ANALELE ŞTIINŢIFICE
ALE
UNIVERSITĂŢII „ALEXANDRU IOAN CUZA”
DIN IAŞI

(SERIE NOUĂ)

SECŢIUNEA II

a. GENETICĂ ŞI BIOLOGIE MOLECULARĂ

TOMUL XII, fascicula 1 **2011**

Editura Universităţii „ALEXANDRU IOAN CUZA” Iaşi

EDITORIAL OFFICE

Universitatea „Alexandru Ioan Cuza", Facultatea de BIOLOGIE
Laboratorul de Biochimie şi Biologie Moleculară
Bulevardul Carol I, Nr. 20A, 700506
Iaşi, România
gbmpapers@yahoo.com

CONTENT

BIOCHIMIA LA ANIVERSAREA A 150 DE ANI DE LA ÎNFIINŢAREA UNIVERSITĂŢII „ALEXANDRU IOAN CUZA” DIN IAŞI

Vlad ARTENIE[1*]

În a treia decadă a lunii octombrie 2010, Universitatea „Alexandru Ioan Cuza” din Iaşi a celebrat un secol şi jumătate de la momentul fondării sale. Potrivit decretului de înfiinţare din 26 octombrie 1860, Universitatea din Iaşi în acelaşi timp marchează momentul inaugural al învăţământului superior românesc. Chiar de la înfiinţarea sa ca primă instituţie de învăţământ superior a României şi până la acest moment aniversar din octombrie 2010, Universitatea „Alexandru Ioan Cuza” a menţinut viu spiritul progresist. Aşa cum arăta distinsul şi regretatul academician profesor univ. dr. Gheorghe Platon „…chiar de la întemeierea ei, Universitatea din Iaşi a simţit nevoia de a se încadra în istorie, de a-şi însuşi spiritul istoriei pentru a-şi aprofunda specificul şi a răspunde mai eficient funcţiilor sale”. Graţie marilor personalităţi care s-au perindat la conducerea universităţii, facultăţilor, departamentelor, catedrelor şi disciplinelor şi care au îmbrăţişat progresul, numărul facultăţilor şi disciplinelor a crescut în conformitate cu necesităţile specifice unei societăţi în perpetuă transformare, permiţându-se astfel ridicarea Universităţii ieşene la nivelul instituţiilor europene similare. Păstrând tradiţia umanistă şi ştiinţifică, universitarii ieşeni au introdus în programele de învăţământ ale facultăţilor discipline care la un moment dat exprimau tendinţa de dezvoltare a unei anumite ştiinţe.

În cele ce urmează ne vom referi la locul biochimiei şi evoluţia cercetării biochimice în cadrul Universităţii „Alexandru Ioan Cuza” din Iaşi în cea de a doua jumătate a secolului al XX-lea şi în prima decadă a secolului al XXI-lea.

Viaţa este cel mai frumos şi cel mai complex fenomen de pe planeta noastră. Nu există probleme mai incitante şi mai ciudate de care mintea omenească să fi fost întotdeauna fascinată decât numeroasele mistere privind viaţa, indiferent de formele pe care le îmbracă şi de nivelurile la care aceasta se află.

Biochimia joacă un rol esenţial în cunoaşterea multiplelor faţete încă neelucidate ale vieţii, originii şi dezvoltării ei pe Terra sau pe alte planete. Biochimia sau chimia biologică reprezintă chimia vieţii. Cu alte cuvinte biochimia studiază structura, proprietăţile şi transformările specifice componenţilor chimici ai organismelor vii. Ansamblul transformărilor suferite de aceşti componenţi chimici în organismele vii, cunoscut sub numele de metabolismul substanţelor, se află la baza vieţii.

Pe plan mondial cercetările biochimice încep să se contureze încă din cea de a doua jumătate a secolului al XIX-lea. În Universitatea „Alexandru Ioan Cuza” din Iaşi biochimia s-a individualizat ca disciplină de sine stătătoare mult mai târziu. Înainte de Cel de al Doilea Război Mondial, problemele de biochimie s-au abordat sporadic prin eforturile cadrelor didactice de la alte specialităţi. În primul rând trebuie menţionate cercetările marelui medic şi savant Constantin I. Parhon(1874-1969), pionier al endocrinologiei româneşti, care în anul 1912 este numit profesor de neuropsihiatrie la Facultatea de Medicină (înfiinţată în anul 1879) din cadrul Universităţii din Iaşi. Profesorul C. I. Parhon va onora această disciplină la Iaşi până în anul 1934, când va fi transferat la conducerea Catedrei de Endocrinologie Clinică de la Facultatea de Medicină din Bucureşti. În timpul şederii la Iaşi, profesorul C. I. Parhon a reuşit să atragă în jurul său numeroşi elevi şi să formeze ceea ce s-a numit „Şcoala Parhon de la Socola”, caracterizată printr-o abordare complexă, clinică şi experimentală, a interrelaţiilor dintre glandele endocrine, bolile neurologice şi psihice, în care se urmăreşte rolul hormonilor în aceste patologii.

Între cercetările biochimice din prima jumătate a secolului al XX-lea, trebuie reţinută ipoteza interesantă referitoare la structura proteinelor elaborată în anii 1935-1936 de profesorul univ. Haralamb Vasiliu (1880 – 1953), ctitorul Catedrei de Chimie Agricolă (1906) în Universitatea din Iaşi. Unele din elementele acestei ipoteze se regăsesc în concepţiile formulate mult mai târziu (1953) de Linus Carl Pauling şi R. B. Corey asupra legăturii peptidice şi structurii secundare a proteinelor, pentru care profesorul Pauling a primit Premiul Nobel pentru chimie în anul 1954. De asemenea, trebuie amintite cercetările efectuate de academicianul profesor univ. dr. Radu Cernătescu(1894-1958) care a studiat acţiunea bacteriană *in vitro* a compuşilor clorodici ai diferitelor baze organice cu azot în nucleu.

În decadele 4 – 5 ale secolului al XX-lea, se înregistrează pe plan mondial un avânt deosebit al cercetărilor ştiinţifice în multe domenii ale biologiei, chimiei, medicinii, agriculturii etc. care determină schimbarea radicală a valenţelor biochimiei. În acest context, în anul 1948 are loc în România reforma învăţământului care prevede introducerea biochimiei ca disciplină de învăţământ pentru studenţii de la Facultatea de Ştiinţe Naturale care a luat naştere în acelaşi an, 1948, din fosta secţie de Ştiinţe Naturale a Facultăţii de Ştiinţe din cadrul Universităţii „Alexandru Ioan Cuza”. În anul 1959, Facultatea de Ştiinţe Naturale se transformă în Facultatea de Ştiinţe Naturale-Geografie, prin unirea colectivelor de cadre didactice ce predau ştiinţele biologice cu cele ce predau ştiinţele geografice. Facultatea de Ştiinţe Naturale-Geografie îşi schimbă denumirea în anul 1963 în Facultatea de Biologie-Geografie care se întregeşte în anul 1977 în Facultatea de Biologie-Geografie-Geologie, ca urmare a dezvoltării Secţiei de Geologie. În anul 1990 s-a constituit Facultatea de Biologie în cadrul căreia, la momentul actual, funcţionează cicluri de licenţă şi studii de masterat în domeniile de Biologie, Biochimie şi de Ecologie şi Protecţia Mediului. Biochimia, de asemenea, a fost introdusă, ca disciplină facultativă sau opţională şi în planul de învăţământ al Facultăţii de Chimie care a devenit de sine stătătoare în anul 1948 din Secţia de Chimie a Facultăţii de Ştiinţe din cadrul Universităţii „Alexandru Ioan Cuza” din Iaşi. Trebuie să reţinem că pentru învăţământul biologic biochimia a avut un statut de disciplină obligatorie de la introducerea ei în planul de învăţământ.

Cadrele didactice care au onorat disciplina de biochimie la cele două facultăţi menţionate mai sus şi Laboratorul de biochimie au făcut parte de la început din structura organizatorică a Facultăţii de Chimie. În anul 1974 are loc un eveniment total nedorit şi iraţional, anume unificarea Facultăţii de Chimie de la Universitatea „Alexandru Ioan Cuza” cu Facultatea de Chimie Industrială de la Institutul Politehnic „Gh. Asachi” şi contopirea celor două facultăţi în

Facultatea de Chimie şi Inginerie Chimică care a trecut în cadrul Institutului Politehnic din Iaşi, aşa cum a fost comanda dată de „marea chimistă” a ţării, Elena Ceauşescu. În aceste condiţii, cadrele didactice care predau disciplinele de Biochimie şi Chimie Generală la alte facultăţi decât Facultatea de Chimie rămân mai departe în cadrul Universităţii „Alexandru Ioan Cuza”, fiind transferate la Facultatea de Biologie-Geografie, unde le-au prins şi evenimentele din decembrie 1989. În urma plecării laboratoarelor de la Facultatea de Chimie în noua clădire a Facultăţii de Chimie şi Inginerie Chimică(pentru care în anul 1977 este adoptată denumirea de Facultatea de Tehnologie Chimică) de pe Splaiul Bahlui, o parte din spaţiul eliberat a fost ocupat de laboratoarele de lucrări practice cu studenţii la disciplinele de Biochimie şi Chimie Generală, precum şi de laboratoarele de cercetare ale cadrelor didactice din Colectivul de Biochimie şi Chimie Generală. În aceste laboratoare s-au desfăşurat activităţile didactice cu studenţii şi cercetarea ştiinţifică la disciplinele biochimice şi de Chimie Generală, din 1974 şi până în anul 2002, când Colectivul de Biochimie şi Chimie Generală din Catedra de Biochimie-Genetică-Microbiologie s-a mutat în spaţiul Facultăţii de Biologie. Pe această linie, beneficiind de înţelegere şi sprijin din partea rectorului prof. univ. dr. Dumitru I. Oprea şi a decanului prof. univ. dr. Gheorghe Mustaţă, din acea perioadă, am reuşit organizarea a două laboratoare pentru lucrările practice la disciplina de Chimie generală şi la disciplinele biochimice, precum şi a două laboratoare de cercetare în domeniul biochimiei, în felul acesta condiţiile pentru biochimie devenind mai favorabile.

Primul titular al disciplinei de biochimie în Universitatea din Iaşi, a fost profesor univ. dr. Elisabeta Văscăuţeanu (1897-1989), care a fost numită conferenţiar la această disciplină la 1 decembrie 1948. Numirea Elisabetei Văscăuţeanu la disciplina de biochimie s-a făcut pe baza rezultatelor obţinute în cercetările sale anterioare care aveau o puternică orientare spre biochimie.

Un deosebit interes prezintă cercetările efectuate de Elisabeta Văscăuţeanu în cadrul doctoratului, efectuat sub conducerea ştiinţifică a renumitului savant academician profesor univ. dr. Radu Cernătescu. În anul 1938, Elisabeta Văscăuţeanu susţine teza de doctorat cu titlul „*Studiul asupra acizilor 5,5-dietil- şi 5,5-fenil-etil-barbituric şi asupra sărurilor sale*”, aceşti compuşi având utilizări în terapeutică şi în practica biochimică. Merită să fie amintite, de asemenea, cercetările efectuate de profesor dr. Elisabeta Văscăuţeanu în colaborare cu Elena Bogdan, Mărioara Motaş, Olga Vicol, Valentina Jurca asupra complecşilor chininei, chinidinei şi cinconinei cu clorurile de cobalt(II) şi cupru(II), compuşilor acidului nicotinic cu acidul fosforic, respectiv asupra sărurilor acidului ascorbic cu diferiţi alcaloizi şi nitrofuranul, unele din aceste săruri având capacitatea de a inhiba slab creşterea unor germeni patogeni.

Pe linia cercetărilor coordonate de academician prof. dr. Constantin I. Parhon, cu privire la compoziţia chimică a lichidelor biologice din organismul uman, Elisabeta Văscăuţeanu s-a ocupat de variaţia conţinutului de sodiu sanguin în raport cu vârsta, datele obţinute fiind citate de profesorul francez P. Boulanger în monografia sa despre biochimia sodiului şi potasiului.

Un domeniu de cercetare, atacat de profesor dr. Elisabeta Văscăuţeanu şi colaboratorii săi, este cel al biochimiei animale. Profesor Elisabeta Văscăuţeanu şi Valentina Jurca au studiat dinamica proteinelor, calciului şi fosforului în sângele crapului galiţian şi crapului lausitz, crescuţi în apele iazurilor din Moldova, în strânsă corelaţie cu calitatea apei şi condiţiile de furajare. În ideea de a veni în sprijinul producţiei piscicole, profesor Elisabeta Văscăuţeanu a publicat şi date despre compoziţia chimică şi calitatea apelor din unele iazuri de pe teritoriul Judeţului Iaşi. Profesor Elisabeta Văscăuţeanu şi Valentina Jurca au arătat că nivelul de

carotenoide şi fosfor în ouăle găinilor crescute în libertate este mai mare decât în ouăle găinilor crescute în padoc. Valentina Jurca, şef de lucrări la disciplina de Biochimie, a abordat studiul electroforetic al proteinelor în embriogeneză la unele ciprinide de cultură. În cadrul doctoratului, Valentina Jurca a investigat influenţa unor microelemente şi a vitaminelor din complexul B asupra metabolismului diferitelor forme ale fosforului şi activităţii fosfomonoesterazelor alcalină şi acidă din sângele păstrăvului curcubeu (*Salmo gairdneri*), crescut în bazine experimentale. Valentina Jurca a cercetat, de asemenea, acţiunea ionilor de litiu, sodiu şi potasiu *in vitro* asupra activităţii fosfomonoesterazei alcaline şi acide din diferite organe de la şobolani albi, precum şi din plasma şi globulele roşii din sânge uman, apoi comportarea peroxidazelor la unele plante tratate cu insecticide împotriva dăunătorilor.

În alte cercetări de biochimie animală, profesor Elisabeta Văscăuţeanu, Vlad Artenie, Elvira Tănase şi alţii, au urmărit separarea şi identificarea pe cale electroforetică a glicoproteinelor, albuminelor şi globulinelor din serul sanguin, de asemenea, glicemia şi proteinemia la găinile purtătoare de *Salmonella pullorum*.

Importante contribuţii aduce profesor dr. Elisabeta Văscăuţeanu prin abordarea unor probleme de biochimie vegetală. În acest context, împreună cu Vlad Artenie şi Elvira Tănase, a studiat particularităţile biochimice ale unor soiuri de soia create la Staţiunea de Cercetări Podu Iloaiei, Judeţul Iaşi.

Cercetarea biochimică în Universitatea „Alexandru Ioan Cuza” a înregistrat un nou suflu, după anul 1967, când titularul disciplinei de biochimie devine Vlad Artenie. Acesta a efectuat un *stagiu de doctorat cu frecvenţă* şi a obţinut titlul de doctor în ştiinţe biologice, specialitatea biochimie animală, susţinând în mai 1966 teza de doctorat „*Izolarea, purificarea şi proprietăţile colinacetilazei*”, elaborată sub conducerea academicianului prof. S. E. Severin, Şeful Catedrei de Biochimie Animală a Facultăţii de Biologie-Pedologie de la Universitatea de Stat „M. V. Lomonosov” din Moscova, Rusia. O parte din rezultatele cercetărilor efectuate de Artenie în cadrul tezei de doctorat, au fost folosite de academicianul prof. Severin în lucrarea „The enzymes of acetylcholine biosynthesis” (la care Vlad Artenie este coautor), prezentată la Cel de al VII-lea Congres Internaţional de Biochimie care a avut loc în anul 1967, la Tokio(Japonia). Preluând disciplina de biochimie, în calitate de lector(1967-1969) şi de conferenţiar(1969-1990), Vlad Artenie a iniţiat şi a dezvoltat direcţii noi de cercetare ca enzimologia, biochimia microorganismelor, biochimia nutriţiei salmonidelor în condiţii de creştere intensivă în viviere flotabile etc. Totodată, au fost extinse unele cercetări de biochimie vegetală.

Cercetările de **enzimologie** au fost orientate de Vlad Artenie în mai multe direcţii. Una din acestea a urmărit izolarea, purificarea şi caracterizarea unor enzime sintetizate de microorganisme şi plante. Astfel, putem menţiona purificarea şi caracterizarea catalazei sintetizate de *Penicillium chrysogenum* sau a proteazelor din frunzele de *Plantago* (Vlad Artenie, N. D. Topală, Dumitru Cojocaru şi alţi). 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 s-a axat pe obţinerea de biocatalizatori heterogeni prin imobilizarea de enzime pe diferite suporturi organice sau anorganice, reuşindu-se imobilizarea pepsinei şi tripsinei pe Biozan R, a ureazei pe carboximetilceluloză, a catalazei pe xanthan sau fibre de celuloză etc.(Vlad Artenie, Dumitru Cojocaru, Elvira Tănase şi alţi).

În general, cercetările privind **biochimia nutriţiei salmonidelor în condiţii de creştere intensivă**, 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(Vlad Artenie, Ionel Miron, Klaus Battes, Costică Misăilă, Elvira Tănase, Elena-Rada Misăilă, Maria Apetroaiei).

Importanţă deosebită au cercetările referitoare la **biochimia microorganismelor** care 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 posibilitate 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 populaţii locale, soiuri şi hibrizi de porumb, ale unor varietăţi de nuc, ale unor populaţii de fasole, ale unor plante medicinale (dracila, vinca), evidenţiindu-se calităţile plantelor respective şi importanţa lor practică(Vlad Artenie, Elvira Tănase, Mandache Leucov şi alţi). 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(Vlad Artenie şi alţi), modificarea amilazelor şi catalazei din plante sub acţiunea unor pesticide(Vlad Artenie) etc.

Un moment important în evoluţia cercetării biochimice în Universitatea „Alexandru Ioan Cuza” din Iaşi a fost marcat de înfiinţarea, în anul 1990, a specializării **Biochimie**, alături de cea de Biologie, respectiv de Ecologie şi Protecţia Mediului, în cadrul Facultăţii de Biologie. Un an mai târziu, adică în 1991, a fost înfiinţată specializarea **Biochimie Tehnologică** în cadrul Facultăţii de Chimie care a revenit după anul 1990 la Alma Mater de la care a fost smulsă în anul fatidic 1974. Disciplinele de biochimie din planul de învăţământ al specializării de Biochimie Tehnologică au fost predate mulţi ani de cadre didactice de la Facultatea de Biologie. Astfel, au apărut condiţiile creării, începând cu anul 1991, a **Catedrei de Biochimie-Genetică-Microbiologie (B-G-M)** în cadrul Facultăţii de Biologie ca răspuns la creşterea numărului de studenţi şi de discipline biochimice, genetice şi microbiologice, ceea ce a presupus organizarea de noi laboratoare de cercetare.

Primul şef al Catedrei de B-G-M a fost profesorul dr. Vlad Artenie, care a contribuit efectiv la consolidarea învăţământului biochimic în Universitatea „Alexandru Ioan Cuza”, în această acţiune nebucurându-se totdeauna de sprijin din partea conducerii Facultăţii de Biologie,

respectiv de susţinere din partea celorlalţi şefi ai catedrelor din facultate. Înfiinţarea Catedrei de B-G-M a imprimat cercetării biochimice o nouă dimensiune.

În cercetările ştiinţifice efectuate de cadrele didactice din Colectivul de Biochimie al Catedrei de B-G-M au fost reluate şi extinse direcţii de enzimologie, biochimie vegetală, biochimia microorganismelor, biochimie animală, la care se adaugă biochimia clinică, iar din anul 2004 şi biologia moleculară. Diversitatea cercetărilor biochimice se poate explica şi prin pregătirea de specialişti pe calea doctoratului, conducere de doctorat în domeniul biologiei, specialitatea biochimie având din anul 1982(reconfirmată în anul 1990) profesor dr. Vlad Artenie şi din anul 2008 profesor dr. Dumitru Cojocaru. Din cei nouă membri ai actualului Colectiv de Biochimie din cadrul Laboratorului Profesional de Biochimie şi Biologie Moleculară(şef profesor dr. Dumitru Cojocaru) de la Facultatea de Biologie a Universităţii „Alexandru Ioan Cuza” din Iaşi, şapte şi-au susţinut doctoratul sub conducerea ştiinţifică a profesorului Vlad Artenie, iar doi au finalizat teza de doctorat în cotutelă cu Laboratorul de Fiziologia Plantelor al Academiei de Ştiinţe din Republica Moldova şi respectiv cu Universitatea de Ştiinţe şi Tehnologii, Lille, Franţa. În tabelul 1 sunt specificate titlurile tezelor de doctorat ale membrilor din Colectivul de Biochimie care au obţinut titlul ştiinţific de doctor în **biologie**, specializarea **biochimie**.

Tabelul 1. Titlurile tezelor de doctorat ale membrilor Colectivului de Biochimie care au obţinut titlul ştiinţific de doctor în **biologie**, specializarea **biochimie.**

Nr. crt.	Titlul tezei de doctorat	Autorul, locul şi anul susţinerii tezei de doctorat
1	Cercetări privind imobilizarea enzimelor şi celulelor microbiene cu aplicaţii în biotehnologie	Dumitru COJOCARU, Universitatea „Alexandru Ioan Cuza”(UAIC) Iaşi, 10 mai 1990
2	Bilanţul şi patternul polipeptidelor în procesul de stratificare a seminţelor de măr	Ovidiu C. TOMA Universitatea de Stat din Chişinău, Republica Moldova, 29 iunie 1994 (Cotutela între Institutul de Fiziologie a Plantelor al Academiei de Ştiinţe a Republicii Moldova şi UAIC Iaşi)
3	Implicarea unor enzime în metabolismul energetic la speciile *Torulopsis candida* M1 şi *Claviceps purpurea*	Zenovia OLTEANU, UAIC Iaşi, 14 noiembrie 2003
4	Cercetări asupra unor procese metabolice la unele specii celulozolitice în diferite condiţii de creştere	Lăcrămioara-Anca ANTOHE, UAIC Iaşi, 16 februarie 2005
5	Imobilizarea unor proteaze pe diferite suporturi, cu eventuale aplicaţii practice.	Anca-Mihaela HUMĂ, UAIC Iaşi, 18 februarie 2005 (Cotutela cu Universitatea de Ştiinţe şi Tehnologii, Lille, Franţa)
6	Studiul amilazelor şi α-glucanfosforilazei la unele graminee cultivate şi spontane	Elena CIORNEA UAIC Iaşi, 26 ianuarie 2008
7	Unele implicaţii moleculare ale plasmidului pAO1 în metabolismul microorganismului *Arthrobacter nicotinovorans*	Marius I. MIHĂŞAN UAIC Iaşi 14 septembrie 2009

Nr. crt.	Titlul tezei de doctorat	Autorul, locul şi anul susţinerii tezei de doctorat
8	Studiul particularităţilor metabolismului proteic în germinaţia seminţelor la unii arbori de importanţă forestieră	Eugen V. UNGUREANU UAIC Iaşi 29 ianuarie 2010

În ianuarie 2011, numărul **doctorilor în biologie** care şi-au trecut doctoratul sub conducerea ştiinţifică a profesorului univ. dr. Vlad Artenie s-a ridicat la 63.

Cercetările de **enzimologie**, axate în principal pe imobilizarea de enzime(pepsină, tripsină, catalază etc.) şi celule microbiene pe suporturi reprezentate de diferiţi polimeri organici (Vlad Artenie, Dumitru Cojocaru, Elvira Tănase şi alţi) au stat la baza stabilirii Acordului de Colaborare 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 până în anul universitar 2008-2009 profesor dr. Didier Guillochon, care în anul 2005 a primit titlul de Doctor Honoris Causa al Universităţii „Alexandru Ioan Cuza”din Iaşi. În urma acestei colaborări au fost susţinute două teze de doctorat în cotutelă(Anca Mihaela Humă în februarie 2005 şi Elena-Loredana Ţicu în septembrie 2006), având ca subiecte studiul imobilizării pepsinei pe oxid de aluminiu în scopul obţinerii de peptide biologic active prin scindarea hemoglobinei.

În domeniul **biochimiei vegetale** se abordează studiul electroforetic al proteinelor din seminţele unor genotipuri de măr, supuse procesului de stratificare în diferite variante experimentale(Ovidiu Toma), studiul proteinelor din seva unor soiuri de viţă de vie(Vlad Artenie, Iulia Dascaliuc), variaţia nivelului de clorofile şi carotenoide la diverse specii de plante în cele patru anotimpuri anuale(Vlad Artenie, Anca Humă, Eugen Ungureanu), dinamica activităţii unor enzime şi conţinutului de pigmenţi fotosintetici la unele specii de plante spontane şi cultivate, tratate cu erbicide din clasa paraquatului(Vlad Artenie, Elvira Tănase, Anca Humă, Antoanela Patraş şi alţi), cu radiaţii gamma sau microunde(Vlad Artenie şi alţi), dinamica amilazelor şi glucan fosforilazei în germinaţia seminţelor unor graminee spontane şi cultivate(Elena Ciornea, Dumitru Cojocaru, Sabina Ioana Cojocaru, Gabriela Vasile) etc.

Aria cercetărilor de biochimie vegetală s-a lărgit considerabil prin abordarea unor teme pe bază de contracte, având ca directori membri din Colectivul de Biochimie. Unele din aceste teme sunt exemplificate în tabelul 2.

Tabelul 2. Teme de cercetare de biochimie vegetală, contractate cu diferiţi beneficiari.

Nr. crt.	Denumirea contractului	Director de grant	Anii de realizare	Valoarea (RON)
1	Caracterizarea unor principii bioactive de origine vegetală şi fungică cu acţiune citostatică, imunomodulatoare, metabolică şi neurotropă şi valorificarea lor în alimentaţia funcţională	Prof. dr. Dumitru Cojocaru	2005 - 2008	1.500.000
2	Valorificarea biotehnologică a potenţialului productiv la *Hyppophae rhamnoides* ssp. c*arpathica* prin caracterizarea complexă a soiurilor, sursă de recolte ecologice pentru o agricultură durabilă	Conf. dr. Zenovia Olteanu	2006 - 2008	350.000

Nr. crt.	Denumirea contractului	Director de grant	Anii de realizare	Valoarea (RON)
3	Ameliorarea potenţialului genetic şi caracterizarea complexă a biotipurilor din grupa plante de viitor, cu impact asupra dezvoltării ecologice şi durabile în pomicultură	Conf. dr. Zenovia Olteanu	2008 - 2011	82.203
4	Selecţia şi cultivarea *Rhodiola rosea* prin metode moleculare, fitochimice si fiziologice	Prof. dr. Ovidiu Toma	2006 - 2008	

Din tabelul 2 se poate constata că după anul 2005 cercetările de biochimie vegetală s-au axat pe mai multe direcţii cu importanţă teoretică şi implicaţii practice :

- evidenţierea unor proprietăţi şi roluri ale extractelor vegetale şi fungice asupra unor procese fiziologice, biochimice, imunologice şi citogenetice pe celule animale normale şi tumorale(Dumitru Cojocaru, Zenovia Olteanu, Lăcrămioara Oprică, Elena Ciornea, Eugen Ungureanu, Anca Humă, Marius Mihăşan, Sabina Ioana Cojocaru şi alţi) ;
- caracterizarea biochimică complexă a diferitelor soiuri de *Hyppophae rhamnoides* ssp. c*arpathica* şi studiul influenţei factorilor de mediu asupra principalilor componenţi biochimici din frunzele şi fructele soiurilor de cătină(Zenovia Olteanu, Lăcrămioara Oprică, Vlad Artenie, Dumitru Cojocaru, Elena Ciornea) ;
- investigarea particularităţilor biochimice ale fructelor de *Cornus mass*, *Rosa sp*., *Lonicera caerulea* şi *Aronia melanocarpa* pentru aprecierea calităţilor nutritive, în vederea selectării biotipurilor valoroase(Zenovia Olteanu, Lăcrămioara Oprică, Dumitru Cojocaru, Elena Ciornea).

Posibilitatea realizării contractelor menţionate în tabelul 2 a permis organizarea unui laborator cu specific pentru cercetările de **enzimologie**, precum şi dotarea celorlalte două laboratoare de cercetare în domeniul biochimiei şi a celor două laboratoare pentru lucrările practice cu studenţii la disciplinele biochimice şi Chimie Generală cu aparatură modernă (balanţe electronice, pH-metre, centrifuge performante, spectrofotometre UV şi vizibil din ultimele generaţii, aparate de electroforeză cu cuve verticale şi orizontale, autoclav de masă Certoclav, omogenizator Poter, sonicator VibraSonic, sistem HPLC Bischoff, aparat PCR Biometra, sistem electroporare Biorad GenePulser etc.). Această nouă şi modernizată dotare a laboratoarelor de cercetare şi a laboratoarelor pentru lucrările practice cu studenţii la disciplinele biochimice şi Chimie Generală a permis extinderea cercetărilor din domeniu, în acelaşi timp cu realizarea unei afirmări tot mai puternice a biochimiei de la Facultatea de Biologie din Iaşi pe plan naţional şi internaţional.

Cercetările de **biochimia microorganismelor** au fost concretizate prin studii referitoare la comportamentul unor oxido-reductaze şi hidrolaze participante la căile generatoare de energie în celulele de *Torulopsis candida* şi *Claviceps purpurea*(Zenovia Olteanu, Vlad Artenie şi alţi), răspunsul metabolic al speciilor *Chaetonium globosum* şi *Alternaria alternata* la diferite surse de carbon, vitamine hidrosolubile, oligoelemnte, lichide magnetice, microunde de joasă intensitate(Lăcrămioara-Anca Oprică, Vlad Artenie şi alţi).

Tot în domeniul biochimiei microorganismelor se înscrie şi cercetarea postdoctorală, realizată de dr. Ovidiu Toma pe bază de contract cu Institutul de Cercetări Biotehnologice din Montreal-Quebec, Canada, între anii 1998-2000 şi care vizează expresia şi producţia biotehnologică a domeniului extracelular al receptorului factorului de creştere transformaţională(TGF β sRII) în *Pichia pastoris*.

De un deosebit interes sunt, de asemenea, cercetările care urmăresc valorificarea potenţialului de biosinteză al unor substanţe bioactive la tulpinile de *Claviceps purpurea* (tabelul 3).

Tabelul 3. Teme de cercetare contractate în domeniul biochimiei microorganismelor.

Nr. crt.	Denumirea contractului	Director de contract	Anii de realizare	Valoarea (RON)
1	Obţinerea unor tulpini submerse de *Claviceps purpurea* cu preferenţiala si înalta capacitate glucanosintetica si stabilirea domeniilor de valorificare biomedicala a unor preparate glucanice autohtone	Conf. dr. Zenovia Olteanu	2008-2011	82.203
2	Caracterizarea complexa a unor extracte citostatic active din tulpini de *Claviceps purpurea* obţinute prin biotehnologii de hibridare parasexuala, in vederea valorificării în terapeutica veterinara	Conf. dr. Zenovia Olteanu	2008-2011	57.426

În aceste cercetări pe bază de contracte se abordează probleme fundamentale cu implicaţii practice evidente :

- studiul biosintezei glucanilor la tulpini de *Claviceps purpurea* din flora spontană şi la tulpini ameliorate ale acestei specii(Zenovia Olteanu, Dumitru Cojocaru, Lăcrămiuoara Oprică) ;
- caracterizarea biochimică complexă a unor tulpini de *Claviceps purpurea* în condiţii determinate de cultivare, în vederea elaborării unei biotehnologii de obţinere a unor compuşi bioactivi de uz veterinar(Zenovia Olteanu, Lăcrămioara Oprică, Sabinma Ioana Cojocaru, Ovidiu Toma).

Investigaţiile de **biochimie animală** efectuate după anul 1990 au urmărit particularităţile sistemelor genetico-biochimice ale rasei de ovine Karakul de Botoşani în corelaţie cu potenţialul ei morfo-productiv şi reprezintă o direcţie de cercetare abordată, între 2002-2005, pe bază de contracte, de către profesorul Vlad Artenie în colaborare cu specialişti de la Staţiunea de Cercetare şi Dezvoltare pentru Ovine Popăuţi, Judeţul Botoşani, sub conducerea cercetătorului ştiinţific principal gradul I Gheorghe Hrincă, doctor în biologie (specialitatea Biochimie) al Universităţii „Alexandru Ioan Cuza” din Iaşi din anul 1996. Merită să fie menţionate şi studiile consacrate unor aspecte referitoare la stresul oxidativ în procesele de învăţare şi memorare la şobolanii trataţi cu antagonişti şi agonişti specifici sistemelor neurotransmiţătoare colinergic şi catecolaminergic(Vlad Artenie, Lucian Hriţcu, Alin Ciobîcă şi alţi).

În cercetările de **biochimie clinică** 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, valorile unor parametri

biochimici care exprimă disfuncţiile ficatului în hepatitele virale la pacienţii cu hepatită cronică etc. (Vlad Artenie, Anca Mihaela Negură, Ovidiu Toma şi alţi).

Cea mai nouă direcţie de cercetare pentru Colectivul de Biochimie o constituie aspectele de **biologie moleculară** care au putut fi abordate în urma colaborării, pe de o parte, cu Laboratorul de Biochimie şi Biologie Moleculară condus de profesor dr. Roderich Brandsch din Institutul de Biochimie şi Biologie Moleculară al Universităţii „Albert-Ludwig” din Freiburg, Germania, iar pe de altă parte, cu membrii Laboratorului condus de profesor dr. Stéphane Bouquelet de la Universitatea de Ştiinţe şi Tehnologii din Lille, Franţa.

Colaborarea cu Laboratorul profesorului Roderich Brandsch, care continuă şi în prezent, a fost deschisă în anul 1996 de către conferenţiar dr. Ovidiu Toma, după care a fost reluată şi amplificată de profesorul dr. Vlad Artenie şi profesorul dr. Ovidiu Toma în anul 2000.

În momentul de faţă, colaborarea cu grupul profesorului Roderich Brandsch (Profesor de Onoare al Universităţii „Alexandru Ioan Cuza” din Iaşi) cuprinde cercetări iniţiate şi realizate în cadrul Laboratorului de Biochimie de la Facultatea de Biologie din Iaşi şi care se axează 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 investigarea aspectelor moleculare şi cinetice ale unui număr de cinci enzime cantonate pe megaplsmidul pAO1, aceste enzime fiind implicate în metabolismul nicotinei, respectiv în metabolizarea glucidelor de către *Athrobacter nicotinovorans* (Marius Mihăşan, Vlad Artenie, Roderich Brandsch). Unele dintre aceste probleme au constituit obiectul a două granturi de cercetare(Tabelul 4).

Tabelul 4. Teme de cercetare din domeniul biologiei moleculare.

Nr. crt.	Denumirea contractului	Director de contract	Perioada de derulare	Valoare (RON)
1	Unele implicaţii moleculare ale megaplasmidului pAO1 în metabolismul bacteriei *Arthrobacter nicotinovorans*	Asistent drd. Marius MIHĂŞAN	2007-2009	40865.33
2	Clonarea si caracterizarea ORF32 si ORF40 de pe megaplasmidul pAO1 din *Arthrobacter nicotinovorans* – potenţiale modele de studiu ale interacţiunii tagatoză-proteine	Asistent dr. Marius MIHĂŞAN	2010-2012	
3	Studiul molecular al implicării genelor BRCA în predispoziţia ereditară la cancerul mamar şi ovarian, la unele familii cu risc din nord-estul României	Şef lucrări dr. Anca Mihaela HUMĂ	2007-2008	
4	Optimizarea şi implementarea unor tehnici de biologie moleculară în depistarea predispoziţiei ereditare la cancerul mamar şi ovarian	Conf. dr. Anca Mihaela NEGURĂ	2008-2011	

Cercetările realizate împreună cu echipa profesorului Stéphane Bouquelet vizează caracterizarea prin metode moderne de biochimie, biologie moleculară şi bioinformatică a fructozokinazei la bifidobacterii, bacterii Gram pozitive, capabile să metabolizeze

fructooligozaharidele. Aceste cercetări s-au finalizat prin elaborarea, sub conducerea profesorilor Artenie şi Bouquelet, a unei teze de doctorat în cotutelă, prezentată public de către asistent Iuliana Cristina Căescu, în data de 16 iulie 2004, în cadrul Universităţii „Alexandru Ioan Cuza” din Iaşi.

Tot în domeniul biologiei moleculare trebuie menţionat studiul început de dr. Anca Mihaela Negură asupra implicării genelor BRCA1 şi BRCA2 în predispoziţia ereditară la cancerul mamar şi ovarian în populaţia din Nord-Estul României(tabelul 4).

Membrii Colectivului de Biochimie din cadrul Laboratorului Profesional de Biochimie şi Biologie Moleculară de la Facultatea de Biologie a Universităţii „Alexandru Ioan Cuza” din Iaşi au făcut cunoscute rezultatele cercetărilor lor la diferite manifestări ştiinţifice naţionale şi internaţionale(sesiunii de comunicări, simpozioane, conferinţe, congrese). Între manifestările organizate chiar de membrii Colectivului de Biochimie menţionăm :

- Sesiunea ştiinţifică „**Biochimie şi biologie moleculară – prezent şi perspective”,** organizată în perioada 24-25 octombrie 2008 la Facultatea de Biologie în cadrul Zilelor Universităţii „Alexandru Ioan Cuza” din Iaşi ;

- **Primul Simpozion de Biochimie medicală şi Medicină moleculară**, cu **participare internaţională,** organizat la Iaşi în perioada 7-9 octombrie 2010, sub auspiciile Universităţii „Alexandru Ioan Cuza” şi ale Universităţii de Medicină şi Farmacie „Grigore T. Popa”, cu ocazia aniversării a 150 de ani de la înfiinţarea Universităţii „Alexandru Ioan Cuza” din Iaşi ;

- Sesiunea ştiinţifică „**Interacţiuni moleculare în lumea vie**”**,** organizată în cadrul *Simpozionului dedicat Anului Internaţional al Biodiversităţii* care a avut loc în zilele de 15-16 octombrie 2010 în cadrul Facultăţii de Biologie, cu ocazia aniversării a 150 de ani de la înfiinţarea Universităţii „Alexandru Ioan Cuza” din Iaşi.

Colaborarea cu Laboratorul de Chimie Biologică al Universităţii de Ştiinţe şi Tehnologii din Lille, Franţa s-a concretizat, pe lângă activităţile de cercetare ştiinţifică şi realizarea de lucrări de doctorate în cotutelă, în organizarea de către profesorul Vlad Artenie, cu sprijinul unora din membrii Colectivului de Biochimie(profesor dr. Dumitru Cojocaru, prof. dr. Ovidiu Toma, şef lucrări Anca Mihaela Humă, şef lucrări Elena Ciornea, şef lucrări Eugen Ungureanu), începând din anul 1995 şi până în anul 2004, în fiecare an, în cadrul Universităţii „Alexandru Ioan Cuza” din Iaşi a **Şcolii de Vară Franco-Română de Biochimi**e, intitulată „*Biologie et Pathologie Moléculares. Biotechnologies*”, condusă de profesorul emerit Jean Montreuil (1920-2010) de la Universitatea de Ştiinţe şi Tehnologii I din Lile. 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.).

