32 protein. 1.6 mg purified ORF32 protein was injected on a HiLoad 16/60 Superdex 200 previosly calibrated using Blue-Dextran, Aldolase (158 kDA, Ovalbumine (43 kDa), Chimiotripsinogen (25 kDa).

ORF32 contains a helix-turn-helix (HTH) domain. A BLAST search performed with the amino acid sequence of ORF32 indicates that the protein belongs to the GntR family of transcriptional regulators. This family consists of several subfamilies (Rigali et al. 2002), but most of the members were described as repressors. Using SwissModeller, a computer generated ORF32 model was obtained. The putative 3D structure follows the same general organization with two domains as the LdlR transcriptional factor from *Corynebacterium glutamicum* (Gao et al. 2008). The N-terminal LdlR region contains a winged helix-turn-helix (wHTH) domain characteristic for GntR family proteins which is in a way conserved in the ORF32 model. The two helices and the connecting turn are present and are forming an HTH domain, but the beta sheets forming the wing are missing (figure 4). This HTH domain is usually responsible for the DNA binding activity which one might expect from a repressor protein. This is in good accordance with the experimentally observed fact that specific UV/Vis spectra of ORF32 do not indicate the presence of a Zn finger.

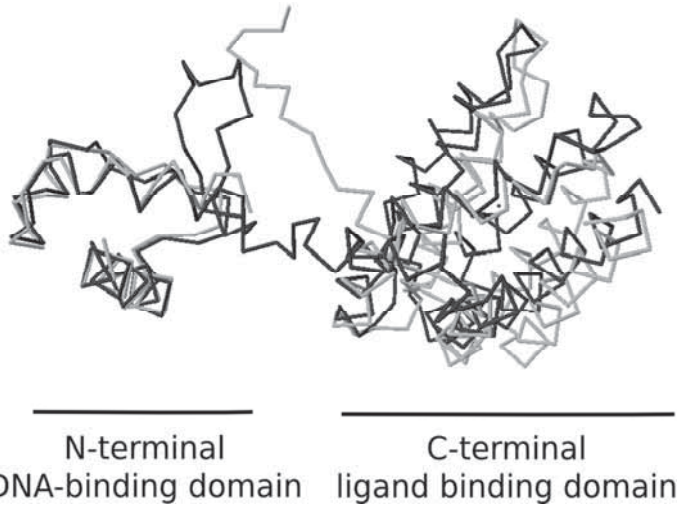

Figure 4. Superimposition of the putative transcriptional factor ORF32 (green) and LdlR transcriptional factor from *Corynebacterium glutamicum* (black, 2di3)

ORF32 is a continuously expressed in *Arthrobacter nicotinovorans pAO1*+ cells. As a DNA/binding assay could not be performed in order to directly address the function of this protein, an indirect approach was chosen. In a series of experiments, the conditions in which this protein is expressed *in-vivo* were studied. Cell free extracts of *A. nicotinovorans* pAO1+ cells grown on citrate medium supplemented with various sugars were separated by SDS-PAGE and the levels of ORF32 proteins were detected by Western-Blot using in the house raised antibodies. In all tested conditions, in the presence or in the absence of the sugars, the ORF32 protein was expressed in equal amounts. This is understandable, as a repressor protein should always be expressed in order to efficiently inhibit the transcription of operon. When the inducer appears, the DNA binding capacity of the repressor is abolished and the transcription can take place.

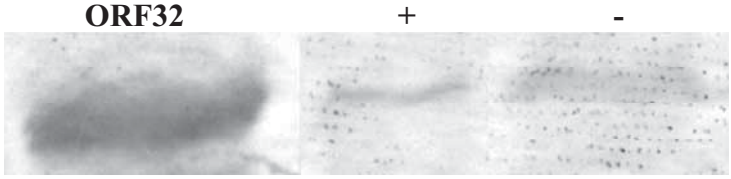

Figure 5. Detection of ORF32 by Western-Blot in the cell free extracts of *Arthrobacter nicotinovorans* pAO1 growth on citrate media (-) and on citrate media supplemented with D-xylose (+)

CONCLUSIONS

The ORF32 from pAO1 was cloned, expressed and purified to homogeneity. It consists of a HTH repressor protein of 29 kDa, which surprisingly is a monomer in solution.

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INFECTION WITH HUMAN PAPILLOMA VIRUS IN CERVICAL NEOPLASIA

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Keywords: human papilloma virus, cervical neoplasia

Abstract: The purpose of this study was to establish if the infection with human papilloma virus (HPV) presents a potential irreversible evolution towards malignancy. Materials and methods. The study was made on a number of 1885 patients that were suspected to have cervical neoplasia, which were monitored between 2001-2010 in „Elena-Doamna” Clinical Hospital of Obstetrics and Gynecology in Iaşi, the Military Hospital Galaţi, the County Hospital Galaţi and the Emergency Hospital Buzau. Results and discussions. The study proved that the risk of contacting a genital infection with HPV and cervical cancer is influenced by the sexual activity, the risk of getting infected with HPV during a person' s lifetime is at least 50% for those sexually active. Conclusions**.** The patients benefited from colposcopy and biopsy only if the repeated cytology suggested more severe changes. The conservative conduct is represented by a repeated cytology when the patients are admitted into the lot (the initial cytology is performed before this moment).

INTRODUCTION

There have been 35 types of HPV described for humans, classified according to the sequence of the nucleotides from the viral ADN by using methods of recombining them (Southern Blot Hybridisation) (10). Types 16, 18, 31 and 35 are oncogenic. About 1-2% of the women who are sexually active have a HPV cervical infection (Bernstein, 1985).

Branca (1995) correlates the risk degree of HPV cervicitis with the association with HIV (Human immunodeficiency virus); 47% of the women who are HIV positive had HPV lesions at their colposcopic and/or cytologic examination, and for 40% of them there was some form of CIN (cervical intraepithelial neoplasia): CIN I, CIN II and CIN III; 37% of the cases with CIN I and CIN II developed rapidly towards CIN III. In the cases with negative HIV only 23% had HPV lesions, and 26% of these cases were associated with CIN I, CIN II or CIN III; only one case had a fast development (after a year) from CIN I to CIN III.

Remmink (1995) monitored a group of women for 3-4 months by cervical cytology, colposcopy and HPV tests. The cases that had progressive lesions of CIN were always positive for HPV, and the biopsy confirmed the stage of CIN III. The author thinks that the effect of HPV on the progression of CIN type lesions is also increased by other risk factors (age, number of sexual partners, smoking, etc.).

The present stage of the research creates premises for selecting the cases of CIN with an irreversible evolutionary potential towards malignancy separately from those with a benign evolution. The infections with oncogene types (16, 18, 31, 35) would require a more aggressive treatment with the purpose of blocking the progression towards CIN types with high malignancy (1, 10).

The literature of speciality underlines the fact that the risk of getting an HPV genital infection and cervical cancer is influenced by the sexual activity, the risk of infection with HPV during a lifetime for people who are sexually active being of at least 50% (1, 7). Although most infections are eliminated by their own immunity, the infected people are not aware of the HPV presence and they can spread the virus. HPV is transmitted through direct contact between tegument - mucous. That is why this infection can occur in the case of virgins with sexual exposure, too. The infection can also be transmitted in other ways than sexually, for example by fomites (8).

The phenotypic expression of HPV infection depends on 3 factors:

1. types of virus – the simple presence of an infection with oncogene types does not mean. For a woman to develop cervical cancer some other additional factors need to act together.
2. local factors of environment
    a. exposure of basal and parabasal cells to the virus
    b. trauma

    In the case of immuno-depressed patients (kidney transplant, HIV, treatment with cortisone) the probability of developing a persistent infection and a cancer respectively is higher.
3. the immune response of the host – the immune system

    The primary immune response of the host has a decisive importance in the evolution of infection. In the case of HPV infection we are talking about cellular immunity; the immunity of the host being the one that is able to eliminate most infections. When a person‘s own immune system is not able to eliminate the

infection, the persistence of the viral oncogenic strains in the cervical mucus can lead to the appearance of pre-cancerous lesions.

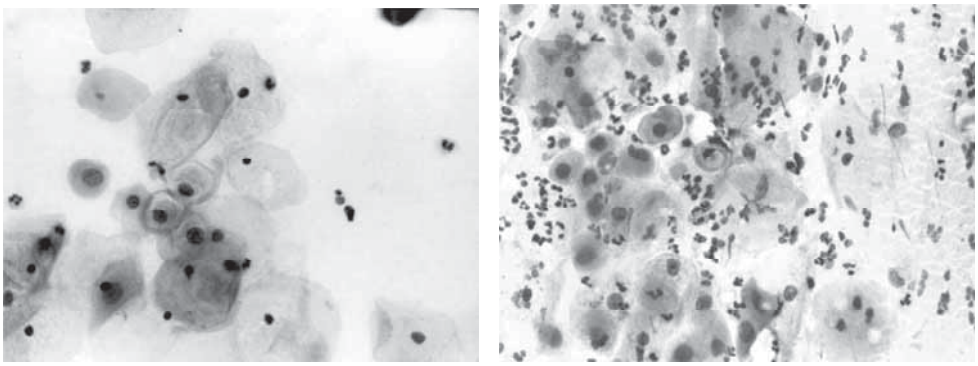

Figure 1. LSIL - Low grade squamous intraepithelial lesion with cytopathic effect HPV (col. Pap x 20)
The Collection in the Pathologic Anatomy Lab
"Elena Doamna" Clinical Hospital of Obstetrics Gynaecology Iasi

There are many types of HPV infection:

1. latent forms in the inferior genital tract without any clinical or colposcopic manifestations;
2. subclinic forms: abnormal smears, colposcopic lesions, histopathologic lesions revealed in biopsy;
3. clinical forms: ano-genital warts (acuminate).

THE PURPOSE OF THE STUDY

The purpose of this study was to establish if the infection with human papilloma virus (HPV) has a potential irreversible evolution towards malignancy.

MATERIAL AND METHODS

During 2001–2010, 1885 women were investigated through specific examinations for suspicion of cervical neoplasia.

The data of the present study come from a thorough analysis of the case files or the observation sheets of the patients diagnosed with such a pathology, where we focused on/researched the following aspects according to a previously established protocol:

- epidemiologic (the distribution of cases on years of study, age groups, sex, area of residence, combination of co-morbidities, HPV infection);
- addressability to a doctor, analysis of statistical indicators in Family planning offices in Iasi, Galaţi and Buzău;
- establishing the profile of the patient with pre-invasive cervical lesions;
- possibilities of detecting the pre-invasive lesions of the cervix by cytology, colposcopy, biopsy;
- therapeutic possibilities(prophylactic and curative conduct);
- clinical-progressive manifestations;
- studying the prognostic factors.

RESULTS AND DISCUSSIONS

According to the cases we studied, the HPV infection was present in 7,1% of the patients in the present group that is studied.

In terms of epidemiological characteristics sought we ascertained the following aspects (fig. 2):

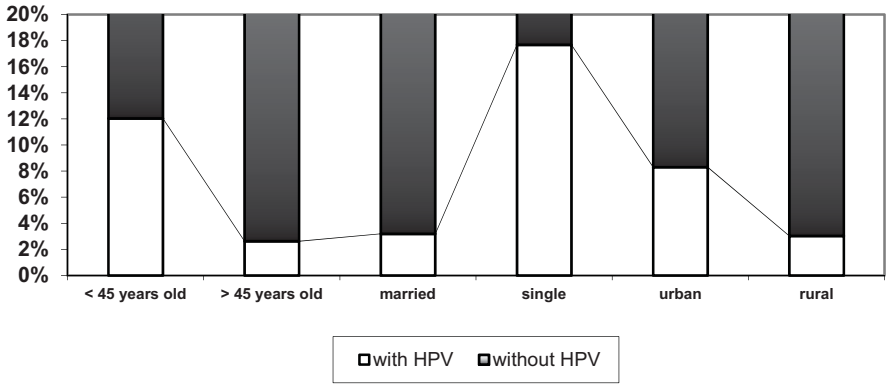

Figure 2. The distribution of the patients in the group that is studied with HPV according to the epidemiologic characteristics

- HPV is present with a predictive positive value of 80,6% for the patients under 45 years old, for whom it represents a risk that is relatively 4,58 times higher than for the patients over 45 (RR=4,58; IC95%: 3,01÷6,95);
- For the single patients the predictive positive value of HPV presence is of 67,2%, with a relative risk 5,53 times higher when compared with married patients (RR=5,53; IC95%: 3,91÷7,81);
- The patients coming from towns or cities have a positive predictive value of the HPV presence of 90,3%, with a relative risk 2,73 times higher than for the patients coming from the rural area (RR=2,73; IC95%: 1,56÷4,80).

Table I. The distribution of cases according to the vaginal examination with valves

Vaginal examination with valves (VEV) This type of examination was performed to all the patients in the group studied.
➢ Soft parts:
▪ episiotomy – 1617 cases (85,8%);
➢ vagina:
▪ condiloma/warts – 145 cases(7,7%)
▪ cystorectocele – 397cases (21,1%) ▪ vaginitis – 1521 cases (80,7%) ▪ stenosis – 1 case (0,05%)
➢ cervix:
▪ multiparous cervix – 481 patients (25,5%) ▪ nulliparous cervix – 127 patients(6,7%) ▪ chronic cervicitis – 378 cases (20,1%) ▪ bleeding exo-cervicitis – 366 cases (19,4%)
➢ discharge/secretion:
▪ purulent – 175 cases (9,3%); ▪ serosanguineous – 69 cases (3,7%).

Table II. Distribution of cases according to the digital vaginal examination

Digital vaginal examination (DVE) The digital vaginal examination is recommended to be used cautiously for the patients who are bleeding, and that is why this kind of examination was only performed to 1282 patients (68%), with the following results:
➢ uterine body:
▪ fibroid uterus – 300 cases (15,9%);
➢ cervix
▪ multiparous cervix – 481 cases (25,5%)
➢ vagina
▪ cistorectocele – 397 cases (21,1%)
➢ annexes
▪ normal – 102 cases (5,4%)

As an immediate result of realizing the gravity of the illness and the long term consequences that are caused by cervical neoplasia following the screening performed (2, 3, 4, 11), during 2010 – 2011 274 people accepted having a vaccine (14,5%), as it follows:

- 224 with Silgard vaccine
- 50 with Cervarix vaccine

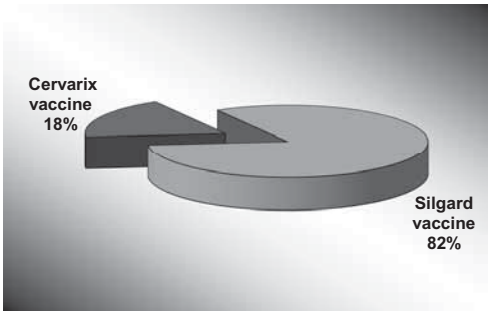

Figure 3. The distribution of patients who accepted vaccination according to the type of the vaccine

The distribution of the vaccinated patients on age groups showed the maximum frequency for the group between 31-35 years old:

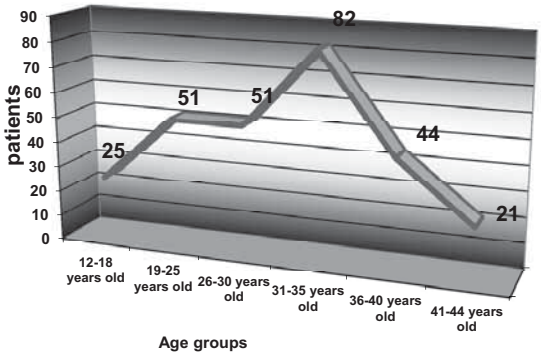

Figure 4. The distribution of vaccinated patients on age groups

There were 35 people from the rural area (12,8%) significantly less than those coming from the urban area (87,2%) (**p=0,00003**), and 152 people were married (55,5%), a significantly increased share when compared with those who are not married (**p<0,001**).

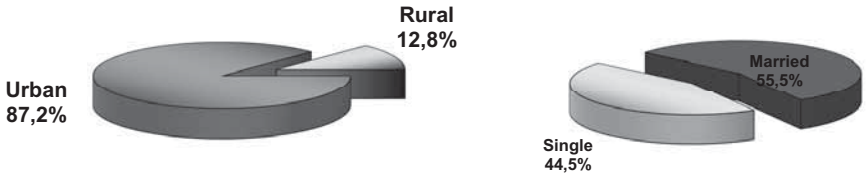

Figure 5. Distribution of vaccinated patients according to their area of origin and marital status

In simple words, the lesions with a low degree are transitory HPV infections that can cause certain cellular changes, but which in most cases disappear spontaneously. Sometimes the HPV infection persists and generates more cellular and tissular abnormalities, which are the high degree lesions. Their presence shows that there was no spontaneous disappearing of the viral infection and hence these lesions must be recognized and treated in order to stop the possibility of developing a cervical cancer (2, 3, 5, 6).

If there is no lesion present, we recommend, if this is possible, to review all the cytologic, colposcopic and eventually histopathologic evaluations made (1, 2, 9).

The patients with ASCUS (Atypical squamous cells of undetermined significance) cytology, who are in the post menopause period, can repeat the cytology at about a week after they have followed local estrogenic treatment. If the patients show clinical or cytological signs of atrophy and do not have any restrictions/contraindications they will use estrogenotherapy. In this case, the clinician can meet the following situations:

- the repeated cytology is negative for a malignant intraepithelial lesion – it is recommended to repeat the cytology every 4-6 months; after 2 negative cytologies the patient will enter a programme of routine screening;
- one of the repeated cytologies is ASCUS type or even more significant than that – colposcopy is recommended here.

For the patients with ASCUS type cytology and also with immunodepressive syndrome, no matter what the viral charge is, antiviral associated therapy is the recommended attitude, followed by an immediate colposcopy.

According to Bethesda 2001 indications regarding clinical conduct, we recommend specifically to investigate ASCH type cytology (Atypical squamous cells - cannot exclude) identical with H-SIL(High grade squamous intraepithelial lesion) smears. However, when the patients with ASCH have a negative histology which does not confirm the existence of the intraepithelial lesion cytologically suspected (because of the equivocal nature of the category ASCH), the doctor must re-assess the colposcopic and histologic aspects (3) before moving on to a more aggressive treatment (electro-surgical procedure of excision).

When facing a cytologic result - type ASCH the recommended conduct is to send it for a colposcopic assessment immediately, no matter if the doctor uses the conventional technique or cytology in a liquid environment.

If the presence of a CIN is confirmed by biopsy, the therapeutic procedure about to be applied will be established according to the severity of the intraepithelial lesion.

If the pluri-disciplinary re-assessment of the case leads to different interpretations, the conduct must change depending on these results.

If the interpretation of the cytologic smear is H-SIL type again, or in the case when it is not possible to re-assess the smear, it is better to choose an excisional diagnosis procedure, especially for the patients who are not pregnant.

The omission of sampling for assessing the endo-cervix is acceptable when it is intended to use an excisional diagnosis procedure immediately.

For women with H-SIL, where the colposcopy suggests a lesion of high degree, the initial assessment made using an excisional diagnosis procedure is also acceptable (British version „*see & treat"* - treatment at first consult following an abnormal cytology) (5).

The sorting that uses either the repeating of the cytology or ADN-HPV determination is not acceptable. The exception to these recommendations are the young patients, of reproductive age (with a satisfactory colposcopy, without the confirmation of a high degree CIN by biopsy and with a negative endocervix assessment), who accept and take the risk of having an occult lesion present that can be supervised for 1 year by colposcopy doubled by cytology repeated every 4-6 months (8, 12).

The excisional diagnosis procedure is recommended if, during one of the examinations, the colposcopy shows a lesion which progresses towards a suggestive aspect of high degree lesion or if the cytology continues to be H-SIL type.

CONCLUSIONS

HPV represents a relative risk 3 times bigger for women under 45 years old, single, coming from the urban area.

The patients benefited from colposcopy and biopsy only if the repeated cytology suggested more severe changes. The conservative conduct is represented by performing a new cytology when accepting the patients in the group (the initial cytology is performed before this moment).

HPV sorting was used in association with a cytologic result, in order to appreciate its effectiveness in selecting the patients with ASCUS, who need colposcopy.

If the cytology showed more severe changes and if the presence of HPV with a high oncogenic risk was proven, the patients were sent to have a colposcopy.

During 2010-2011, following the screening performed, as an immediate result of the fact that people realized the seriousness of this illness and the long term consequences caused by the cervical neoplasia, 274 people accepted vaccination (14,5%).

Considering the scientific evidence concerning HPV infection, the international practice guides and the situation of the morbidity and mortality by cervical cancer in Romania, an ideal screening should cover all women, after a maximum of 3 years after the moment they start their sexual life.

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THE TREATMENT AND EVOLUTION OF CERVICAL CANCER

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Keywords: cervical neoplasia, cytology, colposcopy, biopsy, histopathology

Abstract: The purpose of this study is to establish the evolution of cervical cancer after applying a conventional treatment. Materials and methods. The study was performed on a number of 1249 patients who were suspected of having cervical neoplasia, and who were monitored between 2006-2010 in „Elena-Doamna” Clinical Hospital of Obstetrics and Gynecology in Iaşi, the Military Hospital Galaţi, the County Hospital Galaţi and the Emergency Hospital Buzau. Results and discussions. The study proved the effectiveness of the conservative treatment for the patients who were diagnosed using cytology, colposcopy, biopsy and histopathology, with or without HPV viral infection. Conclusions**.** The patients with an early diagnose have a 15% higher surviving probability. The patients who responded to the conservative preoperative treatment well are more likely to survive than the patients who did not respond favourably to the conservative preoperative treatment.

INTRODUCTION

The activity of early detection of cervical carcinoma is not well organized yet, and that is why we can see that in everyday practice most cases come to the doctor when the cancer is already in a relatively advanced stage, which requires complex laborious interventions and generally having unsatisfying results. When the disease is detected in due time and treated accordingly, the results are very good and lead to a decrease in the specific mortality and make it possible to avoid the mutilating surgical procedures. This way the doctors are able to accomplish the modern medicine goal - *biologic healing of the disease without the physical and/or psychic mutilation of the patient* (1, 4, 6, 7).

According to the data supplied by the Computer and Sanitary Statistics Centre in Romania, over 66% of the new cases of cancer that were detected in 2005 are between 35 and 55 years old, and about 70% of the deaths caused by cervical cancer are registered for women aged between 45 and 70 (11).

Cervical neoplasia is a serious chronic disease of great medical and social importance, with a severe evolution when detected in its advanced stages, being one of the most complex and difficult problems of human pathology (8).

A cytologic result - type H-SIL (High Grade Squamous Intraepithelial Lesion) shows a significant risk for the patient in cause of getting a pre-invasive cervical lesion or even invasive cancer. According to some studies that were analyzed when the recommendations Bethesda 2001 were made - regarding the conduct when meeting H-SIL cytology, the chance for a patient with H-SIL cytology to have a CIN II/III (cervical intraepithelial neoplasia) biopsy of confirmation is around 70-75%, and the chance to have invasive cancer is about 1-2% (10).

THE PURPOSE OF THE STUDY

The purpose of this study is to establish the evolution of cervical cancer after applying a conventional treatment.

The study tries to establish some correlations between elements of epidemiology and the histologic diagnosis for every batch of cases, underlining the risk factors that are frequently met in cervical neoplasia.

We want to see the clinico-progressive particularities according to the pathogenic mechanism, associated comorbidities, doctor addressability, precocity of diagnosis and establishing of treatment and we will decide the prognostic factors involved in causing cervical cancer.

MATERIAL AND METHODS

The batch to be studied was made of 1249 patients, all monitored for suspicion of cervical neoplasia in „Elena-Doamna” Clinical Hospital of Obstetrics and Gynaecology Iaşi.

The criteria to be met for being accepted in this group: a deteriorated health state; suspicion of cervical neoplasia; free consent of the patient; willingness to come back for a subsequent medical check-up; access to a telephone and emergency transportation; willingness to support/have a surgical procedure if the medication fails to have positive results; lack of allergic reactions to the substances used in the study.

The conservative conduct is represented by the fact that patients repeat the cytology when admitted into the batch (the initial cytology was made before this moment).

That is why, the traditional conduct which is recommended as optimum in the case of an H-SIL cytology consists of performing a colposcopy associated after that with the cervix evaluation/examination.

The purpose of all these steps is to see if:

- There is or there is not a lesion that can be colposcopically identified which is also susceptible of suggesting changed cells that correspond to an H-SIL cytology;
- The colposcopy is or is not satisfactory
- The immediate diathermal excision is suitable or not for the case.

RESULTS AND DISCUSSIONS

The share of the patients who were treated in a conservative manner shows the following aspects (tab. I):

Table I. Statistical differences for the patients with a conservative treatment according to the epidemiologic characteristics and diagnosis

Parameters analyzed	Conservative treatment				Statistical significance
	yes		no		
	n	%	n	%	
Age group					
< 45 years old	568	30.1	329	17.5	χ^2=6.36; GL=1; **p=0.012**
≥ 45 years old	681	36.1	307	16.3	
Area of origin					
Urban	921	48.9	536	28.4	χ^2=26.07; GL=1; **p=0.0000003**
Rural	328	17.4	100	5.3	
Marital status					
Married	857	45.5	519	27.5	χ^2=35.41; GL=1; **p<0.001**
Single	392	20.8	117	6.2	
Viral testing					
HPV (+)	89	4.7	45	2.4	χ^2=0.0; GL=1; p=0.956
HPV (-)	1160	61.5	591	31.4	
Cytologic examination					
High degree	848	45.0	412	21.9	χ^2=1.71; GL=1; p=0.191
Reduced degree	401	21.3	224	25.3	
Colposcopic examination					
Yes	272	14.4	87	4.6	χ^2=17.40; GL=1; **p=0.00003**
No	977	51.8	549	29.1	
Biopsy					
Yes	704	37.3	154	8.2	χ^2=174.36; GL=1; **p<0.001**
No	545	28.9	482	25.6	
Histopathologic examination					
Yes	707	37.5	138	7.3	χ^2=206.21; GL=1; **p<0.001**
No	542	28.8	498	26.4	

- 54.5% patients are over 45 years old;
- 73.7% patients come from cities or towns (urban area);
- 68.6% patients are married;
- for 67.9% of the patients treated in a conservative manner the cytodiagnosis was high in degree;
- 21.8% of the patients had colposcopy performed;
- biopsy was performed for 56.4% of the patients treated in a conservative way;
- 56.6% of the patients treated in a conservative manner were histopathologically diagnosed

The effectiveness of the conservative treatment for the patients diagnosed by cytology, colposcopy, by biopsy and histopathology, with or without viral infection with HPV, is shown by drawing the ROC curve (tab. II, fig. 1).

Table II. Comparative analysis of accuracy according to the conservative treatment

Diagnostic	VPP (%)	VPN (%)	Sensibility (%)	Specificity (%)	Accuracy	p
HPV (+)	7.1	7.1	13.1	3.7	8.4	0.956
H-SIL	67.9	64.8	79.1	50.7	64.9	0.191
Colposcopy	21.8	13.7	33.1	8.2	20.6	0.00003
Biopsy	56.4	24.2	59.4	22.0	40.7	<0.001
Histopathology	56.6	21.7	58.7	20.3	39.5	<0.001

ROC curve shows better accuracy of the treatment after establishing the cytologic diagnosis of high degree (64.9%) and also in the case of biopsy (40.7%). The statistical analysis according to the HPV viral infection, for the patients treated in a conservative manner, did not lead to any significant conclusions that could allow the extrapolation of results.

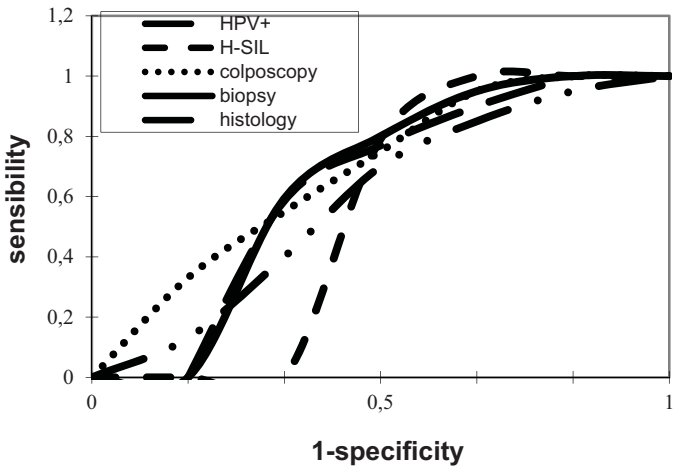

Figure 1. ROC curve - the assessment of conservative treatment effectiveness

The evolution of cases with cervical neoplasia diagnosis

Usually the colposcopic evaluation, following immediately after an H-SIL cytology leads easily to the identification of the high degree cervical or vaginal lesion, the positive predictive value of the colposcopy being really high in this situation (4, 5, 6, 9).

But there are situations when the high degree cervical or vaginal lesion cannot be identified **colposcopically**. So, after the colposcopy and the evaluation of the endo-cervix the following clinical situations can occur:

-There is a cyto-colpo-histologic discordance where we have:

- H-SIL type cytology
- a satisfactory colposcopy, but one that does not identify the presence of a lesion
- negative biopsy or showing changes that would qualify for CIN I at most;

-it is advisable to review the cytologic and histologic smear whenever this is possible and also to perform the colposcopic examination.

-There is a cyto-colposcopic discordance where we have:

- H-SIL type cytology
- unsatisfactory colposcopy, that does not identify the presence of a lesion
- negative biopsy or showing changes that would qualify for CIN I at most;

-it is recommended to reassess the cytologic and colposcopic results and it is imperative to also have a histologic evaluation.

According to **neoplasia staging** you will notice the following aspects (fig. 2):

- for the patients diagnosed in stage II, the probability of survival decreases in the first year after being diagnosed to 65%, after 2 years it decreases to 30% and is theoretically null after 7 years from the moment when it was diagnosed;
- for the patients diagnosed in stage III, the probability of survival decreases in the first year after being diagnosed to 60%, after 3 years it decreases to 30% and is theoretically null after 6 years from diagnosis;
- for the patients diagnosed in stage IV, the probability of survival decreases in the first year to 50%.

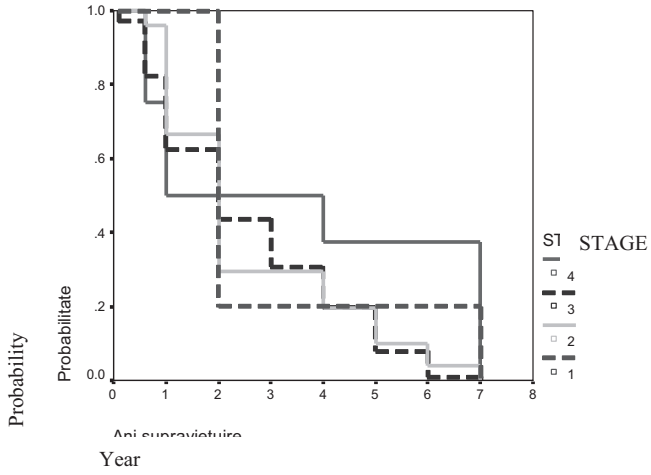

Figure 2. Survival of the patients with cervical neoplasia according to staging

The patients who responded the conservative pre-operative treatment are more likely to survive when compared to the patients who did not respond favourably to the conservative pre-operative treatment; the latter have a probability of survival of at most 5 years after being diagnosed with cervical neoplasia (fig. 3).

The probability of survival decreases to about 45% in the first year after diagnosis for the patients with post-operative complications (fig. 4).

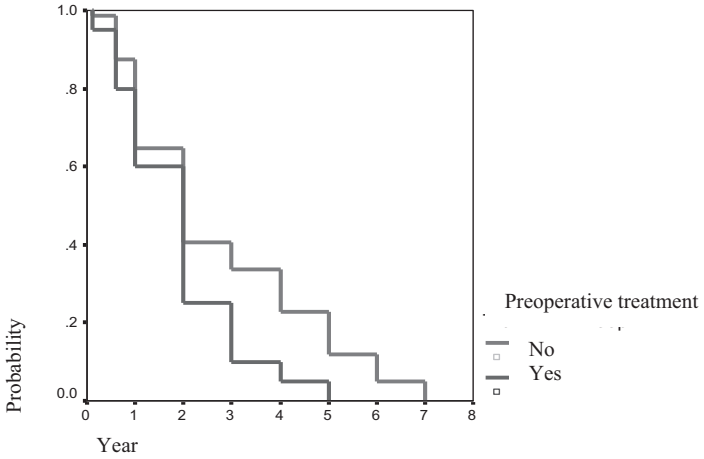

Figure 3. Survival of the patients with cervical neoplasia according to the conservative preoperative treatment

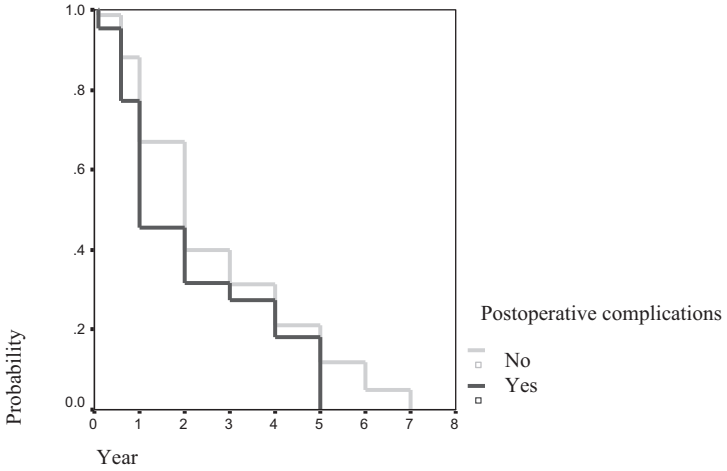

Figure 4. Survival of the patients with cervical neoplasia according to the postoperative complications

CONCLUSIONS

The conservative conduct is represented by a repeated cytology when the patients are admitted into the batch of study (the initial cytology is performed before this moment). The patients benefited from colposcopy and biopsy only if the repeated cytology suggested more severe changes.

The immediate colposcopy, followed by biopsy when needed, represents an aggressive manner of therapeutic conduct.

The sorting of HPV (Human immunodeficiency virus) was used in association with a cytologic result in order to appreciate its effectiveness in selecting the patients with atypical squamous cells with an undetermined significance that need colposcopy. If the cytology showed more severe changes or if it found the presence of a high oncogenic risk HPV, the patients were directed towards colposcopy.