În perioada premergătoare evenimentelor din anul 1989, şi mai ales după anul 1990, membrii Colectivului de Biochimie au elaborat numeroase monografii, cursuri şi îndrumătoare de lucrări de laborator care au fost litografiate sau tipărite în Editura Universităţii „Alexandru Ioan Cuza” şi în alte edituri locale sau centrale acreditate CNCSIS. De asemenea au fost publicate numeroase articole ştiinţifice cotate *Web of Science*, în reviste clasificate CNCSIS B+, B şi BDI, în volume ale unor conferinţe naţionale şi internaţionale etc., unele dintre aceste lucrări fiind citate în literatura de specialitate.

CONCLUZII

Aşa cum se poate constata din cele de mai sus, biochimia la Facultatea de Biologie a Universităţii „Alexandru Ioan Cuza” din Iaşi a depăşit peste 60 de ani de existenţă, perioadă în care interesul pentru această ştiinţă a crescut, permiţând dezvoltarea ei până la dimensiunile actuale.

În deceniile 5 – 8 din cea de a doua jumătate a secolului al XX-lea, dezvoltarea biochimiei în Universitatea „Alexandru Ioan Cuza” din Iaşi s-a făcut într-un ritm influenţat de condiţiile modeste oferite de modificările politice şi sociale generate în România de încheierea Celui de al Doilea Război Mondial în anul 1945.

Abia după anul 1990, dezvoltarea biochimiei la Facultatea de Biologie din Iaşi a intrat pe un făgaş normal, permiţând, pe de o parte, lărgirea ariei disciplinelor biochimice necesare pentru pregătirea viitorilor biochimişti, iar pe de alta, crearea unei platforme de lansare a unor cercetări de perspectivă, unele din rezultatele acestor cercetări fiind evidenţiate mai sus. În general, rezultatele obţinute de cercetare biochimică în cadrul Facultăţii de Biologie din Iaşi a permis o integrare a Colectivului de Biochimie în circuitul naţional şi internaţional actual al acestui domeniu de importantă perspectivă ştiinţifică.

[1] „Alexandru Ioan Cuza” University of Iaşi, Faculty of Biology, Laboratoy of Biochemistry and Molecular Biology, B-dul Carol I, Nr. 20A, 700506, IAŞI, ROMANIA

* vartenie@uaic.ro

TRANSCRIPTS QUANTIFICATION BY USING IN-HOUSE MADE RT-qPCR STANDARDS

LUCIAN NEGURĂ[1*], DRAGOŞ PEPTANARIU[1], ANCA NEGURĂ[2]

Keywords: quantitative Real-Time PCR, absolute quantification, standard calibration curve, re-amplification.

Abstract: Quantitative Real-Time PCR is the most widely used technique for detecting and quantifying nucleic acids in biological samples. It is the most sensitive method for the detection and quantitation of gene expression levels. Two general methods for the quantitative detection of the amplicon have been established: gene-specific fluorescent probes or specific double strand DNA binding agents. The levels of expressed genes may be measured by absolute or relative quantitative Real-Time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve. Calibration curves must be highly reproducible and allow the generation of highly specific, sensitive and reproducible data. We imagined a simple, reliable, easy-to-make method for generating standard for absolute Real-Time quantification, using only common laboratory equipment and reagents. Our method is based on re-amplification of purified amplicons, therefore assuring specificity and reproducibility of the reactions.

INTRODUCTION

Quantitative Real-Time PCR is nowadays standard practice in detecting and quantifying nucleic acids in all biological samples. As a common variant, reverse transcription (RT) followed by polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of mRNA. Real-Time RT-qPCR is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range [Orlando et al., 1998; Lockey et al., 1998; Bustin et al., 2000]. Theoretically, there is a quantitative relationship between amount of starting target sequence and amount of PCR product at any given cycle. In practice, though, it is a common experience for replicate reactions to yield different amounts of PCR product. The development of real-time quantitative PCR has eliminated the variability traditionally associated with quantitative PCR, thus allowing the routine and reliable quantitation of PCR products. The first practical qPCR technology was the 5’-nuclase assay established in 1993, which combined the exponential PCR amplification of a specific transcript with the monitoring of newly synthesized DNA in each performed cycle [Higuchi et al., 1993; Heid et al., 1996; Gibson et al., 1996]. It is the most sensitive method for the detection and quantitation of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue samples and for the elucidation of small changes in mRNA expression levels [Pfaffl et al., 2001; Pfaffl, 2000].

Fidelity of Real-Time RT-PCR is associated with its “true” specificity, sensitivity, reproducibility and robustness and, as a fully reliable quantitative method, it suffers from the problems inherent in RT and PCR, as amplification of unspecific products, primer-dimers, amplification efficiencies, heteroduplex formation, etc. [Freeman et al., 1999]. A most important point in succeed a RT-qPCR is to choose the right detection chemistry. Two general methods for the quantitative detection of the amplicon have been established: gene-specific fluorescent probes or specific double strand DNA binding agents [Ginzinger et al., 2002]. The best-known probe-based system is TaqMan™, which makes use of the 5’-3’ exonuclease activity of the Taq polymerase to quantitate target sequences in the samples. Probe hydrolysis separates fluorophore and quencher and results in an increased fluorescence signal [Livak, 2001]. The alternative is a non-sequence specific fluorescent intercalating dsDNA binding dye, as for exemple SYBR Green I or even ethidium bromide. For single PCR product reactions with well-designed primers, SYBR Green I can work extremely well, with spurious non-specific background only showing up in very late cycles [Zipper et al., 2004]. Among the Real-Time detection chemistry, SYBR Green I and TaqMan™ assays produce comparably dynamic range and sensitivity, while SYBR Green I detection can be more precise than the TaqMan™ probe detection if reaction specificity is perfected [Schmittgen et al., 2000].

The quantification strategy is the principal marker in gene quantification. Generally two strategies can be performed in Real-Time RT-PCR. The levels of expressed genes may be measured by absolute or relative quantitative Real-Time RT-PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA expression levels. The reliability of an absolute real-time RT-PCR assay depends on the condition of ”identical” amplification efficiencies for both the native target and the calibration curve in RT reaction and in following kinetic PCR [Souaze et al., 1996]. Relative quantification is easier to perform than absolute quantification because a calibration curve is not necessary. It is based on the expression levels of a target gene versus a housekeeping gene (reference or control gene) and in theory is adequate for most purposes to investigate physiological changes in gene expression levels. The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple Real-Time RT-PCR experiments [Orlando et al., 1998]. Under certain

circumstances, absolute quantification models can also be normalized using suitable and unregulated references or housekeeping genes.

In absolute quantification, samples are compared to a standard curve obtained with known concentrations by progressive dilutions. Calibration curves must be highly reproducible and allow the generation of highly specific, sensitive and reproducible data [Bustin et al., 2000; Pfaffl et al., 2001]. The external calibration curve model has to be thoroughly validated as the accuracy of absolute quantification in Real-Time PCR depends entirely on the accuracy of the standards. Standard design, production, determination of the exact standard concentration, and stability over long storage is not straightforward and can be problematic. The dynamic range of the performed calibration curve can be up to nine orders of magnitude from $<10^1$ to $>10^{10}$ start molecules, depending on the applied standard material [Pfaffl et al., 2001]. The calibration curves used in absolute quantification can be based on known concentrations of DNA standard molecules, as recombinant plasmid DNA, genomic DNA, RT-PCR products or commercially synthesized big oligonucleotides [Reischl et al. 1995]. Plasmid DNA or *in vitro* transcribed RNA are commonly used to prepare absolute standards. Concentration is measured by A_{260} and converted to the number of copies using the molecular weight of the DNA or RNA. Stability and reproducibility in qPCR depends on the type of standard used and depends strongly on good laboratory practice. The following critical points must be considered for the proper use of absolute standard curves:

- The standard DNA or RNA must be a single, pure species. For example, plasmid DNA is often contaminated with RNA, increasing the A_{260} value and inflating the copy number determined for the plasmid.
- Accurate pipetting is required because the standard must be diluted over several orders of magnitude. Plasmid DNA or *in vitro* transcribed RNA must be concentrated to measure an accurate A_{260} value. This concentrated DNA or RNA must be diluted up to 1000 fold to be at a concentration similar to the target in biological samples.
- The stability of the diluted standards must be considered, especially for RNA. Standards should be diluted into small aliquots, stored at −80ºC, and thaw only once before use [Collins et al., 1995].
- Generally, it is not recommended to use DNA as a standard for absolute quantitation of RNA because there is no control for the efficiency of the reverse transcription step.

We imagined a simple, reliable, easy-to-make method for generating standard for absolute Real-Time quantification, using only common laboratory equipment and reagents. Our method is based on re-amplification of purified amplicons, therefore assuring specificity and reproducibility of the reactions.

MATERIAL AND METHODS

Total RNA was extracted comparatively from either solid tissues or cultured cells, using commercial available kits (SV® Total RNA Isolation System, *Promega*™, Genelute® Mammalian Total RNA Miniprep Kit, *Sigma-Aldrich*™, and TRIZOL® Reagent, *Invitrogen*™). Kits were compared regarding quantity, purity and integrity of extracted RNA (data not shown). A DN-ase treatment step was added to all extraction protocols. RNA was rehydrated in nuclease-free H_2O, imediately retrotranscribed into cDNA, or aliquoted and kept at -80C until use. RNA quantity was estimated by spectrophotometry, measuring absorbance at 260 nm, the ratio 260/280 and with background correction at 320 nm. RNA integrity was estimated by electrophoresis in agarose TAE gel stained with ethidium bromide, in order to visualise clear 18s and 28s rRNA bands. 1 to 5 µl RNA were reverse transcribed to cDNA, either using ImProm-II® Reverse Transcription System, *Promega*™, or the M-MLV Reverse Transcriptase, *Sigma-Aldrich*™. Random hexamers or oligo-dT primers were used for RT reactions. cDNA was stored at 4°C for immediate amplifications or at -20°C for further applications. Standard curves for absolute quantification in Real-Time PCR were obtained as described below.

RESULTS AND DISCUSSION

Here we describe our in-house method for generating standard curves for interferon-gamma (IFN-γ) gene of interest and for beta-actin (β-actin) reference gene. Both curves were used in a SybrGreen I detection system and we strongly believe our system can be optimized and be functional for any interest or reference gene. For comparison, an example of TaqMan™ detection system with standard curve for GAPDH reference gene is also presented.

The first step is to generate pure amplicons from cDNA. Specific primers were designed for amplifying IFN-γ, β-actin or GAPDH genes. Primers were designed to span exon-exon junctions only available for hybridization in cDNA, thus avoiding any cross-amplification of undigested genomic DNA. We used the following primers:

- For IFN-γ : (437 bp amplicon)

Forward : GGCTGTTACTGCCAGGACCCATATGT
Reverse : GATGCTCTTCGACCTCGAAACAGCAT

- For β-actin (661bp amplicon)

Forward : TGACGGGGTCACCCACACTGTGCCCATCTA
Reverse : CTAGAAGCATTTGCGGTGGACGATGGAGGG

- For GAPDH (349 bp amplicon)

Forward ATCATCCCTGCCTCTACTGG
Reverse : TGCTGTAGCCAAATTCGTTG

In all cases, amplification was performed in a final volume of 50 µl containing 5 µl cDNA, 1,25 units of GoTaq® Flexi DNA Polymerase (*Promega*™) in apropriate 1X buffer, 1,5mM $MgCl_2$, 0,4 µM each primer, and 0.2mM each dNTP. Cycling program was composed of an initial denaturation of 15 min 95ºC, followed by 45 cycles of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min, plus a final extension of 10 minutes at 72ºC. This common PCR program allows amplification of all three amplicons.

In the case of IFN-γ and GAPDH genes, unspecific products may appear when using these cycling conditions, which is not the case for the β-actin. For detecting such events, 20 µl of each PCR reaction are migrated in a 1X TBE agarose gel, for 30 minutes at 7V/migration distance. Visualizing gels stained with ethidium bromide allows identification of specific amplicons and unspecific products. When only a specific band is observed (case of β-actin), the amplicon can be purified from the remaining 30 µl PCR product, by using the Wizard® PCR Preps DNA Purification System (*Promega*™), following producer's indications for "purifying PCR products directly from PCR reactions". When unspecific products are either observed in migration profile, the specific band is cut from the gel under UV, and specific amplicon is purified from the gel using the Wizard® PCR Preps DNA Purification System (*Promega*™), following producer's indications for "purifying PCR products from agarose gels". In both cases, the amplicon DNA is eluted in 50 µl H_2O. Its concentration can be measured by spectrophotometry at 260 nm, which was nor possible for cDNA.

Knowing the size of the amplicon in base pairs and its concentration in ng/µl, any easy internet available convertor, such as the *Promega*'s Math calculator, can be used to calculate the molarity of amplicon solution obtained. Molarity can be used to estimate the number of molecules present in a given volume of the same solution, as is presented in Table I.

Table I. Estimating the number of molecules in solution for each amplicon

Amplicon	DO_{260}	DO_{280}	DO_{260}/DO_{280}	Concentration (ng/µl)	Size (bp)	Molarity (pmol/ µl)	Molecules / µl
IFN-γ	0,062	0,035	1,775	3,1	437	10,63	$6,4 * 10^9$
GAPDH	0,173	0,101	1,714	8,65	349	37,66	$2,3 \times 10^{10}$
β-actine	0,376	0,223	1,684	7,52	661	17,191	10^{10}

The last operation to do is to dilute each amplicon solution progressively (10 X dilutions, 10 µl with 90 µl H_2O), in order to obtain a known series of concentrations (molecules). Even if the estimations are slightly similar for each amplicon in Table 1, the range of dilutions used for Real-Time PCR was different in each case, depending on the chemistry and the efficiency of each reaction. Briefly, 5 µl purified amplicon (from the concentrated solution, or from each dilution obtained) served as template in a Real-Time PCR amplification, using the same forward and reverse primers as shown before, and using a SybrGreen I detection system based on the SensiMixPlus® SYBR Kit, *Qantance*™, following producer's instuctions.

In figure 1 is presented the quantitative analysis for IFN-γ standards amplification, ranged from 50 to 6400 template molecules. It is a very good standard curve able to quantify little

quantities of IFN-γ mRNA as it really happens *in vivo*. Figure 2 shows a melting curve obtained from the same amplified products, which demonstrate the specificity of the Real-TIme PCR reaction. However, one may easily observe below 400 template molecules (standard sample no. 5) the apparition in the melting curve of a noise, left-sided from the main specific product (Tm = 85ºC). This corresponds to a primer dimer appearing when not enough template is available. Negligible at the beginning, this unwanted product becomes intrusive for our last standard sample (no. 8). Therefore we cannot recommend using standards below 50 molecules copies, as primer dimer background could false the fidelity of the standard curve and of the quantification.

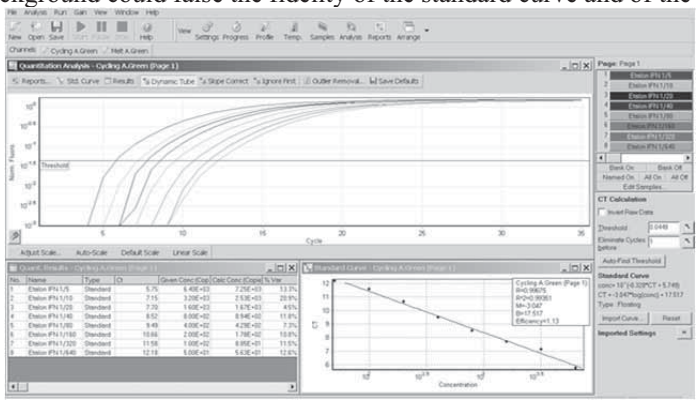


Figure 1. Quantitation analysis for a standard curve generated for IFN-γ cDNA.

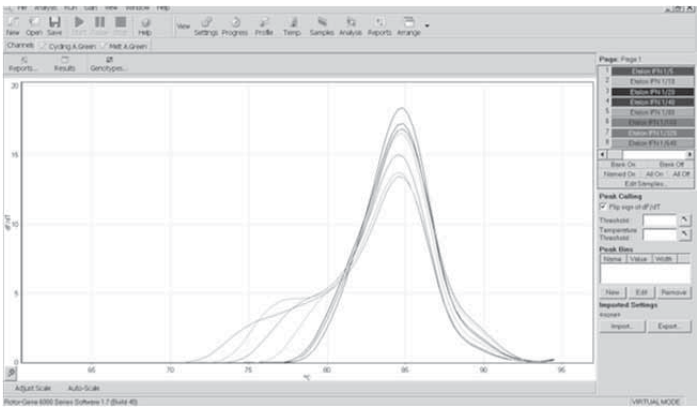


Figure 2. Melting analysis for a standard curve generated for IFN-γ cDNA.

In figure 3, we show the example of analysis obtained for the β-actin standard curve, with samples ranged from 525 to 1.050.000.000 molecules. The standard curve is, here again, large-ranged, reliable and precise. For standards below 500 molecules, the signal was less intense while the specificity of the reaction remained stable. Contrarily, another problem appears for standards containing more than 100.000 molecules. We can observe, in the melting curve, the apparition of a right-sided unspecific product when template is too abundant, as it really happens *in vivo*. Therefore, we do not recommend using standards with more than 100.000 copies, but rather to dilute samples in order to frame them below this value.

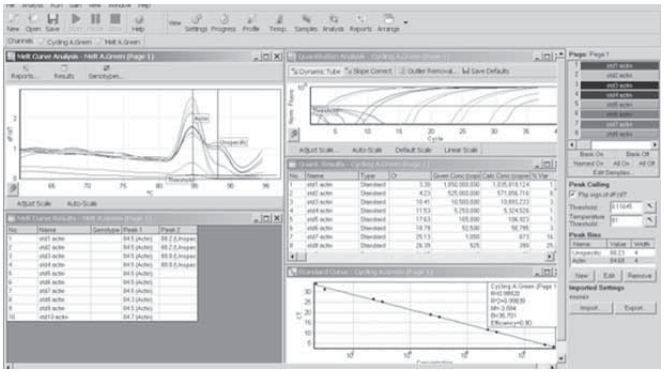


Figure 3. Quantitation and melting analysis for a standard curve generated for β-actin cDNA.

In figure 4, we present a good standard curve generated for GAPDH gene cDNA, with dilutions ranged from 25 to 1.000.000 molecules. Since the detection system used for GAPDH was TaqMan™, melting curve was not possible, although TaqMan™ system ensures by itself the specificity of the Real-Time amplification. Therefore, we do recommend the usage of the curve for the interval mentioned.

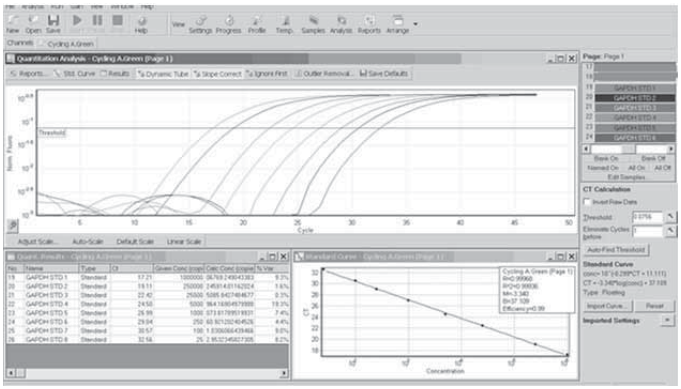


Figure 4. Quantitation analysis for a standard curve generated for GAPDH cDNA.

DISCUSSION

Quantitative Real-Time PCR is extremely useful for measuring gene expression, viral charge, external DNA contaminants, and generally for determining nucleic acid quantities. Absolute and relative quantifications can be used for such approaches. Absolute quantitations always need an external standard curve of known progressive DNA or RNA concentrations. Several approaches were used in time for generating either RNA or DNA standard curves. We presented an in-house, rapid and efficient method for obtaining cDNA standard curves for interest and reference genes, applicable for Sybr Green or TaqMan™ analysis. Our method was validated for IFN-γ, β-actin and GAPDH genes, and we showed the confidence intervals for each concentration ranges.

Certainly, a problem with DNA-based calibration curves is that they are subject to PCR step only, unlike the unknown mRNA samples that must first be reverse transcribed. This increases the potential for variability of the RT-PCR results and the amplification results may not be strictly comparable with the results from unknown samples. However, the problem of the

sensitivity of the RT-PCR to small variations in the reaction setup is always lurking in the background as a potential drawback to this simple procedure. Therefore, quantification with external standards requires careful optimization of its precision and reproducibility (intra-assay and inter-assay variation), in order to understand and assume the limitations for each application.

REFERENCES

Orlando C., Pinzani P., Pazzagli M., 1998, Developments in quantitative PCR. *ClinChemLabMed*, **36**:255-69.

Lockey C., Otto E., Long Z., 1998, Real-time fluorescence detection of a single DNA molecule. *Biotechniques*, **24**:744-746.

Bustin S.A., 2000, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol*, **25**:169-193.

Pfaffl M.W., Hageleit M., 2001, Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time PCR. *Biotechn Lett*, **23**:275-282.

Pfaffl M.W., 2000, Quantification strategies in Real-Time PCR. In *A-Z of quantitative PCR*, Ed. S.A. Bustin, International University Line, La Jolla, CA, USA, Chapter 3, 1-21.

Higuchi R., Fockler C., Dollinger G., Watson R., 1993, Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Biotechnology*, **11(9)**:1026-1030.

Heid C.A., Stevens J., Livak K.J., Williams P.M., 1996, Real time quantitative PCR. *Genome Res*, **6**:986-993.

Gibson U.E., Heid C.A., Williams P.M., 1996, A novel method for real time quantitative RT-PCR. *Genome Res*, **6**:1095-1100.

Freemen W.M., Walker S.J., vrana K.E., 1999, Quantitative RT-PCR: pitfalls and potential. *Biotechniques*, **26(1)**:112-125.

Ginzinger D.G., 2002, Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol*, **30(6)**, 503-512.

Livak K.J., 2001, ABI Prism 7700 Sequence detection System User Bulletin#2 Relative quantification of gene expression. (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Zipper H., Brunner H., Bernhagen J., Vitzthum F., 2004, Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic acids research*, **32(12)**:e103.

Schmittgen T.D., Zakrajsek B.A., Mills A.G., Gorn V., Singer M.J., Reed M.W., 2000, Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal Biochem*, **285(2)**, 194-204.

Souaze F., Ntodou-Thome A., tran C.Y., Rostene W., forgez P., 1996, Quantitative RT-PCR: limits ans accuracy. *Biotechniques*, **21(2)**:280-285.

Reischl U., Kochanowski B., 1995, Quantitative PCR. A survey of the present technology. *Mol Biotechnol*, **3(1)**:55-71.

Collins M.L., Zayati C., Detmer J.J., Daly B., Kolberg J.A., Cha T.A., Irvine B.D., Tucker J., Urdea M.S., 1995, Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays. *Anal Biochem*, **226(1)**:120-129.

1) University of Medicine and Pharmacy « Gr.T.Popa », Iaşi, Romania
2) University «Alexandru Ioan Cuza», Iaşi, Romania
* luciannegura@yahoo.fr

ACKNOWLEDGEMENTS

This study was possible with partial financial support from the Romanian Ministry for Education and Research, by the CNCSIS research grant PN-II-RU-PD-2009, code PD_557/2010, contract 144/2009, financed by UEFISCSU (Unitatea Executivă pentru Finanţarea Învăţământului Superior şi a Cercetării Ştiinţifice Universitare).

OPTIMIZATION OF HETERODUPLEX ANALYSIS FOR THE DETECTION OF *BRCA* MUTATIONS AND SNPs

ANCA NEGURĂ[1], EUGEN CARASEVICI[2], ANDREI CONEAC[3], EUGEN UNGUREANU[1], LUCIAN NEGURĂ[2*]

Keywords: breast/ovarian hereditary cancer, *BRCA* genes mutations, SNPs, pre-screening methods, heteroduplex analysis

Abstract: *BRCA1* and *BRCA2* are tumour suppressor genes whose mutant phenotypes predispose to breast and ovarian cancer. Screening for mutations in these genes is now standard practice for hereditary breast and ovarian cancer (HBOC) cases in Europe, and permits medical follow-up and genetic counselling adapted to the needs of individuals in such families. Currently, most laboratories performing diagnostic analysis of the *BRCA* genes use PCR of exons and intron-exon boundaries coupled to a pre-screening step to identify anomalous amplicons. The techniques employed for the detection of mutations and SNPs have evolved over time and vary in sensitivity, specificity and cost-effectiveness. As a variant for pre-screening techniques, we chose the recently developed Surveyor® heteroduplex cleavage method as a sensitive and specific technique to reveal anomalous amplicons of the *BRCA* genes, using only basic laboratory equipment and agarose gel electrophoresis. Here we present the detection of either mutations or SNPs within the *BRCA1* exon 7, using heteroduplex analysis (HA) by mismatch-specific endonuclease, confirmed by dideoxy sequencing.

INTRODUCTION

Hereditary breast and ovarian cancers are mainly attributable to predisposition genes whose germinal mutations predispose to the disease [Antoniou et al., 2003]. As both pathologies are common in women of western world [Ferlay et al., 2000], identification of such predisposition genes generated an over 15 years candidate-finding race in cancer research. Since the identification of the main breast/ovarian cancer genes, *BRCA1* in 1994 [Miki et al., 1994] and BRCA2 in 1995 [Wooster et al., 1995], a dozen of additional candidates have been reported, some of them being already included in diagnosis tests [Narod et al., 2004]. However, the *BRCAs* are the principal responsible in over 1/3 of hereditary cases, that mean familial agglomerations with 2 or more early onset breast or/and ovarian cancers. Screening for mutations in these genes is now standard practice for hereditary breast and ovarian cancer (HBOC) cases in Europe, and permits medical follow-up and genetic counselling adapted to the needs of individuals in such families. Several problems appear when investigating the *BRCA* status for an individual, with or without a family history.

Firstly, the two genes are very large [NCBI Genbank]. *BRCA1* is composed of 5592 nucleotides and contains 24 exons sparing 100 kb genomic DNA. *BRCA2* is even larger, 10257 nucleotides containing 27 coding exons and sparing 70 kb DNA. As an additional difficulty, both genes possess an unusually large exon 11, of 3,4 kb in *BRCA1* and 5 kb in *BRCA2*. Even though limiting sequencing to exonic regions and exon/intron boundaries, there is a huge amount of work to do when attempting to completely investigate *BRCA1* and *BRCA2*. That will comprise a total of 84 amplicons to be forward and reverse sequenced, which means a very expensive and time consuming approach.

Secondly, *BRCA* mutational spectrum has not been entirely characterized, and not all sequence variants are clearly pathogenic. Over one thousand small sequence variations have been reported by now in the Breast Cancer Information Core (BIC) database. More than half of these mutations cause the loss of function by premature protein synthesis termination, and around 60 % are unique to a family. Other variations include mis-sense alterations and intronic variants with unknown disease relevance. These are classified as benign polymorphisms or unclassified variants (UV) with unknown pathological potential. To date, 43.5 % of *BRCA* variants are of uncertain clinical significance.

Nonetheless, cancer predisposition diagnosis is a hard to assume and delicate problem to manage, so all the resources and means are never sufficient to avoid the mistake. False positives and false negatives are always pending behind, and several different techniques are absolutely necessary in order to bring the error close to null. Besides iterative verifications of a positive result, one should always combine diverse techniques available, and generate a multilevel validated result. Therefore during the last few years, the whole molecular biology scientific community starter a large campaign of searching and comparison of alternative pre-screening methods, with the aim of early identification of mutation bearing susceptible amplicons which will be sequenced. The techniques employed for the detection of mutations and polymorphisms have evolved over time and vary in sensitivity, specificity and cost-effectiveness. The protein truncation test (PTT) has largely been set aside due to low sensitivity and specificity or specificity that is too narrow [Hogervorst et al., 1995]. Currently, most laboratories in Europe performing diagnostic analysis of the *BRCA* genes use PCR of exons and intron-exon boundaries coupled to a pre-screening step to identify anomalous amplicons [Negura et al., 2006]. Denaturing gradient gel electrophoresis (DGGE) or single strand conformation analysis (SSCP) can be used, both of them limited by great number of false positives and difficulties in interpretation of some polymorphisms. Alternatively,

denaturing high pressure liquid chromatography (DHPLC) presents the disadvantage of requiring either repeated analysis using an array of conditions and/or expensive reagents.

Assuming that direct sequencing is too expensive to apply systematically to all HBOC and sporadic cases, and that reliable DGGE, SSCP and DHPLC also require either repeated analysis using an array of conditions and/or expensive, specialized equipment and consumables, we turned our attention to the recently developed Surveyor® heteroduplex cleavage method (*Transgenomic*™) as a sensitive and specific technique to reveal anomalous amplicons of the *BRCA* genes using only basic laboratory equipment. Endonuclease cleavage of mismatch-containing DNA duplexes is a classic method for the detection of mutations and polymorphisms. Unfortunately, the enzymes available to carry out such experiments had widely different efficiencies for cleavage of the different possible mismatches and for the two DNA strands. A newly commercialized mismatch endonuclease isolated from celery, Surveyor® has been shown to be efficient at recognising all possible DNA mispairs as well as bubbles due to insertions or deletions, and to cleave both DNA strands 3' to the unpaired region. Since its first commercialization, Surveyor® enzyme has been successfully used in numerous applications, including mitochondrial DNA mutations analysis [Bannwarth et al., 2006) or SNP genotyping [Mitani et al., 2006]. This enzyme has been used to detect mutations in tumor DNA samples [Janne et al., 2006] using DHPLC to detect cleavage products, though many strategies for the detection of cleavage products are possible.

We implemented heteroduplex analysis with Surveyor® endonuclease, using a peripheral blood DNA protocol, in order of pre-screening *BRCA* mutations. Here we present the detection of either mutations or SNPs within the *BRCA1* exon 7.

MATERIALS AND METHODS

We recruited either HBOC families with three or more breast or ovarian cancer cases within the same family line, or sporadic breast and ovarian cancer cases. All patients agreed by written informed consent. Genomic DNA was extracted from 10 ml peripheral blood by using Wizard® Genomic DNA purification kit (*Promega*™). DNA amount was estimated by spectrophotometry. Polymerase chain reaction amplifying *BRCA* exon 7 was performed in a final volume of 50 µl containing 100 ng genomic DNA, 0.2mM each dNTP, 1,5 mM $MgCl_2$, 20 pmoles of each primer (sequence available on demand), and 0,25 units of GoTaq® Flexi DNA Ploymerase (*Promega*™) in 1X adequate buffer. PCR program was optimized is several steps to generate specific and efficient amplification, as described below.

Formation of heteroduplex molecules and their cleavage by Surveyor® were performed as suggested by the manufacturer (*Transgenomic*™), as explained below, and analysed after electrophoretic separation on agarose gels.

For DNA sequencing, amplicons were verified by electrophoresis on a 1,3% agarose gel, then purified by ExoSap® enzymatic digestion (*Affymetrix*™), following producer's instructions. The product was sequenced in forward and reverse reactions, using the BidDye® Terminator Cycle Sequencing Kit (*Applied Biosystems*™), according to the manufacturer's instructions. Cycle sequencing consisted of an initial denaturation step at 94ºC for 11 min, followed by 30 cycles of 94ºC for 10 sec, 52ºC for 5 sec and 30ºC for 3 min. Sequence analysis was performed using the Seqman® (*DNA Star Inc*™) software. Mutation presence was systematically confirmed by forward and reverse sequencing on a second independent blood sample.

Mutations and sequence variants are described according to HUGO approved systematic nomenclature. The nomenclature for BIC traditional mutations is also indicated.

RESULTS AND DISCUSSION

Heteroduplex analysis by mismatch-specific endonuclease supposes performing the following steps (Figure 1):

1) PCR amplification of the region of interest for all investigated patients (and eventually for references if needed);
2) generation of heteroduplex molecules by denaturation / renaturation of heterogeneous amplicons;
3) cleavage of heteroduplex molecules by the Surveyor® mismatch-specific endonuclease;
4) visualization of digestion fragments by electrophoresis in agarose gel stained with ethidium bromide.

Cleavage of a heterodulpex molecule by the Surveyor® endonuclease indicate the presence of a mismatch (generally, of a impairing due to nucleotide change, or bubbles due to insertions or

deletions), which means heterogeneity of investigated DNA. Since *BRCA* mutations are always heterozygous, the DNA of a carrier will always be heterogenous. In the case of other homozygous sequence variations like common SNPs, heterogenous DNA can be obtained by mixing patient DNA with reference DNA known as wild-type homozygous.

When using heteroduplex analysis as pre-screening method, ideally only one amplicon need be sequenced: the one containing a disease-causing mutation. In practice, samples heterozygous for a polymorphism must be sequenced because of the inability using Surveyor® alone to distinguish between a known polymorphism and a mutation giving rise to similar cleavage products. There is a concrete need for detecting deleterious mutation in diagnosis; there also is a less urgent need for detecting unclassified variants and SNPs for understanding cancer genetics. We tried to imagine a dual system able to identify both mutations and SNPs, and we present an example below for *BRCA1* exon 7.

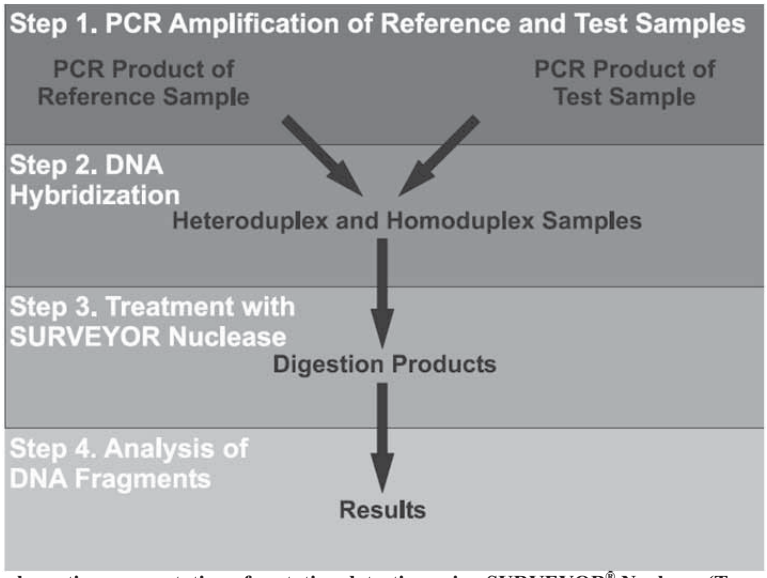


Figure 1. A schematic representation of mutation detection using SURVEYOR® Nuclease (Transgenomic™)

In figure 2 one can observe 325 bp bands corresponding to *BRCA1* exon 7 amplification of genomic DNA from different patients. Following optimization, amplification comprised an initial denaturation step at 94ºC for 5 min followed by 15 cycles of denaturation – 94ºC for 20 sec, annealing – 60ºC for 10 sec and extension – 72ºC for 15 sec, followed again by 25 cycles of denaturation – 94ºC for 20 sec, annealing – 56ºC for 10 sec and extension – 72ºC for 15 sec, and a final extension of 10 min at 72ºC. This touch-down type program is used in order to avoid unspecific product generation that we observed at the beginning. As we can notice in figure 2, amplification after optimization is efficient and specific. Lane 8 represents the no template control, which verifies absence of any contamination.

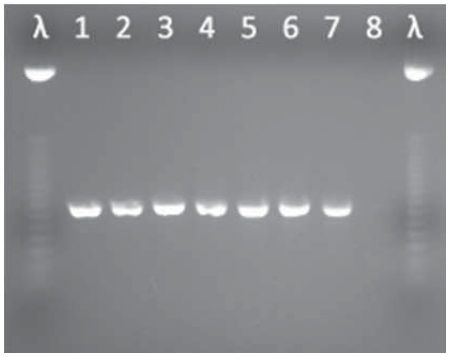


Figure 2. Amplification of *BRCA1* exon 7 from several HBOC patients DNA (λ = 50 bp step ladder).

In figure 3 are represented the results for heteroduplex analysis corresponding to exon 7 amplifications. Concretely, after verification in gel, amplicons were treated by a progressive denaturation / renaturation program (95ºC for 2 minutes, followed by cooling at 2ºC/second until 85ºC, then cooling at 0,1ºC/second until 25ºC). 10 µl of such obtained molecules were treated 20 minutes with 1,25 µl Surveyor® endonuclease (*Transgenomic*™), in the presence of 1,25 µl enhancer solution, following producer's instructions. By optimization of this digestion protocol, we obtained a better resolution for all bands, as is shown in figure 3b comparatively to bands observed in figure 3a. Optimization changes were cooling at 1ºC/second from 95ºC until 85ºC, and endonuclease treatment extended to 30 minutes.

In figure 3a, lane 13 corresponds to an undigested heteroduplex product, while digested molecules were migrated in lanes 1-11. Lane 12 represents the no template control, which verifies absence of any contamination. For three of our patients (lanes 1-3), two digestion bands can be observed around 150 and 180 bp. Those bands are certainly less intense then the undigested 325 bp product, which cannot be digested totally as patients are heterozygous for any deleterious variant. In figure 3b, those heterozygous patients are migrated in lanes 3-5, and we can observe a better resolution due to the diminution of undigested product intensity. That means a better digestion efficiency.

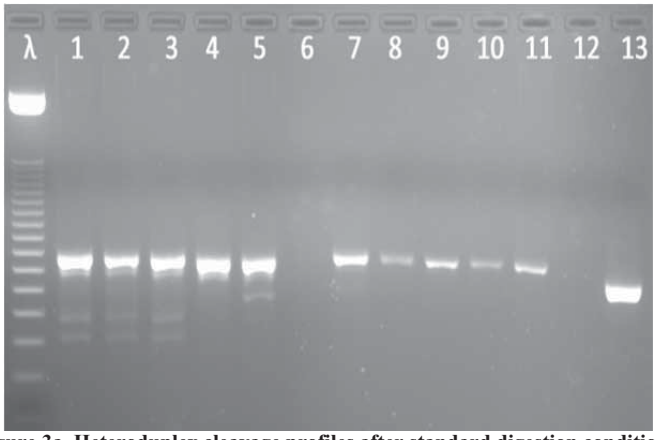


Figure 3a. Heteroduplex cleavage profiles after standard digestion conditions

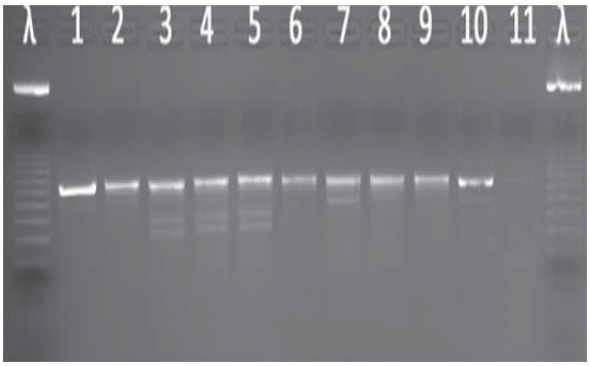


Figure 3b. Heteroduplex cleavage profiles after optimization of digestion conditions

When sequencing the concerned amplicon (figure 4), we detected a deleterious mutation 140 bp from the 3’end. In a heterogenous heterozygote DNA, heteroduplex molecules are formed at this level, and the Surveyor® endonuclease splits the 325 bp amplicon in two distinct fragments of 140 and 183 bp, on the mutant allele. Two nucleotides are missing, as the mutation is a 2-nucleotide deletion called c.342_343delTC in HUGO nomenclature (or 461delTC in the BIC nomenclature). We already reported [Negura et al., 2010a] this rare mutation causing a TGA Stop codon apparition and the premature termination of the protein synthesis after 114 N-terminal aminoacids (p.Pro115Stop). Patients in lanes 1,2,3 in figure 3a and lanes 4,5,6 in figure 3b are in consequence heterozygous for BRCA1 c.342_343delTC, as demonstrated by heteroduplex analysis confirmed by direct sequencing. Patients in lanes 4,7,8,9,10,11 in figure 3a and lanes 2,6,8,9,10 in figure 3b are wild-type homozygous, as no heteroduplex was cleaved by mismatch-specific nuclease. Undigested control product is lane 13 in figure 3a and lane 1 in figure 3b. No template control is lane 12 in figure 3a and lane 11 in figure 3b.

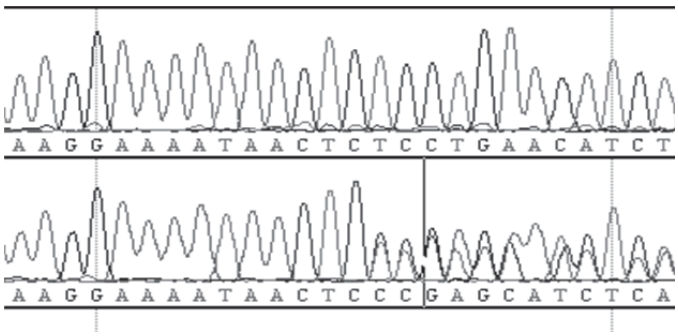


Figure 3b. DNA sequencing detects the c.342_343delTC mutation (down), as heterozygous sequence compared to wil-type homozugous (up)

Patient in lane 5 (figure 3a) or 7 (figure 3b) shows a different digestion profile, with an additional band around 240 bp. In fact, this patient is heterozygous for an unclassified sequence variant within exon 7, called c.427G>C in HUGO nomenclature, or 546G>C in BIC nomenclature. This apparent non-pathogenic UV sequence was described elsewhere [Negura et al., 2010b], and generates a mismatch 226 nucleotides from 5’end, so the nuclease cuts the 325 bp amplicon in a 226 bp (visible) fragment and a 99 bp (non-detectable) fragment.

CONCLUSIONS

Our results prove the feasibility of a rapid inexpensive pre-screening by heteroduplex analysis, in order to identify abnormal *BRCA* amplicons to sequence. As preliminary technique, HA by mismatch-specific endonuclease shows enough sensitivity to detect either deleterious mutations or various benign sequence alterations (SNPs, UVs). When present in a heterozygous context, allelic variations can be detected directly on heterogenous sample DNA; otherwise, an additional step is needed for mixing sample DNA with wild-type reference DNA.

We recommend therefore optimized Surveyor® heteroduplex as good pre-screenig *BRCA* method which greatly reduces the number of amplicons requiring sequencing.

REFERENCES

Antoniou A. et al., 2003, Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: a combined analysis of 22 studies. *Am J Hum Genet*, 72(5), 1117-1130.

Miki Y. et al., 1994, A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*, 266, 66–71.

Wooster R. et al., 1995, Identification of the breast cancer susceptibility gene BRCA2. *Nature*, 378, 789–791.

Narod S.A. and Foulkes W.D., 2004, BRCA1 and BRCA2: 1994 and beyond. *Nat Rev Cancer*, 4(9), 665-676.

NCBI Genebank (http://www.ncbi.nlm.nih.gov/gene).

Breast Information Core (BIC) database (http://research.nhgri.nih.gov/bic/).

Hogervorst F.B. et al., 1995, Rapid detection of BRCA1 mutations by the protein truncation test. *Nat Genet*., 10(2), 208-212.

Negură L. et al., 2006, Méthodologie actuelle de la biologie moléculaire pour la détection des mutations. *Analele ştiinţifice ale Universităţii «Alexandru Ioan Cuza» Iaşi (Serie nouă), Secţiunea Genetică şi Biologie Moleculară*, Tom VII, Fasc.1, 13-20.

Bannwarth S. et al., 2006, Rapid identification of unknown heteroplasmic mutations across the entire human mitochondrial genome with mismatch-specific Surveyor Nuclease. *Nat Protoc*, 1(4), 2037-2047.

Mitani N et al, 2006, Surveyor nuclease-based genotyping of SNPs. *Clin Lab*, 52(7-8), 385-386.

Janne PA, et al. A rapid and sensitive enzymatic method for epidermal growth factor receptor mutation screening. Clin Cancer Res. 2006. 12(3):751-8.

Human Genome Variation Society (HGVS) Nomenclature for the description of sequence variations (http://www.genomic.unimelb.edu.au/mdi/mutnomen/).

Negură L. et al., 2010(a), Complete BRCA mutation screening in breast and ovarian cancer predisposition families from a North-Eastern Romanian population. *Familial Cancer,* 9(4), 519-523.

Negură L. et al., 2010(b), Unclassified sequence variants (UVs) and genetic predisposition to cancer. *Analele Ştiinţifice ale Universităţii "Alexandru Ioan Cuza" Iaşi (Serie nouă). Secţiunea Genetică şi Biologie Moleculară*, Tom XII (1), *in press*.

1) University «Alexandru Ioan Cuza», Iaşi, Romania
2) University of Medicine and Pharmacy « Gr.T.Popa », Iaşi, Romania
3) University of Medicine and Pharmacy « Iuliu Haţieganu », Cluj-Napoca, Romania
* luciannegura@yahoo.fr

ACKNOWLEDGEMENTS
This study was possible with partial financial support from the Romanian Ministry for Education and Research, by the CNCSIS research grant PN-II-ID-PCE-2008, code 1990/2008, contract 1199/2009, financed by UEFISCSU (Unitatea Executivă pentru Finanţarea Învăţământului Superior şi a Cercetării Ştiinţifice Universitare).

DETECTION OF HPV 16 AND HPV 18 VIRAL LOADS BY REAL TIME PCR IN WOMEN WITH CERVICAL DYSPLASIA

RAMONA GABRIELA URSU [1*], MIRCEA ONOFRIESCU [2], DRAGOŞ NEMESCU[2], ROXANA NEMESCU[3], LUMINIŢA SMARANDA IANCU[1, 4]

Keywords: viral load, qPCR, HR HPV types, biomarker.
Abstract. Viral load of high risk HPV types 16 and 18 can be useful biomarkers in detecting women with cervical dysplasia in early stage, prior to development of cervical cancer. Purpose of the study was to assess the clinical utility of viral load determination for HPV 16 and 18 in relation with the severity of cervical dysplasia and the association of classical risk factors. The HPV 16 and 18 positive women were selected from a cohort study, using one HPV DNA test for screening. METHODS: Viral load quantification was performed with Real Time PCR MX3005P. RESULTS: The viral load for HPV 16 was between 3.45 - 7.177 x 10^6 copies / µl, and 1.138 x 10^3 to 7.119 x 10^4 for HPV 18. CONCLUSIONS: Viral load of high risk HPV types seems to be one sensitive and objective biological marker in detecting women at risk for developing cervical cancer. No significant association was fount for the risk factors investigated.

INTRODUCTION

Human papillomaviruses (HPV) are the cause of cervical cancer, and among them, HPV 16 and 18 are the leading types, because they alone cause over 70% of cervical cancers (Walboomers JM *et al.*, 1999). Natural history of HPV infection shows that in 80% of High Risk HPV (HR HPV) infections there are transitory infections, in 20% of cases, in 2 - 4 years will appear productive infection and after 10 – 30 years will be developed cervical cancer. To evaluate the risk of HR HPV positive women to develop cervical cancer it was proposed several biological markers: virus related markers, disease markers and cell cycle related markers (Meyer CJLM, 2009). One of the virus related factors is the viral load of HR HPV types and it is presumed that a higher risk for cervical neoplasia is associated with higher viral loads of high-risk HPV types, in particular HPV 16 (WHO & IARC, 2007). The association between viral load and cervical disease varies with the HPV type, the physical state of the virus and the heterogeneity of the cervical lesion (Woodman CB et al., 2007). A few reports stated that viral load of HR HPV (16, 18, 31 and 33) can be used to select women wich need a more aggresive treatment, because they are consider to have high grade cervical intraepithelial lesions (Cricca M *et al*., 2007, Snijders PJ *et al*., 2006). Our aim in this study was to evaluate the clinical usefulness of copies number of DNA HPV 16 and 18 in relation with the severity of cervical dysplasia and classical risk factors known as oncogenic.

MATERIAL AND METHODS

In the period between September 2009 – December 2010 we invited to participate in one HPV prospective genotype prevalence study women with abnormal Pap smear and with colposcopic suspicion of HPV infection. All the study participants have signed the informed consent approved by the Bioethical Committee of “Gr. T. Popa” University of Medicine and Pharmacy, Iasi.

From 267 genotyped samples, we identified 26 (9.7 %) positive for HPV 16 and 9 (3.4 %) positive for HPV 18, using Linear Array HPV Genotyping Test (*ROCHE*®). HPV/DNA was purified with High Pure PCR Template kit (ROCHE®). Viral load was assed with *2 x Precision TM Mastermix*, *Path-HPV16 Real-time PCR detection kit for Human Papillomavirus, Path-HPV18 Real-time PCR detection kit for Human Papillomavirus kits (PRIMER DESIGN®)* and MX3005P instrument (*STRATAGENE*).

The HPV 16 and 18 specific primers and probes mix can be detected through the FAM channel. The primers and probes mix provided exploits the TaqMan principle. During PCR amplification, forward and reverse primers hybridize to the HPV16 and 18 DNA/cDNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on real-time PCR platforms.

Preparation of standard curve dilution series was performed using the positive control in dilution from 2 x 10^6/µl up to 2 copies / µl. Pathogen detection mix contained: 10 µl 2 x Precision TM Master Mix,1 µl Pathogen Primer/Probe mix, 4 µl RNA se / DNAse free water: the final volume was 15 µl. Endogenous ACTB detection mix contained: 10 µl 2 x Precision TM Master Mix,1 µl Endogenous ACTB Primer/Probe mix , 4 µl RNA se / DNA se free water. 15 µl of each of this two mixes was pipetted into each well according to real-time PCR experimental plate set up. 5µl of diluted DNA wad added into each well, according with the experimental plate set up. Amplification Protocol supposed one cycle for enzyme

activation – 10 minutes / 95 ° C and the 50 cycles each one with one denaturation step – 10 s / 95° C and one for data collection using FAM channel - 60 s / 60 ° C.

RESULTS AND DISCUSSION

The mean age of HPV 16 positive women was 36.31 years old (limits 23 – 59). 6 (23.1%) patients had never performed one cytologic exam, 1 (3.8%) had one normal Pap smear and also 1 (3.8%) has atypical cells, 3 (11.5%) had ASCUS *(Atypical Squamous Cells of Undetermined Significance*), LGSIL (*Low Grade Squamous Intraepithelial Lesion*), and ASCH (*Atypical Squamous Cells for which a High-grade lesion cannot be exclude*d), each, and 9 (34.6%) were detected with HGSIL (*High Grade Squamous Intraepithelial Lesion*). Regarding risk factors, 4 (15.4%) women were smokers and 9 of them (34.6%) declared that they used oral contraceptive in the past. 16 (61.5%) patients presented unique infection with HPV 16 and 10 (38.5%) were detected with multiple HPV types infections, besides HPV 16.

The mean age of HPV 18 positive patients was 30.8 (limits 21 – 41 years). Two had one normal Pap test, one was never testes by cytologic exam, and one had ASCUS and two LGSIL. One patient declared having more than 3 sexual partners, another declared 3 abortion and another patients had genital warts.

The parameters of the standard curves was calculated by the Stratagene software: efficiency = 97,3%, RSq (the linearity is denoted by the R squared (Rsq) value - R2 or Pearson Correlation Coefficient) = 0.988, slope = - 3.389 for HPV 16 and efficiency = 93.1%, RSq = 0.993, and slope = - 3.500 for HPV 18. All this parameters was between the normal limits (efficiency: 90 – 110%, RSq: 0.985 – 1.00, linearity / slope: –3.1 and –3.6). The NTC (negative controls) proved that there was no contamination of the experiments. The Ct values for ACTB control (beta actin gene) confirmed that the cervical cells were optimal collected. The amplifications plots for HPV 16 positive samples and the standard curve can be seen in figure 1 and figure 2.

The samples was tested in duplicate. The Ct / Qc (cycle threeshold / quantification cycle) values were between 19.68 and 33.94. The mean of viral load of HPV 16 was 684515,68 copies (limits 3,45 - 7.177 x 10^6 copies). Ct values for HPV 18 were between 25.44 – 46.93, according with input DNA quantity in the samples.

The highest viral loads (10^5 and 10^6 copies number of DNA / HPV 16, respectively) was detected in 40% of LGSIL cases and in 60% in HGSIL HPV 16 positive cases. (fig. 3)

High viral loads of HPV 16 were found in the group age 31 – 40, in percent of 40%. Smoking condition was not associated with high number of copies number, but 80% of oral contraceptive users had viral loads of HPV 16 of 10^6. If we compare the copies number detected in unique infections with multiple HPV type infections, we observe higher viral loads in single HPV 16 infections.

6 patients did not have any cytological test, 1 had one normal Papanicolau test, but all these women had a colposcopic suspicion of HPV infection. Theses remind the type of cervical cancer in our country, which is one opportunistic screening, whith the drawback that population at greatest risk is ofteen not screened and may mask the true risk of cancer in the population (Marks M. *et al*., 2011)

In HPV 18 positive cases, we did not found any direct correlation between viral load and the severity of cytologic result. In both situations – HPV 16 and 18 we had cases with normal Pap smear results and with high viral load of DNA/HPV. This fact is in accordance with

sensitivity of DNA/HPV tests (96%) in comparison with sensitivity of cytology (53%). (Cuzick J. *et al*., 2006)

Some authors sustain that there is one direct relationship between viral load and the CIN severity (high viral loads has the risk 60 times bigger to develop CIN III), in comparison with other papers which states that viral load is difficult to interpret because of integration of HPV genome in the host genome or because of the multiple HPV types infections. Some reports published that high viral loads of HR HPV types are associated with abnormal Pap smear / severe CIN lesion, (Peitsaro P *et al.,* 2002, van Duin M. *et al*., 2002, De Marco L *et al*., 2002, Cricca M *et al*., 2007), while others authors did not found any relations between the viral load and cervical dysplasia severity (Andersson S. *et al.,* 2005, Fontaine J *et al*. 2005).

Cricca M *et al*. established in 2007 that the viral load of HPV 16 is 1.38 x 10^6copii genom/300 ng total DNA, a value that allow detection of high grade CIN lesions.

Snijders PJ *et al*. (2009) concluded that in a cervical screening setting viral load assessment of HPV16, 18, 31 and 33 has no additive value to stratify high-risk HPV GP5+/6+-PCR-positive women for risk of >or=CIN2 or>or=CIN3.

On the other hand, two years later, Marks M. *et al*., (2011) demonstrated that repeated measurement of HPV 16 viral load may be a useful predictor in determining the outcome of early endpoints of viral infection.

Literature data are controversial because the viral load depends on the type of the pathologic product (cervical biopsy, vaginal washing) which is collected from the patients, mainly because they are containing specially cells from the surface of epithelium. Also, the risk for cervical neoplasia is associated with higher copy numbers of different HPV types and the variability in copy numbers is too great for viral load to be used as a predictor of CIN lesions. It is preferable to conclude that low viral copy numbers are associated with a low risk for developing CIN. However, further studies are warranted (WHO & IARC, 2007). Although some authors (Xi LF et *al*., 2009) found one significant association between viral load of HPV 16 and 18 with the current status smoker, in our cases we did not found one significant correlation (only 4 / 26 HPV positive cases was smoker: one had one viral load around 10^3 copies and two had the viral load around 10^5. No significant association was observed with other known HPV risk factors such as oral contraceptive use, and our findings are similar with the reports of Flores R. *et al.* 2006.

CONCLUSIONS

In a prospective study with 267 patients we found one positive correlation between dysplasia severity and viral load of HPV 16, but we have not found the same relation for HPV 18. No significant association was fount for smoking and oral contraceptive use.

Although the initial optimism regarding the clinical value of HPV viral load testing now seems misplaced, robust measurements of type-specific viral load in samples in which the integration status is also defined, could provide useful insights into the natural history of HPV infections and their relationship to disease.

The identification of more robust markers of disease progression requires a more complete understanding of the natural history of type-specific HPV infections.

REFERENCES

Andersson S, Safari H, Mints M, Lewensohn-Fuchs I, Gyllensten U, Johansson B. (2005): *Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (CIN)*, Br J Cancer.; 92(12):2195-200.

Cricca M, Morselli-Labate AM, Venturoli S, Ambretti S, Gentilomi GA, Gallinella G, Costa S, Musiani M, Zerbini M. (2007): *Viral DNA load, physical status and E2/E6 ratio as markers to grade HPV16 positive women for high-grade cervical lesions,* Gynecol Oncol.; 106(3):549-57.

Cuzick J, Clavel C, Petry KU, Meijer CJ, Hoyer H, Ratnam S, Szarewski A, Birembaut P, Kulasingam S, Sasieni P, Iftner T. (2006) *Overview of the European and North American studies on HPV testing in primary cervical cancer screening*, Int J Cancer.;119(5):1095-101.

De Marco L, Gillio-Tos A, Bonello L, Ghisetti V, Ronco G, Merletti F. (2007): *Detection of human papillomavirus type 16 integration in pre-neoplastic cervical lesions and confirmation by DIPS-PCR and sequencing,* J Clin Virol.; 38(1):7-13.

Fontaine J, Hankins C, Mayrand MH, Lefevre J, Money D, Gagnon S, Rachlis A, Pourreaux K, Ferenczy A, Coutlée F (2005): *High levels of HPV-16 DNA are associated with high-grade cervical lesions in women at risk or infected with HIV,* AIDS; 19(8):785-94.

Flores R, Papenfuss M, Klimecki WT, Giuliano AR. (2006), *Cross-sectional analysis of oncogenic HPV viral load and cervical intraepithelial neoplasia.* Int J Cancer.; 118(5):1187-93.

Herbert A. (2009), *Cervical cancer prevention: screening*, 25th International Papillomavirus Conference, Malmo.

Marks M, Gravitt PE, Utaipat U, Gupta SB, Liaw K, Kim E, Tadesse A, Phongnarisorn C, Wootipoom V, Yuenyao P, Vipupinyo C, Rugpao S, Sriplienchan S, Celentano DD. (2011): *Kinetics of DNA load predict HPV 16 viral clearance,* J Clin Virol.; 51(1):44-9.

Meyer CJLM (2009): *HPV testing: requirements for clinical testing,* 25th International Papillomavirus Conference, Malmo.

Peitsaro P, Johansson B, Syrjänen S. (2002): *Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique,* J Clin Microbiol.;40(3):886-91.

Snijders PJ, Hogewoning CJ, Hesselink AT, Berkhof J, Voorhorst FJ, Bleeker MC, Meijer CJ. (2006): *Determination of viral load thresholds in cervical scrapings to rule out CIN 3 in HPV 16, 18, 31 and 33-positive women with normal cytology*, Int J Cancer., 119(5):1102-7.

van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ. (2002): *Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance.* Int J Cancer.; 98(4):590-5.

Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Muñoz N. (1999): *Human papillomavirus is a necessary cause of invasive cervical cancer worldwide*. J Pathol.; 189(1):12-9.

WHO & IARC (2007), *IARC Monographs on the Evaluation of Carcinogenetic Risks to Humans, Human Papillomaviruses*, volume 90, Lyon, France.

Woodman CB, Collins SI, Young LS. (2007): *The natural history of cervical HPV infection: unresolved issues*, Volume 7, Nature Reviews, Cancer

Xi LF, Koutsky LA, Castle PE, Edelstein ZR, Meyers C, Ho J, Schiffman M., (2009) *Relationship between cigarette smoking and human papilloma virus types 16 and 18 DNA load,* Cancer Epidemiol Biomarkers Prev.;18(12):3490-6.

ACKNOWLEDGEMENTS:

"This study was supported by CNCSIS –UEFISCSU, project number PNII – IDEI code ID_1642 / 2008".

Affiliation of authors:

1 - University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, Microbiology Department

2 - University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, Gynecology Department

3 - University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, Infectious Diseases Department

4 - Regional Centre of Public Health Iaşi.

* ursuramona@yahoo.com

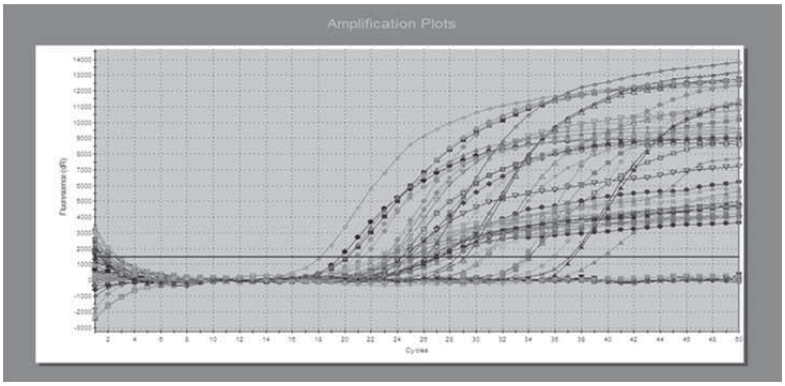


Fig. 1: Amplifications plots for HPV 16 positive samples

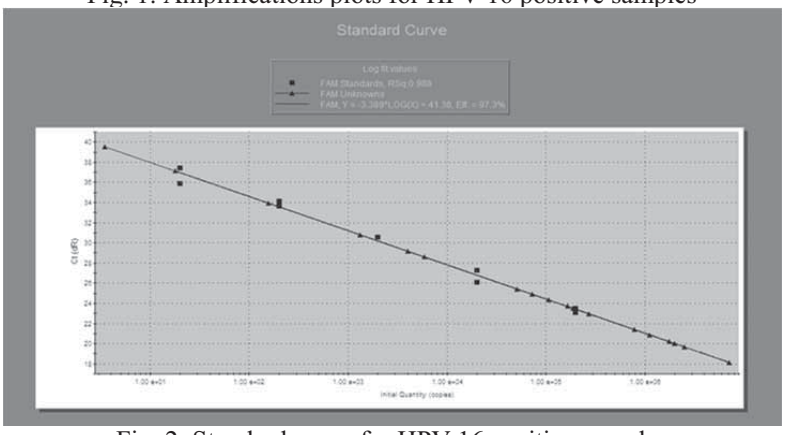


Fig. 2: Standard curve for HPV 16 positive samples

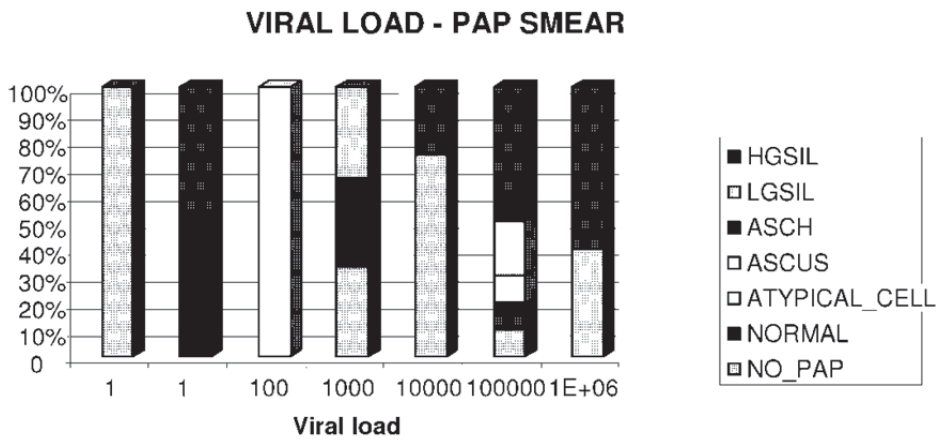


Fig. 3: Correlations viral loads of HPV 16 – Pap smear

THE IMPORTANCE OF THE DOUBLE TEST IN IDENTIFICATION OF HIGH RISK PREGANANCIES FOR CHROMOSOMAL DISEASES DEVELOPMENT

ANDREEA LITEANU[1,2*], MARIUS MIHĂŞAN[1], VLAD ARTENIE[1]

Keywords: prenatal screening, biochemical markers, free beta-human chorionic gonadotropin (free β-HCG), pregnancy-associated plasma protein-A (PAPP-A), trisomy, Down syndrome.

Abstract: The double test plays an important role for the identification of chromosomal diseases and for the prenatal screening in the first pregnancy semester. The current work is focused on the investigation of free beta-human chorionic gonadotropin (free β-HCG) and pregnancy- associated plasma protein-A (PAPP-A) levels (markers part of the double-test) from the blood serum of 132 pregnant women in order to identify the high risk pregnancy for chromosomal diseases development. Also the levels of beta-human chorionic gonadotropin (free β-HCG) and pregnancy-associated plasma protein-A (PAPP-A) were investigated with respect to the maternal age. The interpretation of the results was achieved using the PRISCA v. 4.0 software, considering also the gestational age, smoking, *in vitro* fertilization, diabetic status and the medical history of the pregnant women. All investigated patients were in in the first semester of pregnancy, the specific period for tacking the double test. The biochemical investigations showed that most of the investigated patients presented normal values, within the interval reported in the literature and only a few cases were identified as being with high risk for developing trisomy for the chromosome 21 or 18.

INTRODUCTION

The identification of some suitable serum markers that can be used for assessing the risk of chromosomal aberrations in the first semester of pregnancy lead to development of the double-test (Agarwal, 2003 ; Kagan et al., 2008).

This test combines the two serum markers: the levels of free beta-human chorionic gonadotropin (free β-HCG) and pregnancy-associated plasma protein-A (PAPP-A)

The pregnancy-associated plasma protein-A is a glycoprotein derived from the placenta. During the pregnancy is produced in large amounts by the trophoblast and liberated in the mother’s circulatory system. The serum levels of this protein increase with the gestational age, especially in the last part of the pregnancy. Recent studies showed that the decrease of PAPP-A levels during the pregnancy is associated with chromosomal abnormalities of the fetus (Laborator Synevo, 2006 ; Veduta, Vladareanu, 2007).

The free beta-human chorionic gonadotropin is considered to be an even more relevant marker in the first semester of pregnancy. In pregnancies associated with the Down syndrome, the levels of free beta-HCG are >2 MoM. In the presence of trisomy 18, the levels of free beta-HCG are considerable lowered.

The purpose of this study is the investigation of the markers comprising the double-test (PAPP-A and free beta-HCG) in order to identify pregnancies with high risk for the development of chromosomal maladies.

MATERIALS AND METHODS

All investigations were performed on serum samples from 132 women in the first semester of pregnancy (10-13 weeks), the optimum period for performing the double test. Very important and also required is the fact that both the Ultrasound investigations and the serum sample were performed in the same day. In this manner, the gestational age is the same for both the Ultrasound investigations and the double-test, eliminating errors which might affect the precision of the obtained result. If the date of the Ultrasound is different compared to the blood sampling it must be specifically stated and the errors affecting the result cannot be eliminated. Biological samples were accepted from all the patients with an gestational age between 10 and 13 weeks, no matter of the fertilization method used (natural or *in-vitro*), pregnancy type (mono-fetal or twin), or various abnormalities, dis-functions and diseases identified using Ultrasound. Nevertheless the samples which were not suitable for performing the double test (hemolytic or lipemic) were rejectd. All determinations were performed using the automated analyzer Immulite 1000, belonging to the S.C. MEDICALTEST SRL BACĂU, IAŞI branch and interpreted using the PRISCA v 4.0 software. The PRISCA 4.0 program is able to calculate the corrected multiple of median tacking into account various factors such as: gestational age, mother’s bodyweight, smoking or non-smoking, the diabetic status, the pregnancy type, procedures used for fertilization. Once the corrected MoM was obtained, the similarity ratio is calculated for each of these values. By combining each of these ratios with the risk correlated with the maternal age (an major risk) the final result is obtained (Muller et al., 1999 ; Kevin, 2005).

The results of the biochemical investigations are expressed as mIU/mL for PAPP-A and as ng/mL for free β-HCG. The statistical significance was assayed using the Student test (Văleanu, Hîncu,1990) by grouping the data by mother's age and comparing each group with an random control consisting of the first age group.

RESULTS AND DISCUSSIONS

The 132 pregnant women selected were divided in four group according with their age: 16 in the first age group, consisting of women between 21-25 years old, a second group of 51 women between 26-29 years, 46 women in the third group of age between 30 – 35 years and 19 in the fourth group older then >35 years.

In table 1 are presented the results obtained by measuring the leveled of the two investigated markers PAPP-A and free beta-HCG in the serum of the investigated women from each of the four age groups.

For PAPP-A, the mean value for the first age group was of 2.166 mIU/mL, for the second group was of 2.048 mIU/mL, for the third group the recorded mean value was of 1.942 mIU/mL, and for the fourth group was of 1.568 mIU/mL (table 1).

A previous study on 283 voluntary patients has recorded values situated in the interval of 0.3 şi 10 mIU/mL, with an mean of 3.64 on Immulite 1000 and 3.70 on Immulite 2000 (Siemens Medical Solution Diagnostics ; Wald *et al.,* 1996).

The recorded mean values fort the pregnant women belonging to the four categories are lower, but nevertheless are more close to each other, probably due to an more strict interval on which the results were recorded. Our study was performed only on women in the first semester of pregnancy, making the recorded values interval to be more strict, more specific. Taking into account that in the first age-group no pathological results were encountered, we considered it as the control group. Compared to this group, the results recorded for the second age-group showed no statistically significant differences $p>0.05$(94.5%). Nevertheless, the results recorded for the third age group showed some slight significant differences $0.05>p>0.01$(89.65%), while the forth age-group as expected the differences are highly significant $p<0.001$(72.39%) (table 1, Figure 1).

For the second investigated marker, part of the double test – the free beta-HCG, the recorded levels were as follows: for the pregnant women from the first age-group the mean value was 48.756 ng/mL, for those from the second age-group the mean was 59.53 ng/mL, for the third group 45.689 ng/mL and for the fourth group 59.792 ng/mL(table 1).

The reference values recorded in the literature are between 2 - 200 ng/mL. The studies performed by Siemens using 20 volunteers reported the following values: 60.2 ng/mL from serum, 59.6 ng/mL from heparinized blood, 59.8 ng/mL from blood with EDTA. The levels for the free beta-HCG recorded on 116 pregnant women were between 0.75 şi 129 ng/mL with an average of 27.0 ng/mL on Immulite 1000 and 28.1 ng/mL on Immulite 2000. (Siemens Medical Solution Diagnostics ; Wald *et al.,* 1996).

The data recorded by us are inside the reference interval of 2 – 200 ng/mL and are close to the levels reported in the literature. The differences between values reported in this study and those reported by Siemens could be explained by the fact the interval for ech group of pregnant women in the current study is much smaller.

As stated above, in the pregnancies associated with Down syndrome the levels of free beta-HCG are below 2 MoM (multiple of median). The calculation of MoM for each marker consists of dividing the value with the median of the gestational age. The correction of the MoM is performed by comparing the obtained value for the specific patient with a mean value from a

population of normal pregnant women. In this case the highest values were recorded at women from the last group of age (>35 years), with an increase of over 122.63% compared with the results obtained for the control group (first age-group, 21-25 years), all recorded differences being significant $p<0.001$(table 1 and figure 2). The differences recorded for the pregnant women from the second and third age-group were statistically not significant ($p>0.05$).

The final result of the double test analysis is the risk (low or high) of trisomy development and it is expressed as a function of MoM. The risk level for each subject is based on the combination of the obtained result with the maternal age using an complex mathematical algorithm using software programs such as PRISCA 4.0 (used in our study).

The tests are interpreted as low or high risk based on a cut-off value fixed for each type of trisomy. In the case of 21 trisomy the cut-off is 1/250, and in the case of 18 trisomy the cut-off value is 1/100.

After the determination of the immunological markers, the obtained values were statistically interpreted using the PRISCA v. 4.0 software, DIAGNOSTIC PRODUCTS CORPORATION, SUA. The program is a software application which calculates the statistical value for the risk of development the Down syndrome (trisomy 21) and for the Edwards syndrome (trisomy 18), in the first and the second semester of pregnancy, as well as for neural tube defects in the second semester of pregnancy. The risk calculated using PRISCA software for a given woman is not a confirmation test, but has the role of an additional support in deciding whether a more profound diagnostic procedure is requited or not.

The biochemical risk for developing Down syndrome at birth is calculated on the basis of corrected MoM for each of the two markers and maternal age at birth. PRISCA 4.0 compares the obtained result with the median specific for the age of the pregnancy in order to formulate the final result as MoM, for each of the parameters PAPP-A and free beta-HCG, during the first semester of pregnancy.

Some results from the literature are addressing the double test as a test on its own, and not applied specifically for each markers. In this manner Bellver et al. (2005), analyzing the biochemical screening in the first semester of pregnancy, no matter the fertilization system used conclude that significant differences were due to maternal age, obtaining an $p<0.001$ for patients over 33 years old. Also, the determinations performed by Dr. Elisabeta Kovacs at the 1-st Obstetric and Gynecology Clinic of Emergency District Hospital, Cluj-Napoca, between 2003 – 2005, indicated statistical significant results when the Immulite 1000 automated analyzer was used to perform the double test (Kovacs, 2009). The results obtained by Kovacs, 2009 are in good concordance with the data obtained in our set of determinations, being the differences obtained being statistically significant for the women in the last age-group ($p<0.01$, table 1).

A similar conclusion was reported by the work performed by Adriana Stana (Stana Adriana, Popescu Gabriela**,** 2010), which emphasis that the best method is the simultaneous use of the combined screening and nuchal translucency, the combined risk for trisomy 18 being calculated using the PRISCA software.

All this data are in very good accordance with our observations that patients of age between 18 and 35 years old have a more diminished rate of risk pregnancy compared with those of over 35 years old.

Table 1: Mean values of PAPP-A and free beta-HCG levels determined at pregnant women grouped by age

PAPP-A(mIU/mL)			**free beta-HCG** (ng/mL)		
n=16 (21-25 years)	Mean (M)	2.166	**n=16 (21-25 years)**	Mean (M)	48.756
	Standard error (ES)	0.08489		Standard error (ES)	1.35542
	t	-		t	-
	p	-		p	-
n=51 (26-29 years)	Mean (M)	2.048	**n=51 (26-29 years)**	Mean (M)	59.53
	Standard error (ES)	0.03547		Standard error (ES)	1.89847
	t_1	1.290		t_1	0.68397
	p_1	>0.05		p_1	>0.05
n=46 (30-35 years)	Mean (M)	1.942	**n=46 (30-35 years)**	Mean (M)	45.689
	Standard error (ES)	0.031		Standard error (ES)	0.81503
	t_2	2.479		t_2	1.93929
	t_3	2.236		t_3	0.71231
	p_2	0.05>p>0.01		p_2	>0.05
	p_3	0.05>p>0.01		p_3	>0.05
n=19 (>35 years)	Mean (M)	1.568	**n=19 (>35 years)**	Mean (M)	59,792
	Standard error (ES)	0.099		Standard error (ES)	2.52648
	t_4	4.590		t_4	3,84913
	t_5	4.564		t_5	3,99692
	t_6	3.609		t_6	5,31248
	p_4	<0.001		p_4	<0.001
	p_5	<0.001		p_5	<0.001
	p_6	<0.001		p_6	<0.001
N=132					

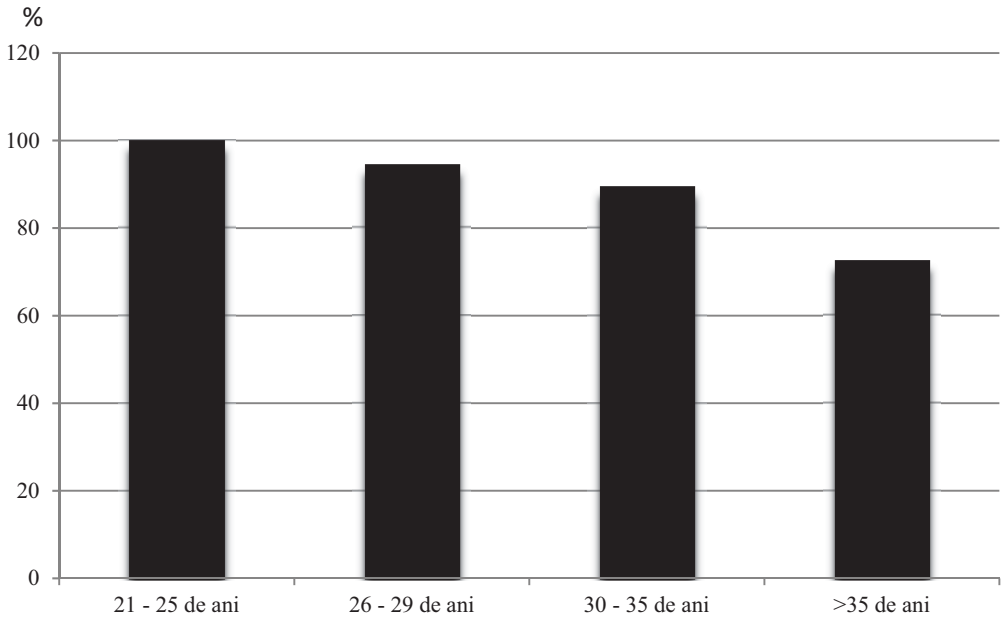


Figure 1. Relative values (%) of PAPP-A levels measured at pregnant women over 35 years old compared with the first, second and third age-group.