The patients who were early detected have a 15% higher survival probability. The patients who responded to the preoperative conservatory treatment have a higher survival probability than the patients who did not respond favourably to the preoperative conservative treatment.

The comparison of the three methods allowed us to find out:

- the effectiveness of each of the conduct options in early detection of the serious changes that can progress to cancer;
- how acceptable each of the conduct options is for the patients;
- which is the ratio cost/effectiveness corresponding to each option.

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INDIVIDUALIZED TREATMENT OF PREINVASIVE LESIONS OF THE CERVIX

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Keywords: preinvasive lesions, cervical cancer
Abstract: The management of cervical preinvasive lesions in primary medical practice is characterized by a wide variety of attitudes, objectives and therapeutic decisions. The purpose of the study**.** To supply new landmarks referring to the preinvasive lesions of cervical cancer from the perspective of the clinician. Materials and methods. The study was made on a number of 16732 patients who were monitored comparatively in the Family Planning offices in the counties of Iaşi and Buzău, between 2007 and 2011. Results and discussions. The treatment was applied according to the type of the lesion. Conclusions**.** The individualized treatment of the preinvasive lesions of the cervix targeted the destruction of the pathogenic agent and the infected tissues and caused a fibroblast proliferation and re-epithelisation.

INTRODUCTION

If we want to have a correct conduct in the preinvasive lesions of the cervix we must have accordance between the colposcopic pictures, the cytologic reflection and the histopathologic substratum. We also need experiences specialists who are able to individualize treatment for every clinical form, to consider the risk factors, the way of communicating with the patient and the possibilities of monitoring her. It is preferable to have a more”aggressive” conduct with the patients who cannot come to a periodical consult, or with the ones having a precarious socioeconomic level. Without standardizing the conduct, we present therapeutic possibilities in different lesions of the cervix.

PURPOSE OF STUDY

The management of the preinvasive lesions of cervical cancer in primary medical practice is characterized by a wide variety of attitudes, objectives and therapeutic decisions. There is a tendency to use the paraclinical data excessively for establishing the diagnosis, and also to misuse the diagnosis methods in first-line therapy, and this imposes the re-analysis of an effective strategy, based on evidence, of diagnosis and treatment.

The present work wants to establish new landmarks referring to the preinvasive lesions of the cervical cancer from the perspective of a clinician; to facilitate the access to health services, including family planning, by using the primary care system – which is one of the main goals meant to improve the quality of life for all the people. We want to prove the effectiveness of the balance cost/benefit by introducing the preventive prophylactic conduct in Family planning offices.

MATERIAL AND METHODS

The study was performed on a number of 16732 patients that were monitored comparatively in Family Planning offices in the counties of Iaşi and Buzău, between 2007 and 2011. The medical treatment, which can be local and general, depending on the pathogenic agent identified, was established as it follows: after a case is accepted for study it is assessed, and a Papanicolaou test is performed, recommending a local trophic therapy and a medical check-up every 6-12 months, or minimally invasive surgical treatment: electrocautery or electrocoagulation with monopolar electrode, if the symptoms persist and the PAP test is changed. The purpose of these methods of treatment is to destroy the pathogenic agent and the infected tissues and to cause a fibroblastic proliferation and re-epithelisation.

RESULTS AND DISCUSSIONS

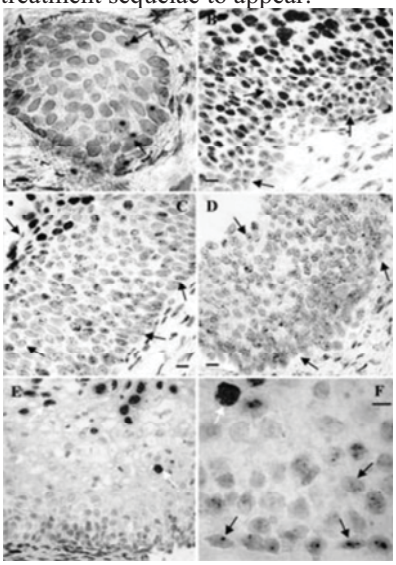
Depending on the type of the lesion identified we applied the following therapeutic schemes:

Lesions type CIN1 (cervical intraepithelial neoplasia)

Under the name LSIL/CIN1 (low grade squamous intraepithelial lesion) you can find: simple dysplasia and plan condiloma, which reflects a benign process, associated to the cervical infection with a heterogeneous group of HPV (1, 4, 5).

The main data that must be considered when choosing the therapeutic alternatives are (7):

- the degree of uncertainty of the diagnosis;
- the biologic potential of spontaneous regression without treatment and a decreased rate of progression towards neoplasia;
- the absence of a certain method for identifying the CIN lesions, which will regress spontaneously, persist or progress;
- the quality of the cooperation with the patient for a systematic surveillance;
- the possibility to develop a cervical lesion after regression; the risk is appreciated to be 23 times higher when compared with patients without cervical lesions;
- the age of the patient and the size of the lesion;
- the patient' s anxiety and the medical responsibility;
- the possibility for post treatment sequelae to appear.



Picture 1. Perinuclear cytoplasmic vascularisation, cytoplasmic membrane thickening, presence of nuclear atypia (big nucleus, increased nucleo-cytoplasmic ratio, hyperchromasia, irregular nuclear membrane, anisocytosis, koilocytosis) (personal collection – Dr. Dorin Neacsu)

Conduct

There are two options: supervision of the patients with treatment or without treatment.

A.Supervision of the patients without treatment.

It is an option that must be supported by a complete clinical and cyto-colpo-histologic balance sheet, in relation with the clinician' s experience and the patient's anxiety from diagnosis, the lesional prognosis.

Supervision of the patient and treatment timing:

- Avoids the useless treatment;
- Consists of repeated cytologic and colposcopic examinations;
- Does not confer prognostic safety for the patients;
- You can recommend the biopsy to be repeated after 6 months.

The classic protocol of surveillance consists of cytologic surveillance every 6-12 months and an associated cytology-colposcopy also every 6-12 months and determining the ADN-HPV after 12 months from the CIN1 biopsy.

The supervision is recommended to last for 2 years, in which interval there is a spontaneous regression or the lesion progresses to CIN2 or CIN3 (6).

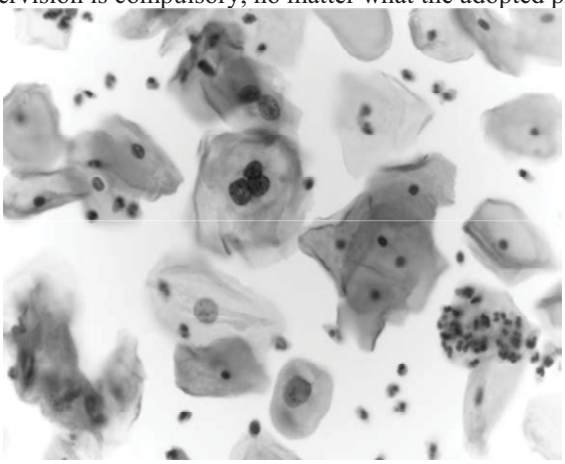
B. Curative treatment

It has as purpose to make sure there is a high rate of long term healing, to reduce the risk of progression of a lesion and also to protect the patient' s psychic.

This treatment recommends the destructive methods: electrocautery, cryotherapy, laser vaporization, excisional procedures like cone biopsy.

Cone biopsy has a more restricted indication in CIN1 type lesions, but in the cases where there are differences among the cytologic, colposcopic and biopsic diagnosis, most authors recommend exerese, and the histopathologic diagnosis to be repeated (5,7). The same conduct is recommended if CIN1 lesions persist after applying another local treatment or if there are any recurrencies.

Postoperative supervision is compulsory, no matter what the adopted protocol is.



Picture 2. Lesions CIN2 and CIN3 type (5, 7)

Altered stratification with the loss of cell polarity; alteration of architecture in the whole thickness of the epithelium; basophilia and alteration of the cytoplasmatic difference; hyperchrome and hypertrophic nuclei; ratio nucleus/cytoplasm in favour of the nucleus; frequent mitoses in the deep and intermediate layers; integrity of the basal membrane.

CIN2 and CIN3 type lesions are also called high degree lesions; they group the moderate and severe dysplasia. These lesions have the highest degree of progression to an invasive cancer. Their frequency increased significantly for the age group 20-29 years old when compared to the age group over 40; it is also unanimously accepted that HPV positivity is over 76%, 8 oncogenic types being incriminated: HPV-16, 18, 31, 33, 35, 45, 52, 58, the most frequent being HPV-16 (2,10).

Cytology can identify smears like: ASCUS (Atypical squamous cells of undetermined significance), LSIL (Low grade squamous intraepithelial lesion), but the most frequent is HSIL (High-grade Squamous Intraepithelial Lesion).

The natural evolution of intraepithelial lesions of high degree that are not treated can lead to regression, persistence, progression.

Badea[14] presents the result of the studies he published, reviewed and quoted in his argument, for Bethesda conduct recommendations, referring to the evolution of CIN2 and CIN3 lesions that are not treated:

	Regression	Progression	Persistence
CIN2	43%	22%	38%
CIN3	32%	14%	56%

We can conclude that CIN lesions of high degree have a higher evolution to progression or persistence rather than to regression. Thus we are justified to recommend an interventionist conduct both for CIN2 and CIN3 type lesions that will be able to perform ablation of the pathologic epithelium.

Conduct

The theory according to which the excision of the lesion is the only fair treatment is unanimously accepted. This ablation can be made by using many procedures (3):

A. *Cone biopsy*

The advantages of choosing cone biopsy are:

- Histopathologic examination of the whole affected area, avoiding the risk of a poor harvest of the tissue specimen from the peripheral area of the lesion and skipping the analysis of a micro-invasive or invasive lesion;
- You avoid the incomplete, local treatment of a sub-evaluated lesion;
- The lesions are extended on a wide area and/or ascended towards the endo-cervix;
- A micro-invasion is suspected based on the previous results of cytology, colposcopy and biopsy;
- It reduces "cervix mutilation" at minimum;
- It protects the function of the endocervical glands and keeps tissue around the endocervical canal;
- It solves the cases that did not respond to other local destructive therapies;
- It can eliminate microinvasion;
- The necessary equipment is cheap, the cost is reduced and it can also be performed outpatient, with local anaesthesia.
- The relapses are avoided if the intervention is performed correctly.

B. Cervix amputation

This extensive technique is indicated when there are also other pathologic elements associated such as:

- cervix lengthening;
- hypertrophy of the vaginal portion of cervix;
- cervix with old, irregular ruptures;
- second degree prolapse that needs to be corrected.

C. Total hysterectomy

Total hysterectomy made the vaginally or abdominally is a rare indication in CIN3 lesions. It is justified in certain circumstances:

- associated benign gynaecologic pathology, uterine fibroids, endometriosis, uterine prolapse;

- microinvasive carcinoma on the cone biopsy piece;
- the difficult supervision of the patients and/or inefficient communication with them;
- extended lesion on the vaginal vault;
- cancerophobia.

D. Systemic medication for increasing immunity (Isoprinosine) with a role in increasing the local T lymphocytes.

Supervision

No matter what therapeutic method the doctor applies, the patients must be instructed to respect the following indications:

- sexual rest for 6 weeks;
- external local hygiene without vaginal lavages;
- avoid physical effort and prolonged standing;
- medical check-up after 6-8 weeks.

The patients must also be informed that in the first 10-12 days they will notice a special vaginal discharge, sometimes with an unpleasant smell, which is part of the healing process of the destroyed tissue that covers with a crust a part of the exo-cervix. It will be eliminated at the end of this interval when it is possible to cause a small bleeding that will not last long. If the bleeding is more consistent the patient must go to the doctor for a consult.

Post-treatment supervision

All the methods used in treating CIN lesions have a margin of error; that is why the patient supervision plays an essential role in assessing the healing and detecting some possible residual or recurrent lesions that are susceptible to become invasive cancer.

The residual lesion is the persistence or appearance of CIN type lesion in the first 12 months after treatment, and the recurrent lesion is the appearance of a lesion many years after a conservative treatment is performed.

There are many supervision protocols, but they have not made a randomized prospective study which to compare their effectiveness.

In order to be realistic, a protocol must consider the therapeutic results in CIN lesions, the possibility of having regular medical check-ups and also compliance (the interest manifested by the patient for a systematic supervision).

The supervision of the cases must be done by clinical, cytologic and colposcopic examination.

The first cytologic examination is recommended to be made after 12 weeks. If it is performed earlier, the metaplasia reparatory processes can be mistaken for some pathologic elements. It is also recommended for the patient to have a colposcopic examination in order to establish the location of the new squamo-cylindric junction. If it is ascended in endocervix it is recommended to make micro-colpohysteroscopy or endocervical curettage of control.

The frequency of the following examinations varies depending on author: 1 check-p every 6 months – minimum 3 years, 3 check-ups every 3 months, then every year for 5 years, after 3 months, 6 months, then every year etc., until obtaining 3 negative cytologic results. Some authors also recommend ADN-HPV at 6 months after treatment (8,10,11,12).

Treatment of cervical preinvasive neoplasias for the patients infected with HIV (Human immunodeficiency virus)

Preinvasive and invasive cervical neoplasia in patients infected with HIV is treated generally after the same principles and protocols, but still there are certain particularities because of the

way the lesions evolve, especially prognosis, because the number of spontaneous regression of dysplasia is small, and that of recurrence and evolution to CI is much bigger.

The standard treatment by ablation is recommended to be first line in LSIL lesions associated with HIV-HPV. Excisional procedures have minimum benefit, because of the high rate of recurrence. Probably immunosuppression makes healing difficult, and the persistence of HPV infection is responsible for the recurrence of dysplasia lesions.

After excision, 77% out of 43 de patients HIV positive and 19% out of 103 seronegative patients have recurred. In the cases when, after excision, the margins of the specimen are positive, recurrence is 100% for the patients who are HIV positive and 32% for the seronegative ones.

Globally, the rate of recurrence of dysplasia for the patients who are HIV positive is of 62% after the first treatment, 43% after the second treatment and 50% after the third excisitional treatment (9).

These adjuvant therapies were proposed:

- 5-Fluorouracil topic (5-Fu) after LLETZ electroexcision (large loop excision of the transformation zone) - recurrence 28% for short term while 47% of the patients treated only with LLETZ;
- Imunomodulator Imiquimod (INN) seems to be more effective as topic treatment;
- HAART (highly active antiretroviral therapy) seems to be the medical therapy that influences the most the evolution of cervical dysplasia for the patients with HIV by partially re-establishing their immunocompetence.

The multivariable analysis of the results of a French study showed that the patients who are not treated with HAART have a risk of recurrence that is twice as high after an excisional treatment; on the other hand this association can increase the global prevalence of CIN for these patients by increasing the life span.

Local medication:

- Antimitocites: Podofiline, Condyline, Colchicine
- Destructive chemical agents: trichloroacetic acid, dicloracetic acid, nitrous oxide;
- Antiviral: Interferon - topic application, intralesional Cidofovir.

CONCLUSIONS

No matter what the applied treatment, every category of patients must be monitored systematically, at an interval of maxim three months.

We noticed that, without organizing and selecting the patients with risk, with preinvasive cervical lesions, so that they can be able to benefit from a confirmation of the diagnosis by colposcopy, biopsy and/or finding ADN-HPV, we cannot apply a proper conduct in accordance with the consensus guides.

Anti-HPV vaccination can be considered a partner of the screening programme for reducing the incidence and decreasing mortality caused by cervical cancer.

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EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND HUMAN PAPILLOMAVIRUS (HPV) L1 CAPSID PROTEIN IN CERVICAL SQUAMOUS INTRAEPITHELIAL LESIONS

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Keywords: EGFR, L1 capsid protein, HPV infection, squamous intraepithelial lesions (SIL)

Abstract: We analyzed the immunohistochemical pattern of epidermal growth factor receptor (EGFR) in cervical squamous intraepithelial lesions (SILs) in correlation with L1 HPV capsid protein, in order to determine the relationship between EGFR expression and the infection status of human papillomavirus (HPV). The study included 40 cases, 24 LSIL (low grade SIL) (CIN1, cervical intraepithelial neoplasia) and 16 HSIL (high grade SIL) (6 cases of CIN2 and 10 cases of CIN3). The immunoexpression of L1 HPV protein was assessed on conventional cervico-vaginal smears and EGFR was immunohistochemically evaluated on the corresponding cervical biopsies. The HPV L1 capsid protein was expressed in 45.83% of LSIL and 25% of HSIL. EGFR was overexpressed in 62,4% of HSIL (58,4% CIN2 and 41,6% CIN3) and 37,6% LSIL. The immunoexpression of L1 HPV has clinical application in the progression assessment of the cervical precancerous lesions without a correlation to the grade of the cervical SIL. EGFR is expressed by all proliferating squamous epithelial cells, thus corresponding with the grade of SIL. The evaluation of EGFR status, correlated with L1 HPV protein expression, can provide useful data of progression risk of cervical squamous intraepithelial lesions.

INTRODUCTION

EGFR (epidermal growth factor receptor) is a potent angiogenic factor present in different tumors, which seems to have, together with c-erbB2 (HER2/neu) and c-myc, an important role in prognostic of advanced cervical cancer. Epidermal growth factor receptor (EGFR) is a member of the ErbB family, the tyrosine kinase receptors with growth promoting effects (Rogers et al, 2005). Human EGFR gene is localized on chromosome 7 and encodes a surface transmembranar glycoprotein which binds EGF (epidermal growth factor), transforming growth factor-α (TGF-α), amphiregulin, and HBEGF (heparin-binding growth factor). HPV-E5 oncogene may be envolved in EGFR activation and this can be done without concomitant increase of receptors’s number (Pim et al, 1992; Gonzales, 2007). HPV-E6 oncogene may determine afterwards the increase of EGFR mARN level and the stabilization of the protein, thus, increasing the signal transduction in the cells. HPV-E5 determines an acceleration of HER2/neu (c-erbB2) gene protein activation. EGFR is expressed in several carcinomas (Janinis et al, 1994; Nakopoulou et al, 1995) and high levels of expression are a common feature of the malignant phenotype in many solid human tumors (Bianco et al, 2007).

HPV L1 capsid protein is expressed in the active phase of the viral infection and is necessary in viral cellular cycle completion. Consequently, viral protein detection, by immunohistochemical reaction is an evidence of active HPV infection in examined tissue (Gu et al, 2007). L1 viral capsid protein is considered a major target of the cellular immune response (Melsheimer et al, 2003). LSIL and moderate SILwithout immunohistochemically detected L1 are correlated, in more than 80% of cases, with dysplasia progression. Moser and co. certify these aspects, evidentiating that minor and moderate lesions without L1 capsid protein expression are significantly more exposed to a progression in comparison to L1 positive cases (Griesser et al, 2004). Most probably, the lack of HPV antigen is determined by a weak protein synthesis, under immunohistochemical test minimum level. As L1 represents the major target of the immune cellular response (Steele et al, 2002), its deficient translation may result in an inefficient epuration of the infected cells, promoting viral DNA integration in host cellular genome and the transformation of immature epithelial cells. The observation that the decrease of the HPV16 capsid positivity in cervical cancer patients serum is an indicator of a poor prognosis sustains the importance of a specific humoral response. Immunohistochemical detection of L1 capsid, on Papanicolau smears, may consequently indicate the defence status locally induced on HPV infection and may offer prognosis information in different squamous intraepithelial lesions.

The purpose of the present study was to analyze the immunohistochemical pattern of epidermal growth factor receptor (EGFR) in cervical squamous intraepithelial lesions (SILs) in correlation with L1 HPV capsid protein, in order to determine the relationship between EGFR expression and the infection status of human papillomavirus (HPV).

MATERIALS AND METHODS

The present study involved 40 women with cytological and histopathological confirmed LSIL (low grade SIL) (CIN1, cervical intraepithelial neoplasia) (n=24) and HSIL (high grade SIL) (6 cases of CIN2 and 10 cases of CIN3) (n=16). The immunoexpression of L1 HPV protein was assessed on conventional cervico-vaginal smears and EGFR was immunohistochemically evaluated on the corresponding cervical biopsies.

The cervico-vaginal smears were fixed and stained with Papanicolaou method. After cytodiagnosis, the cervico-vaginal smears were used to detect HPV L1 capsid protein by immunocytochemistry, using the monoclonal antibodies (Cytoactiv HPV L1 High Risk Set REF SCA0850, Cytoimmun Diagnostics GmbH) in a standardized protocol. Epithelial cells with positive nuclear staining were scored as positive, considering one stained nucleus enough for scoring.

The tissue sections were obtained from the cervical biopsies. The cases were investigated by routine histopathological exam and by immunohistochemistry, using EGFR antibodies. Collected tissues were fixed for 24 hours in buffered formalin and processed for paraffin embedding. Serial sections of 4–5 μm were dewaxed and stained with Hematoxylin–Eosin, or furthermore prepared for immunohistochemistry.

Proteinase-induced epitope retrieval (PIER) technique was performed using Proteinase K (code S 3020, DAKO, Denmark), for 5 minutes at room temperature. After blocking the endogenous peroxidase and non-specific binding, the sections were incubated with the primary antibodies, anti-EGFR mouse monoclonal antibody (clone E30, code M7239, DAKO, Denmark), dilution range 1:50, for 30 minutes, at room temperature. The immune reaction was amplified using the appropriate secondary antibody and the Streptavidin–Biotin– Peroxidase HRP complex (code K5001, DAKO, Denmark). Sections were then developed using 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB, code K5001, DAKO, Denmark), under microscope control. The sections were finally counterstained with Lille's modified Mayer's Hematoxylin and mounted.

Quality control performed by external and internal negative and positive controls was necessary to monitor the accuracy of tissue processing, staining procedures and reagents effectiveness. The primary antibody specificity sought to be assesed by their negative controls.

EGFR immunohistochemical expression was quantified according to the EGFR PharmDx scoring guidelines. Thus, the complete or incomplete circumferential membranous staining in $\geq$1% of squamous cells was considered positive. The absence of membrane or cytoplasmic staining was reported as negative. The immunostaining was scored as follows: 1- weak, 2- moderate, and 3 - strong. The percentage of stained cells was assessed as follows: 1–10, 10–50, and >50%.

RESULTS

In our study, the histopathological diagnoses were consistent with the cytodiagnoses, as follows: 24 cases with LSIL (CIN1) and 16 cases with HSIL (6 cases CIN2 and 10 cases CIN3).

HPV infection was morphologically confirmed by the presence of cytopatic HPV effect (koilocytes) in the smears and biopsies.

From all cervical smears, the HPV L1 capsid protein was expressed in 45,83% of LSIL and 25% of HSIL.

The positive reaction was evidentiated by the strong staining of the whole nucleus, surrounded by a cytoplasm with no background. In most cases, positive reaction for HR-HPV L1 was positive in typical koilocytes or in dyskeratocytes, presenting nuclear characteristics for HSIL (CIN 2 or CIN 3). In LSIL cases, the positivity of the nuclei was presented only in typical koilocytes (figures 3,8).

From all cervical biopsies, EGFR was overexpressed in 62,4% of HSIL (58,4% CIN2 and 41,6% CIN3) and 37,6% LSIL.

The EGFR staining pattern was predominantly membranous with occasional cytoplasmic positivity. Most cases presented heterogeneity of staining, with positive cells admixed with negative cells.

The proportion of biopsies with intense immunoexpression of EGFR increased with the severity of cytological abnormality. EGFR staining was observed in basal and parabasal cells, in koilocytes and in dysplastic squamous cells of the intraepithelial lesions. In HSIL cases, the

staining distribution was as follows: 74% full thickness (figures 5,6,7), 26% basal and intermediate (figure 4). The staining intensity for HSIL cases was strong in 85% (figures 4, 6), moderate in 10% (figures 5,7), and weak in 5% accordingly. The immunostaining was more intense in CIN2 lesions than in CIN3. Regarding LSIL category, the staining distribution was in basal and parabasal cells and in koilocytes. The staining intensity of LSIL cases was strong in 10%, moderate in 68% (figures 1,2), and weak in 22%.

DISCUSSIONS

The sequence of morphologic events in the genesis of invasive cancer of the uterine cervix was thoroughly studied. A progression of intraepithelial lesions from slight to marked and furthermore to invasive cancer has been postulated (Koss and Melamed, 2006; Cain and Howett, 2000). Although a transformation of the initial low grade lesions to high grade lesions may occur, it is a relatively uncommon event. Most high-grade lesions develop independently in adjacent segments of endocervical epithelium (Koss and Melamed, 2006).

Cervical carcinoma arises in women who present a persistent infection with a high risk HPV type and progresses through a multistage process of carcinogenesis (Schoell et al, 1999).

L1 capsid protein is expressed in the active phase of HPV infection and is necessary in viral cellular cycle completion. Viral protein detection, by immunohistochemistry is an evidence of active HPV infection in cells and tissues (Gu et al, 2007). LSIL and moderate SIL (CIN2) without immunohistochemical detected L1 are correlated, in more than 80% of cases, with dysplasia progression (Fiedler et al, 2006). Mild and moderate lesions without L1 capsid protein expression are significantly more exposed to a progression in comparison to L1 positive cases (Griesser et al, 2004). Most probably, the lack of HPV antigen is determined by a weak protein synthesis, below the minimum level of the immunohistochemical test.

From all cervical smears, our data revealed that the HPV L1 capsid protein was expressed in 45,83% of LSIL and 25% of HSIL. Expression of L1-capsid proteins was significantly reduced for HPV positive HSIL. In HPV positive LSIL, no significant reduction of L1 capsid protein expression could be demonstrated. As we previously mentioned, because of the low rate of HR-HPV L1 positivity found in LSIL cases in our study, we can admit that HPV is not helpful in grading cervical SIL, which is in accordance with the literature data (Yildiz et al, 2007).

In our study, patients’ mean age was 28 years. This fact is consistent with the literature data, where it has been demonstrated that the prevalence of HPV infection varies with age and geographical region, reaching highest rates below 35 years of age (Cox, 1999).

The presence of L1 immunopositive nuclei of squamous epithelial cells could be correlated with the clinical course. Expression of L1-capsid proteins was significantly reduced for HPV positive HSIL. In HPV positive LSIL, no significant reduction of L1 capsid protein expression could be demonstrated. Mild and moderate dysplastic cervical lesions without immunohistochemical positive reaction of HPV L1 capsid protein are more likely to progress as compared to positive cases (Griesser et al, 2004). Researches consider that lack of detectable HPV antigen in the Pap smears is due to low protein synthesis in squamous epithelial cells below the limit of the immunocytochemical test. The loss of L1 capsid protein immunoexpression can be the result of the integration of the viral DNA into the human genome. Although most of cervical carcinomas show integration of viral DNA, it is detectable only in a small proportion of LSIL and HSIL (Klaes et al, 1999).

The development of HPV capsid antigen L1 depends upon transcriptional factors, which only can be expressed during maturation process from basal epithelial cell to superficial epithelial cell (Gu et a, 2007). In HSIL, the normal structure as well as maturation of the epithelium are disturbed, thus the dysplastic basal squamous cells represent the predominant cell type with reduced L1 capsid protein expression.

In our study, from all cervical biopsies, EGFR was overexpressed in 62,4% of HSIL (58,4% CIN2 and 41,6% CIN3) and 37,6% LSIL. The EGFR staining pattern was predominantly membranous with occasional cytoplasmic positivity. Most cases presented heterogeneity of staining, with positive cells admixed with negative cells.

The proportion of biopsies with intense immunoexpression of EGFR increased with the severity of cytological abnormality, which is in accordance with the majority of reports on EGFR expression (Magkou et al, 2008).

EGFR staining was observed in basal and parabasal cells, in koilocytes and in dysplastic squamous cells of the intraepithelial lesions. This is in accordance with previous studies, which affirm that EGFR is expressed by all proliferating squamous epithelial cells and as such correlates with the grade of SIL (Chapman et al, 1992).

Regarding HSIL lesions, the immunostaining was more intense in CIN2 lesions than in CIN3. These findings are in concordance with the literature data, which assert that the elevated expression of EGFR may be linked to the neoplastic state of the cervical squamous epithelia (Wistuba et al, 1994).

Findings from previous studies confirm that EGFR staining is mainly membranous and observed in basal and parabasal cells, in normal squamous epithelium and atypically proliferating keratinocytes in CIN and nonkeratinizing cells of cervical carcinoma (Mittal et al, 1990) . These are in accordance with our study, indicating a possible role of EGFR in neoplastic proliferation and differentiation of the cervical epithelium.

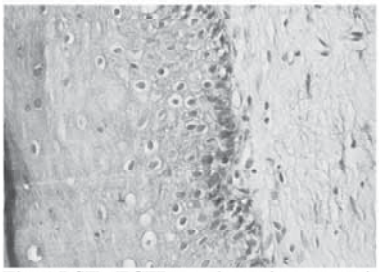

Fig. 1 LSIL, EGFR, moderate immunostaining x 10

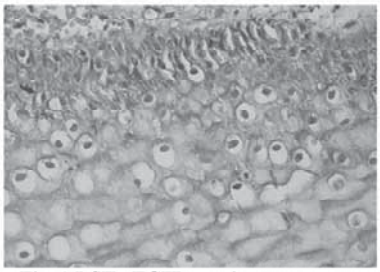

Fig. 2 LSIL, EGFR, moderate immunostaining x 20

Fig. 3 LSIL, HPV L1 positive, x 20

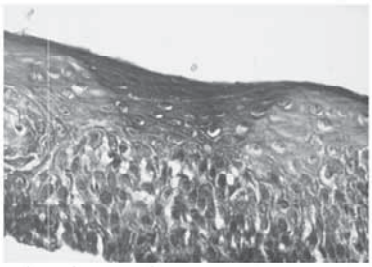

Fig. 4 HSIL (CIN2), EGFR, strong immunostaining x 10

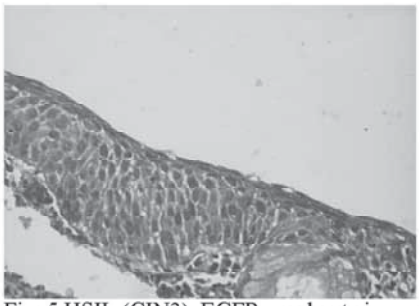

Fig. 5 HSIL (CIN3), EGFR, moderate immunostaining x 4

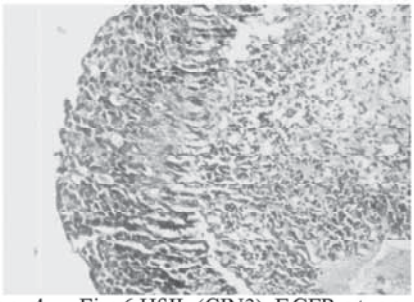

Fig. 6 HSIL (CIN3), EGFR, strong immunostaining x 10

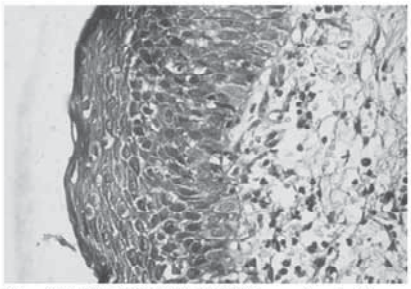

Fig. 7 HSIL (CIN3), EGFR, moderate immunostaining x 10

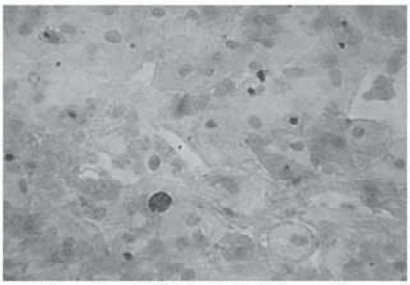

Fig.8 LSIL, HPV L1 positive, x 20

CONCLUSIONS

The immunoexpression of L1 HPV has clinical application in the progression assessment of the cervical precancerous lesions without a correlation to the grade of the cervical SIL. EGFR is expressed by all proliferating squamous epithelial cells, thus corresponding with the grade of SIL. The evaluation of EGFR status, correlated with L1 HPV protein expression, can provide useful data of progression risk of cervical squamous intraepithelial lesions.

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REAL NECESSITIES OF A CONTRACEPTION ALGORITHM IN CASES OF WOMEN SUFFERING FROM SCHIZOPHRENIA. SPECIAL NEEDS FOR FAMILY PLANNING

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Keywords: schizophrenia, female patient, sexual intercourse, contraception, motherhood, family planning, ethics.
Abstract: Schizophrenia has a devastating effect on patient lives all together with their families, changing dramatically the day by day life, affecting thinking, feelings, knowledge and modifying the patient's ability to adapt to society – establishing "boundaries" and "stigma" cause of desasperation, confusion or other symptoms. Objectives: This study wanted to the established an algorithm that concerns contraceptive methods specifically made for schizophrenic female patients according to their needs and to reality in which they live. Material and method**:** the study included 6200 patients at reproductive age that had been hospitalized in Socola Psychiatric unit during 2005 - 2010 and have been investigated by inquiry about age, provenience, marital status, education, number of children, knowledge and attitudes towards methods of contraception failure. Results The majority of the ones that knew about the contraception were from the urban area, age 30-35 having medium education, in a relationship or married. Unfortunately the help providers tend to neglect this "delicate subject" the fertility of schizophrenic patients being a real problem not only for the health care specialist but also costing highly the social assistance, their families, and their own children. Conclusions**.** While the Royal College of Obstetricians from Great Britain affirms that as a criteria for medical eligibility in using contraception in female schizophrenic patients it can be used any method as there are no longer contraindications for each specific case and under a correct counseling the best way is to solve ethical problems offering those patients the right access to family planning.

INTRODUCTION

Schizophrenia has a devastating effect on patient lives all together with their families, changing dramatically the day by day life, affecting thinking, feelings, knowledge and modifying the patient's ability to adapt to society – establishing "boundaries" and "stigma" cause of desasperation, confusion or other symptoms. Never the less, the reproductive aspect for the mentally ill patients was never well done investigated considering that sexuality is a "tabu" subject for this kind of patients assuming that intimate relationships are prohibited (at least in hospital). Despite all this, the historical data and medical literature are showing exactly the contrary – a study that took place in 2007 at Indiana University, Georgia U.S.A. has revealed the difficulties that the female schizophrenic patient are having many occasions to engage to intimate relationships are concerned about pregnancies and diseases, or about the "morality" of having a relationship, feeling deeply the rejection while being "sexually refused" by the other person. (1)

Another study in Maryland by Ritsher, Coursei and Farrel shows that there are more female psychiatric patients sexually active than men.(2)

The 2nd World Congress of Women's Mental Health establish that female patient suffering from schizophrenia(that affects at least 1 percent from general population) and completing with perspectives over the facts about motherhood and its implications on child's safety, female's rights, children's rights and emotional safety of these children.