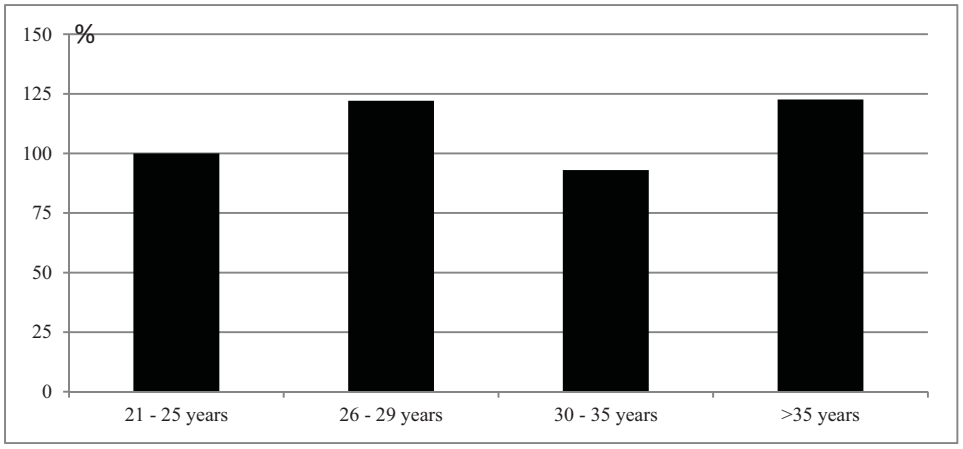


Figure 2: Graphical representations of the relative values for free. beta-HCG levels in serum from pregnant women grouped by age.

As stated earlier, the 132 pregnant women were group according to their age, forming 4 groups. The first group was selected as control group, as no pathological values were detected and the women aged between 21 – 25 do not present an high risk for development of chromosomal maladies.

Out of a total of 51 patients aged between 25 – 29 years, two were detected as presenting a high risk for development of trisomy 21.

Out of a total of 46 patients of aged between 30-35 years, 6 were detected as presenting high risk pregnancies.

The patients from the last group, from a total of 19, 9 were detected as presenting high risk pregnancies and the other 10 (53%) were with low risk. 7 out of the 9 these high risk patients were for trisomy 21, while the other two (10%) for both trisomy 21 and trisomy 18. These last two are more special cases as one patient is 42 years old smoker and the other is 38 years old.

In order to emphasis the importance of the maternal age in the prenatal screening, in figure 3 we present the percentage results of the double test performed on the patients from the forth age group, the one with most pathological cases.

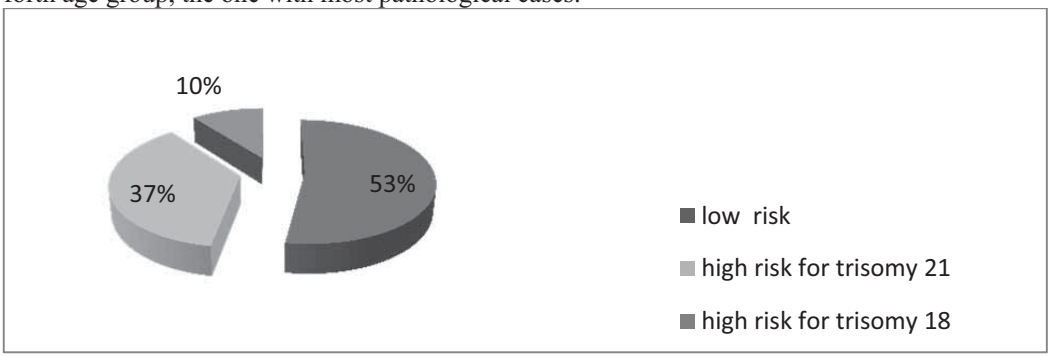


Figura 3: Procentage of the results of the double test performed on women from the fourth age group

CONCLUSIONS

The results obtained in this study, grouped by the age of the pregnant women for PAPP-A are well correlated with those reported in the literature, highlighting the importance, of maternal age in the prenatal screening. Also, according the literature, free beta-HCG levels is a very important marker which, together with PAPP-A levels, makes the double test and assures a very low rate of false positive hits, offering a very good correlation between the biochemical investigations and the ultrasound investigations.

The values obtained by evaluating the biochemical markers, combined with the ultrasound data, maternal age, mothers medical history, is a feasible prenatal screening, fact proved by both our study and the literature. The method of interpretation of the double test results using the PRISCA software is precise and does not require any manual adjusments. Due to the fact that in the first age group (21 – 25 years) no pathological results were recorded and the number of high risk pregnancies is increased as the patient is older, we can conclude that the result depend most on the maternal age.

REFERENCES

Agarwal R., 2003 - *Prenatal diagnosis of chromosomal anomalies:Pictorial essay*. Indian J Radiol Imaging ,13:173-88

Bellver J., Lara C., Soares S., Ramirez A., Pellicer A., Remohi J., Serra V., 2005 - *First trimester biochemical screening for Down's syndrome in singleton pregnancies conceived by assisted reproduction,* Human Reproduction, vol 20, 9:2623-2627

Kagan K., Wright D., Spencer K., Molina F., Nicolaides K., 2008 - *First trimester screening for trisomy by free beta-human chorionic gonadotropin and pregnancy-associated plasma protein –A: impact of maternal and pregnancy characteristics,* Ultrasound Obstet Gynecol, 31:493-502

Kevin Spencer, 2005 - *First trimestermaternal serum screening for Down's syndrome: an evaluation of the DPC Immulite 2000 free β hCG and pregnancy- associated plasma protein-A assays,* Ann Clin Biochem 42: 30 – 40

Kovacs E., 2009 - *Metode biochimice de diagnostic prenatal al afecţiunilor embrio-fetale în trimestrul I vs trimestrul II de sarcină,* Teză de doctorat, Universitatea de Medicină şi Farmacie „Iuliu Haţeganu” din Cluj-Napoca

Laborator Synevo, 2006 **-** *Referinţe specifice tehnologice de lucru utilizate* 2006. Ref Type: Catalog

Muller F., Aegerter P., Ngo A., Beachet A., Giraudet P., Dommergues M., 1999 - *Software for Prenatal Down Syndrome Risk Calculation: A Comparative Study of Six Software Packages,* Clinical Chemistry 45, nr 8: 1278-1280

Stana Adriana, Popescu Gabriela, 2010 - *Evaluarea comparativă a metodelor de screening prenatal pentru aneuploidiile fetale*, Revista Medicală Română, vol. LVII, 4:235-238

Văleanu I., Hîncu M., 1990 - *Elemente de statistică generală*, Editura Litera, Bucureşti, p. 25, 74

Veduta A., Vladareanu R., 2007 **-** *Diagnosticul prenatal al anomaliilor cromosomiale,*www.pressprogineco.ro, Ref Type: Internet Communication

Wald N. J. *et al*., 1996 - *Serum screening for Down's syndrome between 8 and 14 weeks of pregnancy.* Br. J. Obstet. Gynaecol,103:407-12

[1]”Alexandru Ioan Cuza” University of Iaşi, Faculty of Biology, B-dul Carol I, Nr.20A, 700506, IAŞI, ROMANIA
[2*]” Medicaltest” Clinical Laboratory, IAŞI, ROMANIA
* andreeaberbece@yahoo.com

APOPTOSIS IN NORMAL BRONCHIAL RESPIRATORY EPITHELIUM – BETWEEN CERTAINTIES AND UNCERTAINTIES

ADRIANA GRIGORAŞ [1*], IRINA DRAGA CĂRUNTU [1], RALUCA ANCA BĂLAN [1], TRAIAN MIHĂESCU [1], CONSTANTIN CRISTIAN GRIGORAŞ [1], LUDMILA LILIAC [1], EDUARD CRAUCIUC [1], EUGEN UNGUREANU [2], OVIDIU TOMA [2], CORNELIA AMĂLINEI [1]

Keywords: airway epithelium, apoptosis, caspases, Bcl-2

Abstract. The respiratory epithelium lines the conducting airways and functions as a selective barrier interposed between external environment and human body. It is exposed to various aggressive factors such as viral and bacterial microorganisms, or cigarette smoke and other inhaled noxious substances. The normal airway epithelium has its own mechanisms that maintain the integrity of the epithelial barrier and it is relatively refractory to a number of apoptotic stimuli. The up to date data about apoptosis in normal airway epithelium are limited, especially regarding the regulatory factors of this process. The current knowledge concerning the airway epithelium apoptosis regulation needs to be further studied by exploring the Bcl-2 superfamily members, Zn, p21, or peroxiredoxine V and pirine.

Apoptosis or „programmed cellular death” is an essential physiological process in the development of multicellular organisms, keeping constant the tissue cell population during life. The term “apoptosis” was used for the first time in literature by Kerr, Wyllie and Currie (Kerr JF et al, 1972).

Apoptosis involves a series of biochemical events accompanied by morphologic modifications among which the most important are changes in the mitochondrial membrane, chromatin condensation and fragmentation of the DNA. The „decision” of apoptosis comes either from the cell itself or from the neighboring cells in the tissue or from the immune cells (Ameisen JC, 2002).

Caspases have a key role in the apoptotic pathway and this is the reason why the classification of programmed cellular death is currently made according to the apoptosis execution modality through these protein-enzymes (Bratton SB et al, 2000). Caspases are cysteine-dependent aspartate-specific proteases and their catalytic activity depends on the cysteine residue which has a highly conserved site called pentapeptidic site QACRG. Fourteen different types of caspases have been described in mammals until now, while caspase-11 and 12 were only identified in rats. Two groups of caspases are described: a group of proapoptotic caspases that initiate apoptosis, including procaspases-2, -8, -9 and -10 and a group of execution caspases, including procaspases-3, -6 and -7 (Denault JB et al, 2010).

Caspases are synthesized as inactive enzymes called pro-caspases, having at their NH2 terminal a pro-domain that is followed by larger or smaller subunits. Pro-caspases are proteolysed between these subunits and the pro-domain is also frequently removed by cleavage and as a consequence these protein enzymes become activated.

As an intrinsic barrier between outer and inner environment, the respiratory epithelium is directly exposed to inhaled pollutants, viruses and allergens which can induce its direct injury or, through an inflammatory process, an indirect injury.

Histological pattern of the upper respiratory airways epithelium (excepting the olfactory mucosa) and of the inferior ones up to the entrance to the pulmonary lobule (intralobular bronchiole) is a pseudostratified columnar ciliated type, with goblet cells, also called respiratory epithelium.

Six different types of cells compose the normal structure of this epithelium: high columnar ciliated cells (the most numerous), goblet cells (normally rated as one goblet cell for 5

ciliated cells), regenerative cells, brush cells type I and II and endocrine-like cells (Ross MH, Pawlina W, 2010). Through the muco-ciliated barrier, these cells ensure the adhesion and removal of foreign particles and of infectious agents from the inhaled air and moreover, they secrete a series of cytokines and other mediators playing an important role in the regulation of the immune processes and bronchodilation.

By comparison with the alveolar epithelium, the knowledge regarding the apoptosis of normal respiratory epithelium is extremely limited, with only few data on the apoptosis of ciliated cells and that of goblet cells and almost none on that of regeneration cells. Among the most important substances inducing the apoptosis of the respiratory cells most reports are referring to inhaled substances such as cigarette smoke, cytokines (such as transforming growth factor-TGF), infectious agents (such as viruses or bacteria), hypoxia and allergens (Gelbman BD et al, 2007; Zalewski PD, Ruffin RE, 2008).

Beside the participation in the occurrence of epithelial lesions and in the inflammation regulation, the apoptosis of the respiratory cells contributes to the maintenance of the normal amount of cells. Also, apoptosis is the mechanism involved in limitation of viral infections or other epithelial lesions (allergen-induced or induced by other irritants) (Zalewski PD, Ruffin RE, 2008; Tesfaigzi Y, 2008). Moreover, the intensification of the apoptosis phenomenon could represent a marker of epithelial lesion (Zalewski PD, Ruffin RE, 2008) in some respiratory diseases.

The mitochondria play a very important role in the normal cell biochemistry. Alongside its role in mediating and amplifying the apoptotic signals, secondary to DNA injury or other aggressive factors such as physical and chemical stress, mitochondria has a key role in generation and dispersion of cell death signals originating within cells (Kroemer G, Reed JC, 2000; Kroemer G et al, 2007).

Most inducing factors produce a disturbance of the internal mitochondrial membrane potential that results in an increased permeability for molecules with a molecular weight of more than 1.5 kDa. Concomitantly with the increase in mitochondrial internal membrane permeability a water mitochondrial influx, followed by secondary swelling and the possible rupture of the external mitochondrial membrane, contributing to the cytoplasmic release of proapoptotic proteins (such as cytochrome c) and of other factors such as: apoptosis induction factor (AIF), endoG endonuclease and Htr/Omi (Li LY et al, 2001; Verhagen AM et al, 2002). An interest ing fact is that the increased nuclear membrane permeability is always followed by a disturbance in the mitochondrial internal membrane potential but this event is not reversibly (disturbed potential is not followed by an increased membrane permeability); moreover the cytosolic release of cytochrome c is also noted in the absence of mitochondrial internal membrane potential modification.

Up to date several mechanisms involved in mitochondrial membrane permeability increase have been proposed, but most researchers agree on the idea of “permeability pores” development thought to be formed by an adenine nucleotide translocator (ANT) and of a voltage-dependent anionic channel as a central component of the transmembranar pore, called VDAC (Kroemer G et al, 2007).

Normally all cells including respiratory cells have a strict mitochondrial regulatory system, performed by a series of molecules belonging to the Bcl-2 family of molecules. Two apoptosis control modalities involving the members of Bcl-2 family are suggested: regulation of the caspases activity (the antiapoptotic pathway) or favoring the preservation of mitochondrial

integrity, hence the inhibition of the proapoptotic mitochondrial proteins cytoplasmic release (Marsden V, 2002).

Recently, a series of proapoptotic factors (Bax or Bak) were included in the Bcl-2 family. The way Bax or Bak modify the mitochondrial integrity is not completely elucidated. The current hypothesis is that polymerized Bax and Bak initiate themselves the formation of a permeation channel in the external mitochondrial membrane either interact with some components of the mitochondrial membrane pore such as VDAC (Marsden V, 2002).

It is also possible that several antiapoptotic members of the Bcl-2 family may act by sequestering the proapoptotic members of the Bcl-2 family. Thus the inhibition of Bax or Bak activation or their polymerization and the development of the mitochondrial proapoptotic events could be achieved.

Moreover, Bcl-2 is considered an inhibitor of Apaf-1/caspase-9- independent, caspase-7 dependent apoptotic pathways (Marsden V, 2002). Furthermore, nowadays it is believed that a still unidentified Apaf-1 homologue directly regulated by Bcl-2/Bcl-XL may exist.

Most studies regarding the respiratory pathology demonstrated an increase of Bcl-2 expression in respiratory epithelium cells under exposure to ozone, endotoxins, cigarette smoke or allergens inducing metaplasia. All researchers conclusions support the idea that Bcl-2 expression regulation may lead to a decrease in goblet cells hyperplasia (Tesfaigzi Y et al, 2004; Tesfaigzi Y, 2002; Harris JF et al, 2005).

Nevertheless, limited data referring to Bcl-2 as well as to other members of the Bcl-2 family are currently available. Knowledge of the proteins involvement in the protection of the normal respiratory epithelium and in the development of inflammation may develop new regulatory methods of the cellular death in the respiratory epithelium.

An interesting discovery using immunohistochemical studies was made regarding the evident distribution of procaspase-3 in the apical pole of respiratory epithelium cells. If procaspase-3 was proved to be apically localized consequently its regulatory factors (including apoptosis regulatory factors) should be also placed in the neighboring cellular area. These aspects were certified by results of several studies which proved that Cu/Zn superoxide dismutase (with regulatory role in the apoptotic process) is also disposed in the cellular apical pole (Carter JE et al, 2002), and moreover by the intense immunolabeling for inhibitors of apoptosis (IAPs) in the normal bronchial epithelium (Viscioni B et al, 2006).

This unique spatial arrangement of antiapoptotic and propaoptotic factors in the respiratory epithelial cells involves some sequestering mechanisms. An apical cellular cytoskeleton organized as a dense network of cytokeratin filaments and microtubules may be most probably involved in these mechanisms.

Other partially known regulatory factors could be involved beside Bcl-2 family in the normal respiratory epithelium in the apoptosis cascade. Among these Fas, FasL, Zn, p21, stress-response proteins, peroxiredoxin V, pirine and corticosteroids, may be included. Unfortunately, the existent information is poor and incomplete to facilitate the understanding of the regulatory pathways of this mechanism in the respiratory epithelium and of the rather refractory characteristics to different apoptotic stimuli of this epithelium despite multiple aggressive factors.

The Fas important role in the apoptosis initiation in the hematopoietic line is today unanimously recognized. The expression and role of this molecule in the epithelial tissue is still incompletely elucidated. Recently, research results demonstrated the coexpression of Fas/FasL in normal human bronchial epithelium. Fas and FasL are both expressed on basal, columnar ciliated, and goblet cells surfaces. This coexpression of receptor and ligand is rarely observed in

epithelial cells in human body (Zalewski PD, Ruffin RE, 2008; Druilhe A et al, 1998). The role of Fas/FasL is the initiation of apoptosis or the modulation of the cell turnover rate in the respiratory epithelium. However, cellular turnover rate in normal respiratory epithelium is estimated to be 1%, suggesting the intervention of some regulation modalities for Fas/FasL interaction to prevent apoptosis. The regulation modalities may be represented by the separation of Fas receptor from FasL in nonadjacent membrane surfaces, by phosphorylation of Fas receptor, by metalloproteinase involvement in soluble Fas or cell membrane fixed Fas occurence.

A special interest is nowadays directed toward separation Fas and FasL roles in the bronchial epithelium. Although inconclusive data are available Fas expression seems to be important in the injured areas of the respiratory epithelium. Nevertheless FasL expression in bronchial epithelial cells may prevent local infiltration with inflammatory cells expressing Fas (such as eosinophils).

Thus, FasL expression in bronchial epithelium represents a normal cellular status, while epithelial infiltration by inflammatory cells can be attributed, at least partially, to the inactivation of Fas/FasL barrier. This collapse may be a consequence of the alteration of FasL expression or of a genetic variant of FasL expression exhibiting a decreased protection capacity of the bronchial epithelium against inflammatory processes. This aspect could be considered as a genetic predisposition of respiratory epithelium to chronic inflammatory processes (Gochuico BR et al, 1998).

Zn (zinc) can be included among factors contributing to the normal respiratory epithelial cells resilience against apoptosis. In an experimental model, Zn reduced the accumulation of eosinophils in the lamina propria in induced respiratory mucosal inflammation (Lang CJ et al, 2007; Richter M et al, 2003). More than a caspase inhibitor, Zn is also an antioxidant and a membranar stabilization factor (Truong-Tran AQ et al, 2003). Moreover, different authors suggest that the administration of Zn supplements may represent a protection factor against respiratory epithelial cells lesions (Bao S, Knoell DL, 2006). This fact is also supported by decreased Zn concentrations in some chronic inflammatory respiratory illnesses (Zalewski, P. D, 2006).

Another control factor of respiratory epithelial cells apoptosis is p21 or Cip1/WAF1, a cyclin-kinase dependent inhibitor involved in cell proliferation and death regulatory mechanism (Maddika S et al, 2007). Experimental studies performed on genetically modified rats deprived of p21 gene expression demonstrated an increased apoptosis in respiratory epithelial cells compared to normal rats (Blundell R et al, 2004). Supplementary, some researches suggested that p21 could be one of the factors involved in normal turnover of respiratory epithelial cells in chronic inflammatory diseases (such as asthma). p21 expression regulation may be one of the new therapeutic targets mainly as its expression proved to be irresponsive to corticosteroid therapy (Puddicombe SM et al, 2003).

Stress response proteins (such as Hsp-70 shock protein) ensure cells protection against several pathogens. The involvement of these stress-response proteins in the protection of respiratory epithelial cells against proteases release from granulations of neutrophils was quite recently demonstrated (Ito H et al, 2006). Nevertheless, further investigations regarding the role of these proteins in respiratory epithelium are necessary.

Understanding the exact mechanisms of cigarette smoke involvement in bronchial epithelial cells lesions represents a real interest for the researchers, especially finding new modalities to modulate its regulating factors. It was experimentally proved that normal respiratory epithelial cells express important quantities of peroxiredoxine V (PRXV), an

antioxidant protein (Serikov VB et al, 2006). Moreover, other studies proved that pirine could be an intensely expressed protein in human bronchial epithelial cells exposed to substances from cigarette smoke and associated with an important increased cell apoptosis (Gelbman BD et al, 2007). However, the literature data regarding peroxiredoxine V and pirine involvement in bronchial epithelium apoptosis is extremely scarce, so further studies are waited to support current findings.

In the category of apoptotic factors taken nowadays into account in the respiratory epithelium, some drugs, currently used in respiratory pathology, especially in chronic inflammatory diseases requiring the administration of corticosteroids, can be included (Dorscheid DR et al, 2003; White SR, Dorscheid DR, 2002). According to the results of some experimental researches, corticosteroids lead to a perturbation in mitochondrial polarity, to the caspases activation and apoptosis induction in bronchial epithelium, in cell cultures (Dorscheid DR et al, 2006).

Thus, there are numerous researchers which consider that an apoptosis increase noted in some respiratory diseases can be partially attributed to the administration of corticosteroids and not only to the disease itself.

Apoptosis is a normal process unrecognized as a distinct mechanism of cellular death until the second half of the past century. This achievement was logically followed by the evaluation of the importance of this process both in normal tissue physiology and in the physiopathology of some illnesses.

Research and correspondent obtained information of normal apoptosis in bronchial respiratory epithelium is quite recent, being obtained by the wide application of fibrobronchoscopy associated with bronchial biopsy as an investigation method in respiratory pathology. Current data are incomplete especially regarding oxidative stress and hence epithelial repair phenomenon with a special reference to the biology of local growth factors.

Eventually, cellular and molecular biology data regarding respiratory epithelial cells need further researches. Certainly, respiratory epithelial cells communicate and a single cell’s activity can influence the neighboring cells. Thus the bidirectional intercellular physical and biochemical interaction may regulate ciliated and goblet cells death.

All this information will unquestionably contribute to a better understanding of physiopathology notions on respiratory illnesses involving the mucosa and the respiratory epithelium, developing new premises of a precise target of the therapeutic approach, decreasing side effects number and severity, and improving the patient’s quality of life.

REFERENCES

Ameisen JC., (2002): *On the origin, evolution, and nature of programmed cell death: a timeline of four billion years*. Cell Death Differ, 9(4), 367-93

Bao S, Knoell DL., (2006): *Zinc modulates airway epithelium susceptibility to death receptor-mediated apoptosis*. Am J Physiol Lung Cell Mol Physiol, 290, L433-L441

Blundell R, Harrison DJ, O'Dea S., (2004): *p21(Waf1/Cip1) regulates proliferation and apoptosis in airway epithelial cells and alternative forms have altered binding activities*. Exp Lung Res, 30, 447-464

Bratton SB, McFarlane M, Cain K, Cohen GM., (2000): *Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis*. Exp Cell Res, 256(1), 27-33

Carter JE, Truong-Tran AQ, Grosser D, et. al., (2002): *Involvement of Redox events in caspase activation in Zn-depleted airway epithelial cells.* Bioche. Biophys Res Commun., 279, 1062-1070

Denault JB, Salvesen GS., (2002): *Caspases: keys in the ignition of cell death*. Chem Rev, 102(12), 4489-500

Dorscheid DR, Low E, Conforti A, Shifrin S, Sperling AI, White SR., (2003): *Corticosteroid-induced apoptosis in mouse airway epithelium: effect in normal airways and after allergen-induced airway inflammation*. J Allergy Clin Immunol,111, 360-366
Dorscheid DR, Patchell BJ, Estrada O, Marroquin B, Tse R, White SR., (2006): *Effects of corticosteroid-induced apoptosis on airway epithelial wound closure in vitro.* Am J Physiol Lung Cell Mol Physiol, 291, L794-L801
Druilhe A, Wallaert B, Tsicopoulos A, Tillie-Leblond I, et al., (1998): *Apoptosis, proliferation, and expression of Bcl-2, Fas, and Fas Ligand in bronchial biopsies from asthmatics* Am J Respir Cell Mol Biol, 19(5), 747-757
Gelbman BD, Heguy A, O'Connor TP, Zabner J, Crystal RG., (2007): *Up-regulation of pirin expression by chronic cigarette smoking is associated with bronchial epithelial cell apoptosis.* Respir Res, 8:10
Gochuico BR, Miranda KM, Hessel EM, De Bie JJ, et al., (1998): *Airway epithelial Fas ligand expression: potential role in modulating bronchial inflammation*. Am J Physiol, 274(3), 444-9
Harris JF, Fischer MJ, Hotchkiss JR, et.al., (2005): *Bcl-2 sustains increased mucous and epithelial cell numbers in metaplastic airway epithelium*. Am J Respir Crit Care Med, 171, 764-772
Ito H, Sekimura K, Sasaki N, et. al., (2006): *Cytoprotective effects of heat shock protein-70 in bronchial epithelium against neutrophil elastase-induced cell injury.* Arerugi, 5, 820-826
Kerr JF, Wyllie AH, Currie AR., (1972): Apoptosis*: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 26(4), 239-57
Kroemer G, Reed JC., (2000): *Mitochondrial control of cell death*. Nat Med, 6, 513-19
Kroemer G, Galluzzi L, Brenner C., (2007): *Mitochondrial membrane permeabilization in cell deth*. Physiol Rev, 87. 99-163
Lang CJ, Murgia C, Leong M, et.al., (2007): *Anti-inflammatory effects of zinc and alterations in zinc transporter mRNA in mouse models of allergic inflammation*. Am J Physiol Lung Cell Mol Physiol, 292, L577-584
Li LY, Luo X, Wang X., (2001): *Endonuclease G is an apoptotic DNase when released from mitochondria*. Nature, 412(6842), 95-9
Maddika S, Ande SR, Panigrahi S, et al., (2007): *Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy*. Drug Resist Updat, 10, 13-29
Marsden V, O'Connor L, O'Reilly L, et al., (2002): *Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome.* Nature, 419(6907), 634-7
Puddicombe SM, Torres-Lozano C, Richter A, et. al., (2003): *Increased expression of p21(waf) cyclin-dependent kinase inhibitor in asthmatic bronchial epithelium.* Am J Respir Cell Mol Biol, 28, 61-68
Richter M, Bonneau R, Girard MA, Beaulieu C, Larivee P., (2003): *Zinc status modulates bronchopulmonary eosinophil infiltration in a murine model of allergic inflammation.* Chest*,*123(Suppl.3), 446S
Ross MH, Pawlina W., (2010): *Histology – A Text and Atlas*, Sixth edition, 664-677, Lippincott Williams & Wilkins
Schwartz, D. C., Cantor C. R., (1984): *Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis*. Cell, 37(1), 67-75
Serikov VB, Leutenegger C, Krutilina R, et al.. (2006): *Cigarette smoke extract inhibits expression of peroxiredoxin V and increases airway epithelial permeability.* Inhal Toxicol, 18, 79-92
Tesfaigzi Y., (2006): *Roles of apoptosis in airway epithelia*. Am J Respir Cell Mol Biol, 34, 537-547
Tesfaigzi Y, Harris JF, Hotchkiss JA, Harkema JR., (2004): *DNA synthesis and Bcl-2 expression during development of mucous cell metaplasia in airway epithelium of rats exposed to LPS.* Am J Physiol Lung Cell Mol Physiol, 286, L268-274
Tesfaigzi Y., (2002): *The role of apoptotic regulators in metaplastic mucous cells*. Novartis Found Symp, 248, 221-230
Truong-Tran AQ, Grosser D, Ruffin RE, Murgia C, Zalewski PD., (2003): *Apoptosis in the normal and inflamed airway epithelium: role of zinc in epithelial protection and procaspase-3 regulation*. Biochem Pharmacol, 66, 1459-1468
Verhagen AM, Silke J, Ekert PG, et al., (2002): *HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins.* J Biol Chem, 277(1), 445-54
Viscioni B, van der Valk P, Span Ing, et.al., (2006): *Expression and localization of inhibitor of apoptosis proteins in normal human tissues*. Hum Pathol, 37, 78-86
White SR, Dorscheid DR. , (2002): *Corticosteroid-induced apoptosis of airway epithelium: a potential mechanism for chronic airway epithelial damage in asthma*. Chest*,*122(Suppl. 6), 278S-284S
Zalewski PD, Ruffin RE., (2008): *Apoptosis-regulatory factors as potential drug targets in the epithelium of normal and inflamed airways.* Curr Mol Pharmacol, 1, 38-49
Zalewski, P. D., (2006): *Zinc metabolism in the airway: basic mechanisms and drug targets*. Curr Opin Pharmacol, 6, 237-243

[1] "Gr.T.Popa" University of Medicine and Pharmacy, Iasi, Romania / „Elena Doamna" Iaşi Clinical Hospital
[2] "Alexandru Ioan Cuza" University, Iasi, Romania
* a_grigoras6600@yahoo.com

EFFECT ON NERVE STRUCTURES OF FUNCTIONALIZED GOLD-CHITOSAN NANOPARTICLES OBTAINED BY ONE POT SYNTHESIS

ANA CAZACU[1], DANIELA BINDAR[1], LILIANA TARTAU[2], LUCIAN HRITCU[3], MARIUS STEFAN[3], LOREDANA NITA[4], CEZAR IONESCU[2], VALENTIN NICA[1], GEORGE RUSU[1], MARIUS DOBROMIR[1], VIOREL MELNIG[1*]

Keywords: gold-chitosan nanoparticles, synthesis, nerve structures.

Abstract: Gold nanoparticles have potential applications in drug delivery, cancer diagnosis and therapy, food industry and environment remediation. However, little is known about their potential toxicity or fate in the environment. In this study we observed significant effects of functionalized gold-chitosan nanoparticles obtained by one pot synthesis on nerve structures of Wistar rats.

INTRODUCTION

The obtaining of metal nanoparticles immobilized in different materials is extremely important in nanoscience and nanotechnology research, since synergistic and bifunctional effects are expected (Mizukoshi *et al*., 2006). There have been many reports regarding the method of metal nanoparticles preparation, such as chemical reduction (Yi *et al*., 1995), photochemical using UV irradiation (Yonezawa *et al*., 1994), sonochemical (Okitsu *et al*., 2007), sonoelectrochemical, etc (Wang *et al*., 2008). Sonochemical method for obtaining reduced gold ions in chitosan solutions involves radical and/or thermal reactions. Chitosan has excellent biocompatible and biodegradable characteristics and is a naturally occurring polysaccharide. Due to the cationic character as polymer and its gel coating properties, the chitosan has been extensively investigated in the pharmaceutical industry for its potential use in the drug delivery development.

Polycationic nature of chitosan results from polycondensation in the presence of anionic molecules. Chitosan has been used (Yao *et al*., 1995) as a protective agent in the preparation of gold nanoparticles and gold salt could be reduced to a zerovalent gold nanoparticles using chitosan, without any additional reducing agent. Thus, gold nanoparticles (Au NPS) with different size distributions have been obtained using chitosan with different molecular weights as an agent of stabilization/reduction. The obtaining of metal nanocomposites with gold nanoparticles has been intensively studied in biology as markers, dyes, catalysts and sensors (Kusumi *et al*., 1993).

The biomedical studies have highlighted a number of effects caused by the combination of chitosan with gold nanoparticles. Sub-acute and acute toxicity studies on male and female rats showed that the chitosan - gold nanoparticles do not produce toxicity in oral administration, showing optimal levels of compatibility (Pokharkar *et al*., 2009). Also, it was shown that Au NPS control the level of glucose, lipids and serum biochemical in mice blood.

The aim of the present study was to investigate the effects of functionalized gold-chitosan nanoparticles, obtained by one pot synthesis, on nerve structures of rats, close related to neurodegenerative conditions.

MATERIALS AND METHODS

Synthesis of gold nanoparticles

The practical grade chitosan (PG), $HAuCl_4 \cdot 3H_2O$ precursor and CH_3-COOH used were obtained from Sigma Aldrich. All solutions were prepared using Milli-Q deionized water (18.2 MΩ resistance).

Numerical molar mass, M_n, gravimetric molar mass, M_w, polydispersity index, PI, and mass distributions were determined by gel permeation chromatography (GPC) using a Varian PL-GPC 120 chromatograph. Thus, for PG, the following values were obtained: M_n = 97,607 g/mol, M_w = 263,836 g/mol, PI = 2.70.

The nanoparticle size optimization was achieved depending on $HAuCl_4 \cdot 3H_2O$ precursor concentration and injected energy into the system by ultrasonic field.

The chitosan stock solution was prepared from 0.1% (0.1 g/L) chitosan in 1% acetic acid (*v/v*). Precursor gold solutions were prepared by mixing 2, 4, 6, 8 and 10 mL of $1 \cdot 10^{-3}$ M $HAuCl_4 \cdot 3H_2O$ stock solution with 38, 36, 34, 32 and 30 mL respectively of 0.1% chitosan stock solution, all these solutions constituting a lot of 5 bottles. The samples were named: 2PG (38 mL PG + 2 mL HAuCl4), 4PG (36 mL PG + 4 mL HAuCl4), 6PG (34 mL PG + 6 mL HAuCl4), 8PG (32 mL PG + 8 mL HAuCl4), 10PG (30 mL PG + 10 mL HAuCl4). Two lots of solutions were stirred and heated by applying an ultrasonic field of 20 kHz with an amplitude of 50% and 80% respectively for 10 minutes, using a Sonoplus Bandelin device.

The micro- and nanophase structure and the average size of lyophilized powder crystallites were investigated by X-ray diffraction with a Shimadzu XRD 6000 diffractometer using CuKα radiation (1.54060 Å). Crystallites size and network constant were calculated using the Topas Academic program.

The micrographic morphology studies by transmission electron microscopy (TEM) were made with a CM100 Philips microscope. For analysis, the nanoparticle solutions were deposited on formvar-coated copper grid.

The nanoparticles average size was determined by visual comparison of TEM micrographies with a standard scale using NIS Elements Basic Research program (NIS-BR). To determine the size distribution it was used the same statistical program.

The nanoparticles size distribution in solution was evaluated with a Malvern Zetasizer Nano ZS, Zen-3500 model, at room temperature. The stability of nanoparticle solutions as a function of average Zeta potential was evaluated by measuring the Zeta potential with the same device, at room temperature.

The characterization of nanoparticles biofunctional properties revealed the biofunctional properties transfer of polymers used as coating agents and stabilizers of nanoparticles.

Animals and treatment

20 male Wistar rats weighing 200-250 g at the start of the experiment were used. The animals were housed in a temperature- and light-controlled room (22°C, a 12-h cycle starting at 08:00 h) and were fed and allowed to drink water *ad libitum*. Rats were treated in accordance with the guidelines of animal bioethics from the Act on Animal Experimentation and Animal Health and Welfare Act from Romania and all procedures were in compliance with the European Council Directive of 24 November 1986 (86/609/EEC). This study was approved by the local Ethic Committee and also, efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were divided into two groups: control group and experimental group. Experimental group was intraperitoneally injected with gold nanoparticles every day for 7 days, respectively.

Coefficients of the brain

After administration of Au NPS (5μg, 8-12 nm) for 7 consecutive days, all animals were weighed, anesthetized (100 mg/kg body weight, ip, Sigma) and then sacrificed. After weighing the body and brains, the coefficients of brain to body weight were calculated as the ratio of tissues: wet weight (mg) to body weight (g) (Ma *et al.*, 2010, Hritcu *et al.*, 2011).

Statistical analysis

Results were expressed as mean ± S.E.M. The results were analyzed statistically by means of the Student's "t" test (T- test: Paired Two Sample for Means). $p<0.05$ was taken as the criterion for significance.

RESULTS AND DISCUSSIONS

Characterization of gold nanoparticles

Depending on the amount of gold precursor solution and energy injected into the system by ultrasonic field, different shades of blue - indigo - violet - brown - yellow can be obtained (Fig. 1). The last sample, 10PG, was stable for a short time, the solution becoming brown to yellow with a brown precipitate.

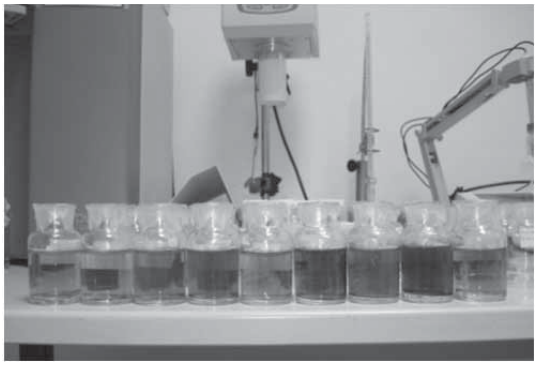


Fig. 1 - Different colors of gold nanoparticles solution.

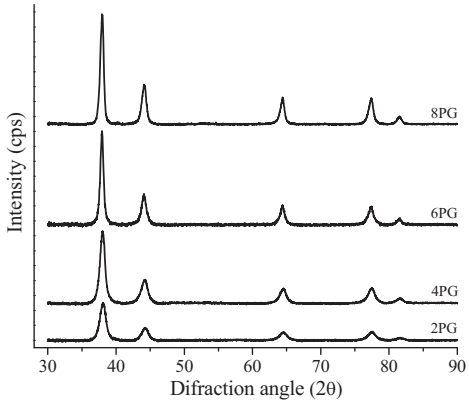


Fig. 2 - Diffractograms for PG with different concentration of $HAuCl_4$.

It is known that the color is strongly dependent on the Au nanoparticles size, the geometrical shape, the mass of polymer covering nanoparticles, etc (Huang and El-Sayed, 2010).

In Fig. 2 are presented the diffractograms for lyophilized powders of 2, 4, 6 and 8 mL 1mM containing gold precursor.

In Table 1 are presented the calculated values for the crystallites size in the direction of Miller planes (hkl), their average size and the network constant (a). Comparing the experimental peaks in Figure 2 with the data specified in the Crystallographic Database we can affirm that the gold particles obtained crystallize in face-centered cubic system (FCC). The crystallites have an average size of 9 – 14 nm and the constant network a = 4.089 Å. For the studies that follow are suitable structures with dimensions as small, so in further tests will be analyzed only the samples 2PG and 4PG.

Table 1. The calculated values for the size of crystallites in the direction of planes (hkl), their average size, and the network constant for the 2PG, 4PG, 6PG, 8PG solutions.

2PG			4PG			6PG			8PG		
Miller (hkl)	Cryst. average size (nm)	a (Å)	Miller (hkl)	Cryst. average size (nm)	a (Å)	Miller (hkl)	Cryst. average size (nm)	a (Å)	Miller (hkl)	Cryst. average size (nm)	a (Å)
1 1 1			1 1 1			1 1 1			1 1 1		
2 0 0			2 0 0			2 0 0			2 0 0		
2 2 0	**9**	**4.084**	2 2 0	**9**	**4.089**	2 2 0	**14**	**4.092**	2 2 0	**13**	**4.091**
3 1 1			3 1 1			3 1 1			3 1 1		
2 2 2			2 2 2			2 2 2			2 2 2		

TEM analysis shows that indeed the obtained nanoparticles have different types of tetrahedral, decahedral, hexagonal, icosahedral, multitwinned, and irregular (Fig. 3).

Size distribution analysis performed with the NIS-BR program (Figure 4) shows that the average sizes of Au nanoparticles is 11.68 nm.

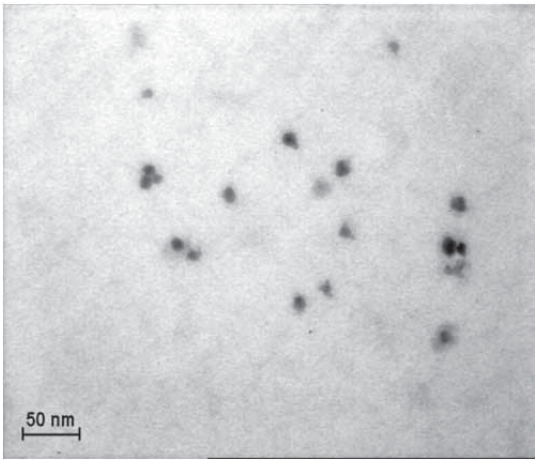


Fig. 3 - TEM micrographs of Au NPS in 2PG sample.

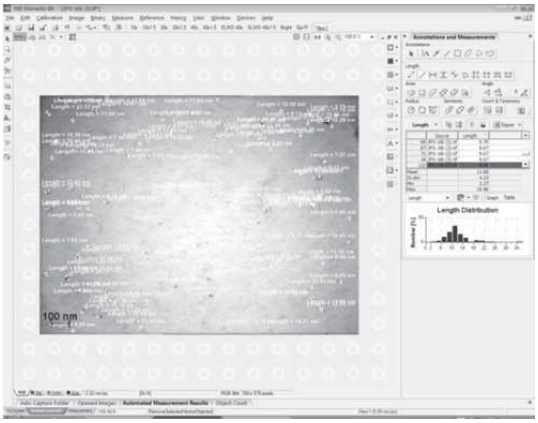


Fig. 4 - Size distributions analyses performed with the NIS-BR program for 2PG sample.

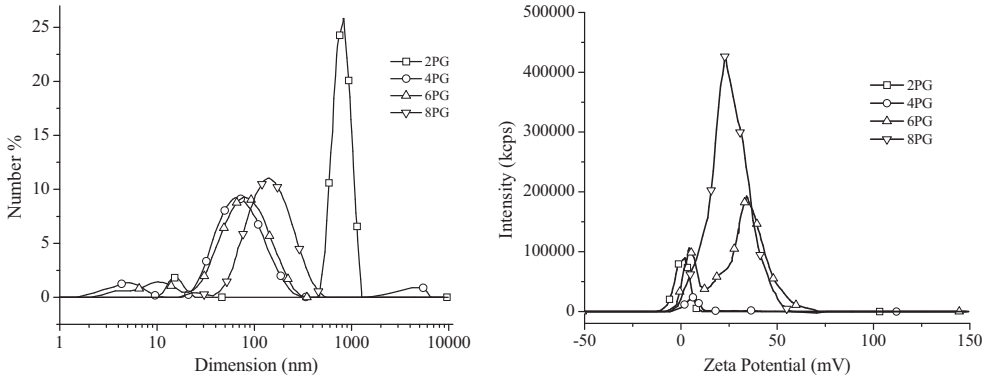


Fig. 5 - The dimension (a) and Zeta potential (b) distributions of Au NPS solutions.

In Fig. 5 are presented the dimentions and Zeta potential distributions for Au NPS coated with chitosan. The Au NPS coated with chitosan average values obtained by LDS measurements are presented in table 2. The values for the size of Au NPS coated with chitosan in aqueous solutions show the same order of magnitude. The average value of Zeta potential is a stability criterion of colloidal solutions (Riddick, 1968). If all the particles in suspension have a large positive or negative potential, they tend to reject them and thus cannot form aggregates. However, if they have small values of Zeta potential, then no force will prevent particle aggregation.

Table 2. The average size and Zeta potential of Au NPS in aqueous solutions.

Sample	Average size (nm)	Average Zeta potential (mV)
2PG	29.9	27.2
4PG	51.5	18.7
6PG	49	29.2
8PG	126	24.9

The results from table 2 show that the stability of obtained Au NPS - chitosan solutions are situated in the category of those who are at the threshold of light dispersion to moderate stability. Experience has shown that these solutions are stable for more than six months. The error in the device decision is due to the fact that there are two distributions: one of the Au NPS nanoparticles coated with chitosan and one of the chitosan micelles (see Fig. 5a).

Coefficient of brain to body weight

Throughout the treatment, animals maintained their weight to normal levels. Daily behavior of animals treated with Au NPS, and food intake, fluid intake was the same as the control animals. Coefficients of brains are shown in table 3 expressed as milligrams (wet weight of tissues)/grams (body weight). No significant differences were found in the body weight of all groups. In the groups treated with Au NPS coefficients of brain significantly decreased compared to control group, suggesting that Au NPS induce degradation of nervous areas involved in memory processes.

Table 3. **Body weight (BW) and coefficients of brain after Au NPS treatment.**

Indexes	Control	Au NPS (5µg)
BW (g)	230 ± 3.16	245 ± 7
Brain/BW (mg/g)	10.5 ± 0,2	8.43 ± 0.5*

Values are means ± SEM, n=10 animals/group, *p <0.05 vs. Control.

CONCLUSIONS

The Au NPS coated with chitosan were obtained by their reduction and nucleation in the chitosan matrix. The nanoparticles were produced in chitosan aqueous solutions by heating under an ultrasonic field, at room temperature. X-ray diffractograms emphasize a structure predominantly FCC with very small grains (crystals): 9 - 10 nm. TEM micrographs show the presence of the NPS in systems, the size and, also, the fact that they have different geometries. AFM micrographs and light scattering analysis on nanoparticles in solution (LDS) show that the

nanoparticles are coated with chitosan. This study suggests that Au NPS are capable of inducing neurotoxicity in rats, close related to neurodegenerative conditions.

REFERENCES

Dhar, P.V., Bhumkar, S., Mali, D., Bodhankar, V., Prasad, S., (2009): B.L.V. Acute and Subacute Toxicity Studies of Chitosan Reduced Gold Nanoparticles: A Novel Carrier for Therapeutic Agents, *J. Biomed. Nanotechnol*, 5(3): 233-239.

Huang, X., El-Sayed, M. A., (2010): Gold nanoparticles: Optical properties and implementations in cancer diagnosis and photothermal therapy, *J. Adv. Res.*, 1: 13–28.

Hritcu, L., Stefan, M., Ursu, L., Neagu, A., Mihasan, M., Tartau, L., Melnig, V., (2011): Silver nanoparticles exposure-induced memory deficits and oxidative stress, *Cent. Eur. J. Biol*. (in press)

Kusumi, A., Sako, Y., Yamamoto, M., (1993): Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells, *J.Biophys,* 65: 2021-2040.

Ma L., Liu J., Li N., Wang J., Duan Y., Yan J., (2010): Oxidative stress in the brain of mice caused by translocated nanoparticulate TiO2 delivered to the abdominal cavity, *Biomaterials*, 31, 99-105

Mizukoshi, Y., Seino, S., Kinoshita, T., Nakagawa, T., Yamamoto, T.A., Tanabe, S., (2006): Selective magnetic separation of sulfur-containing amino acids by sonochemically prepared Au/gamma-Fe2O3 composite nanoparticles, *Scr. Mater,* 54: 609 -613.

Okitsu, K., Mizukoshi, Y., Yamamoto, T.A., Maeda, Y, and Nagata, Y., (2007): Sonochemical synthesis of gold nanoparticles on chitosan, *Letters Materials,* 61 3429 -3431.

Riddick, T.M., (1968). Control of Colloid Stability through Zeta Potential, *Livingston Pub. Co*.

Wang, L., Mao, W., Ni, Di, J., Wu, Y., Tu, Y., (2008) Direct electrodeposition of gold nanoparticles onto indium/tin oxide film coated glass and its application for electrochemical biosensor, *Electrochemistry Communications* 10: 673 -676.

Yao, K.D., Peng, T., Yin, Y.J., Xu, M.X., (1995) Microcapsules/microspheres related to chitosan, *JMS-Re*V. *Macromol. Chem. Phys.*, *C35*: 155 – 180.

Yi, K.C., Mendieta, V.S., Castanares, R.L., Meldrum, F.C., Wu, C., Fendler, J.H., (1995) Gold Particulate Film Formation Under Surfactant Monolayers, *J. Phys. Chem*., 99: 9869-9875.

Yonezawa, Y., Sato, T., Kawabata, I., (1994) Photo-Induced Formation of Gold Metal Film from Metal Salt of Chitosan, *Chem. Lett.*, 23(2): 355 –358.

[1] "Alexandru Ioan Cuza "University of Iasi, Characterization of Molecular Organization in BioMaterials Laboratory, 11A, Carol I Blvd., 700506, Iasi, Romania
[2] "Gr.T. Popa" University of Medicine and Pharmacy, Faculty of Medicine, 16, Universitatii St., 700115, Iasi, Romania
[3] "Alexandru Ioan Cuza "University of Iasi, Laboratory of Microbiology, 20A, Carol I Blvd., 700506, Iasi, Romania
[4] "Petru Poni" Institute of Macromolecular Chemistry, LAMINAST Laboratory, 41A, Grigore Ghica Voda St., 700487, Iasi, Romania
[*] vmelnig@uaic.ro

Acknowledgements
This study was supported by CNCSIS – UEFISCSU, 509 PNII–IDEI 1996/2008.

LEAD-INDUCED GENOTOXICITY IN WHEAT

ELENA TRUŢĂ[1*], CRĂIŢA MARIA ROŞU[1], IULIA CSILLA BĂRA[2]

Keywords. Lead acetate, lead nitrate, chromosome aberrations, genotoxicity
Abstract. The changes induced in cytogenetic parameters from root meristems of *Triticum aestivum* cv. *Maruca* seedlings have been studied after treatment with lead acetate and lead nitrate solutions, at four concentrations (10, 25, 50, 100 µM) containing 2.07, 5.18, 10.36, respectively 20.72 µg ml^{-1} Pb^{2+}. Lead induced mitosis disturbances in root meristematic cells of wheat seedlings, expressed mainly in decrease of mitotic index and changes in preponderance of division phases. This heavy metal has genotoxic effects, expressed in the occurrence of many chromosomal aberrations in all Pb^{2+} treated variants. Pb^{2+} nitrate shows a more pronounced genotoxic potential than lead acetate trihydrate.

INTRODUCTION

Lead is naturally found in small amounts in the earth crust and is largely used in the production of containers of foods, stills, batteries, paints, and leathers. Human activities like burning of fossil fuels, mining, and manufacturing are lead sources. Its use as tetraethyl and tetra methyl additives in gasoline to increase octane rating has transformed lead into one of the metals of high toxic risk. In 1965 – 1990 lead consumption increased in the world to 5.6 x 10^6 tones, its concentration in biosphere being 1,000 – 100,000 times higher than the natural level. Since the half-life in biological systems is one of the longest among metals (150 – 5000 years), the consequences of lead pollution can be devastating. Increases in lead content of soils are registered near industrial areas. Lead-contaminated soils induce diminutions in crop productivity.

The primary effect of lead toxicity in plants is a rapid inhibition of root growth, probably due to inhibition of cell division in root tips. Important alterations have been reported in the structure, biochemistry and physiology of plant cells in lead excess. In *Helianthus annuus* L., Pb^{2+} showed the highest phytotoxicity comparatively with Al, Cd, Cu, Ni, Pb and Zn (Chakravarty and Srivastava, 1992). This metal denatures the proteins (Rathore et al., 2007) and alters photosynthesis (Akinci *et al.*, 2010). It causes changes in lipid composition of thylakoid membranes and modifies membrane permeability (Stefanov *et al*., 1995). Root elongation, plant growth, seed germination, transpiration, photosynthesis, mineral nutrition, plant water status and enzymatic activities can be also negatively influenced by lead treatment (Pinero *et al.*, 2002; Kaznina *et al*., 2005; Akinci *et al*., 2010; Jiang and Liu). Increasing concentration of Pb reduced DNA, RNA and protein synthesis in embryo axis and endosperms of germinating rice seedlings (Maitra and Mukherji, 1977).

Some sources (Carruyo *et al*., 2000) consider lead as probable carcinogen for humans, but relatively few data are available on lead genotoxicity in plants and positive as well as negative results have been registered on genotoxic potential of lead in these biological systems, so the mechanisms of lead-induced genotoxicity still need more experimental research. Lead binds strongly to a large number of molecules including DNA and RNA; it disrupts DNA synthesis and alters the transcriptional process and mitotic activity. Genome alterations consist in depolymerizations, generation of abnormal nitrogenous bases, DNA strand breaking, DNA – DNA cross-links, DNA – protein cross-links. DNA damage may result in the production of abnormal bases such as thymine glycol and 8-hydroxyguanine or to strand breakage through a series of reactions initiated by the abstraction of a 4'-hydrogen atom from a ribose residue (Babior, 1997). Indirectly, like other heavy metals, lead can inhibit DNA repair enzymes or DNA replication; consequently they can act as co-clastogens or co-mutagens. This heavy metal binds to SH groups of cell tubulins, modifying the typical arrangement of metaphase chromosomes (Liu *et al*., 2009).

Wheat is a plant of a worldwide economic importance, a main link in trophic chain and a pathway of pollutant ingestion for animals and humans and, like other plant systems, it can be used as plant test in the quantification of effect induced by various xenobiotic factors on its genetic material. The main objective of the present investigation is to evaluate the genotoxic potential of Pb^{2+} provided as lead acetate and lead nitrate, in *Triticum aestivum* L. cv. *Maruca*, by analyzing the frequency and types of mitotic chromosome disturbances in wheat root tips.

MATERIALS AND METHODS

Biological material is represented by wheat seeds (*Triticum aestivum* L. cv. *Maruca*), Agricultural Research Station, Podu Iloaie, Romania). The seeds were 4 h treated with:

1. *Lead acetate trihydrate* $Pb(C_2H_3O_2)_2.3H_2O$, mol. weight=379.33 g/mol
2. *Lead nitrate* $Pb(NO_3)_2$, mol. weight=331.20 g/mol

Four concentrations (10 µM, 25 µM, 50 µM, 100 µM) were prepared for each lead compound and were used for seed treatment. The lead concentration (µg ml^{-1}) in each solution is presented in Table 1. In control, distilled water was used.

Table 1. Lead concentration in tested solutions.

variant	molar concentration of salt solution	lead concentration ($\mu g\ ml^{-1}$)
Control – distilled water		
Lead acetate trihydrate	10 μM	2.07
	25 μM	5.18
	50 μM	10.36
	100 μM	20.72
Lead nitrate	10 μM	2.07
	25 μM	5.18
	50 μM	10.36
	100 μM	20.72

For *cytogenetic analysis*, wheat roots (10 – 15 mm in length) were firstly fixed for 24 h in ethyl alcohol:glacial acetic acid (3:1, v/v), at room temperature, then washed and stored in 70% ethyl alcohol. The plant material was 10 min hydrolyzed in 50% HCl, and then stained in modified charbol fuchsin solution. To prepare the slides – five for each variant - the meristematic regions were carefully squashed into a drop of 45% acetic acid. 10 microscopic fields were microscopically analyzed on every slide. A Nikon Eclipse 600 light microscope was used for this analysis. Photos were taken with a Nikon Cool Pix 950 digital camera, 1600x1200 dpi resolution.

Mitotic indices (MIs), frequencies of mitotic phases (prophase, metaphase, anaphase and telophase indices), ana-telophase chromosome aberrations and metaphase abnormalities were calculated and were used as endpoints for determination of lead-induced genotoxic effects.

These indicators were calculated according to the following formulas:

Mitotic Index = TDC x100/TC

PI% = prophase cells x 100/TDC

MeI% = metaphase cells x 100/TDC

AI% = anaphase cells x 100/TDC

TC% = telophase cells x 100/TDC, where

TC = total (dividing and non-dividing) cells, and TDC = total dividing cells.

The percentages of ana-telophase chromosome aberrations ($A\text{-}T_{CA}\%$) and of metaphase abnormalities ($M_{abn}\%$) were also calculated in relation to the number of cells in mitosis:

$A\text{-}T_{CA}\% = A\text{-}T_{CA} \times 100/TDC$

$M_{abn}\% = M_{abn} \times 100/TDC$

RESULTS AND DISCUSSIONS

Behaviour of mitotic index. Mitotic index is a reliable predictor of cell proliferation in tissue indicating the frequency of dividing cells. Based on the gravity of effects induced on mitosis, Patra *et al*. (2004) classified the heavy metals in three groups. Pb^{2+} is included in the category of relatively low active metals, together Mg, V, As, Mo, Ba.

In this experiment, we found that Pb^{2+} induces mitosis disturbances in root meristems of wheat seedlings (Fig. 1). The lowest tested concentrations of lead acetate and lead nitrate (10 μM) have slight stimulatory effect on cell division (8.58±0.42 %, respectively 8.83±0.34 %), comparatively to untreated control (8.22±0.62, in terms of %). All the other concentrations – except 50 μM lead acetate – influenced in a negative manner the mitotic index. Other studies also confirmed mitodepressive and mitotoxic effects of lead in different plant species (Samardakiewicz and Wozny, 2002; Glinska *et al*., 2007; Rathore *et al.*, 2007). No direct correlation between lead concentration and MI was established in our experiments. Carruyo *et al*. (2008) found that the correlation between lead content and mitotic index was not significant ($p>0.05$) and that the exposure time is a more important factor in induction of mitosis disturbances than lead concentration.

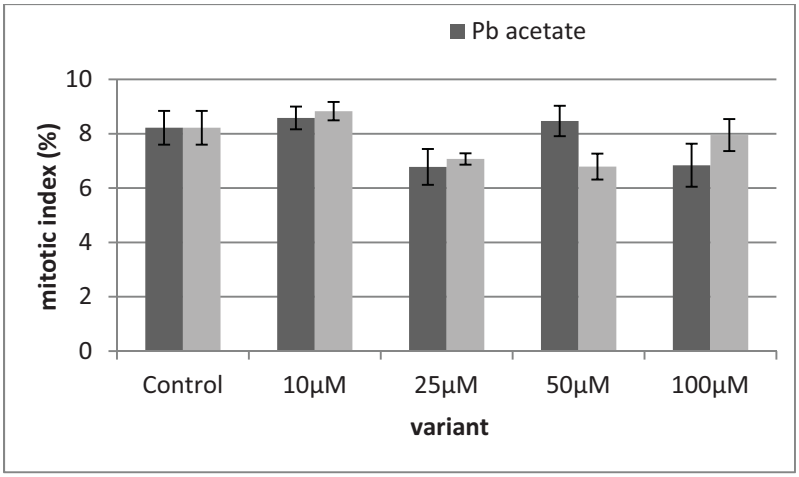


Fig. 1 Graphic representation of evolution of mitotic index (x±SE) in wheat root meristems, after lead treatment (x±SE).

The precise action of lead on cell division is still incompletely known. It is possible that higher MI values in Pb^{2+} exposure to result from extending mitosis rather than from promoting cell entrance into mitosis, while its depression can result from the inhibition of DNA synthesis or of some proteins essential for mitotic cycle (Wozny and Jerczynska, 1991).

Another Pb^{2+}-induced change consists in percentage modification of division phases (Wierzbicka, 1999; Glinska *et al.*, 2007). In this study, in wheat root meristems a decrease of prophase index was induced in all lead-treated variants, whereas metaphase index had values generally close to control (Fig. 2). The decline of prophase index could be a proof that lead prevents mitosis beginning, by stopping interphase cells to enter into prophase. Concerning metaphase index, only in 25 µM lead acetate and 25 µM lead nitrate treated variants this parameter shows slight increase comparatively to control. Ana-telophase indices are higher than control in all variants, but significant increases (over 35%) exhibited those variants exposed to 100 µM lead acetate, 25 µM and 50 µM lead nitrate. Higher values of metaphase and ana-telophases indices could be the effect of lead action on division spindle resulting in stop of division in these stages.

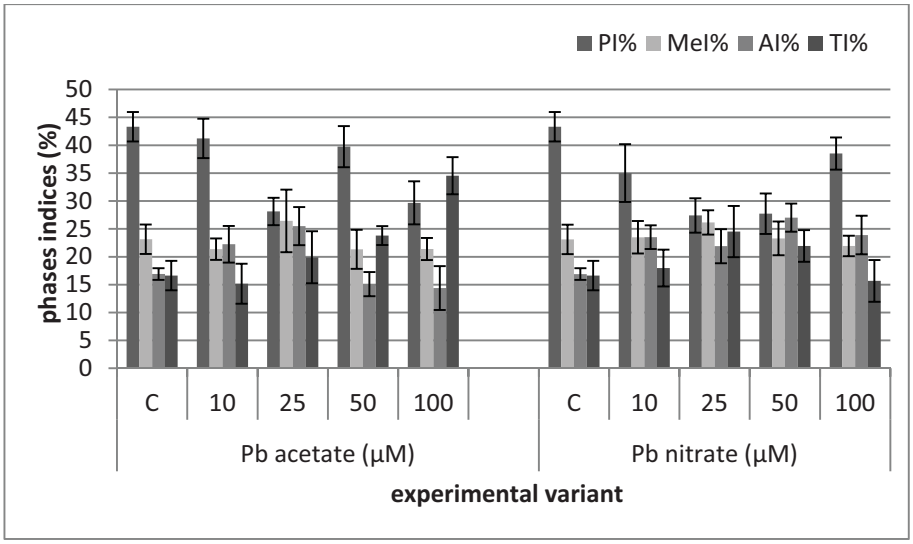


Fig. 2. Graphic representation of frequency of division phases in wheat root meristems, depending on lead compound and lead concentration (x±SE).

Metaphase disturbances. Lead is, at certain doses, an effective turbagen due to the affinity for thiol groups, inducing various types of spindle disturbances (Patra *et al*., 2004). In our study, total aberrant metaphases registered smaller values than control in variants treated with lead acetate (Fig. 3).

In the case of lead nitrate, the minimum tested concentration has values close to control, but the other three surpass control group, slightly at 50 and 100 µM lead nitrate and significantly for 25 µM lead nitrate, where the percentage of anomalous metaphases is 1.80 times higher than control**.** The abnormal metaphases with abnormal configurations are mainly represented by *metaphases with one or more expelled chromosomes* from equatorial plate and *C-metaphases*. Delayed centromere division can induce colchicine-like chromosome configurations which can result in the formation of cells with elevated degrees of ploidy. C-metaphases, result of spindle inactivation, are followed by chromosome scattering in cell. The metaphases showing expelled chromosomes from equatorial plate are numerically predominant, in all lead treated variants.

*Ana-telophase chromosome aberrations***.** Relatively few data exist on lead influence on plant genetic material. Lead is genotoxic itself or it enhances the effect of other DNA-damaging agents. Generally, at high Pb^{2+} doses the antioxidative capacity of plant systems is overcame and the generated ROS can combine with DNA, determining unreliable inter-cross connections and duplications in DNA which result in chromosome aberrations. Pb^{2+}-induced clastogenic effects can cause cell death due to the blocking of genome repair enzymes; the cell cannot recover from the produced damage and thus the number of aberrations increases as a new cycle begins.

Although lead is considered to be rather a co-mutagen or a weak genotoxic agent, our results reflect a considerable genotoxic potential of this metal (Fig. 4), fact confirmed too for other species (Wierzbicka, 1999; Mansour and Kamel, 2005; Glinska *et al.*, 2007). The clastogenic effect was materialized in induction of an important number of ana-telophase chromosome aberrations in wheat root meristems for both compounds and at all tested concentrations. This increase may be explained by preponderant accumulation of lead in plant roots - about 90% of

Pb^{2+} is accumulated in roots, only a small fraction of lead being translocated upward to the shoots and other plant parts (Patra *et al*., 2004; Gichner *et al*., 2008; Jiang and Liu, 2010).

In this study, Pb^{2+} concentration is the same in the two compounds, at correspondent variants (Table 1), but the effect amplitude is different. In lead acetate, the frequency increase is moderate (1.1 – 1.7 times higher than control), whereas in lead nitrate variants the genotoxic effect is higher - the frequency of chromosome aberrations observed in mitotic ana-telophases exceeds 1.7 – 2.0 times the control value. For lead acetate, the 25 µM-treated variant exhibits the highest percentage of ana-telophase aberrations (21.11 %), while for lead nitrate the 50 µM-treated variant shows the most numerous chromosome aberrations (24.86 %). 100 µM lead nitrate variant has 3.06 times more complex aberrations so confirming the strong genotoxic effect of this heavy metal; 10 µM lead acetate variant also shows a percentage of complex aberrations 2.0 times higher than control.

A variety of chromosome abnormalities was registered in wheat root tips (Fig. 5), fact indicating the damage amplitude at chromosome level: laggards, bridges, multipolar A-T, A-T with expelled chromosomes as well as an important number of complex aberrations (A-T with bridges and laggards, A-T with bridges and expelled chromosomes, multipolar A-T with chromosome bridges and expelled chromosomes, A-T with expelled and lagging chromosomes etc). In all variants, the highest preponderance belongs to chromosome bridges followed by laggards. This is a proof that lead alters the normal function of mitotic spindle, so that the chromosome movement to the cell poles is disturbed. Laggards are a potential source of aneuploidy because they lost the ability to attach by spindle fibres; they do not participate to the normal division and cause genetic disequilibriums between daughter cells. The complex chromosome aberrations have more severe repercussions at genetic level and on subsequent plant growth and development.

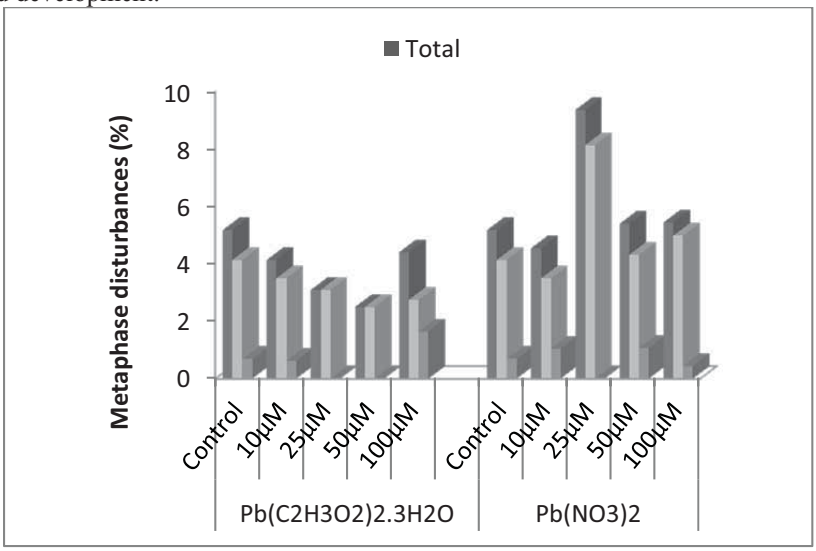


Fig. 3 Percentual preponderance of main types of metaphase disturbances in the total of abnormal metaphases, after lead treatment.

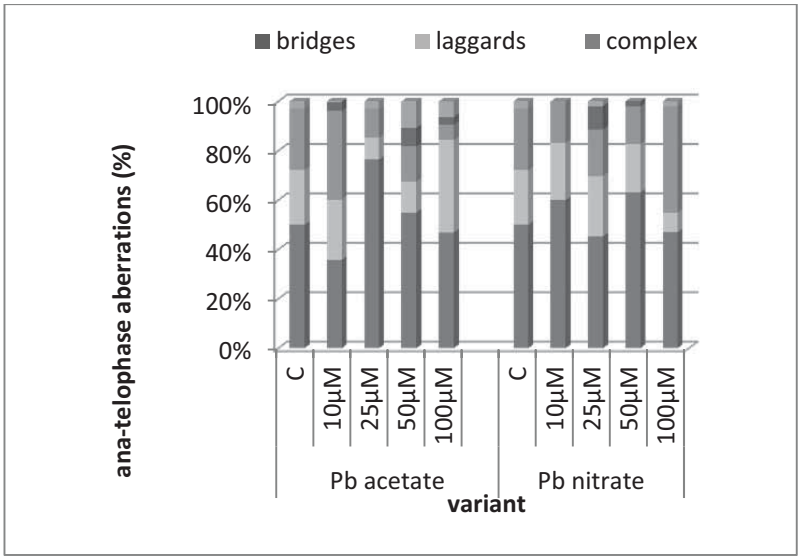


Fig. 4 Percentual preponderance of main types of ana-telophase chromosome aberrations in the total of aberrant ana-telophases (considered 100 %), after lead treatment.

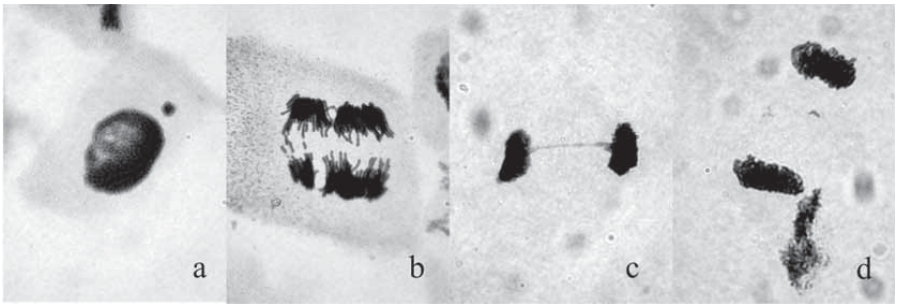


Fig. 5 Types of mitotic disturbances induced by lead in wheat root tip meristems. a. interphase with micronucleus (lead nitrate, 25 µM); b. tetrapolar anaphase with laggards (lead nitrate, 25 µM); c. telophase with bridge (lead nitrate, 10 µM); d. telophase with laggard (lead acetate, 10 µM).

Out of main categories of chromosome aberrations, micronuclei (1.43% in 50 µM Pb^{2+} acetate; 1.62% in 50 µM Pb^{2+} nitrate) (Fig. 5a), fragments, and polar deviations (in the variants treated with 50 and 100 µM Pb^{2+} acetate) represented other mitotic irregularities encountered in our study, but not at significant values. Insufficiently condensed chromatin material was observed in 50 µM Pb^{2+} acetate, 50 µM and 100 µM Pb^{2+} nitrate. Mainly in variants treated with lead nitrate lysis zones of chromatin material and dissolution of chromosomes have been evidenced.

Based on obtained results, we can conclude that lead nitrate was more clastogenic than lead acetate but the aberration frequency was not concentration dependent. If some literature data establish a gradual increment of chromosome aberrations with lead concentration increase (Kumar and Tripathi, 2008, in grass pea), in other approaches a Pb^{2+} dose-dependent relation was described only at small concentrations of metal, whereas at higher Pb^{2+} concentrations a

significant decrease of DNA damage was observed such as in lupin after Pb^{2+} nitrate treatment (Rucinska *et al.,* 2004) or in Pb^{2+} acetate treated tobacco plants (Gichner *et al*., 2008).

Amplitude of lead-induced genotoxic effects depends on metal oxidizing state, exposure duration, the plant parts used for exposure, metal concentration, pH of solution, lead compound type, plant species, features of chromosome set (Patra *et al*., 2004; Azmat and Haider, 2007). The pathways of lead genotoxicity may involve the interaction of Pb^{2+} with DNA, either directly or indirectly *via* oxidative stress, but the mechanism of this interaction is not fully understood (Cenkci *et al.*, 2010).

According to some opinions, the chemical form of lead affects only transport of the heavy metal from medium into the plants and all forms had similar effects on mitosis (Patra et al., 2004). In plant systems *in vivo*, water solubility of the salt is of primary importance. The degree of dissociation and the availability of cations influence the aberration number. The lead compounds showing lower water solubility have greater toxic and mutagenic effects than those moderately soluble perhaps because the more soluble compounds dissolve completely in the solution and are supplied as ions, rather than molecules as in the cases of those low soluble (Radecki *et al.*, 1989). Although the water solubility of lead nitrate and lead acetate trihydrate, at 20ºC, is not strongly different [52 g/100 ml, for $Pb(NO_3)_2$; approximately 45 g/100 ml, for $Pb(C_2H_3O_2)_2.3H_2O$] (Hilber *et al*., 2001), and contrary to the opinions considering lead nitrate as a weak mutagen, the effects of lead nitrate concerning the rate of induced chromosome aberrations are more pronounced in our study than those induced by lead acetate. The high genotoxic effect of lead nitrate was also evidenced in other species by molecular studies using multiple biomarker systems such as random amplified polymorphic DNA (RAPD) profiles. These markers indicate that genomic template stability was significantly affected at all Pb^{2+} concentrations (Cenkci *et al*., 2010), in *Brassica rapa* L. Lead acetate is very toxic but the published results are inconsistent concerning its mutagen, clastogen and carcinogen effects.

In this study, heterogeneous responses have been obtained concerning the behaviour of cytogenetic parameters. Although the problem of Pb^{2+}-induced mutagen effects remains in discussion, many authors confirmed the genotoxic potential of this heavy metal, expressed in occurrence of chromosome aberrations and other mitotic disturbances, and their persistence during next generations. However, few works reported production of viable plants carrying lead-induced chromosome abnormalities (Kumar and Tripathi, 2008).

CONCLUSIONS

Pb^{2+} treatment caused mitosis disturbances in root meristematic cells of wheat seedlings, expressed mainly in decrease of mitotic indices and changes in preponderance of division phases. The occurrence of many chromosomal aberrations in all Pb^{2+} treated variants clearly indicates that this heavy metal has genotoxic effects in root meristems of wheat seedlings.

Lead nitrate shows a more pronounced genotoxic potential than lead acetate trihydrate in the studied wheat cultivar.

It was not established a direct relationship between Pb^{2+} concentration and aberration frequency.

REFERENCES

Akinci, I. E., Akinci, S., Yilmaz, K., (2010): *Response of tomato (Solanum lycopersicum L.) to lead toxicity: Growth, element uptake, chlorophyll and water content*. Afr. J. Agric. Res., 5(6), 416-423.

Azmat, R., Haider, S., 2007. *Pb stress phytochemistry of seedlings of Phaseolus vulgaris and Lens culinaris.* Asian J. Plant Sci., 6(2), 332-337.

Babior, B. M, (1997): *Superoxide: a two-edged sword*. Braz. J. Med. Biol. Res., 30(2): 141-145.

Carruyo, I., Fernández, Y., Marcano, L., Montiel, X., Torrealba, Z., (2008): Correlation of toxicity with lead content in root tip cells (*Allium cepa* L.). Biol. Trace Elem. Res., 125(3), 276-285.

Cenkci, S., Ciğerci. I. H., Yildiz, M., Özay, C., Bozdağ, A., Terzi, H., (2010): *Lead contamination reduces chlorophyll biosynthesis and genomic template stability in Brassica rapa L*. Environ. Exp. Bot., 67(3), 467–473.

Chakravarty, B., Srivastava, S., (1992): *Toxicity of some heavy metals in vivo and in vitro in Helianthus annuus*. Mutat. Res., 283(4), 287-294.

Gichner, T., Žnidar, I., Száková, J., (2008): *Evaluation of DNA damage and mutagenicity induced by lead in tobacco plants*. Mutat. Res., 652: 186–190.

Glińska, S., Bartczak, M., Oleksiak, S., Wolska, A., Gabara, B., Posmyk, M., Janas, K., (2007). *Effects of anthocyanin-rich extract from red cabbage leaves on meristematic cells of Allium cepa L. roots treated with heavy metals*. Ecotox. Environ. Safe, 68(3), 343-350.

Hilber, T., Marr, R., Siebenhofer, M., Zapfel, W., (2001): *Solid/liquid extraction of zinc from EAF-dust.* Separ. Sci. Technol., 36(5-6), 1323 — 1333.

Jiang, W., Liu, D., (2010): *Pb-induced cellular defense system in the root meristematic cells of Allium sativum L.* BMC Plant Biol., 10, 40-47.

Kaznina, N. M., Laidinen, G. F., Titov, A. F., Talanov, A. V., (2005): *Effect of lead on the photosynthetic apparatus of annual grasses*. Biol. Bull., 32(2): 147–150.

Kumar, G., Tripathi, R., (2008): *Lead-induced cytotoxicity and mutagenicity in grass pea*. Turk. J. Biol., 32, 73-78.

Liu, D., Xue, P., Meng, Q., Zou, J., Gu, J., Jiang, W., 2009. *Pb/Cu effects on the organization of microtubule cytoskeleton in interphase and mitotic cells of Allium sativum L*. Plant Cell Rep., 28, 695-702.

Maitra, P., Mukherji, S., (1977): *Effect of lead on nucleic acid and protein contents of rice seedlings and its interaction with IAA and GA_3 in different plant systems*. Indian J. Exp. Biol., 17, 29-31.

Mansour, M. M., Kamel, E. A. R., (2005): *Interactive effect of heavy metals and gibberellic acid on mitotic activity and some metabolic changes of Vicia faba L. plants*. Cytologia, 70(3): 275-282.

Patra, M., Bhowmik, N., Bandopadhyay, B., Sharma, A., (2004): *Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance*, Environ. Exp. Bot., 2(3), 199-223.

Pinero, H. J. L., Maiti, R. K., Star, M. J. V., Diaz, G. G., Onzalez, A. N., Avila, M. L. C., Orough-Bakhch, R., (2002): *Effect of Pb and Cd on seedling growth, chlorophyll and protein content of common bean (Phaseolus vulgaris L.), alfalfa (Medicago sativa), avena (Avena sativa) and ryegrass (Lolium multiflorum) selected as hyper accumulator of heavy metal*. Crop Res., 3(3), 473-480.