Most of all a child's safety implies his emotional safety, and mother's diagnostic should not be the only base to take this decision – most likely the right observation of mother and child interaction should be important.

Maternal abilities might be affected by having severe difficulties in appreciating the children's needs (basing or caressing), or his intention or mother's authority on older children. Much more these abilities might be transformed by disillusion judgments or psychotic symptoms asserted to schizophrenia.

It is hard to make a choice between the positive aspects of motherhood: pride motivation , feeling "important" and negative aspects: stress concerning the evolution of the psychiatric diseases loosing custody or stopping the medication to stay awake witch makes the patients uncomfortable in seeking help when she has relapses. Financial problems, problems with the partner might complicate her situation and increases substances abuse due to poor education and psychiatric illness. Most sadly it is the fact the their children are developing health and mental problems and they have psychological difficulties as adults, partially explained by genetic heritage but also determined if the children grow somewhere else so the help their mother received should include not only psychiatric care but also family counseling, family planning, social assistance and help needing therapy unit for mother and child. (9)

OBJECTIVES

This study were to established an algorithm that concerns contraceptive methods specifically made for schizophrenic female patients according to their needs and to reality in which they live.

MATERIAL AND METHODS

The study included 6200 patients at reproductive age that had been hospitalized in Socola Psychiatric unit during 2005 - 2010 and have been investigated by inquiry about age, provenience, marital status, educations, number of children, knowledge and attitudes towards methods of contraception failure.

Contraception methods focused on psychiatric patient's are generally classified:

- According to gender female or male
- According to reversibility (temporary/ permanent)
- According to methods

We use Peal index to define the real effectiveness of these methods:

$$Pearl\ index = \frac{number\ of\ unwanted\ pregnancies\ (accidental)}{total\ number\ of\ exposures\ to\ pregancies} x1200$$

Results are expressed by number of failures per 100 women/year

Ideal qualities for a contraceptive method are:

1. More safety
2. Efficacy
3. Lack of secondary effects
4. Reversibility
5. Low price/free
6. Less needs for medical check up
7. Not depending on moment of sexual intercourse
8. Acceptability.

Taking in to account that none of all these contraceptive methods known until now have advantages and disadvantages and side effects applying for a certain method should be an individual option tightly connected to patient's motivation obtained by counseling.

Specific counseling includes talks that offer information and support on contraception methods and individual circumstances for the person that deeds contraception. It also includes accepting contraception and discussing methods, easy access to contraception, electing the real specific methods by assuming benefits and risks and enhancing the correct utilization.

Contraceptive methods are:

a. Natural methods (coitus interrupts , abstinence)
b. Barrier methods
c. Hormonal methods
d. Intrauterine device (IUD)
e. Sterilization (10)

A short history about the creation of the pill is revealing the forgotten women behind it, the women who gave the founding to researcher Gregory Pincus. Catherne McCormick was a wealthy here who's husband died from schizophrenia and she decided to turn her attention to the birth control movement with Margaret Sanger. Margaret Sanger watched her mother die at a early age, witch was partly due to the stress of bearing 11 children. After her mother's death she has worked as a nurse in New York City and saw many women die from child birth and self-induced abortion. The horrors that she witnessed there caused here to devote much of here time to promoting birth control for women. She set up the first clinic in 1916 and founded The American Birth Control League in 1921. She had also envisioned a birth control pill that would be much easier to use than the diaphragm. Her exhaustive efforts paid off in 1960 when the pill was finally approved and sold on the market.

A) Natural contraceptive methods:

Coitus interrupts: is the most well known contraceptive method.

Advantages:

- Can be used any time
- No costs
- It doesn't interfere with antipsychotic medication

Disadvantages:

- Implies a strong motivation
- Needs a special male self control, and considering the sexual high risk behavior in case of women suffering of schizophrenia (multiple sexual partners engaging sex without sexual education, being abused …) it is hard to belive that failure rate will be lower than in general population (12-25 pregnancies per 100 women per year)

Periodical abstinence: it is a method based on understanding of menstrual cycles phases and the right moment of ovulation

Advantages:

- No expenses
- It does not interfere with antipsychotic medication

Disadvantages:

- It is only indicated for the patient with regular menstrual cycles
- It needs a good understanding of the moment of the ovulation which is hard to be done with the patients in psychotic status; one of the most mentioned reasons to confirm lack of contraception being (“I didn’t know how it is used”)

During the lactation period natural contraception is hard to be used, the psychoses being enhanced by the postpartum period and also by social factors (loosing custody)

B) Barrier contraception: it represents the use of steroids hormones introduced in 1960 by Gregory Pincus, a biologist, fighting against Comstock Law (1873) the highest opponent against family planning.

C) Hormonal contraceptive methods

- Oral contraceptives:
- Combined estroprogestative pill (COC)
- Mini pills (only progestagen)
- Monthly pill
- Injection contraception
- Implant contraception
- Vaginal ring contraception

1. COC

Advantages: during the premenstrual psychotic exacerbation or during the menstruation, the pill has a positive influence on the psychiatric disease evolution reducing psychotic symptoms and enhancing the treatment efficacy; it increases the concentration of Diazepam or other benzodiazepine in blood and is not recommended to the patients which are using this mild tranquilizing. In case of Fenitoin, Carbamazepin and Fenobarbital could accelerate the steroid metabolism causing failure to contraception. The best would be to use monophasical pill 21 days and then 7 days pause most of schizophrenes preferring to take the pill continuously to avoid the menstrual period during witch they feel “dirty” and “neglected”.

Disadvantages: lack of compliance (patient and partner – as in condom use) ; needs of correct administration – the same hour day by day, correct information (hard to obtain because their lack of interest and also because special services do not include special needs)

2. Injection contraception

Advantages:

- does not need special compliance
- have higher efficacy compare to COC (there is no forgotten pill)
- check up at a long period of time

Disadvantage:

- vaginal bleeding
- menstrual hygiene difficult to keep
- discomfort and fear

3. Implants

Advantages:

- high acceptability
- lack of toxicity

Disadvantage:

- genital bleeding
- needs to be administered by special person

4. Vaginal rings

Disadvantages:

- genital bleeding

- lack of compliance for a patient that has occasional sexual contacts
- difficulty of utilization

D) Intrauterine device

Disadvantages:

- needs of special anamnesis and gynecological examination
- risks of multiple sexual partners
- occasional sexual intercourse
- BTS
- inflammation
- chronic abdominal pain

E) Sterilization: is a legal act in many states while in Romania there are no specifications intern of informed consent. A difficult choice regarding patient's decisional autonomy might be taken under negotiation, adequate counseling and informed consent. No one should be sterilized without understanding all the risk and finalities becoming from this action.

RESULTS AND DISCUSSIONS

Epidemiological distribution:

- 52% were from urban environment while 48% from country;
- 33% have been given birth to 1 child; 24% to 2 children; 11% more than 3 children; 22% no children;
- 46% were married; 22% in a relationship; 22% single,
- 13% have been to gynecologist to ask for a contraceptive method while 87% never been.

From the married group 62% were using coitus broken/periodical abstinence, while 33% had IUD, 5% using hormonal contraceptives. From the ones being in a relationship/occasional sex only 7% have been using condoms, 72% using natural contraception. From the single group witch reported occasionally sexual intercourse – no contraception mostly, occasionally condom use 6% or coitus interrupts 12%, no other use or other methods.

Most of all, the prevalence of birth shows without any doubt that schizophrene women are sexually active: 143 births/ 1000 schizophrenic patients during 5 years are expressing the fact that most likely there is a possibility that any sexual contact might end up in pregnancy, the conclusion is that sexual contact are quite frequent even for hospitalized patients.

The sexual freedom launched in 1960 and the changes that appeared in psychiatrically practice have been "reflected" in so many opportunities for carnality in time of our days. Leaving the hospitals and being in a community, offered higher possibilities to have sexual intercourse: 52% of patients have been sexually active during last months and 62% last year (Calgary, Canada). The same results have been obtained in New York City: 45% of female schizophrenic patients being sexually active during the last 6 months.(3)

Considering all these facts it was decided that the pattern of sexual behavior for the schizophrenic female patients is to be engaged in occasional intercourse having unprotected sex, with multiple partners (witch increases the risk for transmitting BTS) and implies a multitude of risk factors as: social circumstance, substance abuse, differences concerning the sexual practices between women and men.

Even initially was accepted that in schizophrenia the sexual impulse is intensified at the debut of the disease and that is decreasing during the evolution of the disease the real surprise was revelation that these patients are constantly sexual active during the psychiatric illness, never minding the hospitalization through witch they have been having the same sexual behavior .

The concern regarding “homosexuality” – have been postponed since DS IV sharpen the criteria for diagnosing schizophrenia, the supposed sexuality being just an assumption of social all-time realities.

While medication is mostly responsible for the – institution and by reducing symptoms and increasing life quality and adapting the patients to community life it is also responsible for increased opportunities for sexual contact.

Medication also influences the libido having effects that are on over not so investigated as the risk for BTS (there has not been studied yet the effect of psychiatric medication on genital infection – ex. Candida as in antibiotics or contraceptives).(4)

During psychiatric treatment for schizophrenia it was proved that it occurs hiperprolactinemia witch is the cause for different symptoms that include not only amenorrhea and also galactoreea. (5,6)

Strong antipsychotics (ex.: Flufenazina, Haloperidol, Risperidona) are associated with high growth of prolactinemia which concludes in high risk for osteoporosis and breast cancer – unfortunately an accurate study on the influence regarding the sexual function cannot be mentioned, 52% of treated patients are relating sexual dysfunction when asked, female patients aparing more affected than men. (7)

There is an obvious relationship between unprotected sex, substance dependence and having a psychiatric disease – endemically superposed with the one of syphilis, reflected in the increased number of the sexual partners and the decreased use of the condoms (Chiasson et all, 1981, Sussen et all, 1995) witch confirms reality that sex is often used in change for drugs to this specific category of patients.

Increased alcohol use is associated to their sexual behavior although not well studied; alcohol is acting as a desinhibant, this fact having a specific importance to female patients suffering more than men from alcohol dependence (Wetermeyer 1996) (6)

The realities we have exposed are reflecting the needs for family planning for mentally ill women, many of whom do not use contraception and are a high risk for unwanted pregnancies taking an adequate sexual history is the 1st step in assessing patient needs for family planning services. Patient education, including instruction about physiological processes and contraceptive methods or assertiveness training is the most important component of these services. Offering family planning services in the mental health center has many advantages including better communication between mental health care providers’ needs and enhanced opportunities for the integrating family planning with other programs such as parenting classes, substance abuse treatment and services for preventing sexually transmitted disease. (7)

The family planning knowledge’s attitudes and practices in women with schizophrenic spectrum disorders is based on three hypotheses about family as compared to demographically comparable non-mentally ill control women: that they (1) report at least as much unprotected intercourse while not desiring pregnancy; (2) have less knowledge about contraception: and (3) perceive more, and different, obstacles to obtaining or using birth control. A semi structured Family Planning Interview was administered to subjects (n=44) with Research Diagnostic Criteria diagnoses of schizophrenia and schizoaffective disorder and not to non-mentally ill control subjects (n=50). The participants had high rates of unprotected intercourse, as did non-mentally ill controls. They had significantly less reproductive and contraceptive knowledge than the control subjects, and were more likely to perceive birth control was that they did not expect to have sex, while that given by non-mentally ill subjects related to side-effects of birth control. Important obstacles to family planning in women with schizophrenia could benefit form long-

acting, reversible contraception, many may be aware of those options and/or may find them difficult to obtain. Integrating family planning with mental health care might better address the unique needs of this population.

A semi-structured interview was used to gather data in testing the three hypotheses about family planning in women with schizophrenic spectrum disorders, as compared to demographically comparable non-mentally-ill control women: 1) that they report at least as much unprotected intercourse while not desiring pregnancy; 2) that they have less knowledge about contraception; and 3) that they perceive more, and different, obstacles in obtaining or using birth control. A total of 44 women with Research Criteria diagnosed of schizophrenia and schizoaffective disorder, and 50 non-mentally-ill control subjects were administered with the Family Planning Interview. The interview elicited detailed information about sexuality, pregnancy history, education and communication about family planning, and birth control knowledge, practices and attitudes. Results revealed that the participants had high rates of unprotected intercourse, as did non-mentally-ill controls. They had significantly less reproductive and contraceptive knowledge than the control subjects, and were more likely to perceive birth control as difficult to obtain. The reason most commonly endorsed by the psychotic disorders had to do with not expecting to have sex, and not thinking about birth control while having sex. It also provides support for the hypothesis that difficulty planning ahead was a major obstacle to the use of birth control methods. These findings underscore the importance of gearing family planning programs to the particular needs of mentally ill women.(8)

The result of these risky sexual behavior and variable responsibility is motherhood, a high demanding situation; if there is a consent about what it means to be “a bad parent” there is not a certain definition about the real meaning of a “good parent” – being a parent is a complex and exposure job and for these cases all the help provided by the social services is mostly needed.

But experience during childhood create difficulties in adulthood, these mothers tend to be reluctant in receiving help from others meanwhile the social health providers tend to ignore their role as a parent considering that this problem belongs to Social Services.

A study made by Haldberg University using video cameras to evaluate the interaction between 30 mothers suffering from schizophrenia and their children revealed that mothers were suffering from deficit attention and are severely affected by negative symptoms (absences and paranoia) that can influence the natural born maternal sensitivity and intuitive competence. The maternal reactions to new born demands are extremely low.

On the other hand the University of Toronto showed of that 50% of schizophrenic patients are becoming mothers that are half of the number to those who experienced motherhood in mentally healthy population. One third o these patients are loosing custody in favor of family member, ex partners, social assistance services or even adoption. Very few are maintaining the custody witch is a hard to take decision for the social workers, whom need to take in to account not only the mother’s rights to bread her own child but also the children’s right to be next to his own mother.

CONCLUSIONS

The majority of the ones that knew about the contraception were from the urban area, age 30-35 having medium education, in a relationship or married. Unfortunately the help providers tend to neglect this “delicate subject” the fertility of schizophrenic patients being a real problem not only for the health care specialist but also costing highly the social assistance, their families,

and their own children. While the Royal College of Obstetricians from Great Britain affirms that as a criteria for medical eligibility in using contraception in female schizophrenic patients it can be used any method as there are no longer contraindications for each specific case and under a correct counseling (11) the best way is to solve ethical problems offering those patients the right access to family planning.

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CHANGES IN SOME BIOCHEMICAL PARAMETERS IN PATIENTS WITH KAHLER RUSTITZKI DISEASE

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Keywords : Kahler Rustitzki disease, uraemia, bilirubinaemia, renal insufficiency, creatinine.
Abstract: This paper aims to carry out research into the changes in some biochemical parameters in 10 patients diagnosed with plasmacytoma. The parameters have been obtained in the laboratory of “Elena Beldiman” Emergency Hospital Barlad, and in the”Dr Stoian –Dr. Ungureanu”Private Medical Practice, between 2005 – 2011. The analyses results, which are in accordance with those from the scholarly literature, show that the changes in blood indicators may occur as a result of the influence of various factors which negatively modify the patient’s health. The 10 cases surveyed during 2010 also emphasize the frequency of this disease in the sense that they have the tendency of equalization under the aspect of the two genders (masculin and feminin) in comparision with the years before 2010.

INTRODUCTION

The experimental research from the last years have showed the possible existentence of a connection between the inflammatory and immunological processes and and the emergence of plasmocytomas, at first reactive and then having a neoplastic feature. Once the sternal puncture was introduced in the clinic, the disease was better studied and diagnosed, and in the last years due to the use of the electrophoretic study of the serum proteins, the protein genesis disorders found in this disease were properly described. As a result of the changes in the dispersion of proteins, the synthesis of the enzymes was heavy to be made because of their main component which consits of protein fractions with a special physical and chemical structure.Most of the patients are aged between 50 and 60, acknowledging the fact that in the disease development there is an asymptomatic period which varies from 10 to 15 years. Acute renal insufficiency may be a consequence of dehydration, hypercalcemia,and hyperuricemia which represent the biological parameters characteristic of multiple myeloma development. What is outstanding in what that concerns the biochemical parameters is the fact that a precocious paraclinic marker is the increase of the serum value of the uric acid, which will lately determine the presence of a higher procent of immature myeloma cells in the bone marrow (MUT POPESCU, D., 2003).

MATERIALS AND METHODS

The research were carried on a casuistry which consisted of a number of 10 patients of different ages diagnosed with plasmacytoma between 2005 and 2011. The venous blood was sampled in vacutainers for biochemistry, and then spun out in the „EPPENDORF”5804 centrifuge. The samples were operated with the help of “RX IMOLA” TM RANDOX , the wet biochemistry auto-analyzer (with liquid reagents). During the comparative study of the researched cases, the values of the following biochemical markers were correlated with the age and the gender of the patients: the amount of urea (mg/dl), the amount of creatinine (mg/dl), the amount of total bilirubin (mg/dl). The amount of urea (mg/dl), the amount of creatinine (mg/dl), the amount of total bilirubin (mg/dl) were defined with “RX IMOLA” TM RANDOX, biochemsitry auto-analyzer. Rx Imola is a clinical chemistry analyzer fully automated with an analyzer software. The software functions of the analyzer include the facility of interaction with the host computer in order to directly download the selection details of the assay method for individual samples. A barecode system is used for a rapid identification of the patient samples, reagents and QC samples. The Incubation Reaction Unit platform cuvette holds 90 cuvettes. It spins and brings the designated samples in the place where the samples/ reagents are divided to Reagent Pipette Unit. The Detector Unit measures the solutions absorbance durinf the reaction process (mixing and incubation) in cuvettes. The light form the halogen lamp is dispersed using an active measurement of diffraction of 12 various wavelengths. The Sample Pipette Unit aspires the sample from Auto Sampler Unit using Sampling Pump Unit and operates it in a cuvette (in Incubation Reaction Unit) and / or in Ion Selectable Electrode Unit. The Reagent Pipette Unit aspires a reagent from a reagent bottle (in Reagent Container Unit) using Reagent Pump Unit, and then it dispense it in a cuvette (in Incubation Reaction Unit). After a sample and reagent are dispensed in a cuvette, Mixing Stirrer Unit brings the stirrer paddle and mixes the stirs the mixture in the cuvette. The Auto Sampler Unit has 72 sample tubes (normal and emergency samples) and 20 sample cups (standard samples and Ion Selectable Electrode Unit washing solution) and brings the designed samples in Sample Pipette Unit pipetting position through turntable rotating. The Reagent Container Unit holds a maximum 60 reagent bottles on the reagent tray and brings the designed reagent in the Reagent Pipette Unit pipetting position. The measurements are made on each 9 seconds during a period of 10 minutes. This results in a maximum rate of 400 photometric tests per hour. RX Imola analyzer has a cycle of 9 seconds. During each cycle, the

system either adds samples, reagents, mixes or take measurements. The measurements can be taken at one or two wavelengths, depending on the chemistry parametres specific to the test.

RESULTS AND DISCUSSION

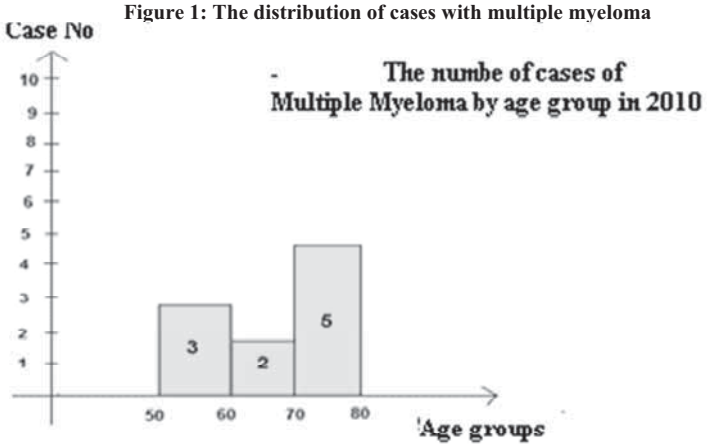
In the present paper we have analyzed a casuistry which consisted of patients belonging to both genders, aged between 54 and 79 years throughout 2010, whose diagnosis was plasmacytoma, as primary and secondary diagnosis, with a more or less favorable development. As a consequence, the irregularity degree of the studied biochemical parameters varied more or less from one patient to another.

Tabel 1: Gender distribution of the studied casuistry

Case no.	Name Initials	Gender	Age
1	C.D.	M	71
2	P.G.	M	54
3	S.M.	F	54
4	Ş.R.	M	70
5	B.E.	F	62
6	C.M.	M	74
7	C.V.	M	59
8	M.A.	F	74
9	P.I.	F	75
10	Z.I.	F	79

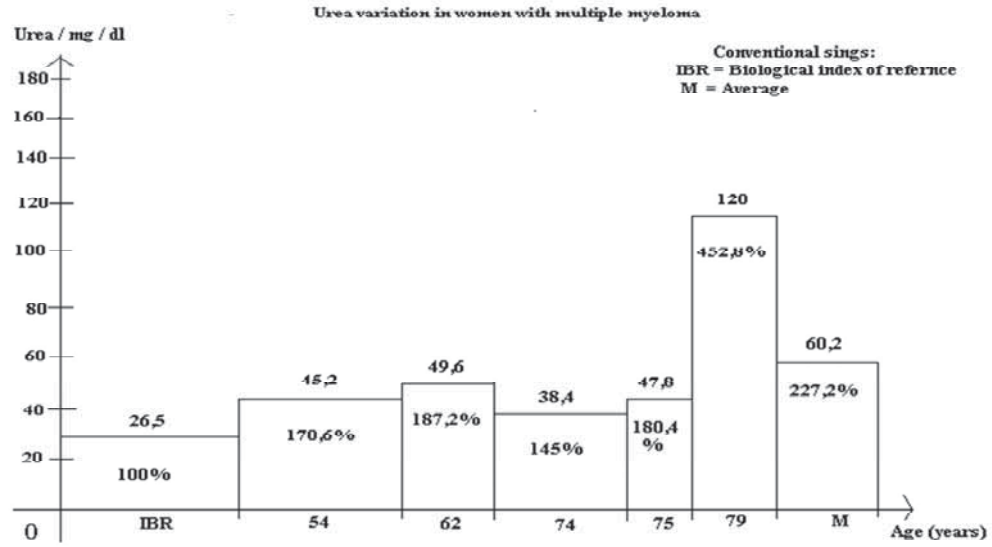
Conventional signs: F – female cases with Multiple Myeloma
B – male cases with Multiple Myeloma

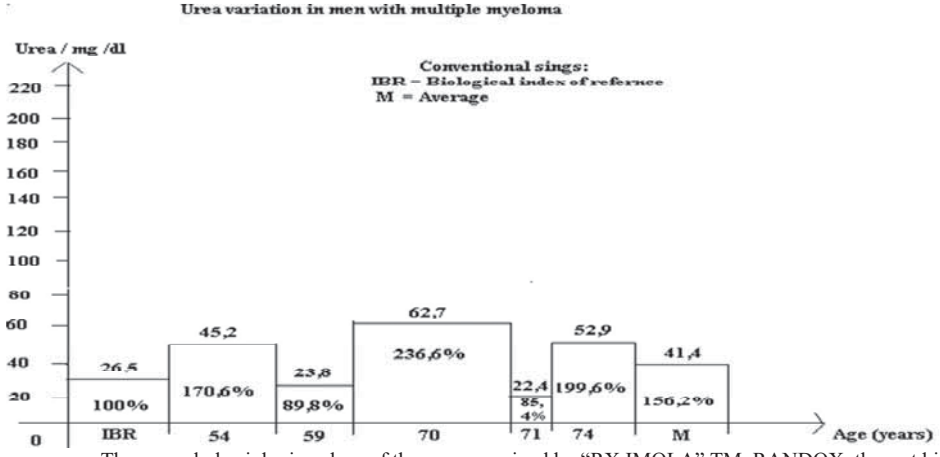
Figure 1: The distribution of cases with multiple myeloma



Urea – being an organic compound (sanguineous non-proteinaceous nitrate class) as well as creatine – creatine, bilirubin, it results in a final product of amino acids degradation (MIHELE, D., 2007). It is exclusively synthetized in liver and is eliminated via kidney. The achievement of a balance between the liver's function (where it is synthetized) and the kidney's function (through where it's eliminated) determines the maintaning of the urea concentration within the normal physiologic limits.

Figures 2 and 3: Changes in the urea values in patients of both genders suffering from multiple myeloma

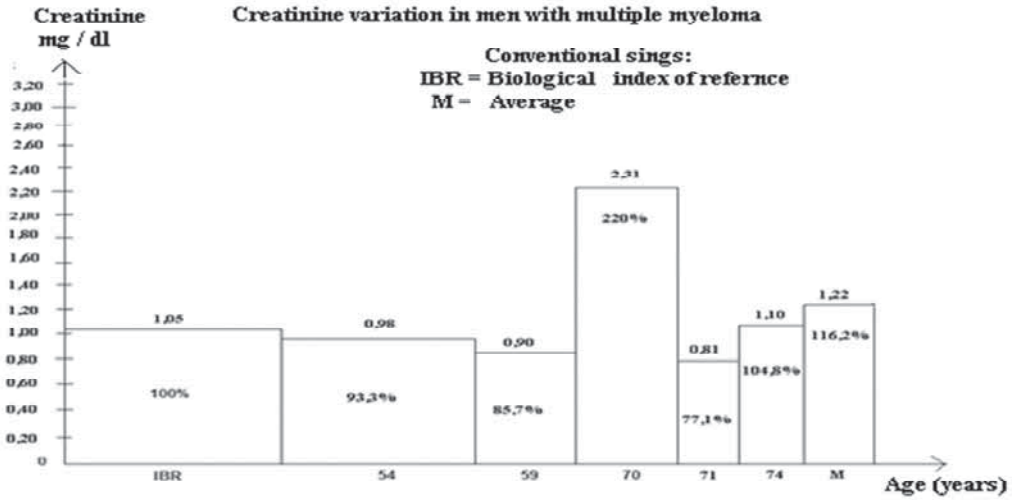


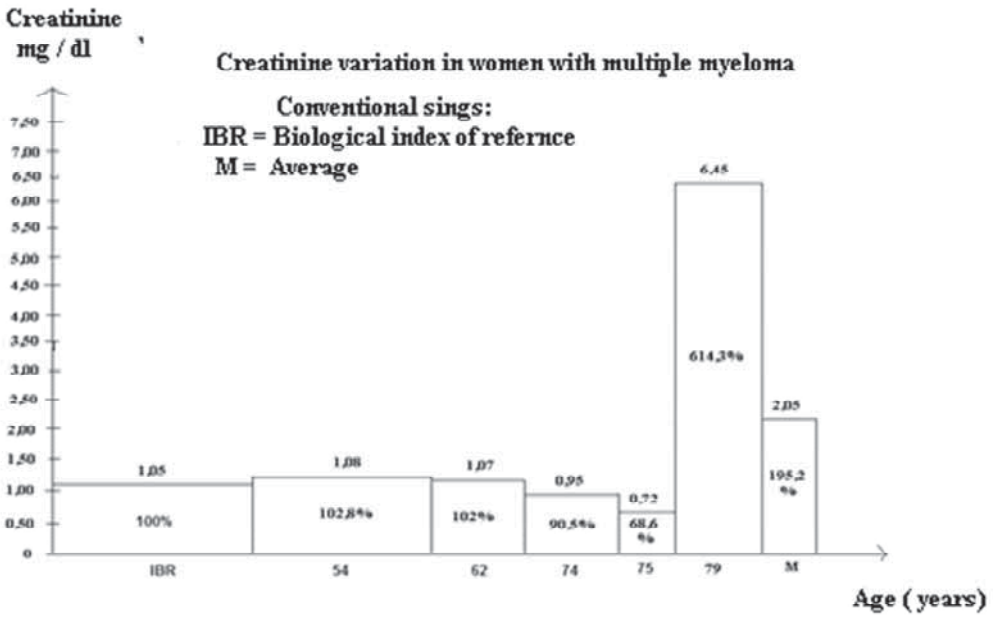


The normal physiologic values of the urea perceived by “RX IMOLA” TM RANDOX ,the wet biochemistry analyzer (with liquid reagents), for both females and males situate between 10-43 /mg/dl with an average of 26,5 / mg/dl.

Creatine and serum creatinine – creatine(the methyl guanidine-acetic acid) is obtained in tissues after the biosynthesis between arginine and glycocoll takes place. Its outcome is represented by glycocyamine which changes into creatinine through methylation. Creatinine represents the creatine’s anhydride, being a secretion product of creatine. Although most of the patients with Multiple Myeloma have as finality the renal insuficiency (IRA) or chronic (IRC), the creatinine can’t be considered a sensitive marker in the case of light to moderate renal impairments.

Figure 4 and 5: Changes in creatinine values in the multiple myeloma for both genders

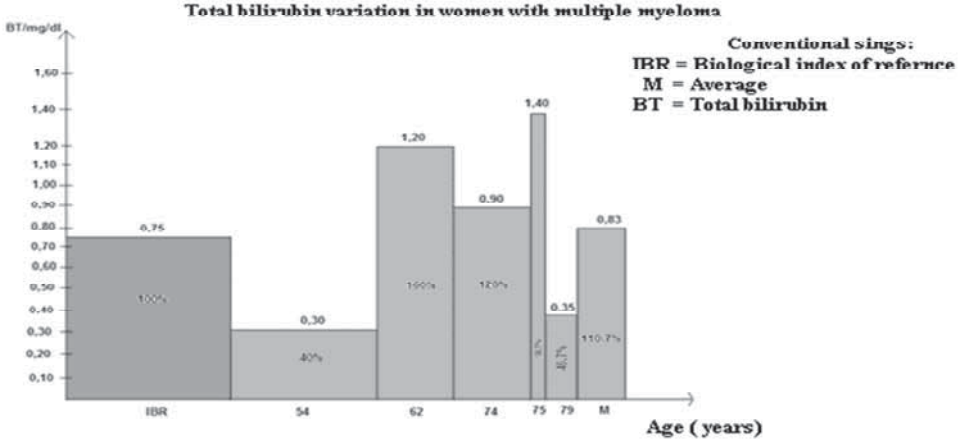


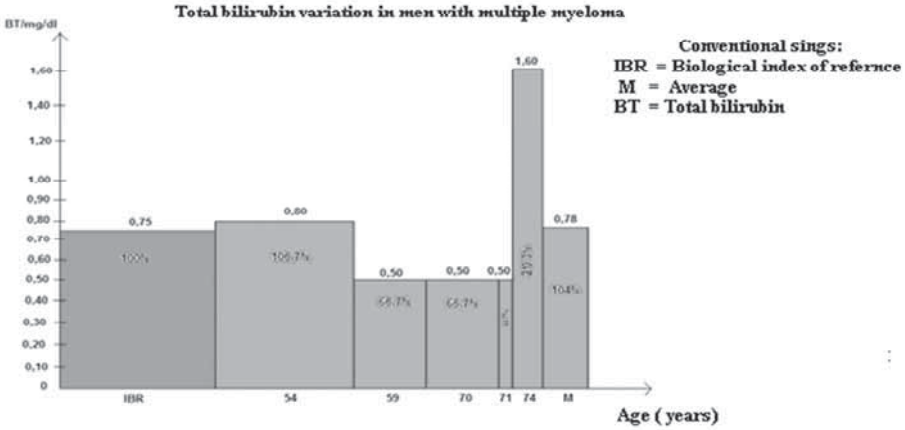


The normal physiologic values of the creatinine perceived by "RX IMOLA" TM RANDOX, the wet biochemistry analyzer (with liquid reagents) for both genders situate between 0,60 – 1,50/mg/dl with an average of 1,05 / mg/dl.

Total bilirubin – it results after the hemoglobin catabolization, circulating in the blood plasma, bound to the serum albumins. The boold contains both free bilirubin (indirect, unconjugated) and bound bilirubin (direct, conjugated). An important role of these direct and indirect bilirubin reactions allow us to make a difference between various types of jaundices (mechanical, haemolitic, hepatocellular). In this way, in the mechanical jaundice the value of the direct bilirubin grows, in the haemolitic one the value of the indirect bilirubin grows, and in the hepatocellular jaundice the values of both conjugated and unconjugated bilirubin grow.

Figure 6 and 7: Changes in total bilirubin values in multiple myeloma for both genders





The normal physiologic values of the total bilirubin perceived by “RX IMOLA” TM RANDOX, the wet biochemistry analyzer (with liquid reagents) for both genders are situated between 0,20 – 1,30 /mg/dl with an average of 0,75 / mg/dl.

CONCLUSION

The research emphasized the frequent incidence of the multiple myeloma at individuals aged between 50 and 80 years.
The urea values on men exceed the normal physiologic limits of the biological benchmark, and two of five cases show values of this mark under the normal physiologic limit.
In what that concerns the feminin gender, all the cases revealed urea values higher than the normal physiologic limit of the biological benchmark.
By relating the cases to the average of the values acquired by these, it could be noticed that the urea values were higher in just one case.
The presence of a creatinine value higher than the normal physiologic value could be noticed at both men and women in just one case , as well as when it was related to the average.
Changes in the total bilirubin values that were above the normal physiologic range for both men and women took place in just one patient’s case.

The mean of the total bilirubin value that was refered to values aquired by female patients shows an ascending tendency for more than a half of cases, in comparision with men where this tendency existed in less than a half of the cases.

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THE STUDY OF SOME BIOCHEMICAL PARAMETERS IN PATIENTS WITH MULTIPLE MYELOMA

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Keywords: serum proteins, multiple myeloma, hyperproteinemia,hypercalcaemia, renal impairment.
Abstract: This paper presents the results of some biochemical parameters obtained in the laboratory of “Elena Beldiman” Emergency Hospital Barlad, and in the”Dr. Stoian –Dr. Ungureanu”Private Medical Practice on 10 patients diagnosed with multiple myeloma during 2005-2011. Numerous researches over serum proteins with the help of various methods (refractometry) have shown the presence of some changes in protein fractions of blood serum. These changes express the change of the normal ratio between these fractions, that means disorders in colloidal structure of blood. The results of the electrophoresis analysis, also in accordance with those from the scholarly literature, show that the changes in the condition of the blood serum are due to the increase in the globulins fractions which is linked to the growth of the immunological processes, the antibodies synthesis being especially closely linked to gammaglobulinic fraction.