Radecki, J., Banaszkiewicz, T., Klasa, A., (1989): *The effect of different lead compounds on mitotic activity of maize root tip cells*. Acta Physiol. Plant., 11(2), 125–130.

Rathore, H., Punyasi, R., Joshi, P., Rathore, D., Bhatnagar, D., (2007): *Studies on the reversal of lead induced mitostatic effect in Allium cepa root tip cells with myrobalan (fruit of Terminalia chebula, Retz, Combretaceae)*. Internet J. Altern. Med., 4(1), *http://www.ispub.com/ostia/*.

Rucińska, R., Sobkowiak, R., Gwóźdź, E. A., (2004): *Genotoxicity of lead in lupin root cells as evaluated by the comet assay*. Cell. Mol. Biol. Lett., 9, 519-528.

Samardakiewicz, S., Woźny, A., (2005): *Cell division in Lemna minor roots treated with lead.* Aquat. Bot., 83(4), 289-295.

Stefanov, K., Seizova, K., Popova, I., Petkov, V. L., Kimenov, G., Popov, S., (1995): *Effects of lead ions on the phospholipid composition in leaves of Zea mays and Phaseolus vulgaris*. J. Plant Physiol. 147, 243–246.

Wierzbicka, M., (1999): *The effect of lead on the cell cycle in the root meristem of Allium cepa L*. Protoplasma, 207, 186–194.

Wozny, A., Jerczynska, E. (1991): *The effect of lead on early stages of Phaseolus vulgaris L. growth in in vitro conditions*. Biol. Plantarum, 33(1), 32-39.

1 – Biological Research Institute, Lascar Catargi 47, 700107 Iaşi, Romania
2 – University "Alexandru Ioan Cuza", Faculty of Biology, Carol I Bd. 20A, 700506 Iaşi, Romania
* - *trutaelena@yahoo.com*

Acknowledgements. This study was realized in the framework of project BIODIV 09-36-301/2009 from National Research Program of Romania - National Authority for Scientific Research.

IMPLICATIONS OF VARIOUS INTENSITIES OF RADIO FREQUENCY ELECTROMAGNETIC RADIATION (462 MHZ) IN THE INDUCTION OF OXIDATIVE STRESS DURING THE GERMINATION OF HIPPOPHAE RHAMNOIDES SEEDS

CĂLIN LUCIAN MANIU[1*], EUGEN UNGUREANU[1]

Keywords: electromagnetic field, oxidative stress, reactive oxygen species, superoxide dismutase, catalase, peroxidase.
Abstract. The data accumulated by now shows that the topic of biological effects of electromagnetic radiation is far from being exhausted. Is undoubtedly that a non-ionizing radiation field maintained on a biological entity has some effects on it. To try shaping issues regarding this, this work aims to study the impact of radiation generated by an emission-reception radio station that emits on 462.6875 MHz frequency. For this purpose, were used *Hippophae rhamnoides* L. seeds which germinated in the laboratory, under controlled conditions, concentrically arranged around the radiation source, in which case electromagnetic radiation has a different impact. Seed germination lasted 35 days, while the device has continuously worked, and the seeds were constantly irradiated. It was precisely measured the intensity of the magnetic component of the field in all places where the seeds were placed for germination. It was calculated the percentage of germination and it was determined the enzyme activity involved in eliminating the oxidative stress effects. It was found significant variations of the parameters mentioned above in conjunction with the radiation intensity depending on the distance from the source.

INTRODUCTION

Accelerated and widespread use of different electric and electronic devices increased the exposure to radio and microwave frequency electromagnetic fields (EMFs). These EMFs are classified as non ionizing radiation but they can cause damage depending on the power level, frequency, and the properties of exposed tissue. There is some evidence that microwaves (300 MHz–300 GHz) produces changes in the cell membrane’s permeability and cell growth rate as well as interference with ions and organic molecules, like proteins (Kwee et al., 1998, 2001; de Pomerai et al., 2003; Repacholi, 2001; Pologea-Moraru et al., 2002; Banik et al., 2003). Plants are essential components of a healthy ecosystem and have important role in the living world as main primary producers of food and oxygen; therefore it would be beneficial to investigate their interaction with today’s increased exposure to radio and microwave frequency fields. Additionally, higher plants are useful test organisms for environmental studies because they are eukaryotic multicellular organisms. Many of them are sensitive to different kinds of stresses and are easy to grow in controlled laboratory conditions without too much expense (Wang, 1991). During the years it became more and more interesting to test the effects of EMFs on higher plants (Tkalec et al. 2005, 2007). Considering the increasing interest for the subject, this work focus on the influence of 462.6875 Mhz EMF on the oxidative stress during the *Hippophae rhamnoides* seeds germination. This species was chosen because of the following aspects. Firstly, the period of germination is relatively long, the experiment is held over a period of 35 days, this issue was important because the seeds were irradiated for a long time, unlike other species that germinate very fast (3-5 days). Secondly, sea buckthorn (*Hippophae rhamnoides* L.) is a species which has some interesting biochemical characteristics: vitamins B, C, E, K, carotenoids (the most dominant carotenoid in sea buckthorn, it’s admitted to be associated with reduced risk of breast, stomach, esophageal, and pancreatic cancers), flavonoids (it have been found in controlling arteriosclerosis, reducing cholesterol level, turning hyperthyroidism into euthyroidism and eliminating inflammation), tannins, metallothionein (acts as detoxifying agency for heavy metals and as free radical scavenger for most toxic radical) and 5-hydroxytryptamine (5-HT), a chemical neurotransmitter substances (Lian, 2000; Thomas, 2003).

MATERIALS AND METHODS

To seek evidence of the influence of electromagnetic field (EMF) of radio frequency on oxidative stress, during the germination of seeds, was used a source consisting of two Motorola T5725 emission-reception radio stations that have been programmed to automatically call one another throughout experiment. The communication system frequency is set on channel 6 at 462.6875 MHz with 500mW transmit power. Thus, around the two emission-reception radio stations were delimitated four concentrically levels (different distances from the source), with four groups with five Petri Dishes (A1-A5; B1-B5; C1-C5; D1-D5), in each plate with about 20 seeds. Experiment diagram is depicted in the fig.1 and fig.2.

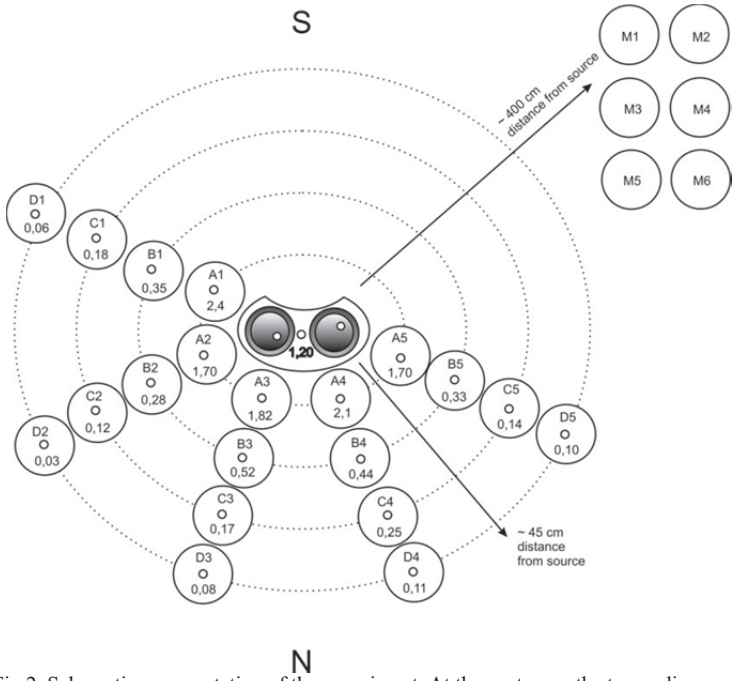


Fig.2. Schematic representation of the experiment. At the center are the two radio stations, around which were arranged the Petri Dishes. In the center of each plate is indicated the magnetic induction in μT.

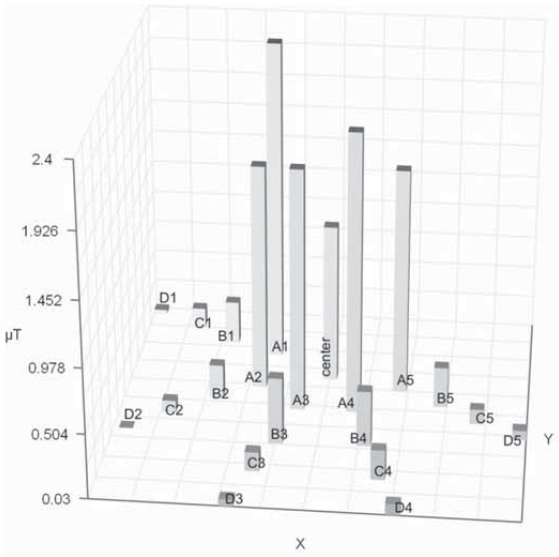


Fig.1. Spatial representation of the intensity of magnetic induction (μT) in relation to the probes arrangement.

The control lots, consisted in six Petri Dishes (M1-M6), were positioned sufficiently far from the EMF source. It was monitored the temperature and the humidity, which were maintained constant in both irradiated and control lots. The magnetic induction (B) of the field was measured with a digital teslameter in the indicated points on the drawing. The values are in μT. After germination period (35 days), the plant material was processed to determine the activity of the superoxide dismutase (SOD), catalase and peroxidase, enzymes involved in the removal of oxidative stress, (Artenie et al., 2008). Also it was determined the total protein synthesis and was calculated the percentage number of the germinated seeds. From each sample was counted the number of germinated seeds and reported to the total number of seeds. Data were represented graphically in the diagrams at the end of the paper, which appear after the statistical processing. On the charts, the vertical error bars shows the 95% (0.05) confidence level for mean. Interval estimates are often desirable because the estimate of the mean varies from sample to sample. The interval estimate gives an indication of how much uncertainty there is in our estimate of the true mean. The narrower the interval, the more precise is our estimate (Kotz et al., 1988-2008).

RESULTS AND DISCUSSION

After investigations, it was obtained a number of results regarding the catalytic activity of SOD, catalase and peroxidase. In mitochondrial electron transport, the participation of flavins, ubisemiquinones, and other electron carriers leads to the formation of superoxide radicals, H_2O_2, and hydroxyl radicals (summarized as ROS, **r**eactive **o**xygen **s**pecies) as by-products. These by-products cause severe cell damage. Since the formation of ROS is especially high, when the components of the respiratory chain are highly reduced, there is a necessity to avoid an over-reduction of the respiratory chain. As regards, the activity of SOD, there was a significant increase in the activity at the A1-A5 and C1-C5 samples, in comparison with the control samples. In case of B1-B5 and D1-D5 samples, it was found no significant differences in relation to the control. It is possible that these variations are due to interference between EMF and the normal light reactions in photosynthesis and electron transport chain, the sequence of reactions is known as generating superoxide ions (O_2^-) (Heldt, 2005; Schulze, Beck, Müler-Hohenstein, 2005). In comparison with the magnetic field induction intensity, it was observed that there is no direct correlation between the induction and enzyme activity. In case of C1-C5 was founded that a small amount of induction caused a significant increase in enzymatic activity comparative to the control as to the A1-A5 lots, undergo on a much higher intensity, being the nearest from the radiation source. The results are presented in the fig 3.

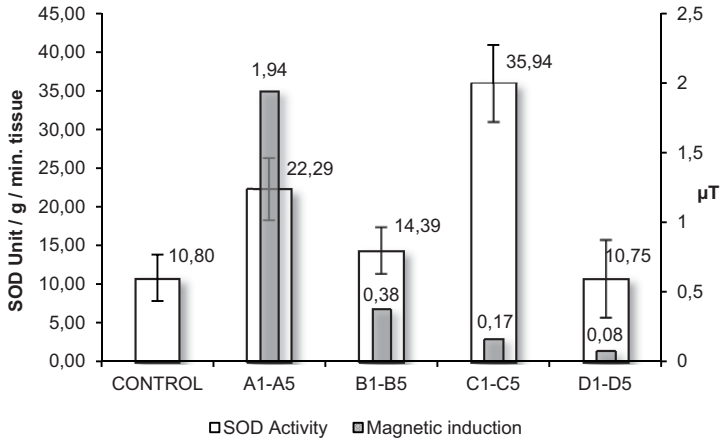


Fig.3. Variations of the SOD activity and magnetic induction.

Catalase is an enzyme present in large quantity in peroxisomes where neutralize H_2O_2 resulting from redox processes. Peroxisomes are a common constituent of eukaryotic cells. In plants there are two important differentiated forms: the leaf peroxisomes, which participate in photorespiration and the glyoxysomes, which are present in seeds containing oils (triacylglycerols) and play a role in the conversion of triacylglycerols to carbohydrates. They contain all the enzymes for fatty acid β-oxidation. (Heldt, 2005). The experiment conducted, shows a significant decrease in the catalase activity in relation to the control (Maniu et al., 2009). The profile of these decrease is similar in both activity expressed in enzyme units at 100g material and for specific activity (enzyme units per 100 mg protein) as is illustrated by the fig. 4 and fig. 5.

Peroxidase, unlike the other two, is an enzyme widespread in all cellular compartments (Bakalovic et al, 2006; Passardi et al., 2007; Koua et al., 2008) where the function is to neutralize hydrogen peroxide using various electron donors (Heldt, 2005). The experiment conducted has shown that activity of this enzyme undergoes major fluctuations, as can be seen in fig. 6 and fig. 7. It might say that this pattern of fluctuation could be due to influences exerted by the different EMF from a cellular compartment to another, which is to have different amounts of enzyme (Ungureanu et al., 2009).

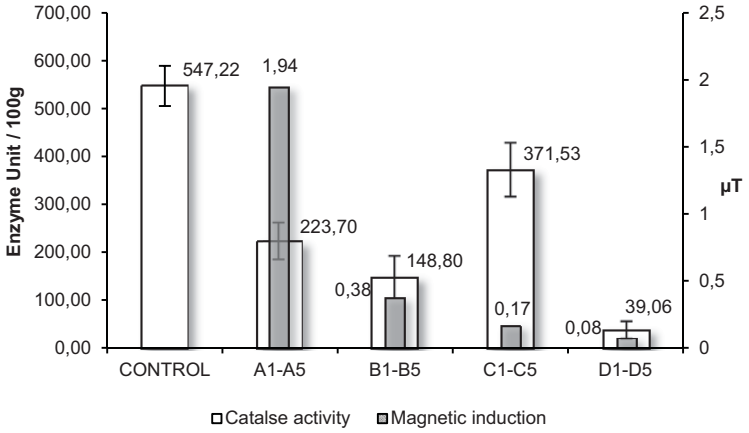


Fig.4. Variation of the catalase activity and magnetic induction.

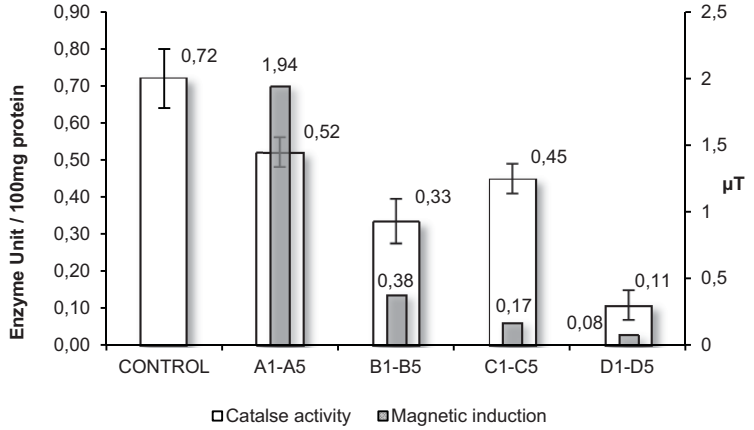


Fig.5. Variation of the catalase specific activity and magnetic induction.

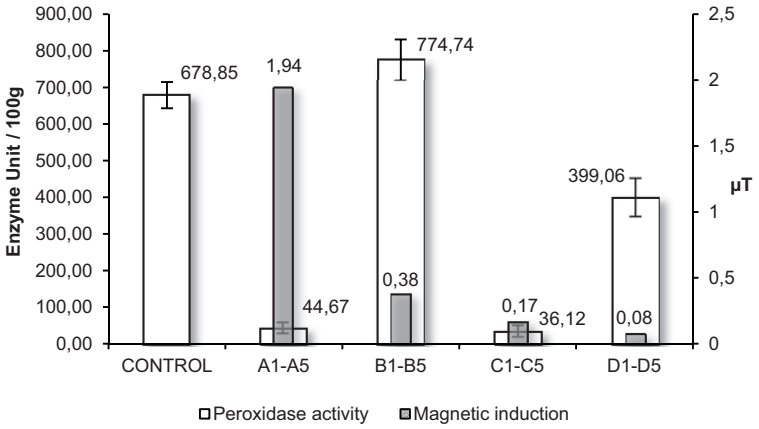


Fig.6. Variation of the peroxidase activity and magnetic induction

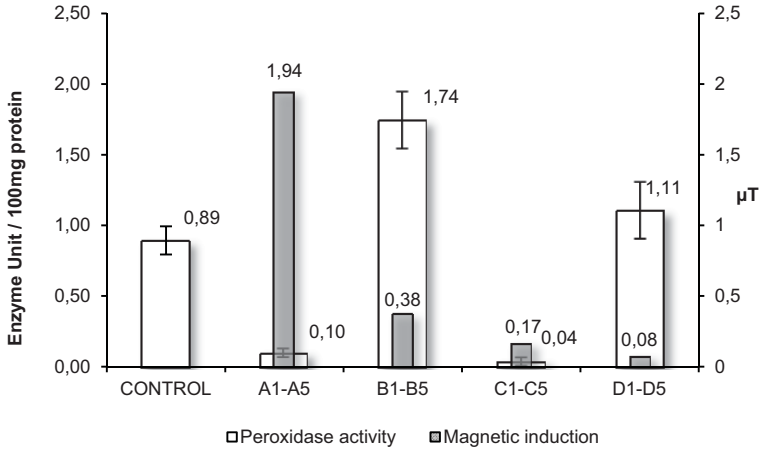


Fig.7. Variation of the peroxidase specific activity and magnetic induction.

The amount of protein highlighted by Bradford method shows a significant variation for A1-A5, B1-B5 and D1-D5 in relation to the control (fig. 8), in such cases were founded decreases. C1-C5 probes, shows an amount of protein approximately equal to control lots. Proteins highlighted in the experiment come both from the reserve proteins in seeds and "de novo" synthesis protein necessary seedlings, especially towards the end of germination.

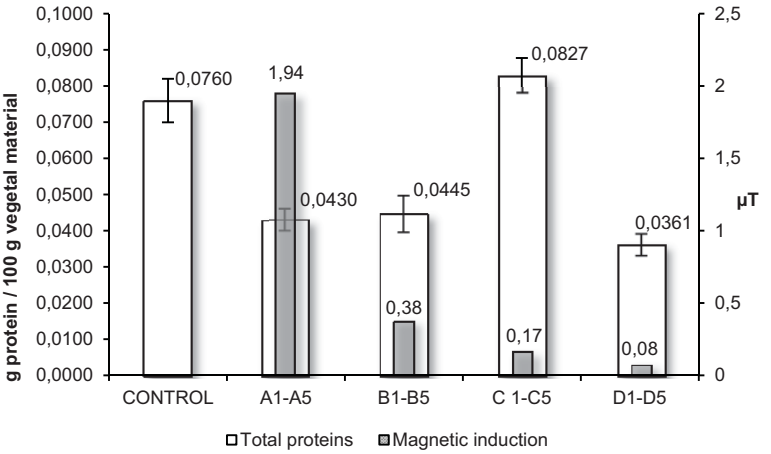


Fig.8. Variation of the total protein quantity and magnetic induction.

The percentage of seeds germinated (fig. 9) during the experiment, indicates that low intensity magnetic induction can have a stimulating effect. This is observed for samples B1-B5 and D1-D5, where the percentage of germination reached very high values, 97% respectively 94%. In the other two cases, compared with the control, there is a negative trend in germination. In case of A1-A5, high intensity magnetic induction does not seem to have affected germination

(percentage difference being only 8% compared to control), where C1-C5 can be considered a decrease by 19%.

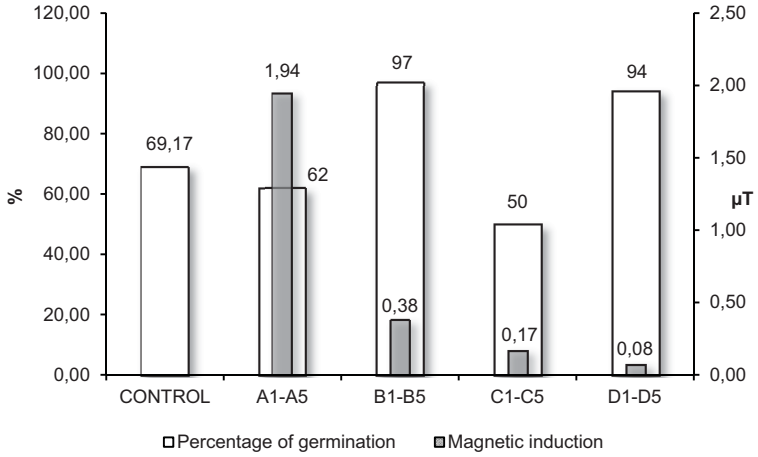


Fig.9. The percentage of seeds germination and the variation of magnetic induction

From germination behavior of toward various intensities of magnetic induction, it may find a correlation between enzyme activities involved in removing the effects of oxidative stress. In cases B1-B5 and D1-D5, a high percentage of germination is correlated with a decreased oxidative stress, due to a weak catalase activity and a normal SOD activity, correlates with a low amount of total protein. Low values identified in the total proteins quantity may be due to a greater consumption of resources during germination. Since peroxidase takes part to other processes, very high activity in cases B1-B5 and D1-D5 in consistency with high rates of germination may be due to the involvement of this enzyme into other metabolic processes closely related to germination and growth (Atak et al., 2007). From this perspective, the low activities recorded in samples A1-A5 and C1-C5 cannot be attributed to the direct effect of EMF. On the other hand, cases A1-A5 and C1-C5, recorded lower rates germination, in line with a higher oxidative stress, indicated by a significant increase in SOD activity. In these cases, catalase activity although lower than the control, is significantly higher than in cases B1-B5 and D1-D5, which shows clearly that it took part in countering the stress in close correlation with SOD.

CONCLUSIONS

The performed experiment, with 462.6875 MHz electromagnetic radiation frequency, obtained from two emission-reception radio stations, has demonstrated that there is no direct correlation between the intensity of induction and the effects caused by the different magnetic induction during the seeds germination. Thus, there are cases where electromagnetic radiation may be used as a stimulating agent since two cases were found with a very high percentage of germination in correlation with a normal SOD activity, low catalase activity and low total protein amount. In these circumstances, it is required more detailed investigations, particularly targeted on that induction values that caused the stimulation of germination.

REFERENCES

Artenie V., Ungureanu E., Negură Anca, Metode de investigare a metabolismului glucidic şi lipidic, Ed. Pim, Iaşi, 2008.
Atak Ç., Çelik Ö., Olgun A., Alikamanoğlu S., Rzakoulieva A., *Effect of magnetic field on peroxidase activities of soybean tissue culture*, Biotechnol. & Biotechnol. Eq. 21/2007/2.
Bakalovic N, Passardi F, Ioannidis V, Cosio C, Penel C, Falquet L, Dunand C., PeroxiBase: a class III plant peroxidase database, Phytochemistry 2006 Mar; 67(6):534-9.
Banik S, Bandyopadhyay S, Ganguly S., Bioeffects of microwave. A brief review. Bioresource Technol, 2003, 87:155–159.
de Pomerai DI, Daniells C, David H, Allan J, Duce I, Mutwakil M, Thomas D, Sewell P, Tattersall J, Jones D, Peter E, Candido M., Microwave radiation can alter protein conformation without bulk heating. FEBS 2003, 543:93–97.
Heldt H.W., Plant Biochemistry 3rd ed., Elsevier Academic Press, 2005.
Kotz, S., Campbell B. Read, N. Balakrishnan, Brani Vidakovic, (Ed. & Co-Ed.), Encyclopedia of Statistical Sciences, vol. 1-9, plus supplements. New York: Wiley-Interscience, 1988-2008.
Koua D, Cerutti L, Falquet L, Sigrist CJ, Theiler G, Hulo N, Dunand C., PeroxiBase: a database with new tools for peroxidase family classification, Nucleic Acids Res. 2008 Oct 23.
Kwee S, Raskmark P, Velizarov S., Changes in cellular proteins due to environmental non-ionizing radiation. I. Heatshock proteins. Electro Magnetobiol 2001, 20:141–152.
KWEE S, RASKMARK P., Changes in cell proliferation due to environmental non-ionizing radiation II. Microwave radiation. Bioelectrochem Bioenerg, 1998, 44:251–255.
Lian Yongshan (Editor-in-chief), Biology and Chemistry of Hippophae. L., Gansu Science & Technology Press, 2000.
Maniu C.L., Ungureanu E., Vântu Smaranda, Creţescu I., Preliminary study concerning the radiofrequency electromagnetic field influence on the catalase activity in the Hippophae rhamnoides seeds germination. Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza" Iaşi, (S.N.) Genetică şi Biologie Moleculară, 2009, Tom. X.
Passardi F, Theiler G, Zamocky M, Cosio C, Rouhier N, Teixera F, Margis-Pinheiro M, Ioannidis V, Penel C, Falquet L, Dunand C., 2007, PeroxiBase: The peroxidase database, Phytochemistry 2007 June;68(12):1605-11.
Pologea-Moraru R, Kovacs E, Iliescu KR, Calota V, Sajin G., The effects of low level microwaves on the fluidity of photoreceptor cell membrane. Bioelectrochemistry, 2002, 56:223–225.
Repacholi MH., Health risk from the use of mobile phones. Toxicol Lett, 2001, 120:323–331.
Schulze E.D., Beck E., Müler-Hohenstein K., Plant Ecology, Springer Berlin – Heidelberg, 2005.
Thomas S.C. Li, Thomas H.J. Beveridge, Sea buckthorn (Hippophae rhamnoides L.): production and utilization, NRC Research Press, Ottawa 2003.
Tkalec, M.; Malaric, K. & Pevalek-Kozlina, B., Influence of 400, 900, and 1900MHz Electromagnetic Fields on Lemna minor Growth and Peroxidase Activity Bioelectromagnetics, 2005, 26, 185-193
Tkalec, M.; Malaric, K. & Pevalek-Kozlina, B., Exposure to radiofrequency radiation induces oxidative stress in duckweed Lemna minor L. Science of the Total Environment, 2007, 338, 78-89
Ungureanu E., Maniu C.L., Vântu Smaranda, Creţescu I., Consideration on the peroxidase activity during Hippophae rhamnoides seeds germination exposed to radiofrequency electromagnetic field influence. Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza" Iaşi, (S.N.) Genetică şi Biologie Moleculară, 2009, Tom. X.
Wang W., Literature review on duckweed toxicity testing. Environ Res, 1991, 52:7–22.
Xia L, Guo J., Effect of magnetic field on peroxidase activity and isozyme in Leymus chinensis. Ying Yong Sheng Tai Xue Bao (The journal of applied ecology), 2000, 11:699–702.
Xiujuan W, Bochu W, Yi J, Defang L, Chuanren D, Xiaocheng Y, Sakanishi A, Effects of sound stimulation on protective enzyme activities and peroxidase isoenzymes of chrysanthemum. Colloid Surface B 27:59–63, 2003.

1"Alexandru Ioan Cuza" University, Faculty of Biology, Address: 20A Blvd. Carol I; 700505; Iaşi, Romania
* cmaniu@uaic.ro

THE ACTIVITY OF SOME OXIDOREDUCTASES IN HORDEUM *VULGARE* L. PLANTS TREATED WITH ETHYL–METHANE-SULFONATE AND *ROSMARINUS OFFICINALIS* L. HYDRO-ALCOHOLIC EXTRACTS

IONELA DACIANA MIERLICI[1]*, ELENA CIORNEA[2], GOGU GHIORGHIŢĂ[3]

Keywords: barley, hydro-alcoholic rosemary extract, ethyl-methane-sulfonate, superoxid-dismutase, catalase, peroxidase

Abstract: This paper focuses on the activity of some oxidoreductases (catalase, peroxidase, superoxide-dismutase) in barley seedlings (*Hordeum vulgare* L.) after 6 hours of seeds treatment with different concentrations (0,01 – 0,50%) of ethyl-methane-sulfonate and 12 hours with hydro-alcoholic 0,5% rosemary (*Rosmarinus officinalis* L.) extract (EHR). The EMS treatments led to an obvious increase of the superoxide dismutase, catalase and peroxidase activity in plants, while the application of the hydro-alcoholic rosemary extract, after the EMS treatment, led to a significant decrease of the activities of these enzymes, since the rosemary extract has an obvious antioxidant effect.

INTRODUCTION

In order to counteract the oxidative stress, the plants have developed intracellular defense strategies. These strategies are represented by an enzymatic and a non enzymatic antioxidant system. The non enzymatic system includes ascorbic acid, ά-tocopherol, carotenes, polyphenols, flavones and the enzymatic system includes superoxide dismutase, catalase, peroxidase, ascorbate oxidase, glutathione reductase and polyphenol oxidase. The function of these antioxidant systems relies in the prevention of formation or in the destruction of toxic radicals formed during the oxidative stress, thus ensuring the survival of plants in improper conditions.

In the last decades there has been a great interest in emphasizing the antioxidant properties of some medicinal and aromatic plants. The antioxidant effect of some aromatic plants would be the result of the presence of the hydroxyl groups from the phenol compounds, (Shahididi and Wanasundara, quoted by Faixova and Faix, 2008). Among the antioxidant compounds, the polyphenols and the flavones represent the object of various plant studies (Blaschek et al., 2007; Wichtl, 2009; Hasani-Ranjbar et al., 2009).

Some Lamiaceae species, such as rosemary, oregano, sage and others, have a strong antioxidant effect, (Dragland et al., 2003; Wang S. Y., 2003). The antioxidant effect of some rosemary active principles has been proven in various studies concerning the volatile oils, phenol compounds, flavonoides and diterpenes (Armatu et al., 2010; Papageorgiou et al., 2008; Stefanovits-Banyai et al., 2003; Schwarz and Ternes, 1992). Also, the use of the rosemary extracts in food industry is based exactly on their antioxidant effect that prevents the degradation of the food products containing fats (Cuvelier et al., 1996; Fadel and El-Massry, 2000; Schwarz and Ternes, 1992).

On the other hand, the ionizing radiations and some chemical mutagens (such as alkylating agents) lead to a strong oxidative stress in living organisms (Ghiorghiţă and Corneanu, 2002) by over producing of reactive oxygen species (ROS).

Considering these facts, our paper focuses on the capacity of hydro-alcoholic rosemary extracts (EHR) to decrease the oxidative stress induced to the barley plants by the treatment with ethyl-methane-sulfonate (EMS). In this respect, we investigated the activity of some oxidoreductases in barley seedlings after the treatment with EMS and EHR.

MATERIAL AND METHODS

To complete the experimental part, the barley seeds (*Hordeum vulgare* L.), *Mădălin* cultivar, have been treated for 6 hours with different concentrations (0,01%; 0,025%; 0,05%; 0,10%; 0,50%) of ethyl-methane-sulfonate solutions (EMS). After these treatments, the seeds were well washed in water, in order to remove the mutagen agent, and then treated for 12 hours with hydro alcoholic rosemary (*Rosmarinus officinalis* L.) extracts (EHR).

The rosemary alcoholic extract was obtained at cold, using the plant/solvent report of 1:7 and a concentration of ethanol of 70%. The barley seeds were then treated with a 0,5% diluted solution of the initial extract and, after the germination and the hydroponics cultivation, the 14 days old seedlings were submitted to enzymatic analyses. The activity of some oxidoreductases (superoxide-dismutase, peroxidase and catalase) was evaluated.

The determination of the superoxide-dismutase was done by the method of Winterbourn, Hawkins, Brian and Carrell, adapted by Vlad Artenie, the catalase activity by iodometric titration and the peroxidase by the ortho-dianisidine method (Artenie et al., 2008; Cojocaru et al., 2009). The results were statistically analyzed, a series of statistic indicators being calculated, such as average, standard error and deviation, variation coefficient, average safety coefficient, superior and inferior limits of the confidence intervals. The results of our investigations are presented in Table 1 – 6.

RESULTS AND DISCUSSIONS

It is well known that, in living organisms, the oxidative stress leads to the production of the reactive oxygen species with severe disruptive effect upon the cellular metabolism and the development of some processes that are often assigned to the alteration of the pattern of gene expression.

In plants, antioxidant enzymes such as superoxide-dismutase, peroxidase and catalase are seen as the „defensive team", playing the role of protecting the cells from the injuries caused by oxidative stress (Mittler, 2002). The increase of the activity of these enzymes represents the most common pathway that leads to the elimination of the reactive oxygen species.

The action of superoxide-dismutase (SOD) consists in converting the superoxide radical in hydrogen peroxide. In our investigations, we observed that the SOD activity obviously increased in barley plants after the single treatments with EMS, regardless the concentration, (Table1), reaching maximum values in case of the samples treated with 0,05 and 0,10% EMS (24,86 - 25,46 USOD/ml/min), as compared to 8,45 USOD/ml/min at control. At higher doses of the mutagen, (0,50%), the SOD activity slightly decreased (21,34 USOD/ml/min).

The intensification of the SOD activity suggests that the EMS has determined the increase of superoxide radicals (O2●-) concentration in the tissues of the barley plants. These radicals are strongly reactive and toxic and must be inactivated Since they cause severe oxidative degradations in the cells, the evolution of all aerobe organisms became dependant to the development of some effective defense mechanisms, meant to remove them.

Table 1. The activity of superoxide-dismutase in the barley seedlings from the EMS treated seeds

Variant	Medium activity (USOD/ml/min)	S $\bar{x}$	S (σ)	CV%	m%	LS	LI
Control plant	8,45	0,30	0,52	5,96	3,34	8,64	8,26
0,01% EMS	22,45	0,29	0,51	0,45	0,26	22,64	22,27
0,025% EMS	22,68	0,30	0,52	0,34	0,19	22,87	22,49
0,05% EMS	24,86	0,15	0,27	0,11	0,06	25,05	24,67
0,10% EMS	25,46	0,91	1,58	0,42	0,24	25,65	25,27
0,50% EMS	21,34	0,21	0,37	3,11	1,79	21,53	21,15

S $\bar{x}$ = average standard error, S (σ) = standard deviation, CV% = average variation coefficient, m% = average precision coefficient, LS = superior limit of the coefficient interval, LI = inferior limit of the confidence interval

Table 2. The activity of superoxide-dismutase in the barley seedlings from the EMS and EHR treated seeds

Variant	Medium activity (USOD/ml/min)	S $\bar{x}$	S (σ)	CV%	m%	LS	LI
Control plant	8,45	0,30	0,52	5,96	3,34	8,64	8,26
0,01% EMS +EHR	9,52	0,26	0,46	4,46	2,57	11,53	7,50
0,025% EMS +EHR	9,45	0,12	0,21	3,14	1,81	11,47	7,44
0,05% EMS +EHR	10,34	0,34	0,59	0,53	0,31	12,36	8,33
0,10% EMS + EHR	10,25	0,15	0,27	0,15	0,09	12,27	8,24
0,50% EMS +EHR	9,23	0,55	0,95	0,22	0,13	11,25	7,22

S $\overline{x}$ = average standard error, S (σ) = standard deviation, CV% = average variation coefficient, m% = average precision coefficient, LS = superior limit of the coefficient interval, LI = inferior limit of the confidence interval

The hydro-alcoholic rosemary extract (EHR) added after the mutagen treatments determined a normalization of the SOD activity in case of all the treatment variants, which makes us appreciate that the EHR had a clear antioxidant effect. The average intensity of the SOD activity varied in case of the combined treatments (EMS + EHR) between 9,23 and 10,34 USOD/ml/min, compared to 8,45 USOD/ml/min in control plants, (Table 2). The role of SOD in the cells was probably taken in this case by other defense mechanisms against superoxide radicals.

For each treatment variant, three parallel determinations were made and the results were statistically analyzed. Thus, the average, the standard error and deviation, the average and safety coefficient there were calculated, as well as the limits of the confidence intervals that, as the values show, are pretty close, which makes us conclude we have kept constant the extraction and determination conditions.

Another objective of this study was the determination of the catalase activity in plants, considering the fact that the value of this biochemical parameter also changes in the oxidative stress.

The results show an increase of the catalase activity in the barley plants after single EMS treatments, which suggests the presence of important amounts of hydrogen peroxide in the plants.

The activity of catalase is 2-3 times bigger than in the untreated plants (8,54 mg H_2O_2/g/min). Although different EMS concentrations (0,01-0,5% EMS) have been used, the differences in the catalase activity are not significant, varying between 20,37 mg H_2O_2/g/min (the sample treated with 0,05% EMS) and 26,69 mg H_2O_2/g/min (the sample treated with 0,50% EMS), (Table 3).

Generally, at high concentrations of mutagen solutions (0,05 - 0,50%), we identified a high catalase activity (24,65 – 26,69 mg H2O2/g/min).

Table 3. The activity of catalase in the barley seedlings from the EMS treated seeds

Variant	Medium activity (mg H_2O_2/ml/30min)	S $\overline{x}$	S (σ)	CV%	m%	LS	LI
Control plant	8,54	0,08	0,14	4,93	2,84	8,66	8,41
0,01% EMS	23,29	0,05	0,08	0,92	0,53	23,33	23,24
0,025% EMS	20,37	0,29	0,51	4,16	2,40	20,52	20,21
0,05% EMS	26,35	0,37	0,65	2,26	1,30	26,77	25,92
0,10% EMS	24,65	0,03	0,09	1,83	1,06	24,69	24,60
0,50% EMS	26,69	0,32	0,56	11,67	6,74	26,84	26,53

S $\overline{x}$ = average standard error, S (σ) = standard deviation, CV% = average variation coefficient, m% = average precision coefficient, LS = superior limit of the coefficient interval, LI = inferior limit of the confidence interval

Table 4. The activity of catalase in the barley seedlings from the EMS and EHR treated seeds

Variant	Medium activity (mg H_2O_2/ml/30min)	S $\overline{x}$	S (σ)	CV%	m%	LS	LI
Control plant	8,54	0,08	0,14	4,93	2,84	8,66	8,41
0,01% EMS +EHR	6,29	0,23	0,41	1,41	0,81	6,54	6,03
0,025% EMS +EHR	6,63	0,44	0,76	1,11	0,64	7,65	5,60
0,05% EMS +EHR	7,31	0,34	0,59	1,43	0,82	8,39	6,22
0,10%+ EMS EHR	7,31	0,19	0,33	0,77	0,44	7,56	7,05
0,50% EMS +EHR	7,31	0,11	0,19	1,23	0,71	8,39	6,28

$S\bar{x}$ = average standard error, S (σ) = standard deviation, CV% = average variation coefficient, m% = average precision coefficient, LS = superior limit of the coefficient interval, LI = inferior limit of the confidence interval

The administration of the hydro-alcoholic rosemary extract (EHR) after the EMS treatment induced an important decrease in the activity of catalase, the values being very close to those registered in the case of the untreated plants. So, considering a catalase activity of 8,54 mg H_2O_2/g/min (in the control plants), in case of combined treatments (EMS and EHR), the catalase activity oscillated only between 6,29 and 7,31 mg H_2O_2/g/min, (Table 4). This behavior can only be explained by the intervention of the antioxidant compounds from the hydro-alcoholic rosemary extract and their scavenger quality.