INTRODUCTION

The study of the protein metabolism in this disease began in 1928 when it could be noticed the presence of a hyperproteinemia; but, huge progresses were made at the same time as the electrophoresis was introduced in the medical practice. The present paper has as main objectiv and aim the study of changes in some biochemical parameters in 10 patients with multiple myeloma as both primary and secondary diagnosis. From a quantitative point of view the variation of these biochemical parameters according to the age and gender was recorded on the patients from Elena Beldiman” Emergency Hospital Barlad and from „Dr. Stoian – Dr. Ungureanu”PMP in 2010. It’s appreciable the the life duration of the patients with multiple myeloma, under various statistics, from several moths to 10-12 years, from the moment the diagnosis was set ((BUTOIANU, E., STĂNICĂ, T., 1973), 10-15 years (MUT POPESCU, D., 2003).

MATERIALS AND METHODS

The studies were carried on a casuistry which consisted of 10 patients diagnosed with multiple myeloma between 2005-2011 (5 women and 5 men), aged between 54 and 79 years. Blood samples were gathered in biochemistry vacutainers, and then spun in „EPPENDORF”5804 centrifuge. The samples were operated with the help of the dry biochemistry analyzer ‚System Chemistry „VITROS 5.1.F”. During the comparative study of the investigated cases, the ages and genders of the patients were correlated with the values of the following biochemical markers: the amount of glucose in blood (mg/dl), and the amount of calcium (mg/dl). The amount of glucose and the amount of calcium were determined with the help of the biochemical autoanalyzer ‚VITROS 5.1.FS”Chemistry System. VITROS 5.1 FS Chemistry System can process discrete photometric methods and can perform automatic dillutions using a sample aliquot from the first tubes collections. In the Special Chemistry Center of VITROS 5.1 FS MicroTip the volume of the liquid reagents is mixed with the volume of the samples in a cuvette and incubated for a certain time period. A second reagent, if is requested, is added and absorbance measurements are performed on selected time periods. Absorbance measurement is converted to concentration through an appropriate mathematic pattern and an associated calibration. Data are processed using a user defined algorithm. This photometric process consists of following some steps: a second metering takes over VITROS Versa Tip; the reagent supply spins until it gets to the most appropriate reagent/ diluent from the metering track; the second metering absorbes R1 from the reagent supply; it dispenses R1 in the cuvette, absorbes the sample from Cuve Tip, it distributes it in the cuvette and performs the mixture. The cuvette is incubated in order to warm up the mixture. A cuvette palette is moved towards the metering place in order to mix the fluid. The second metering seals and dispenses VITROS VersaTip, and takes over FS MicroTip. The aliquoted sample from Cuve Tip is placed for metering by Tip Processing Center. The second metering seals and dispenses Fs Micro Tip, and takes over FS MicroTip or VITROS VersaTip. After the cuvette is read, the unused cells can be used for additional tests or sent to residues, in the case when all the cells have been used. The cuvette is read and the results are calculated. The cuvette’s arm moves the incubated cuvette in order for the position to be read. The cuvette is being incubated until the read is requested. The second metering dispenses R2 liquid in cuvette and performs the mixture; it absorbes R2 from the reagent supple; the photometer places the filter in order to be read. The second metering seals and dispenses Tip. The reagent supply spins toward the most appropriate reagent/ diluent from the metering place.The information about the Reagent Lot must be inserted prior a reagent to be loaded into the system. The reagent is enclosed into a reagent package and a bottle in nthe Edit Protocol Parms screen, when REAGENT Protocol is selected. If the user defined methos has the potentgial of discovering a antigen excess condition, it is recommended that the user to define an appropriate method in order for the

system to discover such a condition. The antigen excess refers to the region of the response curve of the assay dose where the concentration of analyte (antigen) exceeds the effective concentration of reaction antibodies, inhibiting the agglutination reaction.

RESULTS AND DISCUSSION

Mentionable is the fact that these studies were performed on a heterogenous casuistry on patients from both genders, aged between 54 and 79 years during the year 2010, and caught in various tages of disease development having multiple myeloma as main and secondary diagnosis. Consequently, the deviation degre of the studied biochemical parameters values differ more or less from one patient to another.

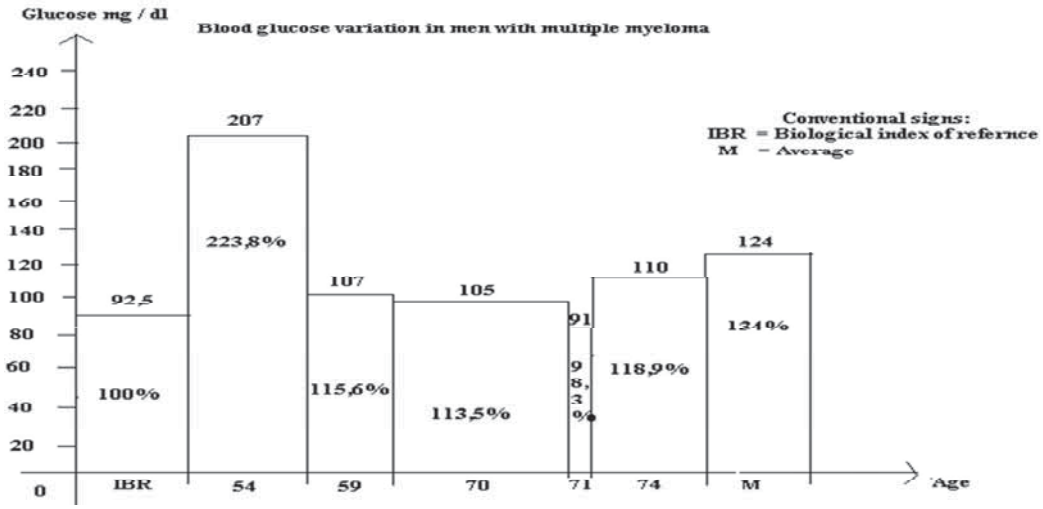
Table 1: Distribution of multple myeloma cases after diagnosis

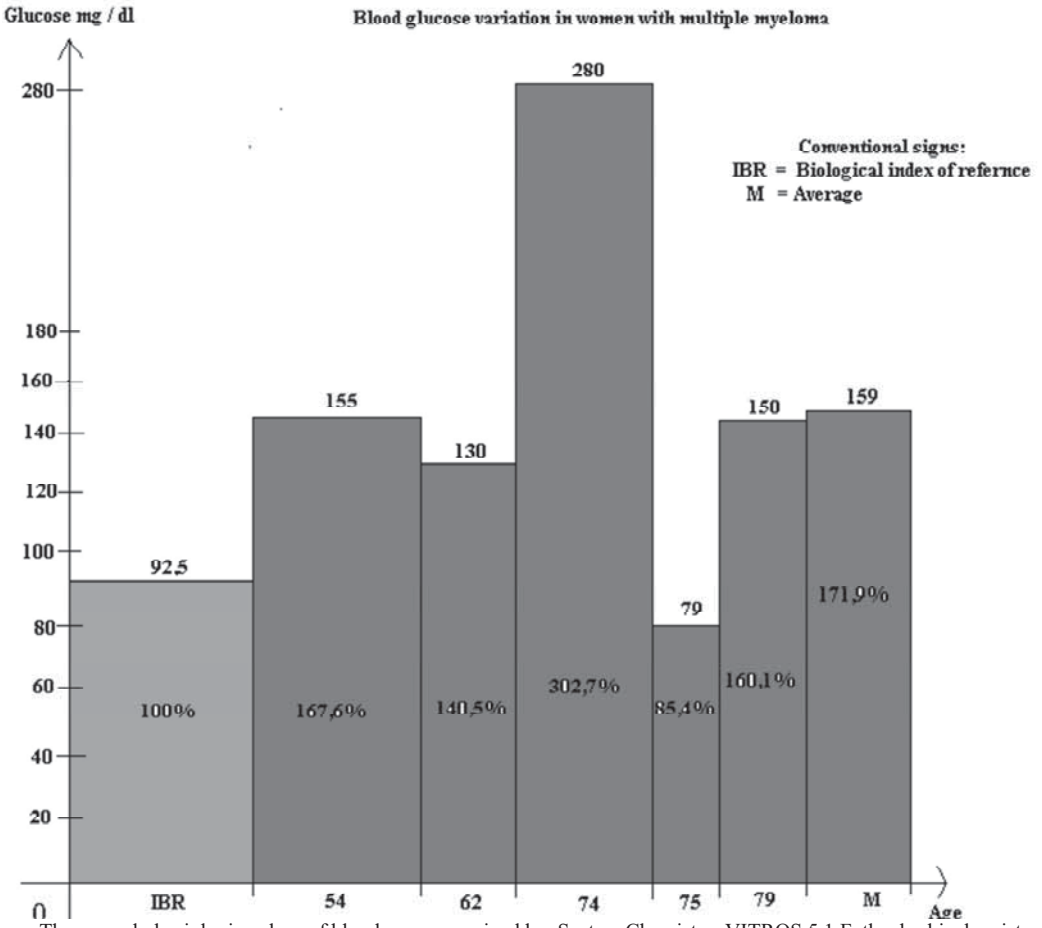
All cases	Main diagnosis	Secondary diagnosis
10	3F + 3B = 6	2F + 2B = 4

Conventional signs: F – female cases diagnosed with multiple myeloma
B – male cases diagnosed with multiple myeloma

Seric glucose - being an organic non nitrate blood component, the sampling for glucose determination was made in vacutainers which contained sodium fluoride in order to prevent coagulation and glycolysis (MIHELE, D., 2007),*hyperglycemia* , representing a main feature of sweet diabetes which is also a cause of cardiovascular complications and renal impairments. *Hypoglycemia* occurs when the alimentary contribution level and digestive absorption level decrease, but also when the catabolism of some hormones decreases. Seric glucose determination is necessary in order to diagnose sweet diabetes or to monitor some impairments.

Figure 2 and 3: Changes of blood sugar values in multiple myeloma for both genders



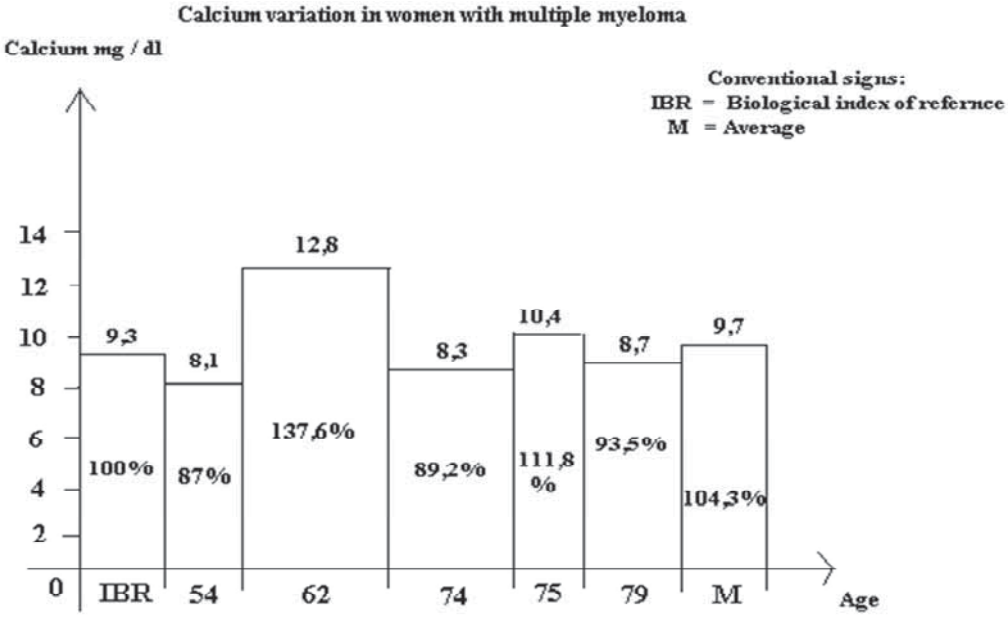


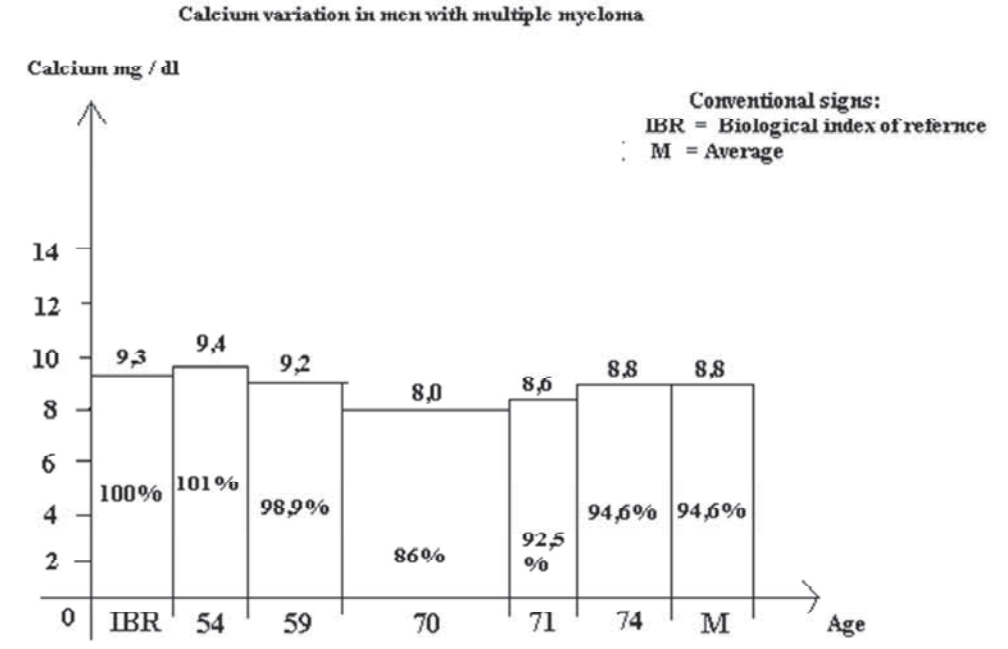
The normal physiologic values of blood sugar perceived by System Chemistry VITROS 5.1.F, the dry biochemistry autoanalyzer for both genders situate between 65 – 110/mg/dl with an average of 92,5 / mg/dl.

Calcium – belonging to inorganic constituents, Calcium is a prevalent extracellular divalent ion. It can be found in plasma, taking both an ionized shape (free) and an unionized shape, bound to the plasmatic proteins (MIHELE, D., 2007). *Hypercalcemia* may be caused by long bed reposes due to a diminution of bone development. This fact leads to a reduction of calcium deposit in bones while the efflux from bone remains constant. When we talk about the healthy subjects we must say that the excess of calcium is being eliminated through urine while when talking about the affected subjects it was noticed that calcium accumulation was taken place within the extracellular compartment, in this way determining hypercalcemia. *Hypocalcemia* may be a cause of the reduction of calcium intestinal absorption, having as main sublevel the reduction of the amount of vitamin D, increase the amount of phosphates in intestine which can couple the calcium. Calcium represents a bone main component (90%), being a reservoir in order to maintain the level of seric calcium.

KDOQI Guide , refering to reference intervals for Ca, recommands keeping the corect calcaemia toward the low limit of the reference interval (8,4 – 9,5 mg / dL) and to not exceed 10,2 mg/dL). NFK – K/DOQI Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease, 2003).

Figure 4 and 5 : Changes of calcaemia values in multiple myeloma for both genders





The normal physiologic values of the calcaemia perceived by System Chemistry VITROS 5.1.F, the dry biochemistry autoanalyzer for both male and female situate between 8,4 – 10,2 /mg/dl with an average de 9,3 / mg/dl.

CONCLUSION

After having carried this casuistry, related to age, it could be noticed that a presence balance of the multiple myeloma for both genders is maintained.
In the case of women’s blood sugar , from those 5 studied cases, a moderate hyperglycemia was recorded in 3 patients, a marked one in one patient and only a glycemia value which was situated between the normal physiologic limits.
If we refer to the average value, a growth of the blood glucose over the average was noted at one from the 5 surveyed cases.
In the case of the male patients, the glycemia values are lightly over the normal range on 3 patients, one of them having a value situated between the normal limits, and in one case an outstanding value of glycemia could be noticed.
Remarkable is the fact that for most of the patients with multiple myeloma the values of blood glucose are high because of the changes in glucidic metabolism.
These changes on glucidic metabolism level are due to a blockage of glucose metabolism and of all the possibilities to be used by cells.
In the case of the female patients it could be noticed a variation of calcaemia values, as it follows: one case of moderate hypercalcaemia, one of a noticeable hypercalceamia, and the rest of them being situated between the normal physiologic range.
In the case of male patients, just in two cases of five it could be noticed a moderate hypercalceamia while the rest of them showed values of calcium which situate between the normal physiologic range.
The changes in calceamia values are due to the presence of the lysis process and bone demineralization process.
The observational studies and lab researches point out the fact that the changes of biochemical parameters in this disease assume the existence of some particular clinico-biological disorders that can be influenced by various factors.
These aspects can be explained by the fact that most of the risk factors are common (diabetes, arterial hypertension, dyslipidemia) that in the end determine the progression or regression of the disease.

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MICROARRAY ANALYSIS REVEALS PATHWAYS AND BIOLOGICAL PROCESSES IN MYELOMA CELL LINE L363 WHICH ARE INFLUENCED BY MICROENVIRONMENT

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Keywords: multiple myeloma, bone marrow stromal cells, microarray, gene expression.

Abstract: Multiple myeloma, also called Kahler disease or myelomatosis is a debilitating and incurable malignancy characterized by proliferation of malignant plasma cells and an increased production of monoclonal paraproteins. In multiple myeloma, the abnormal proliferation of plasma cells within the bone marrow interferes with the production of blood cells therefor leading to anemia, leukopenia and thrombocytopenia. Another characteristic of disease is the activation of osteoclasts which leads to osteolytic lesions accompanied by fractures, bone pain, hypercalcemia and renal failure.

Since a key factor in the pathology of multiple myeloma is represented by the interaction between bone marrow stroma and plasma cells we have designed an *in vitro* experiment where L363 myeloma cells have been grown together with bone marrow stromal cells. The negative control was represented by L363 cells cultured alone. Following coculture, RNA from L363 plasma cells was extracted, revers-transcribed and analyzed by microarray techniques to identify biological processes and pathways which are affected by differentially expressed genes. Among these biological processes we mention regulation of cell cycle, apoptosis, and STAT genes activation.

INTRODUCTION

Multiple myeloma (MM) is cancer of plasma cells which manifests through abnormal elevated levels of monoclonal antibodies called paraproteins and accumulation of malignant plasma cells in the bone marrow. Collections of malignant plasma cell in bones lead to bone destruction and interfere with normal production of blood cells. Because plasma cells are responsible with antibody synthesis, MM patients suffer from immunodeficiency their malignant plasma cells being unable to produce normal antibodies.

Research studies on MM reveals that bone marrow microenvironment is an important player in survival and proliferation of malignant plasma cells and offers protection against therapeutic drugs (Feng *et al.* 2010; Hao *et al.* 2011; Hideshima *et al.* 2002; Nefedova *et al.* 2003; Podar *et al.* 2009; Zlei *et al.* 2007).

Current study was focused on understanding which pathways and biological processes in L363 MM cell line are affected by the presence of bone marrow stromal cells (BMSCs).

To achieve our goal we have elaborated an *in vitro* experiment where L363 MM cells have been cultivated together with BMSCs. Confluent layers of BMSCs were generated first before proceeding to real experiment. Next, L363 cells grown separately where placed in co-culture with BMSCs layers obtained previously.

During co-culture L363 have separated into two groups because some plasma cells became adherent to stroma.

Following co-culture, RNA was extracted from both adherent and non-adherent fractions of L363 cells and was used next for microarray. We have used Gene Set Enrichment Analysis tool from Ariadne Genomics Pathway Studio software to investigate and identify groups of genes significantly changed which takes part in similar biological processes.

Results suggest that most these genes are connected to apoptosis, cell cycle regulation, cell adhesion, transcription, STAT pathways, chromatin remodeling.

MATERIAL AND METHODS

BMSCs where harvested from myeloma patients and healthy volunteers and seeded with Myelocult H5100 media (StemCell Technologies, Vancouver, British Columbia, Canada) in 75 cm^2 flasks (Nunc, Denmark) at 37°C in a 5% CO_2 humidified atmosphere. A volume of 25 ml media per flask was used and it was changed every 3-4 days. With this setup BMSCs cultures have reached confluency within 5 weeks.

The myeloma cell line L363: cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (FCS; Gibco) and incubated at 37°C and 5% CO_2. Media was changed every 2-3 days.

L363 cells prior labeled with 10μM CFSE where co-cultured with confluent layers of BMSCs. Some L363 cells have become adherent to BMSCs while the rest have remained in suspension. The non-adherent fraction of MM cells was collected after 72hrs of co-culture and at the same time point BMSCs with the adherent fraction of L363 cells were

enzymatically detached with a solution containing trypsin and EDTA. Cells were sorted later on to a purity of 90-95% with a MoFlo™ High-Performance Cell Sorter (Dako-Cytomation) based on the CFSE expression in MM cells. Following sorting procedure cells where centrifuged at 300 G and pellets were stored at -70°C.

RNA was extracted from frozen pellets with Qiagen RNeasy extraction kits and Biotin-labeled target cRNA was obtained using Ambion Message Amp II-Enhanced kit. cRNA was hybridized for 16h at 45°C on Affymetrix HG-U133 Plus 2.0 arrays and arrays were scanned using GCOS software.

For data analysis we used the Ariadne Pathway Studio software version 6 where data was imported as Affymetrix files. Find differentially expressed genes tool from the software was applied which briefly it was a two paired correlated T-test with FDR correction Benjamini-Hocheberg (Benjamini *et al.* 1995). The correlated groups were adherent L363 versus control L363 and non-adherent versus control. The algorithm is based on one way ANOVA test to calculate p-value and expression differences. To find group of genes that share similar biological functions or regulation and pathways that these genes have in common we used Gene Set Enrichment Analysis (GSEA) (Mootha *et al.* 2003; Subramanian *et al.* 2005) function of Pathway Studio software with Mann-Whitney U Test as enrichment algorithm with p-value cut-off set to 0.05.

RESULTS AND DISCUSSIONS

During co-culture, L363 cells have separated into two fractions: one fraction remained in suspension, non-adherent to BMSCs and the second fraction strongly adherent to stromal cells (Fig.1).

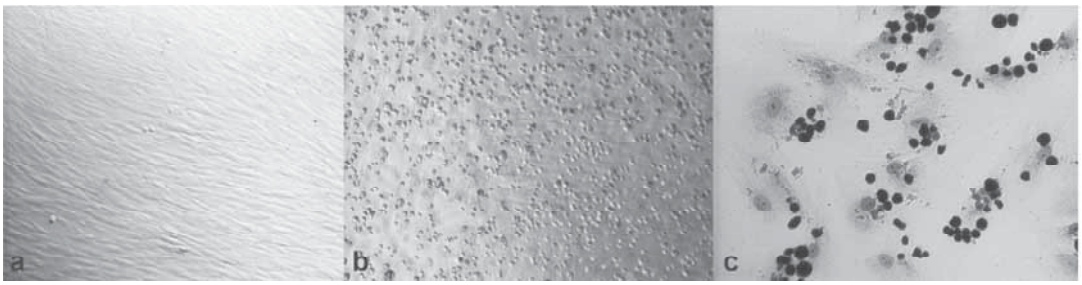

Figure 1. *a)Confluent layers of bone marrow stromal cells in culture (objective x10, phase contrast). b)and c) Co-culture of L363 MM cells with bone marrow stromal cells: b) The entire population of L363 and BMSCs (objective x10, phase contrast). c) BMSCs and adherent population of L363 (objective x40, Dia Panoptic staining)*

The GSEA analysis of these two populations of L363 gave slightly different results (Tables 1 and 2).

For adherent group we could note several important groups of genes involved in: regulation of apoptosis, transcription, cell cycle regulation, cell proliferation, cell-matrix adhesion, Stat pathways and many others, the full list is shown in Table 1.

Table 1. *GSEA analysis for adherent fraction of L363.Groups of genes and pathways are sorted according to p-value.*

Name	Type	p-value	Gene Set Category
protein transport	Group	5.49456e-012	biological_process
Actin Cytoskeleton Regulation	Pathway	7.97844e-011	Ariadne Pathways
regulation of transcription from RNA polymerase II promoter	Group	1.90138e-010	biological_process
transcription	Group	2.47822e-010	biological_process
regulation of transcription, DNA-dependent	Group	1.286e-008	biological_process
EndothelinRb -> AP-1/CREB/ELK-SRF signaling	Pathway	3.42193e-008	Ariadne Pathways
Cell Cycle Regulation	Pathway	4.37057e-008	Ariadne Pathways
protein amino acid phosphorylation	Group	6.13805e-008	biological_process
Focal Adhesion Regulation	Pathway	7.38964e-008	Ariadne Pathways
EndothelinRa -> AP-1/CREB signaling	Pathway	1.4404e-007	Ariadne Pathways
Hedgehog Pathway	Pathway	1.59695e-007	Ariadne Pathways
ubiquitin cycle	Group	1.88078e-007	biological_process
heart development	Group	3.11742e-007	biological_process
apoptosis	Group	4.7738e-007	biological_process
response to DNA damage stimulus	Group	5.29973e-007	biological_process
EDG3/5 -> AP-1/ELK-SRF signaling	Pathway	1.05171e-006	Ariadne Pathways
cell proliferation	Group	1.08143e-006	biological_process
ubiquitin-dependent protein catabolic process	Group	1.55949e-006	biological_process
EphrinR -> actin signaling	Pathway	1.70039e-006	Ariadne Pathways
cell cycle	Group	2.96537e-006	biological_process
B Cell Activation	Pathway	3.51272e-006	Ariadne Pathways
AdrenergicRa -> STAT3 signaling	Pathway	4.86739e-006	Ariadne Pathways
T Cell Activation	Pathway	5.67735e-006	Ariadne Pathways
ICAM1 -> AP-1/CREB/ELK-SRF signaling	Pathway	5.86233e-006	Ariadne Pathways
liver development	Group	6.01069e-006	biological_process
GFR -> AP-1/CREB/CREBBP/ELK-SRF/MYC signaling	Pathway	7.69736e-006	Ariadne Pathways
ProstaglandinIR -> ATF1/ELK-SRF/CREB signaling	Pathway	8.19262e-006	Ariadne Pathways
AngiotensinR -> CREB/ELK-SRF/TP53 signaling	Pathway	8.50676e-006	Ariadne Pathways
regulation of cell cycle	Group	8.90809e-006	biological_process
collagen fibril organization	Group	9.23608e-006	biological_process
protein ubiquitination	Group	9.58185e-006	biological_process
cell adhesion	Group	9.62985e-006	biological_process
VIPR -> CREB/CEBP signaling	Pathway	1.13924e-005	Ariadne Pathways
positive regulation of transcription from RNA polymerase II promoter	Group	1.24272e-005	biological_process
cell-matrix adhesion	Group	1.24424e-005	biological_process
ProstaglandinFR -> ATF1/ELK-SRF/CREB signaling	Pathway	1.24896e-005	Ariadne Pathways
vesicle-mediated transport	Group	2.00596e-005	biological_process
RNA processing	Group	2.17154e-005	biological_process
protein complex assembly	Group	2.39186e-005	biological_process
AdrenergicRa -> ELK-SRF signaling	Pathway	2.51617e-005	Ariadne Pathways
IL8R -> CREB/EGR signaling	Pathway	2.69459e-005	Ariadne Pathways
NTRK -> AP-1/CREB/ELK-SRF/MYC/SMAD3/TP53 signaling	Pathway	3.17944e-005	Ariadne Pathways
intracellular protein transport	Group	3.42739e-005	biological_process
ThromboxaneR -> CREB signaling	Pathway	3.57738e-005	Ariadne Pathways
AdenosineR -> AP-1 signaling	Pathway	4.5588e-005	Ariadne Pathways
Notch Pathway	Pathway	5.07402e-005	Ariadne Pathways
DNA recombination	Group	5.18688e-005	biological_process
PTAFR -> AP-1/ATF1/CREB/ERK-SRF signaling	Pathway	5.46443e-005	Ariadne Pathways
AdrenergicRb -> CREB signaling	Pathway	7.31138e-005	Ariadne Pathways
DNA repair	Group	7.89122e-005	biological_process
TNFRSF1A -> AP-1/ATF/TP53 signaling	Pathway	8.19938e-005	Ariadne Pathways
CholecystokininR -> ELK-SRF signaling	Pathway	9.06278e-005	Ariadne Pathways
endocytosis	Group	9.27979e-005	biological_process
CCR1 -> STAT signaling	Pathway	9.88798e-005	Ariadne Pathways
chromatin modification	Group	0.000103728	biological_process

Like in any cancers malignant cells in MM escape from apoptosis and cell cycle regulation so we can understand these results. The expansion of a malignant populations results when cell cycle is deregulated or apoptosis is inhibited (Nadav *et al.* 2006; Runge *et al.* 2006).

Several pathways involving Stat genes are present. Jak-Stat pathway (Fig.2) is an important mechanism involved in gene activation, proliferation and differentiation of cells (Heinrich *et al.* 1998) and is well documented and targeted for gene therapy in MM (Li *et al.* 2010; Monaghan *et al.* 2011; Zhang *et al.* 1992).

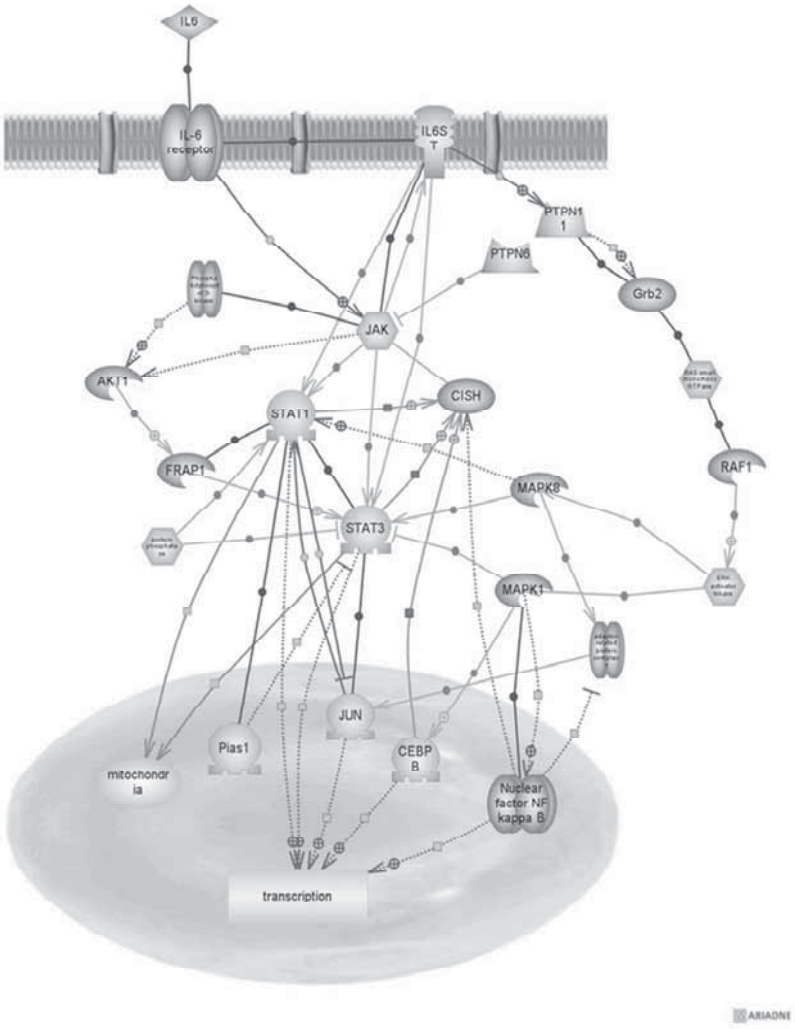

Figure 2. *Jak-Stat pathway starting from Il-6 (Ariadne Genomics Pathway Studio)*

Genes from cell to cell adhesion biological group and focal adhesion regulation pathway in particular (Fig.3) are also present in GSEA analysis of the adherent fraction. These genes could explain why we have an adherent group of L363 cells.

Signaling through adhesion molecules is another important factor for survival and proliferations of MM malignant cells (Runge *et al.* 2006) and we could see here that adherent group of L363 benefit from this.

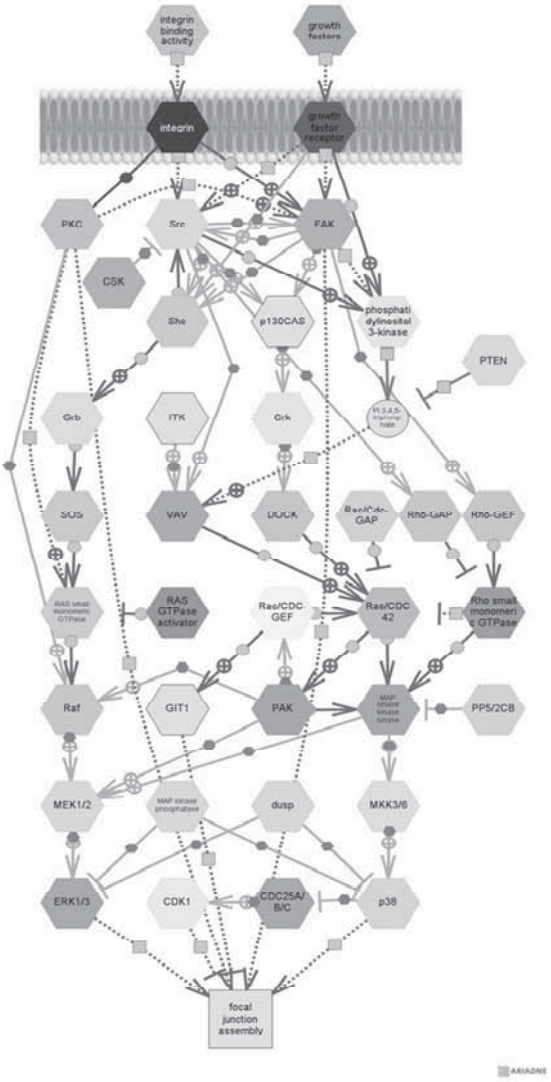

Figure 3. *Focal Adhesion Regulation pathway (Ariadne Genomics Pathway Studio)*

What is interesting is the listing of chromatin remodeling group of genes. We could speculate that during co-culture L363 adhere to BMSCs and this contact is followed by chromatin modifications which should lead to activation and inactivation of other genes.