Another enzyme whose activity has been analyzed in our investigations was peroxidase, enzyme that plays an important role in the detoxification processes, being a regulator of the electronic flow in the cell respiration, but also a „trap" of the free radicals. As we know, peroxidase appears in case of lower quantities of H_2O_2, while catalase is stimulated by higher quantities of this oxidizing agent.

After the EMS treatments, the peroxidase activity increased in the barley seedlings, being about 3 times higher than the untreated plants. Thus, compared to 0,84 UP/g/min in the case of the control, the EMS treated samples had an intensity of the peroxidase activity between 2,54 and 2,71 UP/g/min, (Table 5).

As the analysis of the experimental results shows, the concentration of the mutagen agent did not sensible affect the activity of peroxidase, the differences between the variants being non significant, which makes us suppose that the stress caused by the application of the mutagen agent was extremely strong in all the experimental concentrations.

Table 5. The activity of peroxidase in the barley seedlings from the EMS treated seeds

Variant	Medium activity (UP/g/min)	$S\bar{x}$	S (σ)	CV%	m%	LS	LI
Control plant	0,84	0,13	0,22	1,21	0,70	0,98	0,69
0,01% EMS	2,54	1,72	2,99	10,60	6,12	3,50	1,59
0,025% EMS	2,67	0,11	0,19	3,22	1,86	3,69	1,64
0,05% EMS	2,71	1,66	2,89	2,25	1,30	3,28	2,14
0,10% EMS	2,65	0,87	1,51	1,23	0,71	3,67	1,62
0,50% EMS	2,55	0,38	0,67	1,73	1,00	3,58	1,51

$S\bar{x}$ = average standard error, S (σ) = standard deviation, CV% = average variation coefficient, m% = average precision coefficient, LS = superior limit of the coefficient interval, LI = inferior limit of the confidence interval

For all the analyzed samples we calculated, according to the average values and the standard deviation, the superior and inferior limits of the confidence intervals, based on the critical value t (α, n-1), given by α = 0,05 and n-1 freedom degrees.

From the analysis of the values obtained for the variability intervals of the peroxidase, we can conclude that they generally have pretty small limits, the highest confidence intervals being noticed at the concentrations of the alkylating agent of 0,025%, and 0,1% (1,64 - 3,69 UP/g/min., respectively 1,62 - 3,67 UP/g/min.), while the tightest interval was at the untreated variant (0,69 – 0,98 UP/g/min).

Table 6. The activity of peroxidase in the barley seedlings from the EMS and EHR treated seeds

Variant	Medium activity (UP/g/min)	$S\bar{x}$	S (σ)	CV%	m%	LS	LI
Control plant	0,84	0,13	0,22	1,21	0,70	0,98	0,69
0,01% EMS +EHR	0,64	1,23	2,13	3,13	1,81	0,69	0,58
0,025% EMS +EHR	0,62	0,47	0,82	1,11	0,46	0,85	0,38
0,05% EMS +EHR	0,37	3,97	6,88	1,42	0,82	0,49	0,24

Variant	Medium activity (UP/g/min)	S $\overline{x}$	S (σ)	CV%	m%	LS	LI
0,10%+ EMS EHR	0,54	0,26	0,45	0,53	0,30	0,54	0,54
0,50% EMS +EHR	0,69	1,70	2,95	3,57	2,06	0,72	0,66

S $\overline{x}$ = average standard error, S (σ) = standard deviation, CV% = average variation coefficient, m% = average precision coefficient, LS = superior limit of the coefficient interval, LI = inferior limit of the confidence interval

As well as the other two investigated enzymes, after the combined treatment (with EMS and EHR), the peroxidase activity registered much reduced values. While the peroxidase activity in the control plants was 0,84 UP/g/min, the enzyme activity in the rosemary extract treated samples after the EMS treatment had values between 0,37 (0,05% EMS + EHR) and 0,69 UP/g/min (0,50% EMS + EHR), (Table 6). The effect of the EHR upon the peroxidase activity did not depend on the EMS concentration associated with.

CONCLUSIONS

The investigations related to the effects of the treatments with ethyl-methane-sulfonate (EMS) and the hydro-alcoholic rosemary extracts (EHR) on the activity of some oxidoreductases in barley plants (*Mădălin* cultivar) have led to the following conclusions:

EMS treatments produced an obvious increase in the superoxide-dismutase, catalase and peroxidase activities in plants, as a consequence of the oxidative stress caused by the mutagen agent.

EHR administration after the EMS treatments led to an obvious reduction of the activities of the analyzed enzymes in the barley plants. The similarity with enzyme activities in the control plants, in this case, shows a clear antioxidant effect of the compounds present in the hydro-alcoholic rosemary extract.

The EHR effect on the activity of the investigated enzymes did not evidently vary according to the concentration of the ethyl-methane-sulfonate solutions, probably because the oxidative stress was extremely aggressive even after the administration of small doses of the alkylating agent.

REFERENCES

Armatu A., Colceriu-Mihul S., Bubueanu C., Drăghici E., Pîrvu L. (2010): Evaluation of antioxidant and free scavenging potential of some Lamiaceae species growing in Romania. Roum. Biotechnol. Let., 15(3), 5274-5280.

Artenie V., Ungureanu E., Negură A. M., 2008 – Metode de investigare a metabolismului glucidic şi lipidic. Edit. „Pim”, Iaşi, 182p.

Cojocaru C. D., Toma O., Cojocaru S. I., Ciornea E., (2009): Practicum de biochimia proteinelor şi acizilor nucleici. Ed. „Tehnopress” Iaşi, 261p.

Cuvelier M. E., Richard H., Berset C. (1996): Antioxidative activity and phenolic composition of pilot-plant and comercial extracts of sage and rosemary. J. Am. Oil Chem. Soc., 73, 645-665.

Dragland S., Senoo H., Wake, K., Holte, K., Blomhoff R., (2003): Several culinary and medicinal herbs are important sources of dietary antioxidants. J. Nutr., 133, 1286–1290.

Fadel H. H. M., El-Massry K. F. (2000): *Rosmarinus officinalis*: effect of drying on the volatile oil of fresh leaves and antioxidant activity of their extracts. J. Essential Oil Bearing Plants, 3, 5-19.

Faixova Z., Faix S., (2008): Biological effects of rosemary (*Rosmarinus officinalis* L) essential oil (A Review). Folia veterinaria, 3-4, 135-139.

Ghiorghiţă G., Corneanu G., (2002): Radiobiologie. Edit. „Alma Mater”, Bacău, 307p.

Hasani-Ranjbar S., Larijani B., Abdollahi M., (2009): A systematic review of the potential herbal sources of future drugs effective in oxidant-related diseases. Inflamm. Allergy Drug Targets, 8, 2–10.

Mittler R., (2002): Oxidative stress, antioxidants and stress tolerance. Plant Science, 7, 405-410.

Papageorgiou V., Gardeli Ch., Mallouchos A., Papaioannou M., Komaitis M, (2008): Variation of the chemical profile and antioxidant behaviour of *Rosmarinus officinalis* L. and Salvia fruticosa Miller grown in Greece. J. Agric. Chem., 56(16), 7254-7264.

Schwarz K., Ternes W., (1992): Antioxidative constituents of Rosmarinus officinalis and Salvia officinalis. Z. Lebensm. Unters Forsch., 195, 95-98.

Stefanovits-Banyai E., Tulok H. M., Hegedus A., Renner Cs., Varga Sz. I., (2003): Antioxidant effect of various rosemary *(Rosmarinus officinalis* L) clones. Acta Biol. Szegedensis, 47(1-4), 111-113.

Wang S. Y., (2003): Antioxidant capacity of berry crops, culinary herbs and medicinal herbs. Acta Horticulare, 620, 461–473.

Wichtl M., (2009): Rosmarini Folium, Teedrogen und Phytopharmaka. 5th ed. Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH, 573-575

[1] SC CCPPM „PLANTAVOREL" SA, Piatra Neamt
[2] University "Al. I. Cuza", Iasi, Faculty of Biology
[3] Academy of Romanian Scientists; University "V. Alecsandri", Bacău, Dept. of Biology
* ionela_daciana@yahoo.com

BEHAVIOR OF SOME ENZYMATIC SYSTEMS TO THE ACTION OF THE CYTOSTATIC ACTIVE *EGlCP* GLUCANIC BIOPREPARATION UPON HeLa NEOPLASTIC CELLS

DANIELA GHERGHEL[1], COSMIN TEODOR MIHAI[2], GABRIELA VOCHITA[1], ELENA TRUTA[1], DORINA IUREA[1], PINCU ROTINBERG[1*]

Keywords: *EGlCP* glucanic biopreparation, HeLa neoplastic cells, membranary and intracellular enzymes, enzymatic activity, oxidative stress

Abstract: Interference of an autochthonous cytostatic active *EGlCP* glucanic biopreparation (in dose of 1.5 mg/mL) with the activity of some key enzymes, involved in the development of active transmembranary transport, of the intermediary and energetic metabolism, as well as in cellular answer to the oxidative stress, of HeLa neoplastic cells has been investigated. The study revealed: the intensification of the membranary Na^+-K^+-ATP-ase, of the cellular Mg^{2+}-ATP-ase, of the superoxide dismutase activities; the operating level attenuation of the of catalase, peroxidase, glutathion peroxidase, lactate dehydrogenase, alkaline phosphatase, acid phosphatase; the diminution of the malondialdehyde content. This functional interference with some cell enzymatic biomolecules has also induced the perturbation of the diverse membrane and metabolic processes, which was incompatible with the survival of HeLa tumoral cells

The modulations of the cellular enzymatic equipment activity can be the consequences of the glucanic components direct (with the molecules of the miscellaneous enzymes) or indirect interactions (with membrane or genetic apparatus) with some cell, subcell and molecular structures, implicated in the control and regulation of the biosynthesis and activity of the enzymatic biomolecules. The central element, which induces this enzymatic imbalance, appears to be the excess generation of the free radicals in the tumoral cells’ metabolism aggressed by glucanic constituents.

INTRODUCTION

Always the fungi represented accessible resources for obtaining some extracts used in ethnophytotherapy of many and various diseases. Simultaneous with the development of chemistry were perfomed studies for the identification of the bioactive compounds from fungal extracts as well as for the evaluation of their activity. It has been found that a major part of the biological active substances are belonging to the polysaccharides (Wasser, 2002).

Among the bioactive polysaccharidic compounds we mention the β-glucans, which are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. They occur most commonly as cellulose in plants, the bran of cereal grains, the cell wall of baker's yeast, certain fungi, mushrooms and bacteria. Some forms of beta glucans are useful in human nutrition as texturing agents and as soluble fiber supplements, but can be problematic in the process of brewing.

Therefore, a lot of biologically active compounds are generally glycoconjugates, and particularly glucans, they having numerous and diverse positive pharmacological properties. Thus, it has been highlighted that the glucanic biomacromolecules are behaving as antibacterial, antiviral, antifungal (Gantner et al., 2005), antiparasitical (Veleby et al., 2008), antiallergic Kirmaz et al.,2005), antiarhtritic (Bauerová et al., 2009), cardiovasomotor (Keogh et al., 2003), antiinflammatory (Luhm et al., 2006), antiteratogenic (Kobayashi et al., 1993), antioxidant (Kogan et al., 2005), immunomodulating (Demir et al., 2007; Vetvicka, 2007; Rotinberg et al., 2008b; Solcan et al., 2008), even antitumor and anticancer (Ooi and Liu, 2000; Daba and Ezeronye, 2003) agents.

Also, they are free radical scavengers (Sener et al., 2006, Toklu et al., 2006), metabolic and digestive modulators, nutritional supplements (Kren & Martinkova, 2001; Tian, 2007), antigenotoxic (Tohamy, 2003), antiradiation protectors (Cramer et al., 2006), as well as potentiating agents of some antibiotics (Kernodle et al. 1998).

The therapeutical importance of the glycoconjugates has imposed to the scientific world the necessity of obtaining some efficient bioactive products (Varki, et al., 1999; Wasser, 2002).

Despite the fact that there has been continuous progress in cancer diagnosis and treatment as a result of recent discoveries in cellular and molecular oncobiology, structural and functional genomics, pharmacogenomics and toxicogenomics, proteomics and metabolomics, antineoplastic therapy - which holds pride of place - is still of little effectiveness, fact explained and by its negative impact on the normal cells of the organism under neoplasm aggression and by the development of a resistance phenomenon of the tumoral cells to the cytostatic drugs action (Bronchud, 2000, Lyden et al., 2001; Adams, 2002; Anderson et al., 2002; Abrams, DeVita, 2004, Celis and Moreira, 2010).

So, in the fight against cancer – a real scourge of contemporary times – identification of new, more effective antineoplastic agents, as well as of new ways to decrease cancer cells resistance to cytostatics represent topical and major objectives (Bronchud, 2000; Abrams, 2003; Lodish et al, 2003; DiPiro et al, 2005).

Our previous studies, on appropriate experimental models to the pharmacodynamic or cellular and molecular oncobiology researches, developed either *in vitro*, on various healthy and neoplastic cells (Mihai et al., 2008a), or *in vivo*, on rats bearing various experimental tumoral systems, have highlighted and quantified the significant antitumor property of a biopreparation of glucanic nature (*EGlCP*), extracted from submerged cultures of *Claviceps purpurea* microfungus (Gherghel et al., 2008; Mihai et al., 2008b), as well as the reactivity of some membrane and metabolic processes of cancerous cells to the action of this new potential cytostatic agent (Rotinberg et al, 2007; Rotinberg et al., 2008a).

Thus, it has imposed extending and thoroughgoing investigations necessary to preclinical pharmacological characterization of the product as new oncochemotherapeutic drug, one of very important direction being the elucidation of cellular, subcellular and molecular mechanisms of action, involved in the expression of the pharmacodynamic potential.

Consequently, a first problem investigated in the present work, complementary and explanatory for the functional membrane and metabolic modifications signaled by us in other papers, was related to the interference of the cytostatic active *EGlCP* glucanic biopreparation with the activity of some key enzymes involved in the development of active transmembranary transport, of the intermediary and energetic metabolism, as well as in cellular answer to the oxidative stress.

MATERIALS AND METHODS

The biological material used in the *in vitro* experiments was represented by mycoplasm-negative, stabilized, HeLa cellular cultures of human neoplastic origin, obtained from an uterine cervix carcinosarcoma and cultured in DMEM growing medium (Dulbeco's Modified Eagle's Medium, Biochrom AG, Germany, FG 0415), supplemented with 10.0% fetal bovine serum (Sigma, Germany, F9665), 100 μg/mL streptomycin (Biochrom AG, Germany, A 331- 26), 100 IU/mL penicillin (Biochrom AG, Germany, A 321-44) and 50 μg/mL antimycotic amphotericin B (Biochrom AG, Germany, A 2612), at a density of 2 x 10^6 cells / 300 cm^2 flask, in a humidified 5% CO_2 atmosphere at 37°C (Bissery and Chabot, 1991, Doyle and Griffiths,1998,).

The tumoral cells were incubated for a period of 144 hours, the growing medium being renewed twice in this time frame of cultures development. When the cells reached confluence in the monolayer stage, the cultures were divided into control and glucanic treated cell cultures.

The cytostatic agent used by us in the HeLa neoplastic cells treatment was a biopreparation of glucanic nature (*EGlCP*), specific extracted from submerged cultures of *Claviceps purpurea* microfungus. At the 144 hour old of tumoral cell cultures – the optimum age for achievement of an adequate cellular mass – the culture medium of treated cultures was discarded from the test flasks and replaced with a fresh medium which contain *EGlCP* bioproduct in dose of 1.5 mg/mL, the duration of treatment being of 12 hours. In case of control cultures, the culture medium was replaced with a fresh one.

The layer of tumoral cells was washed with phosphate buffered saline, precisely weighted and then subjected to the steps of obtaining the clarified cellular lysates. Adequate aliquots were used for the biochemical determination of the membranary Na^+-K^+-ATP-ase, cell $Mg2^+$-ATP-ase, lactate dehydrogenase (LDH), peroxidase (Px), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), acid (ACP) and alkaline (ALP) phosphatase activities and of malondialdehyde levels (MDA) (Artenie et al., 2008).

The estimation of the total Mg^{2+}- Na^+ - K^+ and respectively membranary Na^+-K^+-ATP-ases (tATP and mATP) activities, expressed in mg inorganic phosphate/minute/g cellular mass (mg Pi/min/gcm), was based on the amount of inorganic phosphorous released after ATP hydrolysation by ATP-ases from the cellular homogenate. Lactate dehydrogenase activity (μM/min/gcm) was determined through the measurement of NADH oxidation velocity in the case of transformation reaction of pyruvic acid in lactic acid. Peroxidase activity (peroxidase unit, UP, /min/gcm) was estimated by o-dianisidine method, which measures the intensity of the o-dianisidine oxidation product colour.

Glutathione peroxidase activity (μM GSH/ml/min/gcm) was measured on the basis of the reaction of unconsumed reduced glutathione with 2, 2'- dinitro-5, 5'- dithiodibenzoic acid (Merck), which drives to a yellow, photometrable complex.

The evaluation of superoxide dismutase activity (superoxide dismutase unit, USOD, /ml/min/gcm) is based on the enzyme capacity to inhibit the nitroblue tetrazolium reduction by the superoxide radicals generated in reaction medium through riboflavin reduction.

Catalase activity was estimated through spectrophotometric registration of the hydrogen peroxide consumed quantity, being expressed in enzymatic unit (UE/gcm).

Alkaline and acid phosphatases activities (international unit, U.I., /gcm) were determined with para-nitrophenol, which is converted in a spectrophotometrable product, p-nitrophenolat, under the action of phosphatases.

At high temperature and in acid medium, malondialdehyde – product of lipid peroxides degradation – reacts with 2-thiobarbituric acid, leading to a photometrable pink trimetinic adduct (MDA nM/ml /gcm).

Five flasks of cultures have been used for each experimental group, the results being analyzed statistically by means of Student' „t" test (Cann, 2002).

RESULTS AND DISCUSSIONS

The investigation of the consequences of the glucanic treatment upon HeLa cell cultures has conducted to a set of data – shown in figure 1 and 2 – which expresses the modulation of some cellular enzymatic activities by this chemical agent. Thus, the action of *EGlCP* bioproduct upon the activity of different enzymatic systems has materialized, comparatively with the one of the control group, through variations of its sense and amplitude.

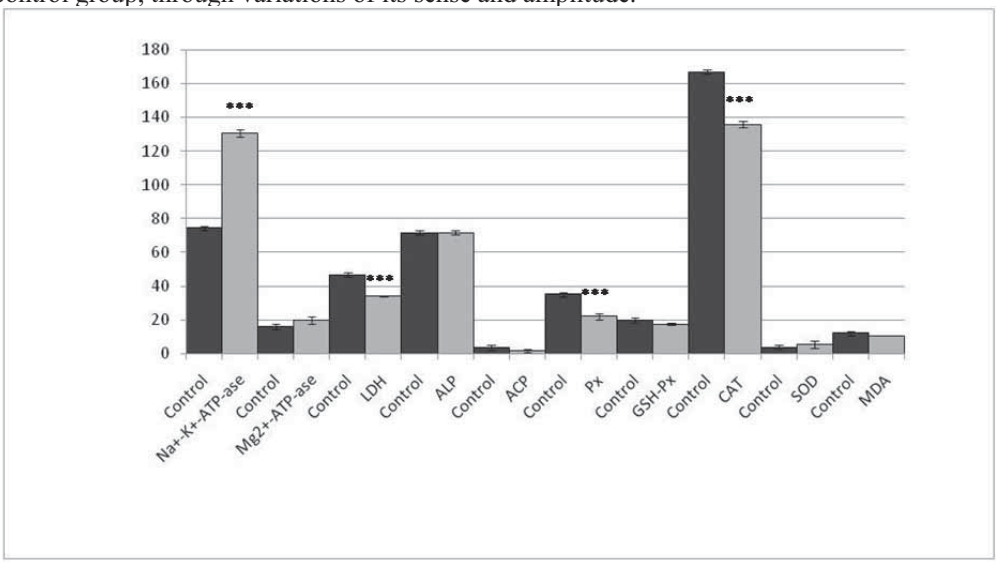


Figure 1. The impact of EGlCP bioextract (1.5 mg/mL) upon the activities of membranary Na^+-K^+-ATP-ase (mg Pi/min/g cell mass), cellular $Mg2^+$-ATP-ase (mg Pi/min/g cell mass), LDH (µM/min), ALP(U.I.) ACP(U.I.), Px(UP/g/min.), GSH-Px (µM GSH/ml/min.), SOD (USOD/ml/min), CAT (UE) enzymes and upon MDA levels (nM/ml) of HeLa neoplastic cells. Significantly different from control: ***p<0.001.

It can be seen, from Figure 1, that in the case of the control HeLa cell cultures, enzymatic activities were of: for membrane Na^+-K^+-ATP-ase, 74.49 mg Pi/min/gcm; in the case of cellular Mg^{2+}-ATP-ase, 16.15 mg Pi/min/g cm; for LDH, 46.7 µM/min/gcm; for Px, 35.28 UP/gcm/min; in the case of GSH-Px, 19.86 µM GSH/ml/min/gcm; for SOD, 3.96 USOD/ml/min/gcm; for CAT, 166.89 UE/gcm; in the case of ACP 3.69 U.I./gcm; for ALP 71.66 U.I./gcm; in the case of lipooxidation enzymes, 12.10 nM MDA/ml/gcm. We considered these quantitative estimations as reference values, necessary for the interpretation of the glucanic impact’ signification upon the activity of the studied enzymes.

As compared to the control group, the interference of glucanic bioextract with enzymatic activities has determined significant functional and statistical modifications of these membranary and intracellular biomolecules. Thus, the impact of the glucanic treatment has been expressed by increases – in case of the membranary Na^+-K^+-ATP-ase (130.72 mg Pi), cellular Mg^{2+}-ATP-ase(19.74 mg Pi), SOD (5.59 USOD/ml/min) – and decreases – in case of the LDH (33.99 mM/min.), Px (22.27 UP/g/min), GSH-Px (17.61 µM GSH/ml/min), CAT (135.9 UE), ACP (1.76 UI), ALP (71.66 U.I./gcm) MDA (10.80 nM/ml) – of the studied enzymes activities.

In comparison with the control enzymatic functionality (see Figure 2), the activity of mem-

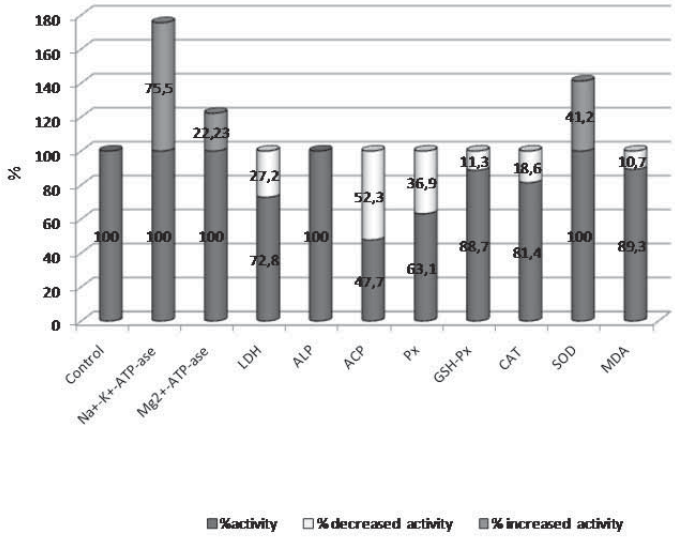


Figure 2. Modulation of the membranary Na^+-K^+-ATP-ase, cellular $Mg2^+$-ATP-ase, LDH, ALP, ACP, Px, GSH-Px, SOD, CAT, enzyme activities and MDA levels (nM/ml) of HeLa cell cultures caused by the EGlCP bioproduct (1.5 mg/mL).

branary Na^+-K^+-ATP-ase has been amplified with 75.5%, while the operating level of cellular Mg^{2+}-ATP-ase has been increased, just with 22.23%.

In the case of the enzymes implied in the response to oxidative stress, we assist to an attenuation of some activities with 36.9% (Px), 11.3% (GSH-Px), 18.6% (CAT), and 10.7% (MDA), respectively, or to an enhancement of SOD's activity with 41.2%.

The activity of lactate dehydrogenase was decreased with 27.2%.

It can be also observed that the operating level of the ALP was not perturbed (100%) and of ACP has registered a major regression (with 52.3%).

The *in vitro* testing on normal and tumoral cell cultures have practical importance in the selection of potential oncochemotherapeutic agents of diverse chemical nature. The cell cultures are also compatible and useful experimental models for preliminary understanding of the action mechanism implied in inducing of pharmacodynamic effect of the bioactive agent (Leiter et al., 1965; Boyd, 1989; Bissery and Chabot, 1991; Phillips et al., 1991).

The animal eukaryotic cells contain self regulation and self control mechanisms which maintain the cell homeostatic status, they being the target of the biologically active substances. The activation of molecular mechanisms of the cellular functional regulation is dependent on the transformation of the extracellular information in an action of cellular response. In this condition, the starting molecular event is logically localized at the level of the environment–cell interface, meaning in the cellular membranes. After this primary interaction between an agent and a cell membrane, there takes place the transfer and traducing of the extracellular signal. Consequently, the intracellular mechanisms of control and the activity of the enzymatic systems will be influenced. These specific modulations would stimulate and would inhibit the different metabolic processes which will exteriorize by global pharmacodynamic effect (Benga 1985; Karp, 1996; Alberts et al., 1998; Stroescu, 1998; Cruce, 1999).

The lack of balance between the structural components of the tumoral cell membranes – the decrease of the membranary fluidity, the modification of the packing degree of the membrane overmolecular structures, the different topographical location and activity of the membrane ATP-ases – is functionally expressed by perturbation of the membranary permeability. The modification of the ionic fluxes leads to the appearance of the transmineralization phenomenon.

This specific feature of the neoplastic cells consists in an abnormal distribution of the ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- etc.) correlated with other ionic ratios in extra- and intracellular compartments and with a decrease of the membrane resting potential. Among other membranary peculiarities of the tumoral cells, it is important to mention the powerful enhancement of the activity of the Na^+–K^+–depending electrogenic pump (Bannasch et al., 1998; Olbe, 1999; Miron, 2000; Owens, 2001).

Our experimental results, registered after treatment of tumoral HeLa cells with exopolysaccharidic *EGlCP* bioproduct have highlighted an increase of the inorganic phosphate, in the membrane and intracellular substratum, fact that suggests a high activity of the membrane Na^+-K^+ electrogenic pump and of the total ATP-ases. Thus, we assist to a stimulatory impact upon the activity of the cell ATP–ases system, comparatively to the one of control, which reveals diverse energetically needs for the insurance of the optimal active transmembranary fluxes of Na^+ and K^+ cations in the glucanic treated HeLa cells.

The stimulatory effect of the glucanic bioextract upon Na^+-K^+ membrane electrogenic pump can be the consequences of it direct interaction with some membrane structures (receptors or Na^+-K^+-ATP-ase biomolecules). This supposition is argued by modulation of the membranary Na^+–K^+–ATP-ase in the condition of *in vitro* treatment of the human HeLa tumoral cells with *EGlCP*, which influences consecutively the membrane permeability, transmembranary ionic fluxes, ionic equilibrium, extra- and intracellular ionic ratios. Our assumption is according to some recent bibliographical data (Akinori, 1996; Mithöfer, 2005), which suggest that the plasma membrane is the site of glucan’s primary action.

We cannot exclude the intracellular penetration of the exopolysaccharidic molecules, due to increased permeability of tumoral cells for the large sizes energogenetic sources, as well as their direct or indirect interaction with the intracell enzymatic systems or intracell receptors.

A key-enzyme of the glucidic intermediary metabolism and a well-known marker of malignant cells is lactate dehydrogenase biomolecule. In the conditions of the cytostatic *EGlCP* treatment, the LDH activity was obviously perturbed, its operating level being inhibited by a probable stimulation of pyruvate or NAD^+ synthesis.

Another enzymatic system, implied in the phosphorylation and dephosphorylation cell metabolic reactions, includes the ALP and ACP phosphatases. In our experimental conditions, the HeLa cells exposure to the glucanic action has not affected the ALP functionality, while the ACP activity was significantly repressed because of some probable changes in intracellular pH due to a very increased intracell production of the free radicals.

The above explications are also supported by our experimental results upon the functional behaviour of the free radicals scavenger enzymatic system (peroxidase, glutathione peroxidase, catalase, superoxide-dismutase etc.) to the glucanic impact on HeLa cells.

Thus, it can be seen that the *EGlCP* has conditioned a nonsignificant attenuation of the Px, GSH-Px and CAT activities, consequence of the possible lack of the specificity to the currently existing substrates in cells. Surprising is the registered simultaneous increase of SOD activity, which justifies a fast elimination of superoxide radical (O_2^-), probably under the influence of the stress generated by the presence of the glucanic extract in the culture medium. This behaviour

leads us to the idea that SOD may be a target of glucanic action, suggesting a promising clinical and experimental way to selectively kill cancer cells (Huang et al., 2000).

The *EGlCP* treatment of the HeLa cells has been correlated with decreased MDA levels, suggesting the inhibition processes which generate the lipid peroxides.

Our experiments have highlighted that the *EGlCP* biopreparation modulates the activities of some oxidative stress enzymes (Px, GSH-Px and CAT), more in an inhibitory manner then in one stimulatory. Therefore, it seems that the glucanic extract – used for cytostatic treatment – has altered probably the unfolding of the metabolic events, generating smaller or larger intracellular amounts of specific free radicals, which have repressed or enhanced the activity of some clearing enzymes by feedback mechanism, generated by modifications of cancerous cells' intracellular medium " homeostasis ". This effect of *EGlCP* – which partially explains its cytostatic action – can be the result of an indirect or direct interaction of the glucanic compounds with the enzymatic biomolecules or with the membranary or intracell receptors.

In this moment of research, we can appreciate – in the light of our results on the behaviour of some enzymatic equipments to the glucanic action – that the oncostatic property of the *EGlCP* natural extract can be also the expression of it capacity to induce profound perturbations of the operating degree of various enzyme systems, these being not compatible with survival of tumor cells, hence the *in vitro* cytostatic action of the autochthonous fugal glucanic extract.

Modification of the tumor cells' specific homeostatic level, no longer is compensated by the specific cell control systems, leads to expression of the cytostatic effect of the glucanic extract. The central element that generates this imbalance seems to be represented by the excess generation of some specific free radicals.

CONCLUSIONS

The *in vitro* glucanic treatment of the HeLa human tumoral cells modulates the activity of some enzymatic systems, located either at membrane level or at intracellular one.

Stimulation of the Na^{+}-K^{+}-ATP-ase, of cellular Mg^{2+}-ATP-ase and SOD enzymes or inhibition of LDH, ACP, Px, GSH-Px, CAT, and those implicated in lipid peroxidation reactions have perturbated membrane and metabolic processes, justifying the cytostatic impact of the glucanic extract.

This cytostatic property of the *EGlCP* is probably due to a primary membranotrop action mechanism and/or metabolic action mechanism of the exopolysaccharide compounds, which can interact with the membrane receptors and/or intracellular ones.

REFERENCES

Abrams, A.C. (2003): *Clinical drug therapy*, Lippincott, Williams & Wilkins

Adams, J. (2002): *Proteasome inhibition: a novel approach to cancer therapy*. Trends Mol. Med., 8, 49–54

Akinori, K., Kazuhiro T., Yuki I., Tetsuji Y. and Tomonori S. (1996): *Specific Response of Partially Purified Cell Wall-Bound ATP ases to Fungal Suppressor*. Plant Cell Physiol., 37(2), 207-214.

Alberts, B., Bray, O., Johnsons, A., (1998): *Essential Cell Biology*, Garland Publ. Inc. N.Y., London

Anderson, S., Chiplin, J., (2002): *Structural genomics: shaping the future of drug design?* Drug Discov. Today, 7, 105–107

Artenie, V., Ungureanu, E., Negură A.M., (2008): *Metode de investigare a metabolismului glucidic lipidic.* Ed. PIM

Bannasch, P., Kanduc, D., Papa, S., Tager, J.M., (1998): *Cell growth and oncogenesis*, Birkhäuser Verlag, Basel–Boston–Berlin

Bauerová, K., Paulovicová, E., Mihalová, D., Svík, K., Ponist, S., (2009): *Study of new ways of supplementary and combinatory therapy of rheumatoid arthritis with immunomodulators. Glucomannan and Imunoglukán in adjuvant arthritis*. Toxicol Ind Health. 25(4-5), 329-35

Benga, G., (1985): *Biologie celulară şi moleculară*, Ed. Dacia, Cluj-Napoca

Bissery, M.C., Chabot, G., G., (1991): *History and new development of screening and evaluation methods of anticancer drugs used in vivo and in vitro*. Bull. Cancer (Paris), 78(7), 587-602

Bronchud, M.H., (2000): *Principles of molecular oncology*, Humana Press Inc

Cann A.J., 2002, *Maths from scratch for biologists*, John Wiley & Sons Ltd

Celis, J.E., Moreira, J.M. (2010): *Challenges and opportunities in oncoproteomics*. Mol Oncol. 4(6):459-60.