The GSEA analysis of non-adherent fraction shows similar results but is a little less enriched in groups of genes and pathways (Table 2).

Table 2. *GSEA analysis for non-adherent fraction of L363.Groups of genes and pathways are sorted according to p-value.*

Name	Type	p-value
Actin Cytoskeleton Regulation	Pathway	9.91471e-013
actin cytoskeleton organization and biogenesis	Group	7.7315e-011
T Cell Activation	Pathway	1.02541e-010
protein amino acid phosphorylation	Group	2.06896e-009
intracellular signaling cascade	Group	2.08824e-009
Focal Adhesion Regulation	Pathway	7.22823e-008
collagen fibril organization	Group	1.02218e-007
antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	Group	3.5503e-007
Notch Pathway	Pathway	1.03183e-006
EphrinR -> actin signaling	Pathway	1.39943e-006
heart development	Group	1.41878e-006
Mast Cell Activation	Pathway	2.80055e-006
negative regulation of transcription from RNA polymerase II promoter	Group	4.4219e-006
NK Cell Activation	Pathway	6.28418e-006
apoptosis	Group	6.72629e-006
GFR -> AP-1/CREB/CREBBP/ELK-SRF/MYC signaling	Pathway	1.26576e-005
GFR -> NCOR2 signaling	Pathway	2.18771e-005
response to cytokine stimulus	Group	2.23038e-005
cell migration	Group	5.00859e-005
FcIgER -> ELK-SRF signaling	Pathway	5.51718e-005
T-cell receptor -> ATF/CREB signaling pathway	Pathway	5.87207e-005
AngiotensinR -> CREB/ELK-SRF/TP53 signaling	Pathway	6.01854e-005
NTRK -> AP-1/CREB/ELK-SRF/MYC/SMAD3/TP53 signaling	Pathway	6.51755e-005
Gonadotrope Cell Activation	Pathway	6.75204e-005
regulation of cell growth	Group	7.11294e-005
regulation of apoptosis	Group	7.49006e-005
IL4R -> ELK-SRF/HMGY signaling	Pathway	7.97987e-005
ICAM1 -> AP-1/CREB/ELK-SRF signaling	Pathway	0.000121752
negative regulation of cell cycle	Group	0.000138059
cell adhesion	Group	0.000141082
KIT -> MITF signaling	Pathway	0.000157054
AdrenergicRa -> STAT3 signaling	Pathway	0.000167192

CONCLUSIONS

This experiment gives an insight of genes and pathways in L363 MM cell line affected by microenvironment represented here by BMSCs. Our results are in concordance with other people findings in this field and offer a good image of effects at molecular level.

We could see that there are many genes involved in this pathology ranging from apoptosis to cell to cell adhesion and interaction, genes that regulates cell cycle, cell migration, cell survival and replication.

There are more genes and pathways affected in the adherent fraction which means that intimate contact of malignant cells with stroma together with signals from soluble factors of microenvironment is more important than paracrine signalling alone.

Knowing what genes and pathways are involved in MM pathology is important for the elaboration of therapeutic strategies.

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THE ANALYSIS OF CATALASE AND PEROOXIDASE ACTIVITY IN SAPROPHYTIC FUNGUS *RHIZOPUS NIGRICANS* GROWN ON MEDIUM WHITH DIFERENT CONCENTRATION OF GRINDED CORN CARYOPSIS

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Keywords: *Rhizopus nigricans*, catalase, peroxidase, corn caryopsis
Abstract. The purpose of this study was to assay catalase and peroxidase activity in the saprophytic fungus *Rhizopus nigricans*, grown on mediums containing grinded corn caryopsis, which, in our experiments have replaced carbon source – sucrose in composition of liquid culture medium Czapeck Dox, resulting in the final three experimental variants: V1 = 20 g/l, V2 = 30 g/l, V3 = 40 g/l, while the control variant composition remained unchanged. Measurements were made at three time intervals: 5 days and 10 days and 15 days after inoculation, using fungus mycelium and culture liquid. Determination of catalase activity was performed using Sinha method (Artenie Vl., *et al.,* 2008), and determination of peroxidase was carried out on the basis of ortho-dianisidine method (Cojocaru D.C., 2009). The results showed significant deferens in dynamic of enzyme activity depending on the concentration of carbon source introduced into the medium and age of the fungus.

INTRODUCTION

The filamentous fungus *Rhizopus nigricans* is an obligate aerobe that is frequently found in decayin of organic matter rich in complex carbohydrates. This organism has de ability to thrive in such environments because of its simple growth requirements and capacity to produce numerous hydrolytic enzymes (Skory, C.D., *et al*, 2009).

Fungal cells must deal with a wide variety of potentially toxic environmental challenges during the course of their proliferation (Moye-Rowley, S. W., 2003).

The use of oxygen as the respiratory substrate is frequently reported to lead to the development of oxidative stress, mainly due to oxygen-derived free radicals, which are collectively termed as reactive oxygen species (ROS) (Qiang, Li *et al*, 2009).

The involvement of oxygen in metabolic processes in fungi is coupled to its activation and formation of number of highly reactive compounds such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical OH^- (Sigler, K., *et al*., 1999).

The development of fungi proceeds in immediate contact with the environment. Therefore, they are constantly subjected to physical and chemical stress factors. All aerobic organisms generate reactive oxygen species, especially through aerobic respiration. Reactive oxygen species (ROS) are formed by fungi in the course of metabolic activity. ROS production increases in fungi due to various stress agents such as starvation, light, mechanical damage, and interactions with some other living organisms. Regulation of ROS level appears to be very important during development of the fungal organism (Gessler N.N. *et al*., 2007).

High reactivity of ROS is responsible for oxidation of proteins, lipids, and acids. Consequently, systems defending against ROS by repair or resynthesis of damaged molecules are present in the cell. Nevertheless, impairment of intracellular redox status, as a result of an increase in generation of oxygen radicals exceeding the cellular capacity to neutralize them, can generate the oxidative stress. Intracellular ROS increase is accompanied by the cessation of growth, and it provokes morphological changes leading to cell adaptation to changes in life conditions as well as the decrease in intracellular oxidants (Belozerskaya T.A.. *et al*, 2006).

Characteristic of the oxidative stress in fungi are a massive protein oxidation with their subsequent degradation, glycosylation, and carbonylation; (Kritskii, M.S., 1982; Hansberg, W., *et al*., 1990; Aguirre J, *et al*. 2005; Gessler, N.N., *et al*., 2006). The metabolic rearrangement leads to the cessation of growth and the synthesis of secondary metabolites in fungal cells, many of which are antioxidants (Bai, Z., *et al*., 2003; Sokolovskii V.Yu. and Belozerskaya T. A., 2000; Yoshida Y. and Hasunuma K., 2004).

Hydrogen peroxyde is the most stable of the oxygene reactive species (ROS) and is a strong nucleofilic oxidant. Hydrogen peroxide is degraded by catalase and peroxidase, enzymes that act synergistically to protect cells.

Fungi are reported to be high producers of catalases (Klotz, M.G. *et al*, 1997; Eremin, A.N., *et al*, 2000; Kurakov, A.V., *et al*, 2001), and different types of catalases and catalase genes have been isolated (Isobe, K., *et al*, 2005).

Also, a large number of peroxidases have been identified in fungal species and are being characterized at the molecular level (Conesa, A., *et al*, 2002).

There are essential nutrients, that participate together with enzymes in antioxidant processes, delaying or totally inhibiting oxidation of the substrate and acting at different levels of oxidative sequence (Halliwell B. and Gutteridge J.M.C., 2007, Sarikurkcu C. et al., 2010). Possessing mechanisms to adapt to oxidative stress (Tanaka C., Izumitsu K., 2010), represented by an endogenous antioxidant system, fungi are able to release exoenzime in the extracellular space to minimize the negative impact of reactive oxygen species.

The objective of this paper, following the line of other research (Manoliu Al. e*t al*., 2005, 2006, 2010) regarding the influence of environmental factors on enzyme activity, aimed at analyzing the activity of these biochemical parameters of oxidative stress in the *Rhizopus nigricans* species grown in laboratory conditions on medium containing different concentrations of grinded corn caryopsis, corn grain representing one of most extensive cultivated cereal species, due to its high nutritional value, being used in human food, animal feed and raw materials for various industries.

MATERIALS AND METHODS

The study was conducted on the species *Rhizopus nigricans*. The fungus has been isolated from germinated wheat cariopses, which were taken from the storage place of the Enterprise of Cereal Products from Chişinău, Republic of Moldova.

Pure culture was obtained after several cycles of growth on PDA solid medium. Identification of *Rhizopus nigricans* species was based on morphological characteristics of the mycelium from culture plates and by making microscopic preparations.

To determine the activity of both enzymes was used Czapek Dox liquid medium with the following composition: sucrose 30 g, NaNO3 2 g, K2HPO4 1 g, KCl 0.5 g, MgSO4. 7H2O 0.5 g, FeSO4. 7H2O 0.01 g, distilled water 1000 ml (Constantinescu O., 1974). The culture medium composition was modified by replacing the carbon source - sucrose, with different amounts of grinded corn caryopsis, resulting in the final three experimental variants: V1 = 20 g/l, V2 = 30 g/l, V3 = 40 g/l, plus a control version, in which composition of medium remained unchanged. Medium was distributed in Erlenmeyer flasks in quantities of 100 ml. In each flask was placed a disk of 8 mm in diameter from 5 days old culture of *Rhizopus nigricans*. The flasks were incubated in the thermostat, set at 28 ˚ C. Enzyme determinations were performed at three time intervals from inoculation of the fungus: at 5, at 10 and 15 days, using fungus mycelium and culture liquid.

Determination of catalase activity was performed using Sinha method (Artenie Vl., *et al*., 2008), and determination of peroxidase was carried out on the basis of ortho-dianisidine method (Cojocaru D.C., 2009).

RESULTS AND DISCUSSIONS

Results on the influence of different concentrations of grinded corn caryopsis, which were introduced into the culture medium, on catalase and peroxidase activity in mycelium and culture liquid of the saprophytic fungus *Rhizopus nigricans*, are shown graphically in figures 1-4.

The dynamics of peroxidase activity in the mycelium (figure 1), recorded, in the first period after inoculation, higher values for all variants compared to the control variant (UP 0.0945 / g / min), enzymatic activity increased in relation to the concentration of grinded corn caryopsis. The maximum value was reached in variant V3 (0.3466 UP / g / min), followed in descending order by V2 version (0.3079 UP / g / min) and variant V1 with a value very close to that of the control value – 0.0984 UP / g / min.

In the second study period, preroxidase activity recorded an increase, the values were much higher than those recorded in the first period. Experimental variants showed higher values compared to the control variant (0.0978 UP / g / min), the maximum enzyme activity occurring in the version with 30 g/l grinded corn caryopsis (1.0689 UP / g / min), followed by a close value in variant V1 with 20 g/l of grinded caryopsis (1.0269 UP / g / min) and the minimum value was found in variant V3 - UP 0.3906 / g / min.

In the third period from insemination, with the ageing of the fungus, there is a decrease of this enzyme activity in all experimental variants (0.2172 UP / g / min - for version V1, 0.6485 UP / g / min – for V2 version and 0.6397 UP / g / min for the variant V3), probably due to

consumption of nutrients from the medium. In all variants were recorded values higher than the value recorded in control variant - 0.0678 UP / g / min.

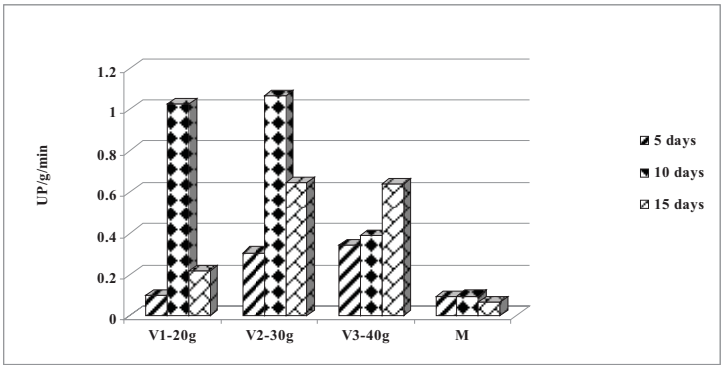

Figure 1. Peroxidase activity in mycelium of *Rhizopus nigricans* species grown on medium with grinded corn caryopsis

Further was determined peroxidase activity in culture liquid at 5, 10 and 15 days, as it is known, fungi are able to synthesize and discharge a series of compounds into the environment, including enzymes.

As shown in figure 2, peroxidase activity recorded at 5 days after insemination of fungus, quite low values for all variants with grinded corn caryopsis, the values are comparable to that of the control version (0.0176 UP / ml / min), ranging between 0.0231 UP / ml / min and 0.0775 UP / ml / min.

At 10 days after inoculation an increase in enzyme activity takes place in all variants, except the control version in which value remained almost unchanged (0.01718 UP / ml / min). The maximum value of peroxidase activity was recorded in V2 variant (0.3109 UP / ml / min) followed in descending order by the variant V3 (0.0718 UP / ml / min) and variant V1 (0.0515 UP / ml / min).

The ageing of fungus entailed a decrease in enzymatic activity at 15 days after inoculation, and in the control variant it was almost completely inhibited (0.0014 UP / ml / min). Maximum value occurred in variant treated with 30 g/l grinded corn caryopsis (0.2478 UP / ml / min), followed decreasingly by the version in which the medium contains 40 g/l corn caryopsis (0.0391 UP / ml / min) and variant with 20 g/l grinded caryopsis (0.0304 UP / ml / min).

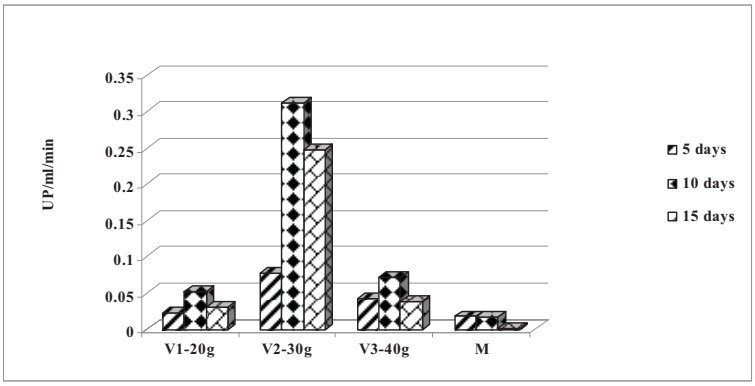

Figure 2. Peroxidase activity in culture liquid of *Rhizopus nigricans* species grown on medium with grinded corn caryopsis

Data on the dynamics of catalase in the mycelium of fungus *Rhizopus nigricans,* grown on medium with different concentrations of grinded corn caryopsis are reproduced graphically in figure 3.

In the first study period, catalase activity in the mycelium showed higher values in all experimental variants compared with control (209.234 cu / g / min). In V1 version was found maximum value (1180.2348 UC / g / min), followed in descending order by variant V3 (763.6217 UC / g / min) and the minimum value was recorded in version V2 (485.5941 UC / g / min).

In the second period catalase activity shows a decrease in all variants, except V2 variant in which it intensifies (798.4831 UC / g / min).

In the third period after inoculation, , with the ageing of the fungus, recorded values in working versions are kept low (762.823 CU / g / min – for version V1 and 408.4513 UC / g / min - for V3 version), except V2 variant in which the enzyme activity continued to increase (3472.7863 UC / g / min).

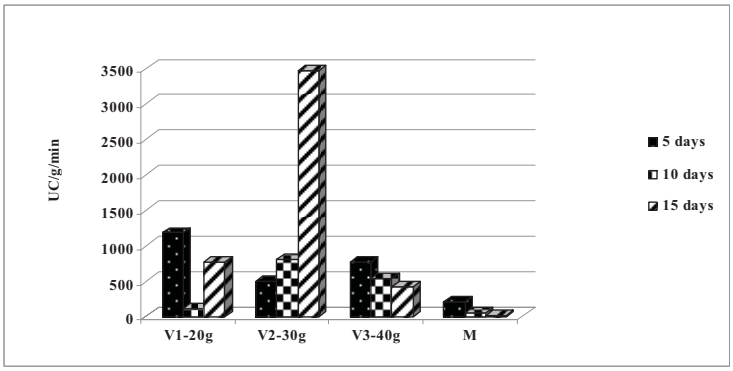

Figure 3. Catalase activity in mycelium of *Rhizopus nigricans* species grown on medium with grinded corn caryopsis

As found in figure 4 catalase activity in culture liquid at 5 days after inoculation of culture medium recorded higher values for all variants (206.2338 UC / ml / min – in the V1 version, 204.9371 UC / ml / min – in the V2 and 208.3618 UC / ml / min - for variant V3) compared with control variant (UC 74.2081 / ml / min), the results are comparable, not being able to detect significant differences depending on the concentration grinded caryopsis of the mediu.

At 10 days after inoculation of the fungus has been a significant increase in enzymatic activity in all variants containing grinded corn caryopsis (probably due to the release of the enzyme in the culture medium), except control version whose value is only slightly elevated (90.3954 UC / ml / min), values were also comparable (V1 - 1129.9807U C / ml / min, V2 - 1090.2004 UC / ml / min, UC / ml / min, V3 - 1142.303 UC / ml / min).

The ageing of fungus and depletion of nutrient soucers from the medium had led to a drastic decrease in catalase activity in the third period in all studied variants (values ranging between 27.8569 UC / ml / min in V3 and 81.1629 in V1), including control variant (13 UC / ml / min).

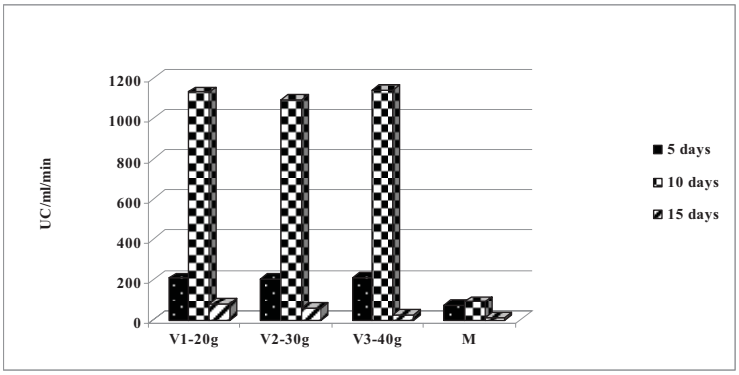

Figure 4. Catalase activity in culture liquid of *Rhizopus nigricans* species grown on medium with grinded corn caryopsis

CONCLUSIONS

After analyzing, the experimental results showed that catalase and peroxidase activity was influenced by concentration of grinded wheat caryopsis and by fungus culture age.

- Peroxidase activity in the mycelium was stimulated in all experimental variants by the introducing into the culture medium grinded corn caryopsis.
- In culture liquid peroxidase activity was stimulated in version V2 in all study periods.
- In the fungus mycelium catalase activity was stimulated in all three periods in the V2 version.
- Catalase in liquid culture was stimulated by the presence of corn caryopsis in all work options in the first two periods, and inhibited in third period, probably due to culture ageing.

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COMPARED ANALYSIS OF CATALASE AND PEROXIDASE ACTIVITY IN CELLULOLYTIC FUNGUS *TRICHODERMA REESEI* GROWN ON MEDIUM WITH DIFFERENT CONCENTRATIONS OF GRINDED WHEAT AND BARLEY STRAWS

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Keywords: *Trichoderma reesei*, catalase, peroxidase, barley straws, wheat straws

Abstract: The purpose of this study was to assess the evolution of catalase and peroxidase activity in *Trichoderma reesei* grown on medium containing grinded wheat and barley straws. Carbon source of cultivation medium - glucose was replaced by various concentrations of grinded wheat and barley straws, finally resulting three experimental variants as follows: V1 = 20 g/l, V2 = 30 g/l, V3 = 40 g/l. În addition to these variants a control sample was added in which composition remainded unchanged. The catalase activity was determined by spectrophotometric Sinha method (Artenie et al., 2008) while peroxidase activity was assesed using the o-dianisidine method (Cojocaru, 2009). Enzymatic determinations were carried out at 7 and 14 days from inoculation, in both fungus mycelium and culture liquid. The enzymatic assay showed significant differences between determinations intervals and work variants. Enzyme activity is influenced by the age of fungus and by the different nature of the substrate used.

INTRODUCTION

Trichoderma reesei is a mesophilic soft-rot ascomycetous fungus producing high levels of cellulases and hemicellulases, commercially used to modify and hydrolyze plant cell walls polysaccharides (Levasseur, 2010). It is an ubiquitos soil dweller, able to transform a wide variety of organic materials of both natural and xenobiotic origin.

All aerobic organisms use molecular oxygen (O_2) for respiration and energy supply. At the same time they have to face the toxic side effects of O_2, the production of reactive oxygen species (ROS), such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-).

Hydrogen peroxide is a by-product of all living organisms which rely on respiration for energy production. The main site of H_2O_2 production is the mitochondrion (Turrens, 2003). Hydrogen peroxide has a cytotoxic effect on the cell due to its ability to damage macromolecules, including lipids, DNA, and proteins (Jamieson, 1998).

Compared with other reactive oxygen species (ROS), H_2O_2 is less toxic, but is able to diffuse into different compartments from its original production sites before reaching its target (Branco, 2004). Detoxification of H_2O_2 is a fundamental aspect of the cellular antioxidant response in which catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) play a major role. These enzymes, commonly designated as hydroperoxidases are involved in the metabolism of hydrogen peroxide (Levy et al., 1991).

The present study followes the line of previous researches on catalase and peroxidase activity of cellulosolytic fungi under the influence of magnetic field (Manoliu et al., 2005a), liquid ferric (Manoliu et al., 2005b), bakery waste industry (Manoliu et al., 2006).

Wheat and barley straws are important agriculture byproducts. These residues represent an abundant, inexpensive and readily available source of renewable lignocellulosic biomass used for the production of alternative fuels. The filamentous fungus *Trichoderma reesei* is used in enzyme pretreatment processes of the lignocellulosic biomass (Rosgaard et al., 2007).

Chemical composition of wheat and barley straws was assessed on previous studies (Antogiovanni, Sargentini, 1991; Graham, Aman, 1984). Wheat straws consist of 35-45% cellulose, 20-30% hemicellulose and 8-15% lignin (Saha, Cota, 2006); barley straws are made of 33% cellulose, 28, 1 % hemicellulose and 14.9 % lignin (Graham, Aman, 1984).

Extracellular hydrogen peroxide has been involved in the degradation of the crystalline cellulose component of plant cell walls (Veness, Evans, 1989).

To determine how the fungus protects itself against detrimental effects of reactive oxygen species, catalase and peroxidase activity was examined on culture medium containing grinded wheat and barley straws.

MATERIAL AND METHODS

Strain and cultivation: Trichoderma reesei was acquired from the Institute Sciétific de Santé Publique, Belgium by Biological Science Research Institute, Iaşi. The fungus was cultivated on potato dextrose agar plates (PDA)

for 7 days at 28°C. For enzymatic assay we used Sabouround liquid medium with the following composition: peptone-10g, glucose-40g and distilled water-1000 ml (Constantinescu, 1974) in which we replaced the carbon source-glucose with different concentration of grinded wheat and barley straws, ultimately resulting four variants for each type of straw: V1-20 g/l, V2-30 g/l , V3-40 g/l and V4 in which the carbon source was not replaced. Wheat and barley straws were collected from a field near Iaşi, in Miroslava. They were kept in polyethylene bags away from humidity. Prior to addition to culture medium, wheat and barley straws were grinded in an electric grinder.

Enzyme assay. Peroxidase and catalase activity was assessed at 7 and 14 days after fungal inoculation in both mycelium and culture liquid. Peroxidase activity was assessed on the basis of ortho-dianisidine method (Cojocaru, 2009), while catalase activity was determined by spectrophotometric Sinha method (Artenie et al. 2008).

RESULTS AND DISCUSSION

The results of catalase activity in the fungus *Trichoderma reesei* grown on medium containing grinded wheat straws and barley straws are depicted in figure 1 and 2. In the fungus mycelium, at 7 days from inoculation the catalase activity was higher in variants containing various concentrations of grinded wheat straws and barley straws compared to the control sample with glucose as a solely carbon source. No increase in catalase activity correlated to grinded straw concentration was recorded. For example, V1 containing grinded barley straws recorded catalase activity of 2461 UC/g/min compared to 2057 UC/g/min in V2.

At 14 days from inoculation all variants showed a slightly decrease in catalase activity. The media variants with grinded wheat and barley straws recorded higher values compared to the control sample.

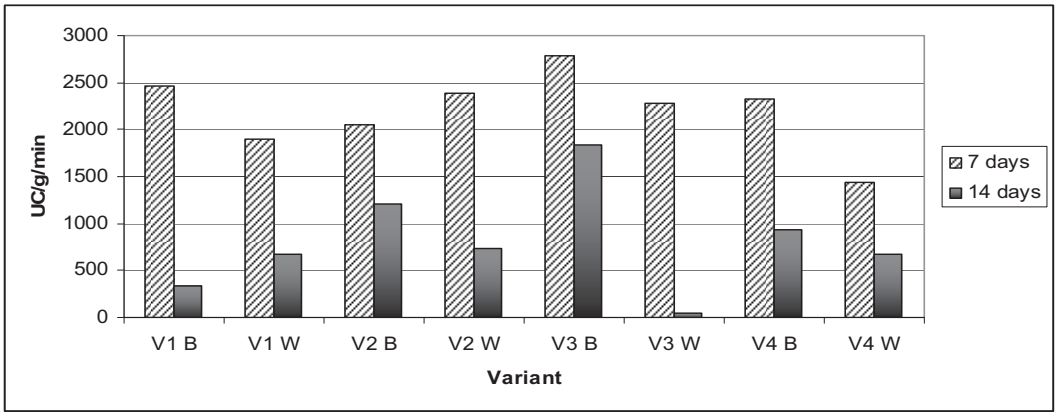

Fig.1 Catalase activity in fungus *Trichoderma reesei* cultivated in medium containing grinded wheat and barley straws-mycelium (B-barley, W-wheat)

Variant 3 medium containing grinded barley straws recorded the lowest value of 49, 14 UC/g/min. In variants containing grinded wheat straw the catalase activity increased simultaneous with carbon source concentration, but was lower than the activity recorded at seven days.

In culture liquid, at 7 days from inoculation, all variants containing grinded barley straws as carbon source recorded lower values of enzyme activity compared to control variant. These results are similar to medium variants with grinded wheat straws, where control sample recorded the highest value of 488 UC/ml/min. In variants with grinded barley straws the activity was constants when correlated with straw concentration. In contrast, in media variants with grinded wheat straws recorded fluctuating values with a value of 192,14 UC/ml/min in V2 variant and a value of 14,92 UC/ml/min in V3.

The catalase activity in culture liquid at 14 days from inoculation was stimulated in medium containing grinded barley straws compared to control sample. Catalase activity also increased compared to values recorded previously at 7 days. In contrast, the catalase activity in control sample decreased, reaching a value of 68, 69 UC/g/min. In variants with grinded wheat straws enzymatic activity incresed compared to datas recorded at 7 days, but decreased in control variant (259 UC/g/min). Grinded wheat concentration did not influenced catalase activity.

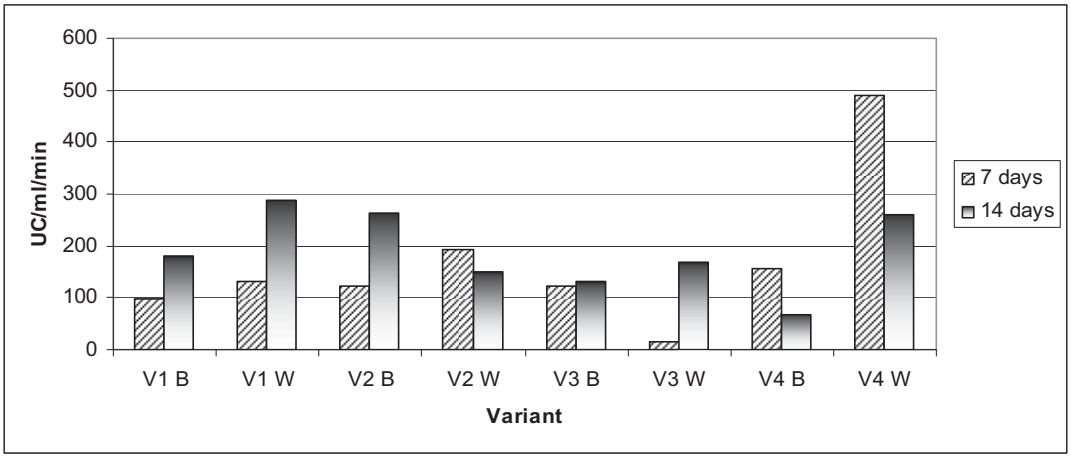

Fig. 2 Catalase activity in fungus *Trichoderma reesei* cultivated in medium containing grinded wheat and barley straws – culture liquid (B-barley, W-wheat)

When comparing datas recorded in both mycelium and liquid culture we conclude that catalase activity increased in mycelium at 7 days from inoculation, and decreased in the second interval. In contrast in liquid culture enzymatic activity decreased at 7 days and increased at 14 days. Even if the overall enzyme assay analysis show a trend in enzymatic activity, the liquid culture recorded low catalase activity when compared to mycelium of *Trichoderma reesei* in both determination intervals.

The peroxidase activity in *Trichoderma reesei* was assessed in both mycelium and liquid culture at 7 and 14 days from inoculation of medium containing grinded barley and wheat straws (Figure 3 and 4).

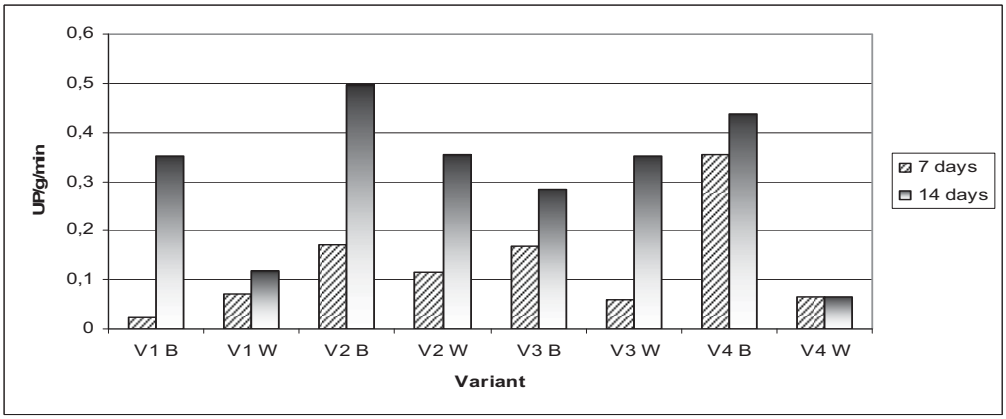

Fig.3. Peroxidase activity in fungus *Trichoderma reesei* cultivated in medium containing grinded wheat and barley straws – mycelium (B-barley, W-wheat)

In mycelium, peroxidase activity in variants with grinded barley straws was lower than in control sample. In contrast, in variants with grinded wheat straws enzymatic activity recorded a lower value in control sample (0, 06 UP/g/min). Peroxidase activity was influenced by carbon source concentration in variants with grinded barley straws. In constrast, media containing various concentration of grinded wheat straws did not effect enzymatic activity.

At 14 days from inoculation enzymatic activity increased in both variants with grinded wheat and barley straws. In variants with grinded wheat straws the peroxidase activity was higher than control variant compared to 7 days enzyme assessment. In medium with grinded barley straws peroxidase activity fluctuated compared to control, with V3 (0, 059 UP/g/min) being higher than control variant (0, 0656 UP/g/min).

Different carbon source concentration added to Sabouround medium did not correlate with enzymatic activity, various values being recorded in both media containing wheat and barley straws. The control sample showed similar peroxidase activities patterns at 7 and 14 days from inoculation.

The peroxidase activity was assessed in liquid culture at 7 days from inoculation and the activity was overall. The values recorded in medium containing different concentration of wheat and barley grinded straws varied, in some cases being higher than control variant (V1 in barley is 0, 1234 UP/ml/min compared to V4 0, 07822 UP/ml/min).

At 14 days from inoculation, the peroxidase activity increased compared to the previous recorded activity at 7 days, reaching its highest in V3 medium with grinded barley straws (0, 12968 UP/ml/min). *Trichoderma reesei* grown on medium with grinded barley straws had an enzymatic activity higher than control, whereas variants with grinded wheat straws recorded just one value above control (V3- 0, 175 UP/ml/min).

Overall peroxidase activity was lower in liquid culture than in mycelium, but in both cases it increased at 14 days from inoculation.

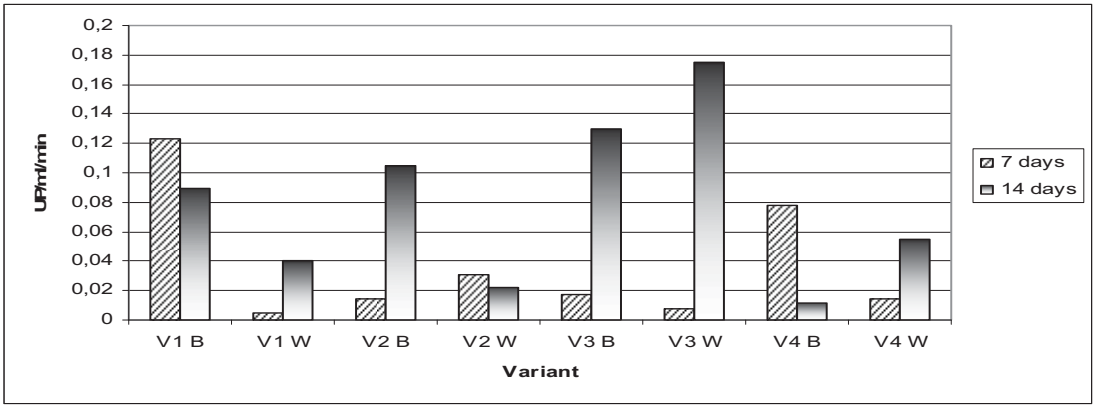

Fig.4. Peroxidase activity in fungus *Trichoderma reesei* cultivated in medium containing grinded wheat and barley straws – culture liquid (B-barley, W-wheat)

CONCLUSIONS

Assessment of catalase activity in the mycelium of *Trichoderma reesei* grown on media with barley and wheat straws showed a slightly increase in enzymatic activity at 7 days from inoculation, and a decrease in activity in the second interval. In contrast, data collected from the liquid culture indicated a decrease in enzymatic activity at 7 days and an increase at 14 days.

The increase in catalase activity from mycelium recorded at 7 days was not correlated with substrate concentration, though at 14 days we recorded an increase in enzymatic activity simultaneously with the carbon source concentration. Enzymatic activity in culture liquid at 7 days from inoculation was lower in all variants compared to control. The catalase activity was stimulated in liquid culture at 14 days in experimental variants with grinded barley straws and in those with grinded wheat straws.

The peroxidase activity recorded from the fungus mycelium at 7 days was lower in variants with grinded barley straws than control. Peroxidase activity was influenced by carbon source concentration in variants with grinded barley straws. At 14 days from inoculation enzymatic activity increased in both variants with grinded wheat and barley straws.

Peroxidase activity recorded in the liquid culture at 7 days was overall low and the data recorded in medium containing different concentration of wheat and barley grinded straws varied. At 14 days from inoculation, the peroxidase activity increased compared to the enzymatic activity recorded at 7 days.

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RESEARCH ON THE INFLUENCE OF H^+ IONS CONCENTRATION ON THE DYNAMICS OF THE ACTIVITIES OF CERTAIN DEHYDROGENASES OF THE KREBS CYCLE IN THE *MONILINIA LAXA* (ADERH. & RUHL.) HONEY FUNGUS PARASITIC ON PLUM TREES

ELENA TUTU*[1], ELENA CIORNEA[1]

Keywords: *Monilinia laxa* (Aderh.&Ruhl.) Honey, dehydrogenases, Krebs cycle, H^+ ions concentration, pH.

Abstracts: During the process of nutrition, thus in that of their growth, microorganisms are subject to the influences of certain environmental factors that condition the microbial activity determining either the growth and reproduction, or the inhibition of activity and the inactivation of microorganisms. A well known means of expressing the H^+ ions concentration in a certain environment is the pH, an important chemical factor that is closely observed when growing ascomycetes, for any alteration of its value entails conformational alterations of their enzymes, the characteristics of the substrate, such that they can no longer interact with the active site of the enzyme or be subject to catalysis. The present study comprises the results of our research on certain oxidoreductase implied in the steps of the Krebs cycle in the *Monilinia laxa* (Aderh.&Ruhl.) Honey, a fungus that parasites the prune. The enzymatic determinations took place at 7 and 14 days from the mycelium of the fungus cultivated in Leonian media, whose pH was adjusted to values between 2.0 and 9.0 by using NaOH 1N and HCl 0,1N solutions. We registered different values of the dehydrogenasic activity, directly correlated with the physiological condition of the fungus (given its age) and with the initial pH value of the culture’s environment.

INTRODUCTION

The cellular metabolism of microorganisms in the nutrition process is influenced by a series of chemical and physical factors, among which the concentration of environmental hydrogen ions. It is well known that pH is an essential means of measuring the concentration of hydrogen ions in biological systems and that it can influence the three-dimensional structure of proteins, including the enzymes that participate in the cellular metabolism, the transport of nutrients and the electrons transfer (Dunca, S. *et al*., 2005, Cojocaru, D. C. *et al.,* 2007).

Several fungi grow on a broad range of pH values (Mehotra, R.S. and Aneja, K.R., 1990, Kawasaki, K. and Suzuki, M., 1993, Naqvi, S.A.M.H., 2004). The alterations of the external concentration of H^+ ions cause small, transitory changes in the intracitoplasmatic pH, that is around 7,6 in most filamentous fungi and whose existence is due to a homeostatic pH mechanism localized intrahifally (Bachewich, C.L. and Heath, J.B., 1997, Bagar, T. *et al*, 2009**)**. The ability of responding to the environmental pH variation in the filamentous fungi is realized by means of a mediation system comprising membranous cytoplasmatic proteins, signal transduction pathways and signal dependant pH transcription factors, acting as gene repressors or activators and their expression represents the answer of the fungal cell and constitutes a key-factor of its virulence, by intervening in the production of mycotoxins and antibiotics, and last but not least in the enzymatic activity (Peñalva, M.A. and Arst, Jr., H.N, 2002, Calcagno-Pizarelli, A. M. *et al*., 2007, Hervas-Aguilar, A. *et al*, 2010, Hua, X. *et al*, 2010)**.**

The present study wishes to be a continuation of certain research on the biology of the *Monilinia laxa* (Aderh.&Ruhl.) Honey fungus cultivated in environments with different pH values (Manoliu, Al. *et al.*, 2010); during the experiments we monitored the activities of four key enzymes of the Krebs cycle: isocitrate dehydrogenase (E.C.1.1.1.41), α – ketoglutarate dehydrogenase (1.2.4.2), succinate dehydrogenase (E.C. 1.3.99.1), malat dehydrogenase (E.C.1.1.1.37). The reasons at the root of this study are related to the following statements: the oxygen’s involvement in metabolic processes within living organisms has to do with its activation and the formation of a large number of very reactive compounds (Gessler, N.N. *et al*., 2007); the respiratory chain, whose reactions have to do with those of the citric acid cycle, is a rich source of oxygen (Turrens, J.F., 1996), the mitochondria thus becoming a vulnerable target of oxidative stress, thus affecting the functioning of the whole Krebs cycle (Hyslop, P.A. *et al*, 1988, Tretter, L. and Adam-Vizi, V., 2000), as well as the fact that the Krebs cycle enzymes are pH-sensitive (Kubicek, C.P., 1988, Papagianni, M., 2007). The central aspects of the present study consists of establishing a degree of susceptibility to various concentrations of H^+ ions of the main dehydrogenasese involved in the four stages of the tricarboxylic acids cycle and the quantification of their activity at different time intervals for the purpose of evaluating their dynamics.

MATERIALS AND METHODS

The *Monilinia laxa* (Aderh.&Ruhl.) Honey strain was isolated in the laboratory, its source being represented by fruits mummified by fungi harvested from genera of *Prunus domestica* from the Experimental Orchard of the Fruit Trees Research and Development Station in Miroslava, Iasi. The pure culture was obtained from the sporodochia previously

washed in distillate water and placed in Petri boxes with a PDA medium, and kept for 7 days in thermostat. The *in vitro* cultivation of the fungus, in submerse conditions, was made in stationary conditions, in the dark and at a constant temperature of 28º C, the fungus being placed on discs with a diameter of 0,8 mm in Erlenmayer flasks, with a Leonian environment. The various concentrations of hydrogen ions in the culture environment were obtained by means of an adequate buffer of NaOH 1N and HCl 0,1N. The pH scale varied between 2.0 and 9.0, thus obtaining 9 probes, of which one was a control probe, whose pH had not been altered. To determine the biochemical experiments, we took samples from the mycelium of the fungus at 7 and 14 days after setting the culture environment.

The method used is mainly based on the dehydrogenases' capacity to transfer hydrogen from various sub layers to the 2,3,5-triphenyltetrazolium chloride that reduces itself and passes to red trifenyl formazan. The intensity of the colour of the resulting formazan was spectrophotometrically determined by means of the Sîsoev and Krasna method (modified by Artenie). The studies indicated significant differences in the dynamics of the enzyme's activity, due on the one hand to hydrogen ions concentration in the environment, and on the other hand to the age of the mycelian culture.

RESULTS AND DISCUSSIONS

The results of the experimental determinations made during the two time frames in the mycelium of the *Monilinia laxa (*Aderh.&Ruhl.) Honey fungusare presented in Figures 1 and 2.

The rigorous analysis of these results indicates a relative proportionality of the values of the four enzymes that catalyse the main stages of the Krebs cycle, suggesting a sort of continuity and a balanced development of the reactions involved in both of the time frames.

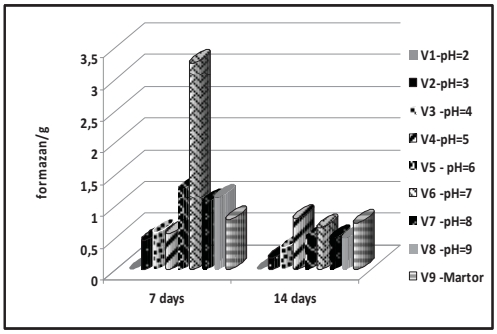

Fig.1. The activity of the isocitrate-dehydrogenase in the *Monilinia laxa* (Aderh.&Ruhl) Honey mycelium cultivated on the media with various H^+ ions concentrations

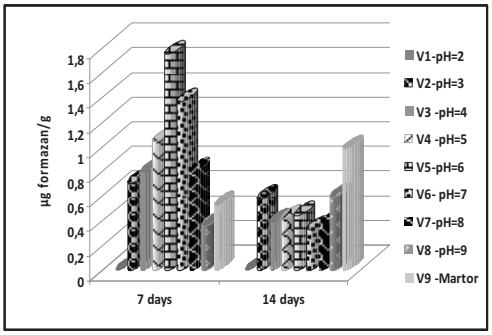

Fig.2. The activity of the α-ketoglutarate-dehydrogenase of the *Monilinia laxa* (Aderh.&Ruhl.) Honey fungus cultivated on the media with various H^+ ions concentrations

A maximum activity of the isocitrate-dehydrogenase was indicated at the day 7 stage (3,2390 µg formazan/g.mat.) in the pH -7 probe, followed by the probe with the initial pH 6, where we registered a value of 1,2601 µg formazan/g.mat.biol. The inferior limits of the endoenzyme's activity in this time frame were registered in the acid interval of the selected pH scale (pH- 2 , pH- 3 – 0,4476 µg formazan/g.mat.), with some growth on the pH- 5(0,5642 µg formazan/g.mat.), which attests to the data in the literature, which situates the optimum value of the pH for the isocitrate dehydrogenase between 7 and 8 (Cjocaru, D.C., 2009), and the inhibition point in the acid extremity of the pH scale (Adrio, J.L. and Demain, A.L., 2005). The ageing of the culture brought a relative uniformization of the activity of the isocitrate dehydrogenase manifested by a diminishing of the activity of the endoenzyme in all probes, except V2 and V3. Thus, at day 14 stage the highest activity was registered environment where the initial pH was 5 (0.7957 µg formazan/g.mat.biol.), followed by the control sample (0,7299 µg formazan/g.mat.biol.). The lowest value of the isocitrate dehydrogenases activity was registered in the pH 3 sample, i.e. probe V2 (0,1743 µg formazan/g.mat.biol.).

In the young culture, 7 days after the incubation, the optimum pH of the α-ketoglutarate-dehydrogenase in the mycelium of the fungus was registered in the V5 probe (1,7504 µg formazan/g.mat.biol.), the endogenous enzyme manifesting an intense activity both at pH 7 (1,3627 µg formazan/g.mat.biol.) and at pH 5 (1,0415 µg formazan/g.mat.biol.), which attests to the data presented in the literature and which places the optimum pH of the enzyme between 7,2 and 7,4 (Hirabayashi, T. and Harada, T., 1971), that is close to our results. Comparatively, at pH 8, pH 4 and pH 3, the α-ketoglutarate-dehydrogenase biosynthesis had lower levels at various levels and at pH 9 the oxoglutarate dehydrogenase was inhibited. The ageing of the culture brought along a diminishing of the activity of the oxoglutarate dehydrogenase in all the probes as compared to the control probe (0,9842 µg formazan/g.mat.biol.).

As for the the activity of the enzyme that catalyses the dehydrogenation of the succinic acid into fumaric acid, at the first interval of biochemical quantitative determinations it a maximum of 1,2994 µg formazan/g.mat.biol. following by the 1,1718 µg formazan/g.mat.biol. in the mycelium of the *Monilinia laxa* cultivated submersely on the medium with an initial value of the pH 3, by the 1,0942 µg formazan/g.mat.biol. in the medium with pH 5, 1,0100 µg formazan/g.mat.biol. in medium with pH 4 and is followed by a level of 0,479807 µg formazan/g.mat.biol., decelated in the initial pH 9 sample.

The running of the enzymatic tests during day 14 since the inoculation indicates a different level of activity of the succinate dehydrogenase in the acid extremity of the chosen pH scale, placing its highest point in the V4 sample (0,7865 µg formazan/g.mat.biol.), followed decreasingly by probes V6 with pH 7, V3 with pH 4 (0,7253 µg formazan/g.mat.biol. and 0,5593 µg formazan/g.mat.biol., respectively), V5 with pH 6 (0,4494 µg formazan/g.mat.biol). The activity of the endogenous enzyme in the mycelium grown on the medium with the initial pH 9 (0,2315 µg formazan/g.mat.biol.) is comparable to the one registered in the medium with pH 3 (0,2241 µg formazan/g.mat.biol), in lower levels, followed by the one in V7 with inital pH 8 (0,1953 µg formazan/g.mat.biol.).

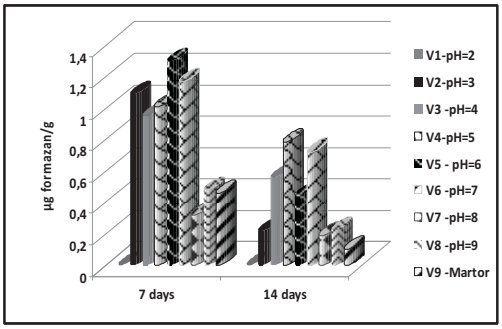

Fig.3. The activity of the succinate-dehydrogenase of the *Monilinia laxa* (Aderh.&Ruhl.) Honey fungus cultivated on the media with various H^+ ions concentrations

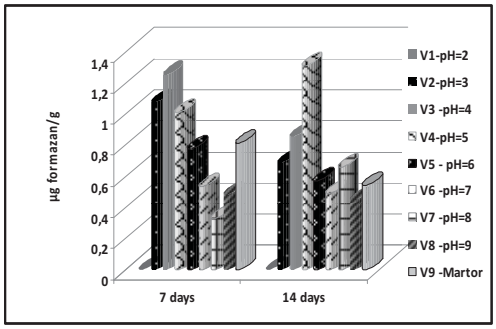

Fig.4. The activity of the malat-dehydrogenase of the *Monilinia laxa* (Aderh.&Ruhl.) Honey fungus cultivated on the media with various H^+ ions concentrations

If we reconsider the determinations from day 7, we notice the existance of an oxalacetate formation rate, catalysed by the intensive malat dehydrogenase in the acid side of the pH scale. Thus, in probe V3 (pH 4) the activity of the endogenous enzyme was 1,2528 µg formazan/g.mat.biol., and in V2 (Ph 3) and V4 (pH 5) the values are 1,0718 µg formazan/g.mat.biol. and 1,0101 µg formazan/g.mat.biol.

A classification of the modulator effect produced by the hydrogen ions concentration n the activity of the malat-dehydrogenase activity in the mature culture maintains the intense conversion rate of the malat in oxaloacetic acid in the acid part of the current experimental pH scale, except its extremity (pH 2), where the enzyme activity is null. Thus, in the V2 sample (pH 3) the regeneration of the oxalacetate rendered by the activity of the endoenzyme reached a level of 1,0718 µg formazan/g.mat.biol., in V4 (initial pH 5) a level of ,0101 µg formazan/g.mat.biol. but the maximum activity was registered in V3 (initial pH 4), i.e. 1,2528 µg formazan/g.mat.biol.

The quantification of the enzymatic activity registered in the two time frames highlighted a series of dynamic changes in the mitochondrial activity, confirming its role in the Krebs cycle, in the conditions of interdependency with the hydrogen ions concentration from the environment, as well as its modulator effect on preserving the redox balance and the involvement of the mitochondria in the antioxidant defensive.

CONCLUSIONS

The investigations run for the analysis of the modulator role of the hydrogen ions concentration on the activity of the enzymes that catalyze the main stages of the Krebs cycle in the mycelium of the *Monilinia laxa* species, have highlighted the following aspects:

- Seven days after the incubation, the activity of the isocitrate dehydrogenase was stimulated by the pH 7, pH 6, pH9 and pH 8, while the α- ketoglutarate dehydrogenase increased the performance of the reaction it catalyzed in the media with pH 6, pH 7, pH 5, pH 8, pH 4 and pH 3. The optimum pH for the succinate dehydrogenase in the mycelium of the fungus was decelated in the probe with pH 6, the enzyme being intensified in initial pH 7 medium, in pH 5 as well pH

4 and pH 3, while the malate dehydrogenase was influenced positively in the media with acid pH, i.e. pH 4, pH 3 and pH 5.

- Fourteen days after inoculating the culture media, the activity of the isocitrate dehydrogenase was faintly intensified in the environments with pH values of 5, while the activity of the α- ketoglutarate dehydrogenase was inhibited in all the probes. In the probes with pH values of 5, 7, 4, 6, 9, 3 and 8, succinate dehydrogenase had an intense activity in this time frame, and malat dehydrogenase manifested an increased activity at an optimum pH of 5, closely followed by pH 4, pH3, pH 8 and pH 6.

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STUDIES CONCERNING THE INFLUENCE OF SOME AMINO ACIDS ON THE DYNAMICS OF KREBS CYCLE DEHYDROGENASES ACTIVITY AT *MONILINIA LAXA* (ADERH.& RUHL.) HONEY PARASITE ON PLUM TREES

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Keywords: *Monilinia laxa,* isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, malate dehydrogenase.

Abstract. The amino acids are metabolised via the enyzmatic reactions of the Krebs cycle, the central mechanism for metabolism in the cell that is generating energy for production of adenosine triphosphate (ATP) molecules. For this reason, in the present paper, the dynamics of the isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase is investigated in mycelium of submerged cultivated strain of *Monilinia laxa* fungus and the analysis of the results concerning the influence of some amino acids as alanine, glutamic acid, aspartic acid, asparagine, cystine, cysteine, histidine, methionine, phenylalanine, valine, leucine, lysine and serine on the Krebs cycle dehydrogenases shows that the profile of the variation curves are dependent by the type of amino acid introduced in culture medium and fungus age.

INTRODUCTION

The filamentous fungi provide their structure and functions (which involve the maintenance of biochemical and functional equilibrium) with a continuous energy input from the outside environments. Numerous data from literature describe a strong relationship between amino acids and the physiological functions of the fungal cell and nitrogen is considered to be essential for the biosynthesis of cellular molecular complexes (Isaac, S., 1992). Most fungal species cannot use inorganic nitrogen sources as well as organic compounds that contain nitrogen (proteins and amino acids) for growth, a wide range of fungi require amino acids for growth, being able to use it better in the presence of carbon sources. Amino acids degradation occurs via oxidation, with oxygen consumption, an biological process improperly called biological oxidation that takes place during the tricarboxylic acid cycle, Krebs cycle being a central point of the metabolism, linked to processes that are part of energy production (Cojocaru, D.C. and Sandu, M., 2004) and in which the carbon skeleton resulting from desamination or transamination is converted into a series of metabolic intermediates (Artenie, Vl., 1991). Amino acids such as alanine, cysteine, cystine, glycocolului, serine, threonine and hydroxyproline, usually enter the citric acid cycle via pyruvate (Lentner, C. quoted by Cojocaru, D.C. and Sandu, M., 2007), through acetyl-CoA. This too is the point of junction with the Krebs cycle of amino acids such as phenylalanine, tyrosine, leucine, tryptophan and lysine, which first forms acetacetil-CoA and phenylalanine and tyrosine are the amino acids that are coupling the Krebs cycle made through fumarate (Kang, J., 2008), while methionine, valine and isoleucine enter the tricarboxylic acid cycle at the level of succinil-CoA (Karp, G., 2009).

Other amino acids such as arginine, proline, histidine and glutamine enter the Krebs cycle by forming in advance, as an intermediary, glutamic acid, which passes through transamination to α-ketoglutarate that represents the point of connection with the citric acid cycle. Biosynthesis of intermediates containing carbon in the Krebs cycle must take into account the metabolism needs of the cell and the energy needs, many biomolecules synthesizing their hydrocarbon skeleton on the account of some intermediaries of the tricarboxylic acid cycle, which enshrines this cycle as a path with amphiboly fingerprint (catabolic and anabolic).

The metabolism of amino acids by fungi and their use in building of protein blocks, enzymes, RNA and DNA is mostly in lag phase, and the number resulting from cell biosynthesis is the maximum in the exponential growth phase, their amount decreasing with the age of the culture, all these procedures being closely linked to the Krebs cycle (Gottlieb, D. and Van Etten, J.L. 1964).

MATERIALS AND MEHODS

The biological material used in this study, represented by ascomycetous *Monilinia laxa* (Aderh. & Ruhl.) Honey was isolated from mummified fruits collected from of *Prunus domestica* varieties from the experimental orchard for Fruit Research and Development Miroslava, Iasi County. The *in vitro* cultivation of the fungus was made using the Leonian medium, shared in Erlenmeyer flasks, in which were added over 0.125 mg of the following amino acids: glutamic acid, aspartic acid, alanine, asparagine, cystine, cysteine, histidine, methionine, phenylalanine, valine , leucine, lysine and serine, working with a control sample without amino acids. The 14 medium were seeded with slices cut from a culture of *Monilinia laxa* (Aderh. & Ruhl.) Honey aged for 7 days and incubated in an thermostat. The experimental measurements performed at 7, respectively, 14 days, were made from the fungus mycelium for each treatment variant three parallel determinations were made, the enzyme activity, followed in dynamics, was determined using Sîsoev and Krasna spectrophotometric method (Cojocaru, D.C., 2009).

RESULTS AND DISSCUTIONS

A detailed analysis of the results obtained from experimental measurements performed in the mycelium of *Monilinia laxa* fungus at 7, respectively, 14 days and graphically presented in Figures 1 and 2, allowed a conclusive framework on which it can be certainty stated that the activity of dehydrogenases enrolled in the study had variations in both time intervals, depending on the type of amino acid added to the culture medium, a correlation with the age of culture mycelia was also found.

So, the isocitrate dehydrogenase during the maturation of the mycelium of *Monilinia laxa* species had an ascending trend over time, modulated by amino acids as methionine, leucine and serine (from 1.7573 µg formazan / g mat. at 1.9778 µg formazan / g mat., from 0.7606 µg formazan / g mat. up to 1.8732 µg formazan / g mat. for serine and from 0.9416 µg formazan / g mat. to 1.3913 µg formazan / g mat. for leucine).

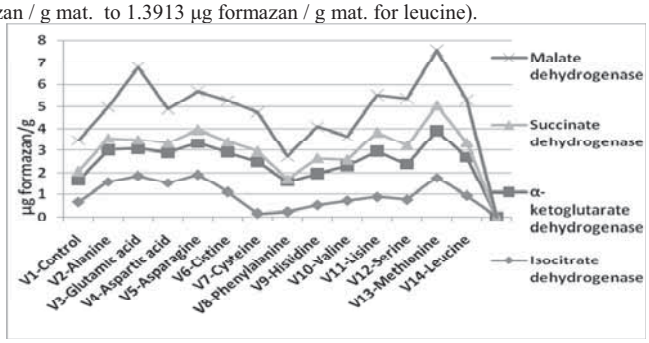

Fig. 1 – The amino acids influence on dehydrogenases of the Krebs cycle in a 7 days old mycelium from *Monilinia laxa* fungus

This suggests that in those cultures catabolic rate is high, respiration is performed at high levels and that's because on the one hand, it is necessary to convert isocitrate to oxoglutarate and, on the other hand, the need for α - ketoglutarate synthesis, essential for other amino acids biosynthesis (possibly among others, methionine, which may derive from e by synthesis "de novo", taking into account the high rates of enzyme biosynthesis in both time intervals).

It is known that by maturation and aging of the mycelia cultures the activity of isocitrate dehydrogenase lowers or cease altogether and it can be concluded that the remaining experimental variants mediums supplemented with amino acids - asparagine (from 1.9072 µg formazan / g mat at 0.9950 µg formazan / g mat.), alanine (from 1.5713 µg formazan / g mat. to 0.8419 µg formazan / g mat), acid glutamic (1.8631 µg formazan / g mat to 0.8350 µg formazan / g mat.) aspartic acid (1.5201 µg formazan / g mat to 0.8549 µg formazan / g mat.), cystine (1.1044 µg formazan / g mat.to 0.7007 µg formazan / g mat.) and valine (0. µg formazan / g mat to 0 , 4854 µg formazan / g mat.) the metabolic activity became slower, as amino acids consumption for various building elements (purine and pyrimidine bases, other enzymes, enzyme cofactors as - thiamine pyrophosphate for example, necessary for the enzyme activity in the Krebs cycle, to fill the needs of the cell (such as active transport of substances) with energy, for other amino acid production or, when the culture came out from trophophase and went into idiophase, for basic functions and for the structures of any secondary metabolism products). Although the values were smaller than those present in version control for other amino acids the endoenzyme dynamic had a upward trend.

Therefore, in the medium with phenylalanine, the enzyme activity increased from to 0.23400 µg formazan / g mat. to 1.0381 µg formazan / g mat, in that with additional cysteine rised from the 0.1540 µg formazan / g mat. to 0.7697 µg formazan / g mat. and histidine supplementation of the culture medium was assisted by a rise isocitrate dehydrogenase from 0.5234 µg formazan / g mat to 0.8365 µg formazan / g mat.

The α-ketoglutarate dehydrogenase activity at 7 days after inoculation compared with isocitrate dehydrogenase activity for the same time, had significant increases in all medium variants of supplemented with amino acids, compared with the version control that had the value of 0.9757 µg formazan / g mat. This confirms the role of α - ketoglutarate dehydrogenase in the detoxification of reactive oxygen species, but also amino acids role in α-ketoglutarate homeostasis, that seemed to be allocated for oxoglutarate production during oxidative stress. Examining of α-ketoglutarate dehydrogenase dynamics shows differences in the amplitude variation curves for oxidoreductase activity at both time intervals depending on the type of amino acid introduced into the culture medium.

Hence, a relatively modest increase had the enzyme activity under the stimulatory action of cysteine - from 2.3224 µg formazan / g mat. to 2.5052 µg formazan / g mat., of cystine from 1.8062 µg formazan / g mat. to 2.2851 µg formazan / g mat., while histidine approximately doubled the oxidoreductase activity in time - from 1.4258 µg formazan / g mat. to 2.3478 µg formazan / g mat, the behaviour also applied to alanine - from 1.4467 µg formazan / g mat. to 2.0885 µg formazan / g mat. and glutamic acid - from 1.2114 µg formazan / g mat. to 2.0419 µg formazan / g mat. The

oxoglutarate dehydrogenase activity showed a slight descent, which coincided with the presence of serine in the culture medium (from 1.6094 µg formazan / g mat. up to 1.9293 µg formazan / g mat.), and of leucine (1.7502 µg formazan / g mat at 1.9261 µg formazan / g mat.), the medium without amino acids (control variant) had the same enzymatic behaviour through time (from 0.9757 µg formazan / g mat to 1.8477 µg formazan / g mat.). Asparagine influenced less the oxoglutarate dehydrogenase activity, varying between the two time intervals from 1.4216 µg formazan / g mat. to 1.4800 µg formazan / g mat. By contrast, the presence of aspartic acid in the submerged culture of *Monilinia laxa* was followed by a slight decrease in oxidoreductase activity over time - from 1.3845 µg formazan / g mat. to 1.2546 µg formazan / g mat., the endoenzyme had a decreasing amplitude in the case of medium variants with phenylalanine (from 1.3457 µg formazan / g mat. up to 1.2668 µg formazan / g mat valine (from 1.5759 µg formazan / g mat.to 0.9920 µg formazan / g mat. lysine (from 2.0674 µg formazan / g mat to 1.1754 µg formazan / g mat.), methionine (2.1559 µg formazan / g mat to 1.5225 µg formazan / g mat. The influence of these amino acids on the enzyme in the mycelium after 14 days of incubation reflected in a much reduced level compared to the first interval and suggests the end of the primary metabolic cycle of events and the start of the next stage.

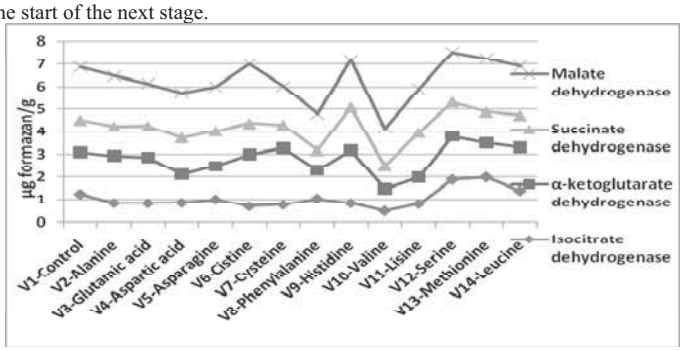

Fig 2 – The amino acids influence on dehydrogenases of the Krebs cycle in a 14 days old mycelium from *Monilinia laxa* fungus

A quantification of the activity of the enzyme that catalyses succinate dehydrogenation in the fungus mycelium, represented graphically shows both continuity and balanced development of the Krebs cycle at this level, but also some significant differences in the activity of succinate dehydrogenase, that has some turns imposed by the age of the culture mycelia and by the type of amino acid on which the culture medium was supplemented.

A careful analysis indicates that the reaction catalysed by the oxidoreductase increased through time for all medium variants supplemented with amino acids, even if they showed some inhibitory effects in the mycelium of *Monilinia laxa* species. Given the fact that, while the fungus is getting older, the activity of α- ketoglutarate dehydrogenase and malate dehydrogenase slowers, while the activity of isocitrate dehydrogenase is stopped, succinate dehydrogenase is the only enzyme whose activity remains high due to its location in the mitochondrial membrane, which lasts as long as there are parts of it, even if the mitochondria begins to conduct the programs related to the cell apoptosis. Under these conditions, the dynamics of succinate dehydrogenase activity shaped when amino acids are used as benchmarks, is upward, the control variant shows an increase from 0.4250 µg formazan / g mat. to 1.4334 µg formazan / g mat., while the culture enriched with alanine, the oxidoreductase has an increase from 0.5467 µg formazan / g mat. to 1.3014 µg formazan / g mat. and in that supplemented with glutamic acid, from 0.4215 µg formazan / g mat to 1.3711 µg formazan / g mat.

During the course of the experimental program, the medium variable containing aspartic acid showed an increase in the respiratory rate and metabolism, the succinate dehydrogenase activity increased from 0.4360 µg formazan / g mat. to 1.6300 µg formazan / g mat., and asparagine, an amide of the aspartic acid acted as an inducer, the biosynthesis levels and oxidoreductase activity increased from 0.6354 µg formazan / g mat. to 1.5640 µg formazan / g mat. Thiol amino acids as cystine and cysteine stimulated the activity of the enzyme that catalyses the third step of the citric acid cycle, its limits going up from 0.4852 µg formazan / g mat. to 1.3955 µg formazan / g mat. respectively and from 0.4799 µg formazan / g mat. to 1.0347 µg formazan / g mat. Although in both quantitative determinations intervals phenylalanine had an inhibitory effect on succinate dehydrogenase, it increased as quantity from 0.1127 µg formazan / g mat. to 0.8769 µg formazan / g mat.

Large variations were found in the medium with the histidine (from 0.7117 µg formazan / g mat to 1.9043 µg formazan / g mat., valine (from 0.2900 µg formazan / g mat to 1013 µg formazan / g mat. and lysine (from 0.8869 µg formazan / g mat at 2.0003 µg formazan / g mat.). Through time, the submerged cultivated mycelium of *Monilinia laxa* fungus in association with methionine activated the succinate dehydrogenase progressively, its limits are marked with 1.1370 µg formazan / g mat., respectively 1.3843 µg formazan / g mat., while the enzyme levels in the culture enriched with leucine jumped from 0.6712 µg formazan / g mat. to 1.3843 µg formazan / g mat.

The malate oxidation with restored oxalacetate, the reaction that ends the Krebs cycle is catalyzed by malate dehydrogenase. What draws the attention to malate dehydrogenase activity after 7 days of incubation is that it is higher than that of succinate dehydrogenase in the same time period, suggesting that a part of the catalyzes substrate by this oxidoreductase enteres the cycle Krebs through other metabolic pathways (eg, in the cultures with glutamic acid or methionine, the difference might be due to a high intensity function of glicoxylate cycle which from the specific isocitric acid leads to glyoxylic acid formation, which by condensation with acetyl-CoA formes malic acid that enteres in the reaction catalysed by malate dehydrogenase). Examination of malate dehydrogenase activity dynamics model at *Monilinia laxa* points out two aspects: the first takes into account the stage in which the organism consumes partially or totally all amino acids from the medium, using them as carbon source and as indicators for oxidoreductase production, the malate dehydrogenase behaviour taking an ascending route for most samples and a downward allure for media supplemented with glutamic acid and methionine. And the second aspect is the fact that, beyond the matching behaviour succinate dehydrogenase for the medium variants with histidine, valine and phenylalanine, even if they had intensification of enzyme activity over time, the inhibitory effect remained present in both time intervals.

The singularity of the enzyme in case of glutamic acid at 7 days after inoculation has seen a fall in the activity throgh 14 days, the values passed from 3.2808 µg formazan / g mat. to 1.8987 µg formazan / g mat. and the presence of methionine in the culture medium was followed by a decrease in malate dehydrogenase activity from 2.5142 µg formazan / g mat. Biol. to 2.3599 µg formazan / g mat. Biol., while the existence of cysteine was followed by a minor decrease (from 1.7957 µg formazan / g mat. to 1.7143 µg formazan / g mat).

The biochemical tests revealed, however, intensification of biosynthesis of oxidoreductase and enzymatic activity results correlate very well with the observations on this behaviour in the case of some amino acids in both time periods. So, malate dehydrogenase activity in the variant without amino acids sources increased from 1.4105 µg formazan / g mat. to 2.3286 µg formazan / g mat., and for cystine from 1.8896 µg formazan / g mat. to 2.5988 µg formazan / g mat. Progressive enzymatic activity in both asparagine and aspartic acid ranged from 1.7286 µg formazan / g mat. to 1.9605 µg formazan / g mat. respectively, from 1.5414 µg formazan / g mat. to 1.9866 µg formazan / g mat.

As time passed, the malate dehydrogenase activity values went from 2.1495 µg formazan / g mat to 2.2176 µg formazan / g mat. under the influence of serine, from 1.9493 µg formazan / g mat to 2.1633 µg formazan / g mat under leucine induction, from 1.4133 µg formazan / g mat. to 2.2368 µg formazan / g mat. The same positive influence was also reflected in the case of lysine, the variation of growth went from 1.6968 µg formazan / g mat to 1.9281 µg formazan / g mat. We could also see from of graphical data analysis that in the case of phenylalanine, histidine and valine the induced activity of the endoenzyme was negative in both periods, despite the advancement operational rates for that stage of the Krebs cycle.

Thus, the value variation was from 1.0602 µg formazan / g mat to 1.9281 µg formazan / g mat in the circumstances of valine present in the submerged culture, from 1.0541 µg formazan / g mat. to 1.6204 µg formazan / g mat. in the case of phenylalanine and from 1.4339 µg formazan / g mat. to 2.0908 µg formazan / g mat. in the mycelium with additional histidine.

CONCLUSIONS

In the young mycelium, aged for 7 days, the isocitrate dehydrogenase activity was stimulated by asparagine, glutamic acid, methionine, alanine, aspartic acid, cystine, leucine, lysine, serine and valine. All amino acid induced the high biosynthesis of α-ketoglutarate dehydrogenase, and the succinate dehydrogenase activity was stimulated by methionine, lysine, serine, histidine, leucine, asparagine, alanine, cystine, cysteine and asparatic acid, while the activity of malate dehydrogenase was stimulated only by glutamic acid, methionine, serine, leucine, cystine, cysteine, asparagine, lysine, aspartic acid, histidine and alanine.

In the mature culture, aged for 14 days, the isocitrate dehydrogenase activity was stimulated by methionine, serine and leucine, the α-ketoglutarate dehydrogenase by cysteine, histidine, cystine, alanine, glutamic acid, serine, and leucine, the succinate dehydrogenase activity by lysine, histidine, asparatic acid, asparagine, serine and the malate dehydrogenase activity by cystine and methionine.

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STUDIES ON THE DYNAMICS OF DEHYDROGENASES KREBS CYCLE ACTIVITY AT *MONILINIA LAXA* (ADERH. & RUHL.) HONEY FUNGUS GROWN ON MEDIA WITH DIFFERENT CARBOHYDRATES

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Keywords: *Monilinia laxa,* isocitrate-dehydrogenase, α-ketoglutarase-dehydrogenase, succinate-dehydrogenase, malate-dehydrogenase, carbohydrates.

Abstract: As ubiquitous organisms, fungi grow on a large number of organic substrate, alive or dead, confronting therefore with a wide variety of carbohydrates and various physical factors, and their versatility to adapt and be able to use a large number of these compounds could provide them the chance to survive. Given that, these fungi have a rich enzyme equipment that allows them to operate on different metabolic pathways, this study aims to monitor the dynamics activity of some Krebs cycle dehydrogenases in *Monilinia laxa* (Aderh & Ruhl.) Honey species parasitic on various species of plum trees. To this end, the fungus was cultivated *in vitro* on media enriched with different carbohydrates and the isocitrate dehydrogenase, α-cetoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase activity in the fungus mycelium was followed, at 7, respectively, 14 days after the inoculation of the culture medium and determined using the spectrophotometric Sîsoev and Krasna method (Cojocaru, D.C., 2009). Data revealed obvious differences depending on the type of carbohydrate introduced into the medium and the age of the culture mycelia.

INTRODUCTION

Mitochondria are involved in many essential cellular processes – the production of ATP by oxidative phosphorylation, it participates in various metabolic pathways, contributes to homeostasis and signalling, also plays a key role in apoptosis (Desagher, S. and Martinou, J.C., 2000) and, as the key pivot in adjusting the relative levels of these proteins that make up the type metabolomics systems, and because all the enzymes involved in the Krebs cycle are found in the mitochondrial matrix, these organelles are able to modulate the entire cellular metabolism (Pon, L.A. and Schon, E.A., 2007) but also those of the substrates, forming a dynamic bio-plant that changes its shape and size depending on the cellular and environmental needs. Most active biomolecules such as proteins in a living cell operates as a complex rather than isolated (Beeckmans, S. and Kanarek, L., 1987) these protein-protein interactions having a great relevance for many biological functions. In addition to the most complexes, an increasingly number of such interactions was established, which form complexes rather transitory. Thus, in the metabolomics represented by the tricarboxylic acid cycle at fungi, four enzymes were found-isocitrate dehydrogenase, oxoglutarate dehydrogenase complex, succinate dehydrogenase, malate dehydrogenase, which catalyses the sequential reactions of the Krebs cycle, interacting with aconitase and fumarase and these with other ones that add anaplerotic pathways linked to the biosynthesis of amino acids from intermediate in the Krebs cycle.

All living organisms require energy to maintain their biologic activities. As quantity, the most important elements necessary for the living cells are oxygen, nitrogen, sulphur, phosphorus and especially, carbon. Since it is the basic constituent for all cellular components, it is, therefore, necessary in increased quantities. Unlike green plants capable of using inorganic carbon as CO2, converted into carbohydrates through photosynthesis, non-chloroplastic organisms such as fungi depend entirely of autotrophic organisms for their carbon requirement, and are characterized as chemoautotrophic. Carbon compounds such as carbohydrate are used in the metabolism of fungi for two functions: the first is to supplement the carbon necessary for the synthesis of compounds in the living cell (proteins, nucleic acids, nutritional reserves are included here, among others deriving from the activity and Krebs cycle) and secondary, oxidation of carbon compounds produce appreciable amounts of energy, derivation of the maximum energy from the carbohydrate necessary is complete oxidation undertaken by the tricarboxylic acid cycle enzymes (Gottlieb, D., 1963), fungi may utilize in this regard a wide range of carbon sources, such as: monosaccharides, oligosaccharides, polysaccharides, alcohols, organic acids and lipids (Kadan, M. and Thind, K.S., 1998).

Glucose occupies a key position in fungi metabolism, the degradation of the carbohydrate and various subsequent intermediaries from the energy supply processes of the terminal respiratory processes is quite important for the economy of the cell. Other hexose such as fructose, galactose, mannose, are also considered "metabolic fuels" (Lupea, X.A., 2007). Sucrose and its derived products from its direct catabolism are preferred as source for generating ATP, for the reducing power and for the C skeleton preferred for biosynthetic pathways connected to the Krebs cycle for fungi that belong to different communities. The ability of a compound to donate or receive electrons, described by the redox potential can imprint some difficulties for the substrate during oxidation or reduction, specifically related to a particular relative affinity for electrons that some substances have. For this reason, we can rightly say that there are compounds with a high redox potential such as dextrose cited above, which contains more energy due to the high degree of structural organization, and

other substances, although they may be completely oxidized, they cannot serve as energy sources although they can accept electrons, which influence the cell physiology at different levels (Husson, F. *et al*. 2006).

The amount of carbon source affects profoundly the status of tricarboxylic acid cycle (Poole, R.K., 2010). High positive correlations are found between the known concentration of carbon source and the Krebs cycle intermediate products such as malate, succinate, fumarate and also their negative correlation with the pH of the culture medium (Tarhan, L. *et al*., 2011) and the dependence on hydrocarbon source introduced into the medium.

The ensemble of works that made the experimental design involved determining the enzymes activity that catalyses various Krebs cycle sequences from the mycelium of *Monilinia laxa* (Aderh. & Ruhl) Honey fungus, their quantification and the analysis, on the one hand, of the relationship between the type of carbon source on which the culture medium was supplemented and endoenzyme activity, and, on the other hand, their evolution over time which involved a graphical and statistical approach, and other, from data reported in the scientific literature.

MATERIALS AND METHODS

The *Monilinia laxa* (Aderh. &Ruhl.) Honey inoculum was isolated from mummified fruits from *Prunus domestica* varieties that were collected from the Experimental Orchard of Fruit Research and Development Resort Miroslava, Iasi County. The fungus was cultivated *in vitro* on Leonian medium (in the formula changed by Bonnar), distributed in a quantity of 100 ml in Erlenmeyer bottles and supplemented with 2 g of the following carbohydrates: pentoses such as xylose, hexoses such as dextrose, fructose, disaccharides such as lactose, maltose, sucrose and celobiose, polysaccharides such as soluble starch and glycoproteins, or combinations such as arabic gum and last but not least, polyols such as glycerol, sorbitol and mannitol. We used a control sample devoid of hydrocarbon sources. The culture media were seeded with 8 mm in diameter rings cut from a culture of *Monilinia laxa* (Aderh. & Ruhl.) Honey aged at 7 days and incubated under stationary conditions at 28^0C in the thermostat. The three consecutive experimental measurements which were held at 7 days and 14 days after the inoculation of the culture medium were carried out in fungus mycelium and the Krebs cycle dehydrogenases activity was determined by Sîsoev and Krasna method, modified by Artenie, Vl. (Cojocaru, D.C., 2009). At the basis of this evaluation method of total microbial dehydrogenase activity are these enzymes and their ability to transfer hydrogen from various substrates to 2,3,5 - trifeniltetrazoliu.chloride which reduces to triphenyl-formazan and colours in red, the colour intensity is proportional to the dehydrogenases activity.

RESULTS AND DISCUSSIONS

The isocitrate dehydrogenase is of particular interest because it controls the carbon flux between the Krebs cycle and bypasses glyoxylate by kinase / phosphatase isocitrate dehydrogenase activation and inactivation (Laporte, D.C, and Koshland, D.E.J. 1982, Laporte, D. C. *et al*, 1985). Thus, activation of the ongoing forces into the Krebs cycle causes a decrease of isocitrate at cellular level and an increase of α-ketoglutarate levels (O'Roy, S. and Packard, T.T., 1998). The concentration of intracellular intermediate metabolites and thus, the enzyme level is dependent on time, the growth phases of submerged culture, and of NADH concentration throughout these phases. The pyruvate is also known as an activator for IDH phosphatase, an enzyme responsible for activating isocitrate dehydrogenase (Gálvez, S., and P. Gadal., 1995). In some microbial cultures with different carbon sources, isocitrate dehydrogenase, the enzyme that reflects the increased respiratory rate, increased in the early exponential growth phase and the plateau phase of culture, but also at the end of this. Also, the same data indicates that depleting the culture hydrocarbon sources is followed by a full depression of isocitrate dehydrogenase activity, to fill other cellular needs, which are maintained at lower levels, but in a state of alert (Roy, S.O. *et al*., 1999).

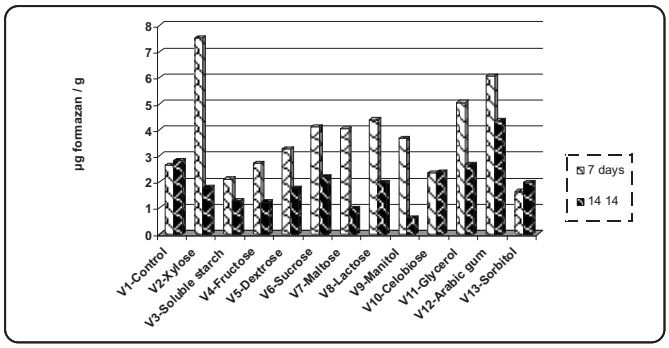

Fig. 1. The influence of carbohydrates on the dynamics of isocitrate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

At a rigorous analysis of the enzyme activity dynamics that catalyses the first step of the Krebs cycle as it is depicted in Figure 1, we found that all medium variants supplemented with carbohydrates, decreased with the aging of mycelia culture, except the witness, where an increase in the activity was observed, that rose to 2.6520 µg formazan / g.mat. to 2.8172 µg formazan / g.mat. In the medium sample enriched with sorbitol the activity of isocitrate dehydrogenase amplified from 1.6255 µg formazan / g.mat. to 1.9709 µg formazan / g.mat., and in the sample were celobiose was added, a tiny increase appeared, from 2.3437 µg formazan / g.mat. to 2.3739 µg formazan / g.mat. Thus, the strong fluctuations of the enzyme in the mycelium were recorded for the following carbohydrates: xylose - from 7.5226 µg formazan / g.mat. to 1.7731 µg formazan / g.mat., maltose - from 4.0352 µg formazan / g.mat to 0.9930 µg formazan / g.mat.and manitol from 3.6551 µg formazan / g.mat. to 0, 6219 µg formazan / g.mat followed by one filled with lactose - from 4.3881 µg formazan / g.mat la 1.9768 µg formazan / g.mat and with glycerol at the 5.0454 µg formazan / g.mat.to 2.6769 µg formazan / g.mat while in the medium enriched with sucrose, the enzyme presented a variation from 4.1032 µg formazan / g.mat.to 2.2096 µg formazan / g.mat, in that with arabic gum resulted a fluctuation from 6.0718 µg formazan / g.mat. to 4.3493 µg formazan / g.mat Dextrose had a similar behaviour in the mycelium on isocitrate dehydrogenase, reducing its activity from 3.2663 µg formazan / g.mat to 1.7503 µg formazan / g.mat. Inhibitory effects between the two time intervals had also fructose, the activity of the endoenzyme decreasing from 2.7209 µg formazan / g.mat. to 1.2243 µg formazan / g.mat but starch determinate a decline in the action of the enzyme that declined from 2.1007 µg formazan / g.mat. to 1.2809 µg formazan / g.mat.

The carbohydrates metabolism via tricarboxylic acid cycle involves the participation of α-ketoglutarate multienzyme complex, that consists of the following enzyme subunit: a ketoglutarate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and dihydrolpoyl dehydrogenase (E3), the complex being responsible for the oxidative decarboxylation of α-ketoglutarate by the intervention of coenzyme A to succinyl ~coenzime A.

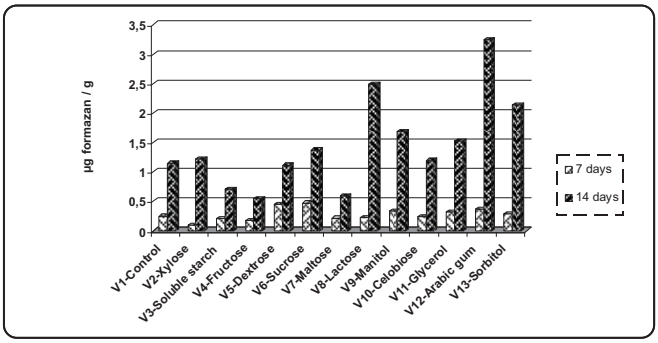

Fig. 2. The influence of carbohydrates on the dynamics of α-ketoglutarate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

The results regarding the activity of oxoglutarate dehydrogenase in the mycelium of *Monilinia laxa* fungus grown on media enriched with different carbon sources are graphically shown in Figure 2. You can see clear differences in the overall activity of the enzyme, suggesting a specific metabolic activity depending on the substrate available for the fungus but also the fact that age of the culture affects both nutrition, respiration and microbial dynamics.

The dynamic profile of this biologically active molecule that catalyses the decarboxylation of α-ketoglutarate is characterized, after careful analysis, by an upward increase in the biosynthesis and enzyme activation, in direct proportion to the aging of the culture of *Monilinia laxa* fungus for all carbohydrates, including the control sample. For the vast majority of medium variations, the increased enzyme activity had the most intense growth found in the variant with gum arabic: from 0.3707 µg formazan / g.mat. to 3.2507 µg formazan / g.mat. In the case of lactose, from 0.2228 µg formazan / g.mat. it reached a peak of 2.4919 µg formazan / g.mat, while sorbitol stimulated the enzyme activity in time from 0.2842 µg formazan / g.mat to 2.1339 µg formazan / g.mat and mannitol from 0.3380 µg formazan / g.mat to 1.6850 µg formazan / g.mat. Glycerol proved to be also a good nutrient, increasing in time the performance of α ketoglutarate dehidrogenase activity that climbed from 0.3219 µg formazan / g.mat to 1.5195 µg formazan / g.mat. and sucrose induced an similar behaviour to the enzyme, its variation going from 0.4696 µg formazan / g.mat to 1.3720 µg formazan / g.mat. Same happened with xylose - from 0.0925 µg formazan / g.mat to 1.2126 µg formazan / g.mat. In the case of the medium with celobiose, oxoglutarate dehydrogenase increased in time, from 0.2367 µg formazan / g.mat to 1.1948 µg formazan / g.mat and in the variant supplemented with dextrose, from 0.4505 µg formazan / g.mat to 1.1159 µg formazan / g.mat. Starch caused a more modest time activity for the endoenzyme, varying from 0.2086 µg formazan / g.mat. to 0.6985 µg formazan / g.mat., while maltose, fructose, determined a similar behaviour as starch, α-ketoglutarate dehidrogenase

activity changed from 0.2133 µg formazan / g.mat. to 1.1159 µg formazan / g.mat, respectively, from 0.1671 µg formazan / g.mat to 0.5401 µg formazan / g.mat.

Because the Krebs cycle enzymes, like those of hexozo-monophosphate shunt and Emden-Meyerhoff-Parnas pathway are respiratory enzymes, the increased oxoglutarate dehydrogenase activity at *Monilinia laxa* suggested in the second interval, an increase in respiratory rate, dependent of the age of the fungus and an intense activity of the enzymes from the antioxidant defense line, knowing that a high metabolic rate is followed by the increase of oxidative stress markers that are responsible for the aging of mitochondria, which are the main source of ROS (Tahar, E.B. *et al*., 2007) due to multiple reactions that transfer electrons. In the electron transport chain, a small amount of electrons are distracted by oxygen to intermediate points, such as complexes I and III that generate superoxide anion radicals, which are converted inside the mitochondria in H_2O and other species of ROS (Kowaltowski , A.J., and Vercesi, A.E. 1999). In addition to the electron transport chain, recent works showed that enzymes soluble in the mitochondrial matrix as pyruvate dehydrogenase and α-ketoglutarate dehidogenase can also generated species of ROS, being the primary site for this (Starkov, A.A. *et al*, 2004), not just a target for oxidative stress. As each source of mitochondrial ROS respond differently to substrates, changes in the energy metabolism, O_2 and tensions (Turrens, J.F., 2003), as a result, each generation mitochondrial apparently, in this study works to lift parameters, regardless the age but with emphasis on the mature one.

Succinate dehydrogenation is catalysed by succinate dehydrogenase, a flavoprotein which, in eukaryotes, is the only enzyme of the citric cycle trapped deep in the internal mitochondrial membrane (Garret, R. and Grisham, C.M., 2005). The enzyme contains succinate dehydrogenase complex, the only complex of the respiratory chain that pumps protons, although it has a transmembrane domain that introduces electrons taken from $FADH_2$ into the respiratory chain whose energy is too high, surrendering CoQ to electron carriers of the respiratory chain (Cecchini, G. 2003). Examination of the course of activity of the enzyme that is converting succinate to fumarate, in time, signals not only an increase in the oxidative stress due to production of reactive oxygen species (ROS), expression of a strong aerobic metabolism in the medium variations, but could reflect a change in the fraction of the active enzyme, due to change of metabolic conditions and an intensification of the respiratory process, which removes the idea that at 4 days after medium inoculation, the mycelium of *Monilinia laxa* fungus is old, since that some data from the literature indicates that this ubiquinone activity decreases with age and nutrient depletion in the culture medium but also because some cellular components are known to be sensitive to oxidative stress, specifically proteins containing Fe-S clusters of succinate dehydrogenase as well, highly sensitive to superoxides resulting from the metabolic activity (Masoro, E.J. and Austad, S.N., 2006). There have been variations of the succinate dehydrogenase activity (graphically seen in Figure 3), that increased from 0.1373 µg formazan / g.mat to 1.3414 µg formazan / g.mat. For version control, to provide a modest increase in medium supplemented with fructose - from 0.2413 µg formazan / g.mat to 0.3360 µg formazan / g.mat. and to have a powerful amplification in the mediums enriched with arabic gum from 0.3785 µg formazan / g.mat to 2.3695 µg formazan / g.mat with sorbitol- from 0.0778 µg formazan / g.mat. to 1.1880 µg formazan / g.mat. and mannitol from 0.2516 µg formazan / g.mat. to 1.3578 µg formazan / g.mat. Succinate dehydrogenase had a similar behaviour in the mediums with lactose supplement, where it increased from 0.1327 µg formazan / g.mat. up to 2.1652 µg formazan / g.mat. and with celobiose where it jumped from 0.0050 µg formazan / g.mat. to 0.6536 µg formazan / g.mat.

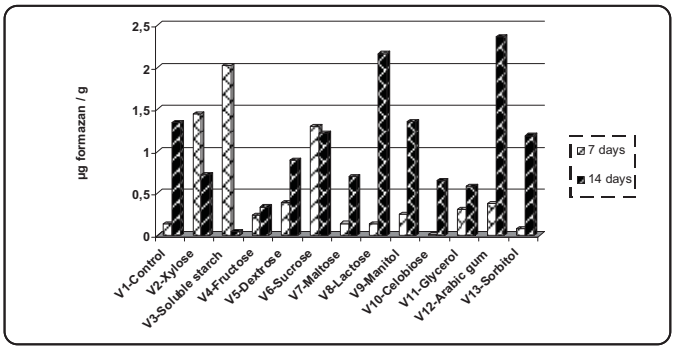

Fig. 3. The influence of carbohydrates on the dynamics of succinate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

The addition of maltose was followed by an increased activity of the endoenzyme from 0.1414 µg formazan / g.mat.. to 0.6967 µg formazan / g.mat. while dextrose (V5) induced an increase of biosynthesis and the enzyme action in time, rised from 0.3857 µg formazan / g.mat. to 0.8900 µg formazan / g.mat.., and glycerin stimulated the enzyme activity from 0.3062 µg formazan / g.mat. to 0.5804 µg formazan / g.mat. Filling the basal medium with sucrose was not followed

by a significant variation of the enzyme specific for the third stage of the Krebs cycle, this had a tiny decrease, from 1.2940 µg formazan / g.mat. to 1.2171 µg formazan / g.mat. More intensive declined succinate dehydrogenase activity levels over time in medium variations with added starch, which decreased from 2.0177 µg formazan / g.mat. to 0.0400 µg formazan / g.mat.. and with addition of xylose, which decreased from 1.4476 µg formazan / g.mat.. to 0.7232 µg formazan / g.mat.

Both maturation and aging of submerged fungal culture in medium with different carbon sources can be followed by the accumulation of toxic metabolites. Typically, the cellular respiration coupled with ATP synthesis decreases during aging, α- ketoglutarate dehydrogenase and malate dehydrogenase decreasing significantly, while the of activity isocitrate dehydrogenase decreases more and is completely inhibited in the oldest culture. By contrast, the literature emphasizes that succinate dehydrogenase is more active. The behaviour of oxidative enzymes and of metabolic pathways are apparently inherent in long-lived cells from the population, selected by genetic fitness during chronological aging (Samokhvalov, V. *et al.,* 2004), depending on genetic background as well as epigenetic regulation through interaction with environmental factors.

The last stage in the Krebs cycle, in which L malate is oxidize to oxaloacetate is catalysed by the malate dehydrogenase. The graphical representation of the enzyme activity as it appears in Figure 4 illustrates a different dynamic to others oxidoreductases from the Krebs cycle, but relatively similar to isocitrate dehydrogenase in the variation curve. The careful examination of malate dehydrogenase activity dynamics helps us to see that there is a tendency to decrease in the of activity malate dehydrogenase in the second time period for the experimental measurements, confirming the data from literature, that show that with the maturation and aging of the microbial cultures, malate dehydrogenase activity decreases.

So, it can be seen that the culture medium with arabic gum, endoenzyme activity remained almost constant, with an infinitesimal variation in the negative direction, down from 3.4901 µg formazan / g.mat to 3.4006 µg formazan / g.mat, while the presence of sorbitol in the medium induced an increase in its activity, while it increased from 1.7935 µg formazan / g.mat to 2.5088 µg formazan / g.mat. The remaining curves of variation regardless the carbon source of glucidic nature introduced in the culture medium, have a descending path. The most powerful decrease was the variant with xylose, the enzyme descending from 5.4696 µg formazan / g.mat. at 7 days after inoculation up to 1.4594 µg formazan / g.mat. in the second period. V8 version (with lactose) showed a degressive variation, moving from 4, 2624 µg formazan / g.mat. at a threshold of 1.0293 µg formazan / g.mat., while the version with glycerol (V11) malate dehydrogenase activity recorded a decrease from 4.2025 µg formazan / g.mat.la 2.2820 µg formazan / g.mat., followed by the sucrose version (V6) of 3.5788 µg formazan / g.mat. to 1.9801 µg formazan / g.mat. and the culture with the addition of mannitol (V9), where values have dropped from 3.2707 µg formazan / g.mat. to 0.8937 µg formazan / g.mat.

Starch induced a decreasing malate dehydrogenase activity from 2.6387 µg formazan / g.mat. to 1.4566 µg formazan / g.mat.., while the control sample, without carbohydrates had activity levels formazan 2.5415 µg formazan / g.mat.at 7 days, respectively, 1.7822 µg formazan / g.mat. at 14 days. The mycelium of *Monilinia laxa* grown on the medium enriched with glucose showed a decreased malate dehydrogenase activity, from 2.4179 µg formazan / g.mat.to 2.0069 µg formazan / g.mat.

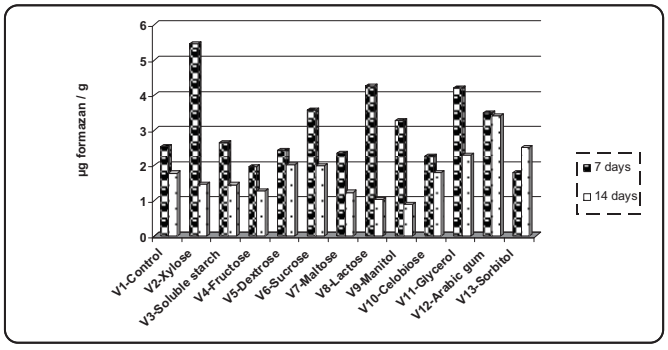

Fig. 4. The influence of carbohydrates on the dynamics of malate dehydrogenase activity at *Monilinia laxa* (Aderh. & Ruhl.) Honey fungus

This enzyme behaviour demonstrates that glucose kept an approximately constant redox potential, the amount of energy released is significantly undiminished through time. With regard to maltose it induced a reduction of malate dehydrogenase activity in the fungus mycelium, while it halved its energy resources that enter in the Krebs cycle from 2.3504 µg formazan / g.mat. to 1. µg formazan / g.mat. Endoenzyme values found under the influence of celobiose also

decreased from 2.2665 µg formazan / g.mat. to 1.8023 µg formazan / g.mat. while, under the action of fructose (V4 version), values went from 1.9563 µg formazan / g.mat to 1.2680 µg formazan / g.mat.

CONCLUSIONS

After 7 days of incubation, the isocitrate dehydrogenase activity was stimulated in media supplemented with xylose, arabic gum, glycerol, lactose, sucrose, maltose, mannitol, dextrose and fructose, and after 14 days, only in the medium with arabic gum.

The α-ketoglutarat dehydrogenase biosynthesis was stimulated in the young mycelium by sucrose, dextrose, arabic gum, mannitol, glycerol and sorbitol, and in the aged culture by arabic gum, lactose, sorbitol, mannitol, glycerol, sucrose, xylose and celobiosys

The succinate dehydrogenase activity was stimulated at 7 days after sowing by starch, xylose, sucrose, dextrose, arabic gum, glycerin, mannitol, fructose, maltose, and at 14 days by arabic,gum lactose and mannitol.

In the 7 days old mycelium, the malate dehydrogenase was stimulated by xylose, lactose, glycerol, sucrose, arabic gum, mannitol and starch, and in the 14 days old mycelium by arabic gum, sorbitol, glycerin, dextrose, sucrose and celobiozã

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RESEARCH REGARDING THE DYNAMICS OF SOME BIOCHEMICAL MARKERS OF OXIDATIVE STRESS AT *MONILINIA LAXA* (ADRH. & RUHL.) HONEY CULTIVATED ON DIFFERENT AMINO ACIDS ENRICHED MEDIA

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Keywords: amino acids, *Monilinia laxa* , catalase, peroxidase, oxidative stress

Abstract: Antioxidants that make up the defense for Ascomycetes still arouses a major interest because of their hypothetical role as virulence and aggression factors and also as the enzymes that play a key role in cellular defense against ROS produced during microbial metabolic activity. A study of catalase and peroxidase activity dynamics of the species *Monilinia laxa* (Aderh & Ruhl.) Honey cultivated *in vitro* on medium supplemented with different amino acids was conducted in order to know the biology of the fungi responsible for the appearance of brown rot at various species of stone fruits. We used for this purpose the Leonian medium (in the formula changed by Bonnar), in each variant being added 0, 125 mg of the following amino acids: alanine, glutamic acid, asparagine, aspartic acid, cystine, cysteine, phenylalanine, histidine, valine, lysine, serine, methionine and leucine. We also used a control variant, without amino acids, in final resulting 14 working versions. To determine the catalase activity Sinha method was used, to monitor the peroxidase activity we used Möller method and the experimental measurements carried out at two intervals, were made both of fungus mycelium and culture fluid. We found notable differences in the activity of two enzymes, microbial culture induced both by the age of the culture medium and the type of amino acid introduced in it.

INTRODUCTION

Ubiquitous organisms, filamentous fungi have a high adaptive plasticity to different environmental changes, one of the fundamental requirements of these microorganisms for surviving is the need to adjust their activities in terms of an aerobic lifestyle, as the metabolic performance in such circumstances is followed by the emergence of reactive oxygen species and, hence, the need for it`s proper management, its absence can lead toward apoptosis and death of eukaryotic cell due to so-called oxidative stress (Avery, S.V. *et al*., 2008). Defined as being an imbalance between reactive oxygen species production and the biological system`s ability of rapid detoxification, followed by the repair and removal of damaged parts resulting from their activity, oxidative stress is characterized by disturbances in the normal redox state of the cells, capable of causing toxic effects by peroxides and free radicals production, equipped to destroy cellular components, including proteins, lipids and DNA (Sies, H., 1991, Dean R.T. *et al*., 1997, Esser, K. and Kues, U., 2006) .

The ways in which organisms are protected from the aggression of reactive oxygen species are related to cell compartmentalisation, to the ability to elaborate adaptive responses inducible in oxidative stress conditions, to repair and turnover processes that help to minimize the damage that occures from reactive oxygen species attack or by the existence of hydroxyl radical scavenger that captures a series of hidroxil radicals, superoxides and organic radicals, which chelate metal ions and prevent certain chemical reactions toxic for the organism, the so-called preventive antioxidants (Gadd, G.M., 2001) and, last but not least, the protection afforded by antioxidant compounds and enzyme systems. The protective enzyme system of eukaryotic cells include: superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, glutathione reductase and glucose-6-phosphate dehydrogenase, these enzymes having a predominantly intracellular localization, the extracellular environment being more exposed to radical attack (Bai, Z. *et al*., 2003, Li, Q. *et al*., 2009).

Present in the medium, some amino acids regulate the activity of fungal enzymes, including the oxidoreductases (Subramanian, K.N. *et al.,* 1968), data from literature indicating that interference between amino acids and the medium are responsible for the catalase inhibition, and the presence of arginine in the culture medium inhibits the catalase activity (Frederick, J.R. *et al*., 2001). Also, experimental studies show the ability of oxidative decarboxylation of some amino acids such as serine, alanine, phenylalanine, tryptophan and methionine. In the presence of dihydrofumarate, this oxidoreductase catalyzes the hydroxylation of various aromatic compounds and reduces nitrate in the presence of some specific donors (Fear, 1976, quoted by Roşu, C.M., 2007). *In vitro* experiments showed that the activity and the thermostability of peroxidase depend, in many cases, by their interaction with amino acids such as proline, tryptophan, valine, β-alanine (Bakardjeva, N. *et al.,* 1999). Shtarkman, I.N. *et al*, 2007 demonstrated that some amino acids present in the medium (methionine, cystine, tyrosine, tryptophan, phenylalalnine, lysine, leucine, arginine and proline) are responsible for protecting the intracellular DNA against damage caused by reactive oxygen species under a moderate oxidative stress, complementing the antioxidant enzyme defense system activity in the eukaryotic cells.

Same authors complete the recent studies that aimed the research on the formation of reactive oxygen species in aqueous solutions, indicating that vary physical factors makes these mediums chemical reactive, the biological molecules,

in their turn, became the sensors of the processes taking place in aqueous mediums and thus, in agreement with the concepts of the day, the reactive oxygen species formed in the cellular and intercellular space plays an ambiguous role, causing on the one hand, damage to biological structures such as DNA, lipids, proteins, etc.. during oxidative stress, and on the other hand, ROS present in specific physiologic concentrations plays an important role as signals, showing the extent to which the liquid medium is suitable for maintaining vital activity and redox regulation of various cellular functions.

This paper is intended to be a time monitoring activity for some biochemical markers of oxidative stress such as catalase and peroxidase in the fungus *Monilinia laxa* (Aderh. & Ruhl.) Honey, parasitic on various varieties of *Prunus* sp. and *in vitro* cultivated on media supplemented with different amino acids.

MATERIALS AND METHODS

The *Monilinia laxa* strain was isolated from mummified fruit harvested from different varieties of *Prunus domestica*, incubated on PDA medium for 7 days and used as inoculum in the Leonian medium (Constantinescu, O., 1974) supplemented with 0.125 mg from the next amino acids: alanine, glutamic acid, aspartic acid, asparagine, cystine, cysteine, phenylalanine, histidine, methionine, valine, lysine, serine, leucine, and we also used a control sample, without amino acids. The cultures were maintained submerged in the dark, at a temperature of 28^0C, and the sampling for biochemical determinations was made at intervals of 7 and, respectively, 14 days after the seeding of the culture medium. The investigations were carried out on biomass and the supernatant resulted from the centrifugation of the culture medium. The methodological support for monitoring the activities of both oxidoreductase have been Artenie Vl. *et al*., 2008 for catalase and o-dianisidine method for peroxidase (Cojocaru D.C., 2009).

RESULTS AND DISCUSSIONS

The *Monilinia laxa* has the ability to biosynthesize catalase in submerged cultures, experimental determination of its activity in the fungus mycelium and in the liquid culture confirming its existence in both time intervals, in all medium variations.

The critical analysis of the graphic details about the dynamics of intracellular catalase activity during growth and development of the mycelium of *Monilinia laxa* (Aderh. & Ruhl.) Honey, as they appear in Figure 1, provides a picture of the trend curves of variation of the enzyme in all variants environment enriched with amino acids (including amino acid-free sample), except the asparagine supplemented culture, where the evolution in time had an descending allure, down from 202.9916 µmoli hydrogen peroxide/min. up to 80.8772 µmoli hydrogen peroxide/min.

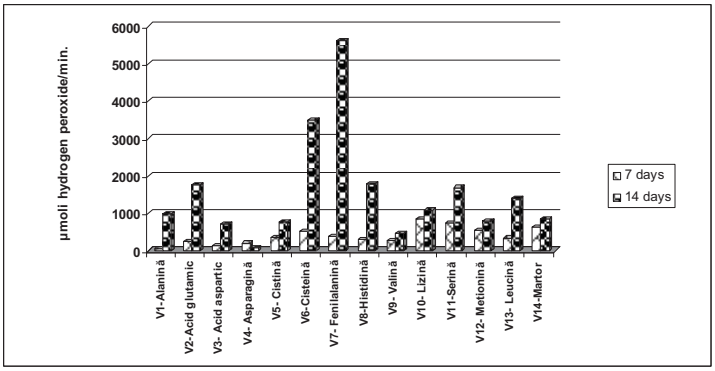

Fig. 1- The dynamics of catalase activity in mycelium of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

One can appreciate that phenylalanine was responsible for the increased biosynthesis rate and the high catalase activity between the two time intervals. The enzyme present in the mycelium illustrates an ascension at a value of 389, 0312 µmoli hydrogen peroxide/min. at 5608.1527 µmoli hydrogen peroxide/min. while cysteine, present in the culture medium, had a variation curve in the two time intervals ranging from maximum 520.0003 µmoli hydrogen peroxide/min. tu minimum 3502, 9601 µmoli hydrogen peroxide/min. The addition of histidine itself undoubtedly influenced the dynamics of enzyme in a progressive direction, moving it to a minimum of 287.8543 µmoli hydrogen peroxide/min. to a maximum 1785.0845 µmoli hydrogen peroxide/min. recorded at 14 days after the inoculation of the culture medium. The same was noted in the mycelia mass developed on the liquid medium enriched with glutamic acid, the catalase having antioxidant effects with a threshold registered at the first determinations interval of 238.7318 µmoli hydrogen

peroxide/min. ., the peak line moving from 1756, 7359 µmoli hydrogen peroxide/min. in the second period of enzymatic determinations. Serine caused a progressive dynamic in time for the endocellulare catalase, the value of 731.1005 µmoli hydrogen peroxide/min. acquiring an additional activity, amounting to 1690.7072 µmoli hydrogen peroxide/min. Serine is essential for catalase activity because, along with a water molecule, formes a hydrogen bonding system, an hydrophobic substrate channel with a narrow base that allows small peroxides to quickly spread at the enzyme active site, guiding histidine located at the C terminus end of catalase N-terminus point. Thus, the histidine imidazole ring, unlike the other hemoprotein in which it is parallel to the heme plane, in the catalase the orientation is parallel to it, which favors it energetically, because intensive interactions of π-π type between the imidazole ring of histidine and porphyrin lower the reactivity of the compound with the reduced substrate (Fita, I. and Rossman, M.G., 1985, Zamocky, M. and Koller, F., 1999).

Leucine induced a similar behavior, the dynamic variation curve going from 341,949 µmoli hydrogen peroxide/min. at 1412.0509 µmoli hydrogen peroxide / min., and alanine caused the emergence of a pattern of behaviour similar to the enzyme, its values moving from one minimum noted at 7 days of 47.9643 µmoli hydrogen peroxide/min. to an upper limit of 998.9287 µmoli hydrogen peroxide/min. at registered 14 days. Alanine, as glutamine, is essential for increasing air hyphae, glutamine and alanin transaminase have an active feature for aerial hyphae (Cárdenas, M.E. and Hansberg, W., 1984), and catalase activity, as a consequence of hiperoxidant status is high during their growth and during the formation of conidia, given that activity in the conidia is 60 times higher than in the mycelium developed in liquid culture medium (Avery, S.V. *et al.,* 2008). Given the fact that the endoenzime activity is related to increased synthesis during the maturation and germination of conidia (Gessler, N.N. *et al.,* 2007), the biosynthesis of the enzyme usually increases at the end of exponential growth phase and early hyphae aggregation (Michán, S.H.. *et al.,* 2002), when the spore biosynthesis begins and, also the cell differentiation and the fungus enters the stationary phase (Hansberg, W. *et al.,* 1993). The oxidoreductase dynamics as an expression of physiological response of the fungal cell to oxidative stress imposed by the presence of amino acids in submerged culture is closely related to the establishment, stabilization, magnitude and metabolism consequences of an aging mycelia culture, the enzyme being a complex mechanism of control for reactive oxygen species, acting at different levels, just to prevent retrograde adaptive responses in the fungal cell and genomic instability.

The fluctuations in the catalase dynamics in the culture fluid indicate several different behaviors of extracellular enzyme activity in the two experimental determinations. So, we note such a situation in which the enzyme had an infinitesimal variation while under the influence of culture medium supplementation with phenylalanine - while remaining practical almost constant at values of as 136.2484 µmoli hydrogen peroxide/min. respectively, 136 099 µmoli hydrogen peroxide / min.

In other cases, the enzyme had increased in value over time, smaller in the environments with added cysteine (from 187.1649 µmoli hydrogen peroxide / min. at 207.6749 µmoli hydrogen peroxide / min.), histidine (from 110.1101 µmoli hydrogen peroxide / min. to 122 647 µmoli hydrogen peroxide / min.), cystine (from 113.9688 µmoli hydrogen peroxide/min. at 158.5504 µmoli hydrogen peroxide/min. , average ups (of about three times) from glutamic acid (from 30.4856 µmoli hydrogen peroxide/min. at 93.1739 µmoli hydrogen peroxide/min. nearly four times more in the case of aspartic acid - from 44.5003 µmoli hydrogen peroxide/min. at 168.4431 µmoli hydrogen peroxide/min. The largest variation curve of catalase activity in liquid culture of *Monilinia laxa* species was recorded in the medium variant enriched with alanine where, although as value, the enzyme activity was low, while the width has increased by about 13 times - from 6.9808 µmoli hydrogen peroxide/min. at 91.7467 µmoli hydrogen peroxide/min. We also found that there were various degrees decreases of the enzyme activity in the extracellular space, the largest variation curve in this direction is noted in the variant without amino acids, where catalase activity in liquid culture dropped six times, going 248.1864 µmoli hydrogen peroxide/min. at a level to 39.5022 µmoli hydrogen peroxide/min. The same significant decrease, similar to the ones registered in the supernatant version control sample were induced by the presence of leucine at the start of the submerged culture, with serine, methionine, asparagine and valine.

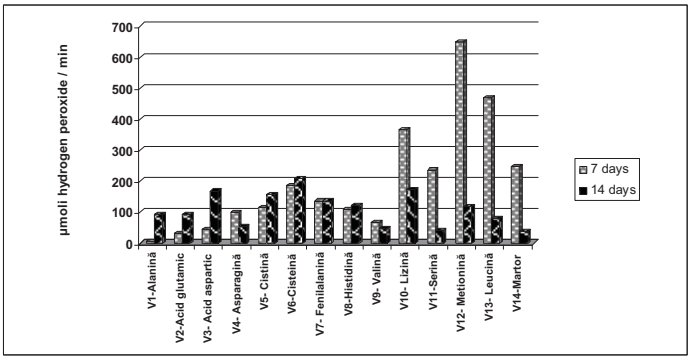

Fig. 2- The dynamics of catalase activity in culture liquid of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

The peroxidase easily operates on low concentrations of hydrogen peroxide, unlike catalase, which has a lower efficacy in such conditions. The experimental results on the modulator effect of amino acids at *Monilinia laxa* (Aderh & Ruhl.) Honey species on peroxidase activity, both at 7 and 14 days after inoculation revealed the activity of this oxidoreductase, its varies depending on the type of amino acid source and on the age and culture mycelia.

In order to achieve a clear image of the in vitro activity of this oxidoreductase in the mycelium of the fungus, we quantified the data from the experiments carried out and the graphical representation of their average values as seen in Figure 3.

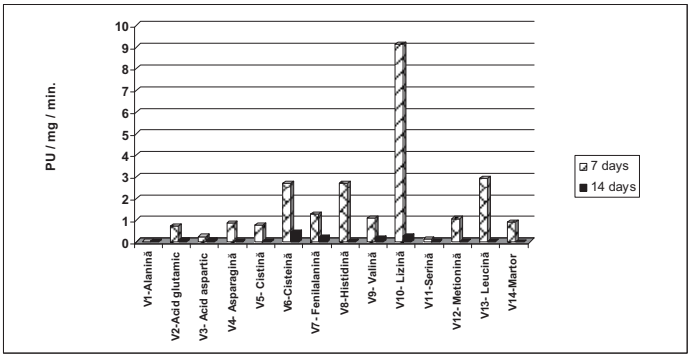

Fig. 3- The dynamics of peroxidase activity in mycelium of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

Lysine plays a constitutive role in the peroxidase molecule, but the metabolic role of this amino acid apears is the oxidative damage mediated by L-lysine-α-oxidase in aqueous medium that leads to the formation of hydrogen peroxide (Lukasheva, E.V. and Berezov, T.T., 2002 , Gomez, D.P. *et al*., 2006). Perhaps this explains the very high intensity of peroxidase activity at 7 days after the sowing of the species mycelium - 9.1424 PU/ mg / min. (ten times higher than the enzyme activity in the control version). Leucine was responsible for the biosynthesis of the enzyme to three times higher than in the version without amino acids - PU 0.9109 / mg / min. We found the intervention of peroxidase in the fungus mycelium developed on medium with histidine and cysteine (2.7193 PU / mg/min respectively, 2.7085 PU / mg / min.) and that the endoenzyme activity was tripled compared to that of control sample, possibly because the culture physiological needs to balance the energy consumption requires a major involving for the redox processes. During the phenylalanine oxidation in eukaryotic organisms, the production rate of hydrogen peroxide is increased (Sauret-Ignaz, G.*et.al.,* 2007).

Peroxidase displays high activity for the substrate represented by phenylalanine, this amino acid being oxidative deaminated via phenylpropanoids pathway with trans-cinnamic acid formation, an intermediate in the phenols biosynthesis (Dixon, R.A. and Paiva, NL.., 1995). Peroxidase is, with polyphenoloxidase responsible for their oxidation. In mycelium of *Monilinia laxa* grown in submerged culture with phenylalanine, the peroxidase rate of production after 7 days of incubation showed a level of value -1.2873 PU / mg / min. By comparison, close oxidoreductase levels were

recorded in the mycelium of the fungus developed on media supplemented with valine and methionine (1.0792 PU / mg / min., respectively, 1.0742 PU / mg / min.). Peroxidase was slightly inhibited in cultures enriched with cystine, asparagine and glutamic acid (0.7861 PU / mg / min, in version V5, PU 0.8519 / mg / min., in the variant V4, 0.7443 PU / mg / min respectively. in version V2. This effect is not necessarily due to the existence of a low rate of cystine oxidation by hydrogen peroxide, for example, in the reaction catalyzed by peroxidase (Stelmaszynska, T. and Zgliczynsky, JM, 1963), but given that this enzyme operates at low concentrations of hydrogen peroxide, reducing its activity or its inactivation occurs when you exceed a certain concentration of the substrate. The same explanation can be given to values recorded by the oxidoreductase in the mycelium grown on media with aspartic acid (0.2470 PU / mg / min.), serine (0.1465 PU / mg / min.) and alanine (0, 0164 PU / mg / min.).

Amplitude variation levels found in the values of peroxidase activity reports made from the mushroom mycelium after 14 days from inoculation of culture media enriched with various amino acids, was different depending on the type of amino acid used, the intensity of oxidoreductase activity in this time interval that is much different from that recorded after 7 days of incubation, in the sense of an enzyme activity reduction in the mycelium. This effect is not due to the existence of a necessarily low oxidation rate, for example, for cystine by hydrogen peroxide in the reaction catalyzed by peroxidase (Stelmaszynska, T. and Zgliczynsky, JM, 1963), but given that this enzyme operates on low concentrations of hydrogen peroxide, reducing its activity or inactivation occurs when you exceed a certain concentration of this substrate or when excessive acidification of the culture medium destabilizes the enzyme structure, inactivating it.

The last stage of the study was the research on the influence that amino acids have on the peroxidase activity in liquid cultures for *Monilinia laxa* (Aderh. & Ruhl.) Honey species, when it was found that the enzyme has different fluctuations over time, its activity being dependent of the nature of the amino acid introduced in the culture medium, as can be seen in Fig. 4, the strongest incentive effect after 7 days of incubation being induced by aspartic acid, asparagine and cystine, and after 14 days by phenylalanine and aspartic acid and that the exoperoxidase dynamics complements the exocatalase one, these two oxidoreductase being significantly marked by oxidative stress generated by the presence in the environment of amino acids as a source for carbon and nitrogen.

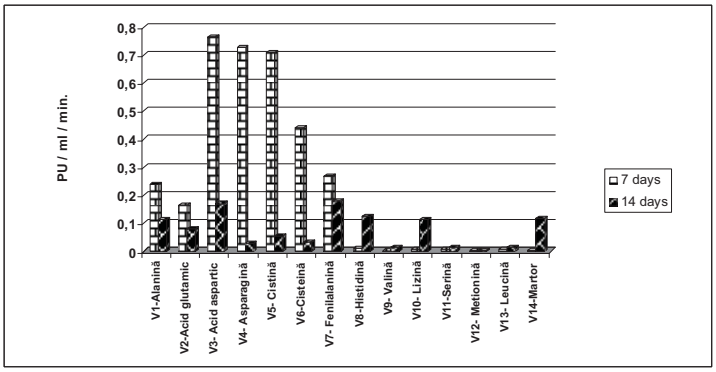

Fig. 4- The dynamics of peroxidase activity in culture liquid of *Monilinia laxa* (Aderh. & Ruhl.) Honey species cultivated on media supplemented with different amino acids

CONCLUSIONS

The studies on the influence of amino acids on catalase and peroxidase activity in the mycelium and the supernatant of *Monilinia laxa* species showed that the activity of the two oxidoreductase was significantly influenced by the amino acid introduced in the medium and by the age of culture.

After 7 days of incubation, the catalase activity in the mycelium was stimulated by lysine and serine and in the 14 days old mycelium the enzyme biosynthesis was stimulated by phenylalanine, cysteine, histidine, glutamic acid, alanine, serine, leucine and lysine. The catalase biosynthesis efficiency in fluid culture medium of *Monilinia laxa* species after 7 days after inoculation was increased in the samples with methionine, leucine and lysine, while in the supernatant of the aged culture, all the studied amino acids had a stimulating effect.

In the young mycelium, aged for 7 days, lysine, leucine, histidine, cysteine phenylanine, methionine and valine induced a very high peroxidase activity and in the aged culture all amino acids increased, to varying degrees, the oxidoreductase activity. After 7 days of incubation in the culture supernatant peroxidase activity was highly stimulated by aspartic acid, asparagine, cystine, cysteine, phenylalanine, alanine and glutamic acid, while in liquid culture at the age of 14 days, the biosynthesis of the enzyme was positively influenced by phenylalanine, aspartic acid and histidine.

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THE EFFECT OF SPRUCE BARK POLYPHENOLS EXTRACT IN COMBINATION WITH DEUTERIUM DEPLETED WATER (DDW) ON *GLYCINE MAX L.* AND *HELIANTHUS ANNUUS L.* DEVELOPMENT

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Keywords: deuterium depleted water (DDW), soybean, spruce bark, sunflower, polyphenolic compounds.

Abstract: The aim of this study was to evaluate the effect of spruce bark aqueous extract and deuterium depleted water (DDW) as bioregulators on the plant growth *Glycine max L.* and *Helianthus annuus.* The following specific parameteres were closely monitorised: germination energy and germination capacity, plants vegetative organelles growth and development and photoassimilatory pigments concentrations. The results have shown that DDW presents different effects depending on tested plant species. In the case of soybean, DDW presented stimulatory effects on both germination energy and capacity, radicles elongation, primary leaves growth and development but inhibitory effects on photoassimilatory pigments. Spruce bark extract reduced the germination capacity of soybean seeds, but accelerated the germination process of sunflower seeds and present stimulatory effects on plantlets biomass accumulation. The combination of DDW with *Picea abies* polyphenolic extract promoted soybean plantlet elongation, especially the rootlets ones and stimulated green biomass accumulation for both soybean and sunflower plantlets. Analyzing the photoassimilatory pigments concentration for sunflower, it can be observed an increasing trend (almost 100% comparing with control) when introduce into the growth medium DDW and *P. abies* polyphenolic extract. DDW and *P. abies* bark extract have shown an important role in plant growth and development, improving photoassimiliation process.

INTRODUCTION

Polyphenolic compounds are the most important classes of secondary metabolites that play an important role in the biosynthesis process. Natural bioactive compounds have a broad spectrum of both the plant as a whole and on tissues and organs, interfering in the metabolic processes (Anghel et al., 2001). Through, the characteristic biological activity, natural polyphenols are essential compounds in the stimulation of plants growth and development. The stimulation or inhibition capacities on the plant growth and development is closely correlated with concentrations of polyphenolic compounds applied. Thus, in some cases the presence of these compounds in low concentrations can have a beneficial effect on the plants development and in other cases, when concentrations are high there is an inhibition phenomenon (Popa et. al., 2007). The aim of this study was to establish the effect of polyphenolic extract from the spruce bark, the DDW and the mixture of polyphenolic extract and DDW, on some physiological processes occurring in plants. The researches undertaken in recent years, which were aimed at finding new biostimulating products compatible with the ambient environment, drew the attention on the possible involvement of aromatic natural products, isolated from biomass to reagent chemicals, in metabolic processes of plant. It was established that spruce bark aqueous extract, rich in polyphenolic compounds, has a stimulating effect in the processes of germination, growth and development of seedlings of rape and soybean (Stîngu et al., 2010, Ignat et al., 2009).

On the other hand, deuterium depleted water or light water is a distilled water microbiologically pure, with an isotopic concentration of 25 ppm, obtained by isotopic distillation, in vacuum, of natural water with an isotopic concentration of 145 ppm D / (D + H) (Somlyai., 2001). In Romania DDW is obtained in two centers: the heavy water plant at Halanga, where daily discharge as waste tons at the DDW and INCDTCI of Ramnicu Valcea where is obtained in special installation get built. Recent research has shown that DDW has a great influence on living organisms, namely in developing cells and tissues and changes that occur in normal water features lead to significant changes in fundamental processes of cells. Some of the main properties of DDW in living organisms are: influence on the development and multiplication of cells, influence on cellular transport, DNA synthesis and also has antioxidant properties (Somlyai., 1993, Olariu et al., 2007). Since the studies on the influence of DDW in plant systems are not representative, it was necessary to develop this subject.

In this context, we analysed the effects of DDW and in combination of it with P. abies extract on bean and sunflower plant growth and development. The following specific parameteres were closely monitorised: germination energy and germination capacity, plants vegetative organelles growth and development and photosynthesizing pigments concentrations.

MATERIALS AND METHODS

Deuterium depleted water was purcheased from INCDTCI Râmnicu Vâlcea, Romania. To obtain an aqueous polyphenolic extract the spruce bark of industrial origin was used as a vegetable raw material. After drying at room temperature and under conditions of normal aeration, the bark was ground, followed by a new stage of drying.

1. Extraction. Ground spruce bark was subjected to extraction using procedure properly on aqueous extraction, namely: 5 g dried vegetal material is brought into a 250 mL Erlenmeyer flask in which there were 125 mL distilled water. Erlenmeyer flask was covered with a watch glass and heated on a water bath so that the temperature in the vessel to be 85-90 ° C. Leave it at that temperature for 45 min., shaking from time to time. The material is allowed to settle and passed the clear solution through a crucible of glass or porcelain funnel. This operation was repeated 3-4 times until a colorless extract was obtained. All extracts are cumulated in a 500 mL volumetric flask and make up to volume mark with distilled water (Rozmarin et al., 1984). Polyphenolic extract was used in two concentrations: 0.5 and 1 g of plant material in 100 ml distilled water. The polyphenolic aqueous extract it was characterized in terms polyphenols total contents. Thus, for 1 g vegetal material in 100 ml distilled water was recorded 130 mg / L total content in polyphenols (Stingu et al., 2010).

2. Germination tests were carried out going through a standard procedure, using a number of 5 Petri dishes for each solution studied (distilled water - control, DDW, extract of spruce, and spruce bark extract in combination with distilled water / DDW). On a filter paper were placed every five soybean seeds, carefully selected to no present major damage. For starters, the vegetal material has undergone a process presterilization, which consisted of submerged seed absolute ethanol for 10 seconds, following the sterilization in the presence of sodium hypochlorite 10% for 20-30 minutes (Cachita et al., 2004). The volume of solution added was 10 mL / dishes. Petri dishes thus prepared were incubated in the dark in a thermostat set at 27 º C. After a period of seven days, Petri dishes were kept in daylight for 3 days to allow the seedlings to synthesize assimilatory pigments. Finally, the biometric and quantitative measurements on components of seedlings (root, stem, primary leaves) and spectrophotometric measurements were carried out to determine the concentration of photoassimilatory pigments.

3. Quantification of assimilating pigments. 0.05 g fresh vegetal material was extracted in 80% acetone by grinding with a spatula tip of quartz sand. Chlorophyll extract was analyzed spectrophotometrically by reading absorbance at various specific wavelengths: 470, 646, 663 nm. In order to determine the concentration of chlorophyll pigments (chlorophyll a and b) and carotenoid pigments were used formula proposed by Lichtenthaler and Welburn (1983):

Chlorophyll a (µg / mL) = 12.21 (A 663) - 2.81 (A 646)
Chlorophyll b (µg / mL) = 20.31 (A 646) 5.03 (A 663)
Carotenoids (µg / mL) = (100 • A 470-3.27 [chl a] - 104 [chl b]) / 22

RESULTS AND DISCUSSIONS

After measurements made on seeds and seedlings of soybean and sunflower it can see the different influence of test solutions according to plant species subjected to experiment. Thus, form resulting energy and capacity germination of soybeans it was observed stimulation of these samples where tested solution was deuterium depleted water. When added to the germination medium, spruce bark aqueous extract or in combination with deuterium depleted water is found a reduction in germination energy and no influence on capacity germination (Fig. 1 and 2).

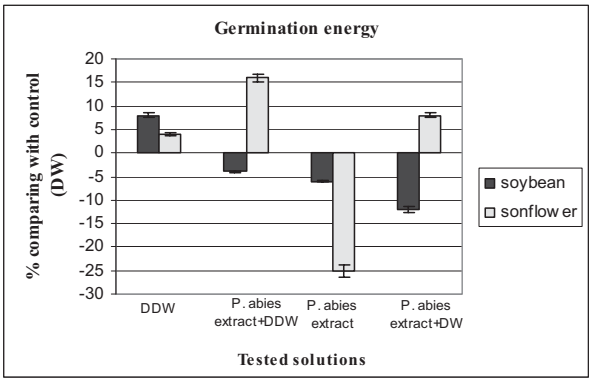

Fig. 1.The influence of DDW and *P. abies* aqueous extract in combination with DDW on seed germination energy of *Glycine max L.* and *Helianthus annuus L.* plants

For sunflower seed there was little different situation. Thus, it was observed that the mixture of DDW and spruce bark aqueous extract (1:1) accelerates seed germination up to 16% and increases the number of seeds germinated by 15% compared to control. Also, stimulation of germination energy and capacity is recorded, and when applied in the germination medium, the spruce bark aqueous extract with 0.5% concentration. When the extract concentration is higher (1%), germination energy and capacity is reduced by 25 and 10 percent for the samples where distilled water was added (Fig. 1 and 2).

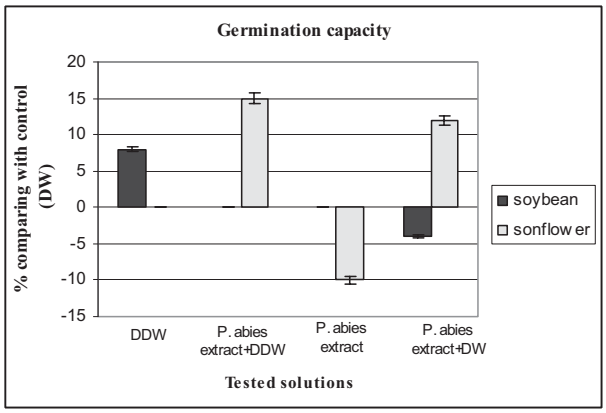

Fig. 2.The influence of DDW and *P. abies* aqueous extract in combination with DDW on seed germination capacity of *Glycine max L.* and *Helianthus annuus L.* plants

Analyzing the influence of test solutions on vegetative organs elongation, there is a stimulating for both plant species considering for experiments. The most significant influence, as shown in figure 4, there was registered for the sample treated with 1% spruce bark aqueous extract. Thus, the percentage of root and stem elongation stimulation of sunflower seedlings, increases to 54% and 31%. In this case, must highlighted differences in the growth of sunflower seedlings were observed, depending on the concentration of polyphenolic extract applied. Therefore, a concentration of 1% of polyphenolic extract, triggers a stimulation of growth process, compared with a concentration of 0.5% for which the influence where reduced, or even more, weak inhibitors was observed. A positive influence on this index was recorded when the distilled water of growth medium, was substituted with DDW or mixed with spruce bark aqueous extract. In this case it was observed a significant stimulation effect for all vegetative organs of sunflower seedlings growth, especially radicle and primary leaves (Fig. 3).

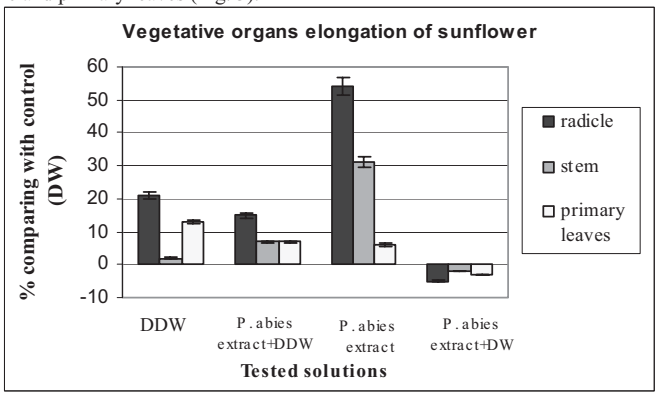

Fig. 3.The influence of DDW and *P. abies* aqueous extract in combination with DDW on vegetative organs elongation of *Helianthus annuus L.* plant.

Spuce bark aqueous extract in combination with deuterium depleted water, increases significantly soybean seedlings elongation, especially radicle (38%). A positive influence is recorded for separate application at the two

solutions, but it is lower than the application in the mixture. Noting the increase in length of soybean seedlings, there is a difference not of it depending on the concentration spruce extract applied samples (Fig. 4).

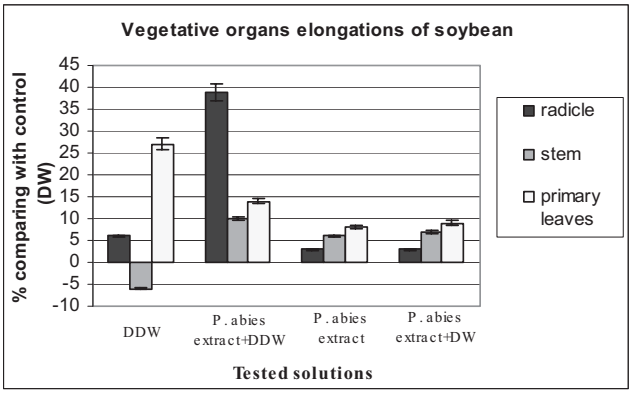

Fig. 4.The influence of DDW and *P. abies* aqueous extract in combination with DDW on vegetative organs elongation of *Glycine max L.* plant.

The observations outlined in the analysis results in increasing the length of vegetative organs which are also confirmed by the results obtained in the case of vegetal biomass for soybean and sunflower seedlings. Analyzing figure 6 it could be observed that the vegetal biomass of sunflower seedlings was significantly higher in the presence of polyphenolic extract, comparing with control (68% - radicle, 30% - stem, 67% - primary leaves). Also, in this case, the difference in concentration of polyphenolic extract is followed by differences in growth and development of sunflower seedlings. The incentive effect is recorded, when DDW, respectively DDW and polyphenolic extract mixture is applying into the growth medium. Spruce bark aqueous extract in combination with deuterium depleted water, increases vegetal biomass accumulation in all vegetative organs of soybean seedlings (17% - radicle and stem, 19% - primary leaves). Soybean seedlings, which were developed in the presence of deuterium depleted water, have accumulated a high amount of primary leaves vegetal biomass with 27% more than the amount accumulated in control seedlings (Fig. 5). The effect of polyphenolic extract, on vegetal biomass accumulation was lower in soybean seedlings. It diminishes proportionally with decreasing polyphenolic extract concetration.

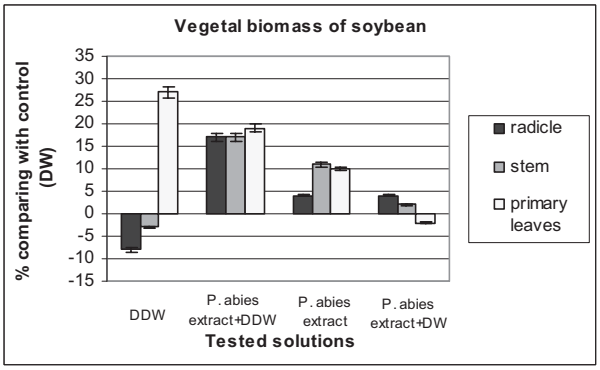

Fig. 5.The influence of DDW and *P. abies* aqueous extract in combination with DDW on vegetal biomass accumulation of *Glycine max L.* plant.

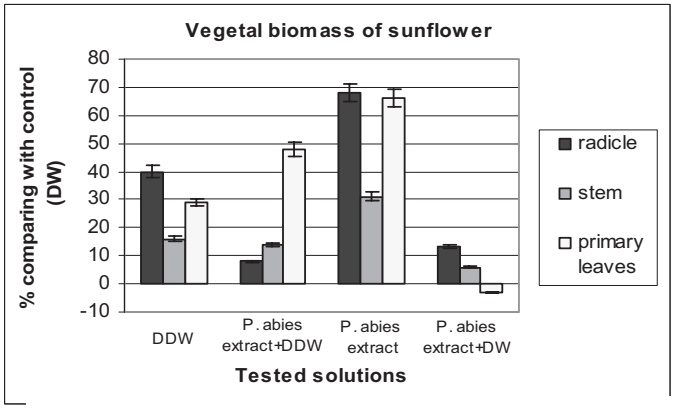

Fig. 6.The influence of DDW and *P. abies* aqueous extract in combination with DDW on vegetal biomass accumulation of *Helianthus annuus L.* plant.

Analyzing photoassimilating pigments synthesis, it was found that it was reduced in the the presence of DDW (Fig. 7). On contrary to sunflower seedlings, deuterium depleted water stimulates the photoassimilating pigments synthesis in a high percentage (45% - chlorophyll "a", 98% - chlorophyll "b", 68% - carotenoids). Following the influence of polyphenolic extract, it was registered a lower stimulation effects for the photoassimilating pigments of synthesis in soybean seedlings (Fig. 7), but a high stimulation effects in case of sunflower seedlings (Fig. 8). For all three tested solutions, charactherized by the presence of spruce bark aqueous extract, which was applied in sunflower seedlings growth medium, it was found a clear increase in photoassimilating pigments content. As it could be observed in figures 7 and 8, the supplementation of soybean and sunflower seedlings growth medium with spruce bark polyphenolic extracts stimulates clorophyll and carotenoids pigments biosynthesis process.

A concentration of 0.5% spruce bark extract into the growth medium increases the amount of chlorophyll 'a' with 54%, chlorophyll "b" with 121% and 55% the total carotenoid pigments concentrations.

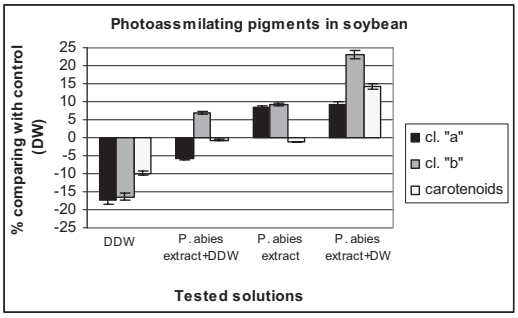

Fig. 7.The influence of DDW and *P. abies* aqueous extract in combination with DDW on photoassimilating pigments accumulation of *Glycine max L.* plant

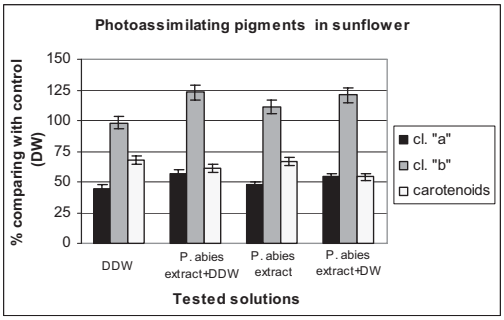

Fig. 8.The influence of DDW and *P. abies* aqueous extract in combination with DDW on photoassimilating pigments accumulation of *Helianthus annuus L.* plant

CONCLUSIONS

The obtained data shown that the deuterium depleted water, stimulates germination energy and capacity, radicle and primary leaves elongation, vegetal biomass accumulation and inhibits photoassimilating pigment synthesis. In

the case of sunflower it was found that DDW stimulates the elongation of all vegetative organs, vegetal biomass accumulation and photoassimilating pigments synthesis.

Spruce bark aqueous extract, reduce germination energy and capacity of soybeans seeds, stimulates accumulation of biomass in soybean seedlings, stimulates elongation for all vegetative organs, accumulation of biomass and photoassimilating pigments synthesis for sunflower seedlings.

Spruce bark aqueous extract in combination with deuterium depleted water stimulates the elongation of all vegetative organs and accumulation of biomass for soybean seedlings. In the case of sunflower seedlings it was observed stimulatory effects on biomass accumulation, photoassimilating pigments synthesis, germination energy and capacity.

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