Cramer, D.E.; Allendorf, D.J., Baran, J.T., Hansen, R., Marroquin, J., et al., (2006): *Beta-glucan enhances complement-mediated hematopoietic recovery after bone marrow injury*. Blood 107 (2): 835–840

Daba, A.S., Ezeronye, O.U., (2003): *Anti-cancer effect of polysaccharides isolated from higher basidiomycetes mushroom.* African Journal of Biotechnology, 2(12), 672-678

Demir, G, Klkein H.O., Mandel-Molinas N., Tuzuner N., (2007): *Beta glucan induces proliferation and activation of monocytes in peripheral blood of patients with advanced breast cancer.* Int Immunopharmacol. 7(1):113-6

DeVita, V.T. Jr., (2004): *Cancer: Principles and Practice of Oncology*, Seventh Edition, De Vita Jr. et al., (Eds.), Philadelpia, Lippincott

DiPiro, J.T., Talbert, R.L., Yee, G.C., Matzke, G.R., Wells, B.G. et al., (2005): *Pharmacotherapy, a pathophysiologic approach*. McGRAW-HILL, Medical Publishing Division, New York

Doyle, A., Griffiths, J.B., (1998): *Cell and Tissue Culture*: *Laboratory Procedures in Biotechnology*. John Wiley & Sons Ltd, West Sussex

Gantner, B.N., Simmons, R.M., Underhill, D.M., (2005): *Dectin-1 mediates macrophage recognition of Candida albicans yeast but not filaments*. Embo J; 23:24(6):1277-1286

Gherghel, D., Rotinberg, P., Mihai C., Truţă, E., Căpraru, et al. (2008): *The in vivo assessment of the preclinical oncochemotherapeutic effectiveness of the polysaccharidic biopreparation*. Lucr. St. USAMV Iasi, Seria Medicina Veterinara, 51(10), 341-348

Huang, P., Feng, L., Oldham, E. A., Keating, M. J. & Plunkett W., (2000): *Superoxide dismutase as a target for the selective killing of cancer cells*. Nature*,* 407, 390-395

Keogh, G.F., Cooper, G.J., Mulvey, T.B., McArdle, B.H., Coles, G.D., et al., (2003). *Randomized controlled crossover study of the effect of a highly beta-glucan-enriched barley on cardiovascular disease risk factors in mildly hypercholesterolemic men.* Am. J.Clin.Nutrition, **78** (4): 711–718

Kernodle, D.S., Gates, H., Kaiser, A.B., (1998)**,** *Prophylactic anti-infective activity of poly-[1-6-beta-D-glucopyranosyl-[1-3]-beta-D-glucopryanose glucan in a guinea pig model of staphylococcal wound infection.* Antimicrobial agents and chemotherapy, **42** (3): 545–549

Kirmaz, C., Bayrak, P., Yilmaz, O., Yuksel, H., (2005): *Effects of glucan treatment on the Th1/Th2 balance in patients with allergic rhinitis*, European cytokine network, **16** (2): 128–134

Kobayashi, H., **Matsunaga K**., **Fujii M**., (1993): *PSK as a chemopreventive agent***,** Cancer Epidemiol Biomarkers Prev., 2(3):271-6

Kogan, G., Stasko, A., Bauerova, K., Polovka, M., Soltes, L., et al., (2005): *Antioxidant properties of yeast (1→3)-β-D-glucan studied by electron paramagnetic resonance spectroscopy and its activity in the adjuvant arthritis*, Carbohydrate Polymers , **61** (1): 18–28

Kren, V., Martinkova, L., (2001): *Glycosides in Medicine: The Role of Glycosidic Residue in Biological Activity*, Current Medicinal Chemistry, 8, 1313-1338

Leiter, J., Abbott, D.J., Schepartz, S.A., (1965): *Screening data from the cancer chemotherapy national service center screening laboratories*, Cancer Res., 25, 20-35

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C.A., Krieger, M., et al. (2003), *Molecular cell biology*, Freeman W.H.&Co

Luhm, J., Langenkamp, U., Hensel, J., Frohn, C., Brand, J.M., et al., (2006): *β-(1→3)-D-glucan modulates DNA binding of nuclear factors κB, AT and IL-6 leading to an anti-inflammatory shift of the IL-1β/IL-1 receptor antagonist ratio*. BMC Immunology, 7(5): 15-19

Mihai C., Rotinberg P., Gherghel D., Capraru G., Truta E., (2008a): *In vitro selection of some potential cytostatic agents from new fungal exopolyglucidic extracts,* Analele Ştiinţifice ale Universităţii “Al. I. Cuza” Iaşi, Secţia II Genetică şi Biologie moleculară, IX(1), 35-41

Mihai, C., Rotinberg, P., Truţă, E., Gherghel, D., Căpraru, G., et al., (2008b): *Highlighting and reproducibility of the antitumoral pharmacodynamic effect of an autochthonous glucanic biopreparation, of fungal origin*. Lucr. St. USAMV Iasi, Seria Medicina Veterinara, 51, 101-107

Miron, L., (2000): *Oncologie generală*, Ed. Egal, Bacău

Mithöfer, A, Ebel J, Felle HH, (2005): *Cation fluxes cause plasma membrane depolarization involved in beta-glucan elicitor-signaling in soybean roots*, Mol Plant Microbe Interact. 18, 983-990
Olbe, L., (1999): *Proton pump inhibitors*, Parnham, M., Bruinvels, J., Eds., Birkhäuser Verlag, Basel-Boston-Berlin
Ooi ,V.E., Liu, F., (2000): *Immunomodulation and anti-cancer activity of polysaccharide-protein complexes*. Curr. Med. Chem. 7 (7), 715–29
Owens, J., (2001): *Something old and something new: taking cancer therapy forward,* Drug Discovery Today. 6(23), 1203–1206
Phillips, R.M., Bibby, M.C., Double, J.A., Loadman, P.M., (1991): *The relationship between the in vitro chemosensitivity of tumor cells and tumor response in vivo in an experimental tumor model.* Int. J. Cell Cloning, 9, 144-154
Rotinberg, P., Gherghel, D., Mihai, C., Neacşu I., Rotinberg H., (2007): *Experimental modulation of HEp-2p tumoral cells reactivity to the action of some autochthonous exopolysaccharidic cytostatics by certain membranotropic and metabolic agents (I)*. Analele Ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi, Secţia II Genetică şi Biologie moleculară, VIII(1), 45-52
Rotinberg, P., Gherghel, D., Mihai, C., Capraru, G., Truta, E., et al., (2008a): *Metabolic behaviour of the HEp-2p neoplastic cells to the action of a bioactive fungal exopolysaccharidic extract.* Analele Ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi, Secţia II Genetică şi Biologie moleculară, IX(2), 23 – 31
Rotinberg, P., Mihai, C., Gherghel, D., Truţă, E., Căpraru, G., et al., (2008b): *Immunomodulatory effects of some new autochthonous natural polyphenolic or polysaccharidic bioproducts of vegetal or fungic origin*. Rom. Biol. Sci, V (1-4), 93 – 100
Sener, G., Eksioglu-Demiraop, E., Cetiner, M., Ercan., F., Yegen, B.C., (2006): *Beta-glucan ameliorates methotrexate-induced oxidative organ injury via its antioxidant and immunomodulatory effects*. Eur.J Pharmacology; 542(1-3), 170-178
Solcan, Gh., Solcan, C., Carp-Carare, M., Rotinberg, P., Boghian, V., (2008): *Some productive and immunitary effects of glucans extracted from Claviceps purpurea in broiler chinckens.* Lucr. St. USAMV Iasi, Seria Medicina Veterinara, 51(10), 528– 534
Tian, Z., Si, J., Chang, Q., Zou, L., Chen, S., et al. (2007): *Antitumor activity and mechanisms of action of total glycosides from aerial part of Cimicifuga dahurica targeted against hepatoma.* BMC Cancer, 7, 237 – 262
Tohamy, A.A., (2003): *Beta-glucan inhibits the genotoxicity of cyclophosphamide, adriamycin and cisplatin.* Mutat. Research. 541(1-2):45-53
Toklu, H.Z., Sehirili, A.O., Velioglu-Ogunc, A., Centinel, S., Sener, G., (2006): *Acetaminophen-induced toxicity is prevented by beta-d-glucan treatment in mice*, European J Pharmacology. 543(1-3), 133-140
Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., Marth, J., (1999): *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press (NY)
Velebny, S., Hrckoya, G., Kogan, G., (2008): *Impact of treatment with prasiquantel, silymarin [milk thistle] and/or beta-glucan on pathophysiological markers of liver damage and fibrosis in mice infected with Mesocestoides vogae (Cestoda [flat worms including tape worms] tetrathyridia).* J. Helminthol, 82(3),1-9
Vetvicka, V., Dvorak , B., Vetvickova, J., Richter, J., Krizan, J., et al.,(2007), *Orally administered marine (1-->3)-beta-D-glucan Phycarine stimulates both humoral and cellular immuni.* Int.J. Biol.Macromol., 40 (4): 291–298
Wasser, S.P, (2002): *Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides*, Appl. Microbiol. Biotechnol., 60, 258-274

[1] Biological Research Institute, Lascăr Catargi Street, 47, 700107, Iaşi, Romania
[2]"Al. I. Cuza" University, Faculty of Biology, Bd. Carol I, 20A, 700505, Iaşi, Romania
* pincu.rotinberg@uaic.ro

COMPARATIVE *IN VITRO* ACTIVITY OF AZITHROMYCIN AND OTHER ANTIMICROBIAL AGENTS AGAINST STAPHYLOCOCCI ISOLATES

ANTONIA POIATA[1], CRISTINA TUCHILUS[1]

Keywords: Staphylococci, azithromycin, susceptibility
Abstract. In the last decades resistance and reduced susceptibility to antimicrobial agents has become a major therapeutic problem. Both *Staphylococcus aureus* and *Staphylococcus epidermidis* are frequently resistant to methilpenicillins and derivatives, including methicillin, oxacillin and nafcillin. One of the antibacterial agents which exhibits a spectrum of activity against Gram-positive bacteria is azithromycin, a macrolide with properties closely resemble those of erytromycin. Azithromycin susceptibility was obtained for 84.3% of *S. aureus* and 87.4% of *S. epidermidis* respectively. Erythromycin resistance was more prevalent in *S. aureus* strains (20.5%) than in coagulase-negative staphylococci (15%). Oxacillin resistance has low and similar resistance rates for staphylococci (between 1.6-1.8%). Meropenem had excellent activity: all strains were susceptible. The results of surveillance of resistance has not been extensive.

INTRODUCTION

An important measure for monitoring the effectiveness of antimicrobial therapy is surveillance of the *in vitro* susceptibilities of bacteria. The ability of staphylococci to develop resistance to antimicrobial agents has been recognised for decades (Ashley and Brindle, 1980).

Staphylococci cause a variety of skin, soft-tissue and invasive infections and in the last decades the increasing resistance to many antimicrobial agents has become a major therapeutic problem (Crosseley and Archer, 1997). Both *Staphylococcus aureus* and *Staphylococcus epidermidis* are frequently resistant to methilpenicillins and derivatives, including methicillin, oxacillin and nafcillin. One of the antibacterial agents which exhibits a good spectrum of activity against Gram positive bacteria is azithromycin, an azalide antibiotic that is derived from the macrolides. This agent is chemically related to erythromycin by the insertion of a methyl-substituted nitrogen into the aglycone ring (Retsema *et al*, 1987; Barry *et al*., 1989). Its activities closely resamble those of erythomycin against Gram-positive cocci but with enhanced potency against Gram-negative and anaerobic organisms (Retsema *et al.*, 1987).

The aim of this study was to investigate the *in vitro* activity of azithromycin against staphylococci, communitary isolates. Other agents (erythromycin, oxacillin and meropenem) were also included in the studies for comparative purpose.

MATERIALS AND METHODS

Bacterial strains

A total of 515 strains isolated during 2007-2008 period were included to evaluate in this study.

The strains studied, originated from nasal swabs from healthy individuals and identified by standard procedures, were the following: *Staphylococcus aureus* (n=127) and *Staphylococcus epidermidis* (n=388).

Sensitivity study

Minimal inhibitory concentrations (MICs) were determined by the agar technique according to the Clinical Laboratory Standards Institute (CLSI) recommendations (2008). An inoculum of 10^5 cfu/ml(colony forming units) was delivered by a multipoint inoculator to a series of Mueller- Hinton agar plates, which obtained the antibiotic in twofold dilutions. Incubation was for 24 h at 35^0C. The MICs were determined as the lowest concentration of antibiotic at which no visible growth or growth <3 colonies were observed.

S. aureus ATCC 25923 was used as standard reference strain for quality control.

Antimicrobial agents

Stock solutions of the following were prepared from their respective powder forms: azithromycin, erythromycin, oxacillin, and meropenem. The range of concentrations used was from 0.06 to 32 mg/l. Resistance rates are reported using the CLSI breakpoints for the fully susceptible category (moderately susceptible isolates are classified as resistant) (Table 1).

Table 1. The value of concentrations used for defining susceptible isolates

Antibiotic	S (mg/L)	R (mg/L)
Azithromycin	≤ 2	≥ 8
Erythromycin	≤ 0.5	≥ 8
Oxacillin	≤ 2	≥ 4
Meropenem	≤ 4	≥ 16

Breakpoint shown is those defining fully susceptible isolates.

RESULTS AND DISCUSSION

Between 2007-2008, a total of 515 strains of staphylococci were obtained from healthy persons from nasal swabs.
Azithromycin MIC results were compared to erythromycin, oxacillin and meropenem. In table 2 the range of MICs, MIC 50, MIC 90 and susceptibility percent are presented.

Table 2. The *in vitro* activity of tested antimicrobial agents against staphylococci strains

Organism (n)	Antibiotic	MIC (mg/L)			Susceptibility ratio
		Range	MIC 50	MIC 90	
S. aureus (127)	Azithromycin	0.25-32	0.5	8	84.3
	Erythromycin	0.125-16	0.25	2	79.5
	Oxacillin	0.25-4	0.5	1	98.4
	Meropenem	0.125-1	0.125	0.25	100
S. epidermidis (388)	Azithromycin	0.25-32	1	4	87.4
	Erythromycin	0.125-8	0.25	2	85.1
	Oxacillin	0.25-8	0.5	1	98.2
	Meropenem	0.25-2	0.5	1	100

Meropenem, a new intravenous carbapenem, approved in 1996, retained excellent potency (MIC 90, between 0.25 mg/L and 1 mg/L); 90% of strains have oxacillin MIC at 2 mg/L. For both *S. aureus* and *S. epidermidis* erythromycin was comparable in antimicrobial activity (MIC 90.2 mg/L) a value that exceed the CLSI erythromycin breakpoint (0.5 mg/L) (Table 2).
Table 3 illustrates the MIC distribution for the surveillance staphylococci strains tested against azithromycin and comparison agents as cumulative inhibition percent.

Table 3. MICs values of staphylococci strains and susceptibility, according to breakpoint defined by CLSI criteria to selected antimicrobial agents

Organism (n)	Agent	Cumulative percent of MICs (mg/L)								
		0,125	0,25	0,5	1	2	4	8	16	32
S. aureus (127)	Azithromycin		28.3	55.9	73.2	84.3	87.4	90.6	93.7	100
	Erythromycin	11	45.7	79.5	86.6	89	94.5	97.6	100	
	Oxacillin		12.6	59	84.3	98.4	100			
	Meropenem	45.7	92.1	96.9	100					
		0,125	0,25	0,5	1	2	4	8	16	32
S. epidermidis (388)	Azithromycin		23.7	44.3	54.9	87.4	90.2	92.3	96.9	100
	Erythromycin	18	24.2	85.1	89.2	91.5	95.4	100		
	Oxacillin		19.6	64.4	85.6	98.2	99.2	100		
	Meropenem		30.9	62.4 99.2	100					

Resistant MIC values are highlighted light gray.

Using the CLSI (2008) breakpoint criteria, the collection of tested staphylococci strains showed susceptibility for meropenem.

Our data show that both *S. aureus* and *S. epidermidis* strains are mostly susceptible to azithromycin (of 84.3% respectively 87.4 %(table 3).

The *in vitro* susceptibility to erythromycin was observed for 79.5% of *S. aureus* and 85% of *S. epidermidis* strains, with slowly increase in resistance by comparison with azithromycin. With regard *S. aureus* strains, erythromycin resistance was more prevalent than in coagulase-negative staphylococci. Erythromycin is active against staphylococci isolates but azithromycin is also more active against these strains.

The analysis of *in vitro* oxacillin resistance in all isolates showed low rates for both *S. aureus* and *S. epidermidis*. The results of oxacillin resistance did not significantly vary between the two strains groups (1.6%-1.8%) (figure 1). The methicillin or oxacillin resistance in staphylococci also predicts resistance to a range of different classes of antibiotics (Archer and Climo, 1994). In accordance with this observation the oxacillin-resistant staphylococci were also resistant to tested antimicrobials.

Phenotypic antibiotic susceptibility was analysed the agar dilution method and selection of the isolates as the “susceptible” and “resistant” was based on their minimum inhibitory concentration to each tested agent. According to the MIC of these isolates, the resistance to azithromycin, erythromycin and oxacillin was observed in 15.7%, 20.5% and 1.6% *S. aureus* isolates, respectively (figure 1), regarding *S. epidermidis*, the resistance was inregistred in 12.6%, 14.9% and 1.8% respectively.

The prevalence of resistance to azithromycin and oxacillin is similarly for both all strains tested. Our data showed an increase erythromycin resistance in *S. aureus* isolates.

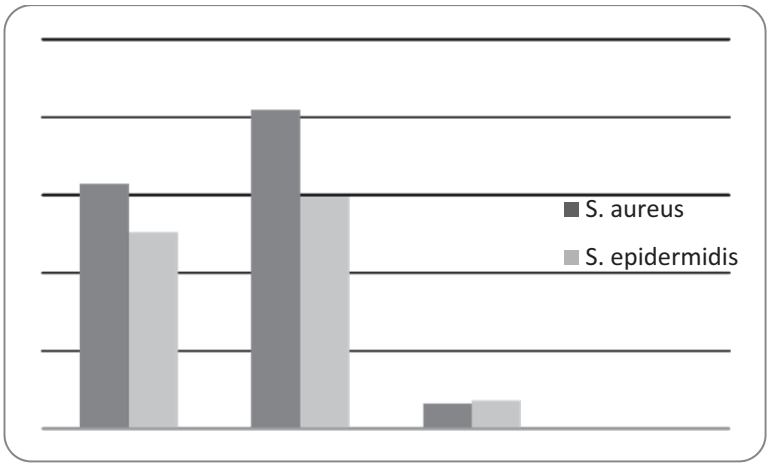


Figure 1. Resistant profile of *S. aureus* and *S. epidermidis* strains

Oxacillin was most comparable in potency with meropenem (resistance rates < 2%).

CONCLUSIONS

Meropenem was the most active of the agents tested and all isolates in the study were susceptible (MICs<2 mg/L). Our results indicate that the potency of azithromycin was slightly superior to erythromycin.

On the basis of these data and in consideration of the facts that azithromycin is usually well tolerated and the duration of treatment is generally short this agent may be recommended as possible alternatives in the treatment of staphylococcal infections by beta-lactamase positive staphylococci.

The data obtained by our surveillance study of the staphylococci strains shows that the emergence of resistance to tested agents has not been extensive.

REFERENCES

Archer, G. L., Climo M. W., (1994): *Antimicrobial susceptibility of coagulase-negative staphylococci.* Antimicrob Agents and Chemother, 38, 2231-2237

Ashley, D. J., Brindle M. J., (1980): *Penicillin resistance in staphylococci isolated in a casually department*. J Clin Pathol, 13, 336-338

Barry, A. L., Thornsberry C., Jones R. N., (1987): *In vitro activity a new macrolide, A-56268, compared with that of roxithromycin, erythromycin and clindamicin.* Antimicrob Agents and Chemother, 31, 343-345

Clinical and Laboratory Standards Institute, (2008): *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically.* Approved standard M07-A8 and Informational Supplement M100-S19, vol. 29. National Committee for Clinical Laboratory Standards, Wayne, Pa.

Crosseley, K. B., Archer G. L., (1997): *The staphylococci in human diseases*, 1st edn New York: Churchill Livingstone.

Retsema, J., Girard A., Schelkly W., Manousos M., Anderson M., Bright G., (1987): *Spectrum and mode of action of azithromycin (CP-62.993), a new 15-memberd-ring macrolide with improved potency against Gram-negative organisms.* Antimicrob Agents and Chemother, 31, 1939-1947

1 Microbiology Dept., Faculty of Pharmacy, "Gr. T. Popa" University of Medicine and Pharmacy, Iasi, Romania

STUDY REGARDING THE SEQUENCE OF ERUPTION OF PERMANENT TEETH AT A GROUP OF CHILDREN FROM BUZĂU

MARIA NICHIFOR [1], MIHAELA MONICA SCUTARIU [1], CONSTANŢA MOCANU [2], EDUARD CRAUCIUC [1*], EUGEN UNGUREANU [3], OVIDIU TOMA [3], MIHAI DINU SCUTARIU [1]

Keywords: dental eruption, permanent dentition, genetic factors
Abstract: Tooth eruption is a physiological process in which the tooth migrates from the maxillary bone towards the oral cavity, at the end of which teeth find their place on the arch creating vicinity relations according to a genetic code characteristic to each individual. Dental eruption begins with the eruption of the first primary teeth around 6 months and finishes at 2 years and a half for primary teeth, and around 18 – 25 years for permanent teeth, when the third molar erupts. The teeth's eruption and development is, usually, related to the child's chronological age, but there can also be some discordances as we refer to a precocious eruption or, on the contrary, to a delayed one. The chronology of dental eruption is submitted to a genetic model that is valid for the entire human population. Nevertheless, the values for the initial and ending moments of each stage present important variations that require the study of the average values and mostly of the variability limits for different characteristic human samples thus to create reliable norms for comparing individual values.

INTRODUCTION

Teeth eruption represents the final stage in teeth formation, being seen as a physiological process in which the tooth migrates from the maxillary bone towards the oral cavity.

At the end of this process, teeth find their place on the arch creating vicinity relations according to a genetic code characteristic to each individual.

Normally, dental eruption starts around the age of 6 months with the eruption of the first primary teeth and finishes at 2 years and a half for primary teeth, and around 18 – 25 years for permanent teeth, when the third molar erupts.

The teeth's eruption and development is, usually, related to the child's chronological age, but there can also be some discordances as we refer to a precocious eruption or, on the contrary, to a delayed one.

Generally, each dental unit covers certain sequences that repeat in a given order starting with the development and the mineralization of the crowns and ending with the root's development and the formation of the apical area. At the same time, the development of each dental group can be related to a certain age period, so that for every moment of the growth period there is a particular representation characterized by the sum of the stages reached by each stage of the dental arch.

The criterion of dental age is very much used by dentists as it has the advantage of a relatively limited individual variability and of some relatively simple possibility of making appreciations.

PURPOSE OF THE STUDY

The study's goal is to evaluate the eruption age of permanent teeth at a group of children, in a longitudinal study and to compare the resulting data with the existent one in literature.

MATERIAL AND METHOD

The study is based on a group of 348 children from Buzău and the coterminous area, aged between 6 and 13 that were evaluated either on request or at the dental offices that exist in schools.

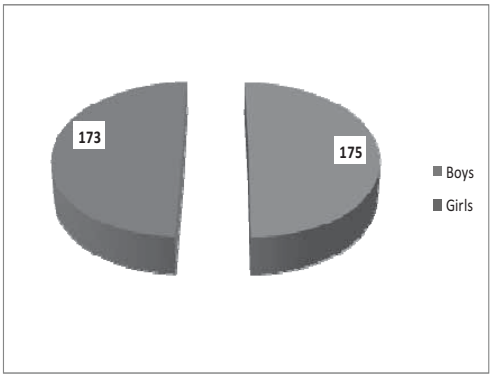
The data were got from the patients' observation charts, after the clinical and paraclinical exams (X-rays, photographies, study models, etc).

The resulting information was statistically interpreted using Excel. The average value and the standard deviation were calculated.

RESULTS AND DISCUSSIONS

Distribution by sex

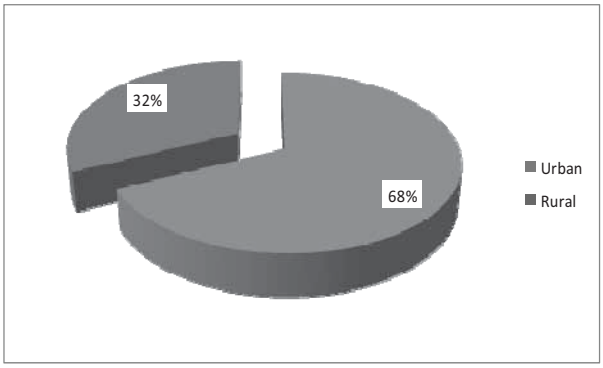
The separation of the initial group members according to their gender reveals a relatively equal number of members in each group (Picture 1).



Picture 1 Distribution by sex of the resulting groups

Distribution by origin

The distribution by origin (Picture 2) shows that most children come from the urban area.



Picture 2 Distribution by origin of the resulting groups

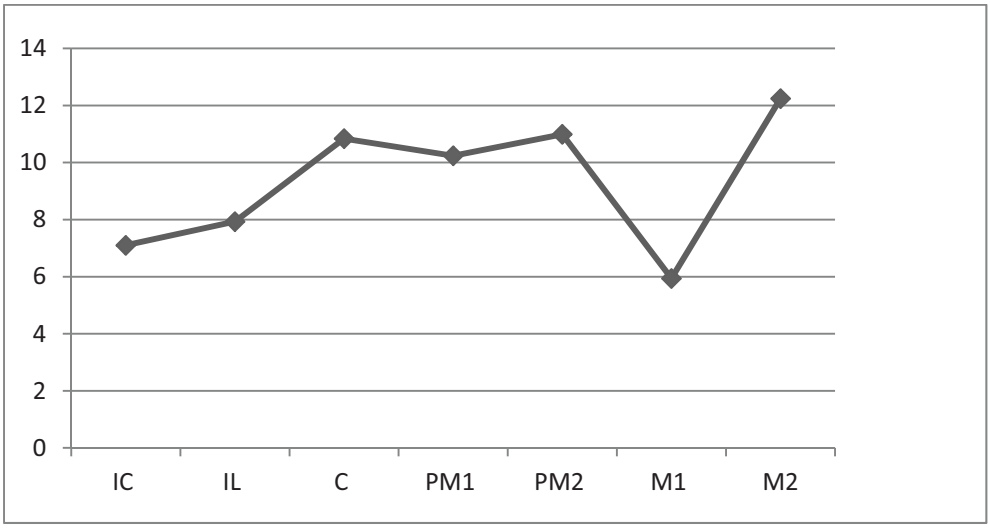
The group of boys

Evaluating the age when teeth appeared at the boys included in the study it resulted the following (charts I, II, pictures 3, 4):

Chart I Evaluation of the age when teeth appeared on boys – Maxillary

Tooth	Average age	SD	2SD (inter arch standard deviation)
CI 11,21	7,10	0,73	1,46
LI 12,22	7,93	0,77	1,54
C 13,23	10,84	1,07	2,14
PM_1 14,24	10,24	1,45	2,90
PM_2 15,25	10,99	1,14	2,28
M1 16,27	6,04	0,85	1,70
M2 17,27	12,24	1,55	3,10

Referring to the group of boys, the first tooth that erupted on the maxillary was the first molar, at the average age of 6.04 age, followed, in order, by central incisor, lateral incisor, first premolar, canine, second premolar, first permanent molar and second permanent molar.



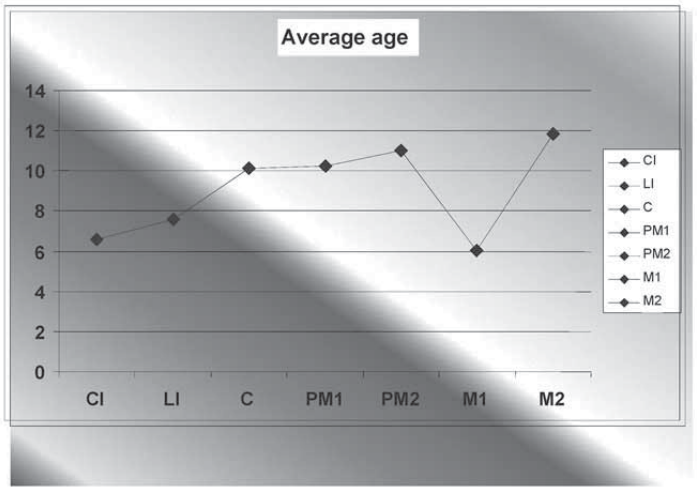
Picture 3 The apparition age of maxillary teeth on boys

Chart II Evaluation of the age when teeth appeared on boys – Mandible

Tooth	Average age	SD	2SD (inter arch standard deviation)
CI 31,41	6,56	0,92	1,84
LI 32,42	7,60	0,87	1,74
C 33,43	10,15	0,65	1,30
PM1 34,44	10,24	1,43	2,86

PM2 35,45	11,01	1,05	2,10
M1 36,46	6,03	0,85	1,70
M2 37,47	11,85	1,40	2,80

At the boys, the first tooth that erupted on the mandible was also the first permanent molar, followed by the central incisor, lateral incisor, canine, first premolar, second premolar and the second permanent molar.



Picture 4 The apparition age of mandibullary teeth on boys

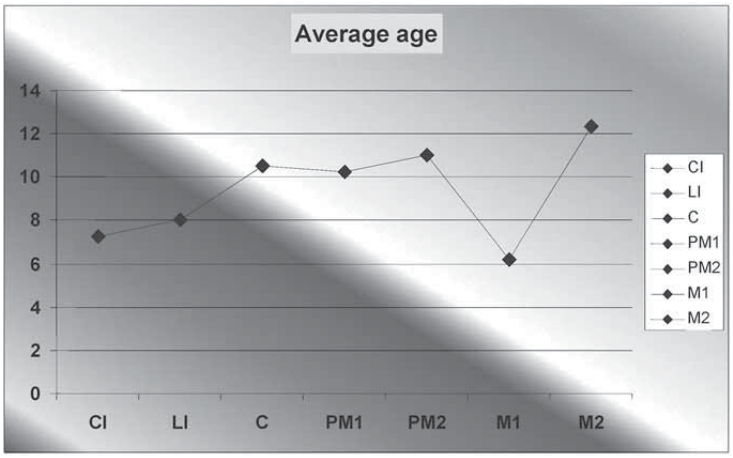
The group of girls

As far as the girls are concerned, the eruption age was as follows:

Chart III Evaluation of the age when teeth appeared on girls –Maxillary

Tooth	Average age	SD	2SD (inter arch standard deviation)
CI 1121	7,26	0,56	1,12
LI 12,22	8,01	0,72	1,44
C13,23	10,49	0,95	1,90
PM114,24	10,26	1,32	2,64
PM2 15,25	11,01	1,06	2,12
M1 16,27	6,17	1,02	2,04
M2 17,27	12,33	1,24	2,48

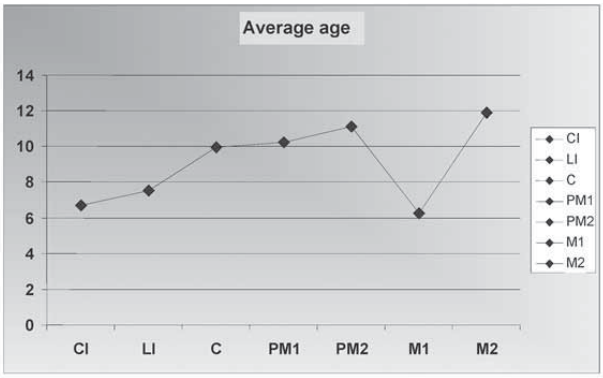
The same eruption sequence is noticed on girls as well, both on the maxillary and the mandible.



Picture 5 The apparition age of maxillary teeth on girls

Chart IV Evaluation of the age when teeth appeared on girls – Mandible

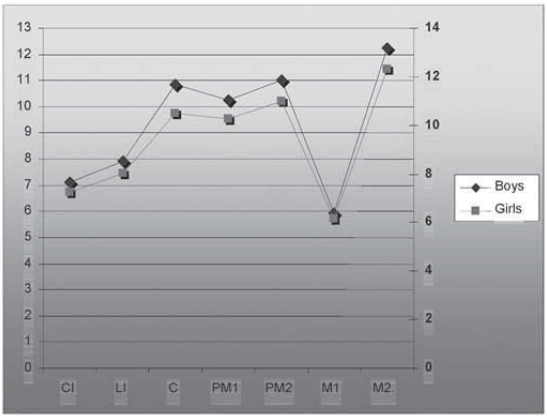
Tooth	Average age	SD	2SD (inter arch standard deviation)
CI 31,41	6,71	0,83	1,66
LI 32,42	7,55	0,70	1,40
C 33,43	9,96	0,93	1,86
PM1 34,44	10,21	1,35	2,70
PM2 35,45	11,12	1,11	2,22
M1 36,46	6,23	1,03	2,06
M2 37,47	11,90	1,08	2,16



Picture 6 The apparition age of mandibullary teeth on girls

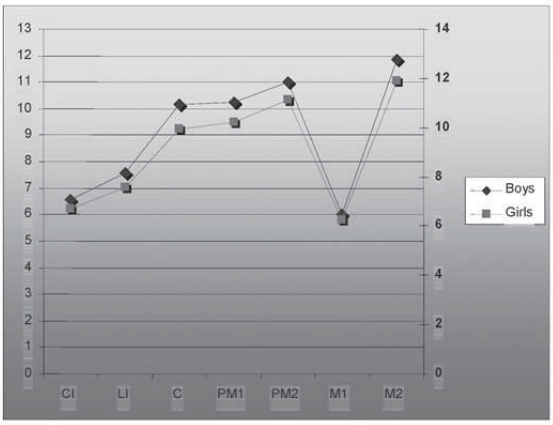
From the data presented above results that there are no significant statistic differences related to the arch (left / right).

Comparing the eruption age of permanent maxillary teeth results that there are some differences between the sexes, dental eruption being precocious on girls than on boys (Picture 7).



Picture 7 Comparison between the eruption age of maxillary teeth on girls and boys

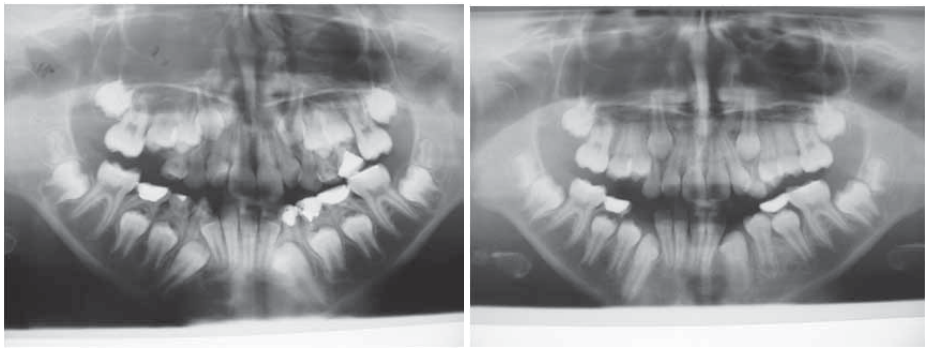
The same tendency is noticed for the mandible too, with differences between the sexes, mandibullary teeth erupting sooner on girls than on boys. Relatively equal values of eruption age resulted only in the case of the first permanent molars (Picture 8).



Picture 8 Comparison between the eruption age of mandibullary teeth on girls and boys

Complementary exams

Complementary exams (radiographic exam) also showed the sequence of eruption:



Picture 9 Sequence of eruption of permanent teeth

The analyzes performed on groups of children from northern countries underlined the existence of some modifications during the last decades, more precisely, the first tooth that erupted was the mandibullary central incisive and not the six years inferior molar.

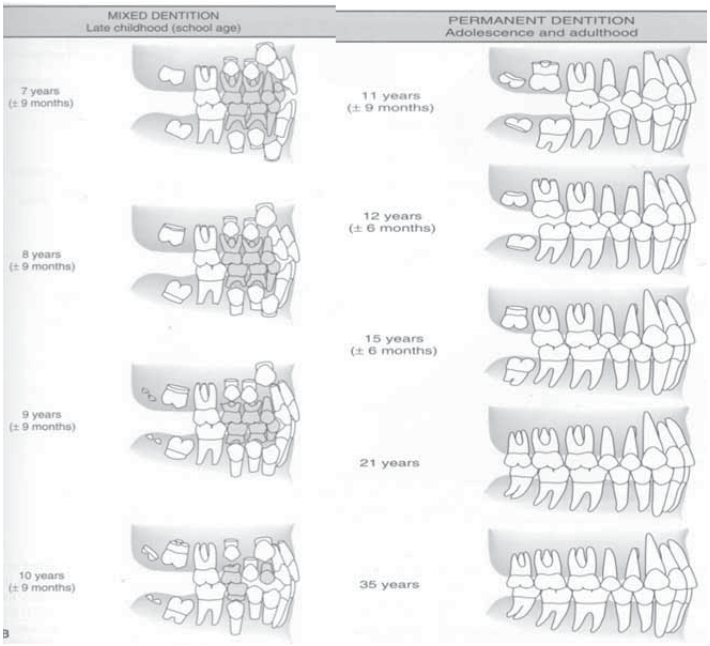
According to Lyselle, Magnusson and Thilender, the eruption of permanent teeth on the populations situated in the N of Europe takes place in the following sequence expressed in years and months (Chart V):

Chart V The eruption of permanent teeth on the populations situated in the N of Europe

Maxillary			**Mandible**		
Tooth	**Boys**	**Girls**	**Tooth**	**Boys**	**Girls**
11;21	7.3	7.1	***41;31***	6.4	6.2
12;22	8.4	8	***41;32***	7.6	7.1
13;23	11.7	11.0	***43;33***	10.8	9.9
14;24	10.4	10.0	***44;34***	10.8	10.2
15;25	11.2	10.9	***45;35***	11.5	10.9
16;26	6.7	6.7	***46;36***	6.6	6.4
17;27	12.7	12.3	***47;37***	12.1	11.7
According to Lyselle, Magnusson and Thilender					

Comparing the data we achieved and the one present in the study mentioned above, we realize that the values are quite close.

Bath-Balogh and Fehrenbach (2010), present the following values for the eruption age of teeth during mixed and permanent dentition (Picture 10).



Picture 10 Sequence of dental eruption – eruption age of permanent teeth (according to Bath-Balogh and Fehrenbach, 2010)

The eruption of permanent teeth expands on a longer period of time, between 6 and 13 years, being submitted to individual variations that are more often and more complex than in the case of temporary teeth.

The different way in which tissues react during the developing process represents an essential factor to differentiate a large number of clinical problems.

The chronology of dental eruption is submitted to a genetic model valid for the entire human population. Nevertheless, the values for the initial and ending moments of each stage present important variations that require the study of the average values and mostly of the variability limits for different characteristic human samples thus to create reliable norms for comparing individual values.

Generally, mandibullary teeth erupt sooner than maxillary teeth, except for the premolars. Apart from some modifications in the order of appearance, in the last decades it was noticed that permanent teeth tend to appear at a younger age. This process is related to the accelerated process of general development and the beginning of puberty at a younger age which are determined by the growth of the life standards and the sudden decrease of rachitism, at least in the European countries. This tendency was noticed for the second molars from both maxillaries and for the canines and the first mandibullary premolars.

It is also true that, in the case of permanent teeth the difference between sexes is obvious; teeth erupt sooner on girls than on boys. Nevertheless, these differences are extremely limited for the first erupted teeth, centrals and first molars, but they are more visible, 8 -12 months, for the teeth that appear later as canine do. The difference in

behaviour between the sexes especially towards the end of the development stage is due to the moment when puberty starts. It is well known that girls enter puberty before boys.

Some deviations from normal refer to the teeth's place and position in relation to the bony substrate.

The sequence of teeth eruption follows a certain pattern but variations on the erupting age can also appear due to general and local factors.

Referring to the sequence of eruption in the case of permanent teeth, a dental group appears in a period of 1 year, unlike the case of temporary teeth, where a dental group erupts at a 6 months interval. The teeth on the inferior arch appear before those on the superior arch, and if we refer to the two sexes, teeth appear sooner on girls than on boys.

Except for the molars, the eruption of permanent teeth takes place in the same time with the resorption of the root of temporary teeth and their exfoliation from the arch. Subsequently, there is a period of time in which, on the arch, coexist both the permanent teeth and the temporary ones – the period of mixed dentition that ends with the exfoliation of the last temporary teeth.

The normal variations of the eruption age are within the limit of one year (6 months sooner or later) in relation to the average age of eruption.

This variability is determined by a series of general factors as the patients sex (on girls dental eruption can take place sooner than on boys with 6 months up to 1 year); race (eruption takes place sooner at black people than on Caucasians); climate (it was noticed that eruption appears sooner in the areas with worm climate than in those with cold climate); social – economic factors (a higher life level and the urban environment favour the precocious eruption of teeth).

Apart from these factors, there are also a series of local and systemic conditions that can influence the sequence of dental eruption.

Local conditions as traumatisms during temporary dentition or cavity complications at temporary teeth can lead to the early loss of the temporary tooth and to the precocious eruption of the permanent tooth that are in this case immature and insufficiently mineralized.

Some systemic conditions can also lead to the variability of the eruption pattern of permanent teeth.

Genetic factors as Down syndrome, osteopetrosis, cleidocraneal dysostosis as well as a series of endocrine diseases can cause delays for the entire permanent dentition: sequence turnarounds, the presence on the arch of some temporary teeth or the presence of over-number teeth.

CONCLUSIONS

The studies on dental eruption that were realized with the help of illustrative groups of people allow in the end to estimate the speed with which dental maturation takes place and more than this can illustrate the levels reached by the general maturation of the children's' organism.

Dental age represents a valuable element in interpreting temporary or permanent differences that may appear during the development of dental occlusion. It allows the

identification of the best moments for initiating orthodontic treatments or selecting the most suitable treatment methods.

Apart from some modification in the order of eruption, in the last decades was noticed that permanent teeth tend to appear sooner, at younger ages, fact that is correlated to the accelerated general development and to the fact that puberty starts earlier; a possible explanation could be the growth of life standards and the sudden decrease of rachitism, at least in the European countries.

In the case of permanent teeth the difference between sexes becomes obvious, permanent teeth erupting sooner on girls than on boys.

REFERENCES

Bath-Balogh, M., Fehrenbach, M.J.: *Dental embryology, histology and anatomy*, 3rd edition, Elsevier, 2010.
Buckler, J. M. H.: *A reference manual of growth and development,* 2nd edition, Blackwell Science, 1996.
Friedrich, R.E., Habib, S., Scheuer, H.A.: *Eruption times of permanent teeth in children and adolescents in Latakia (Syria),* Arch. Kriminol. 2009 Mar-Apr; 223(3-4):84-97.
Gorski, P, Marks, Se.: *Current concepts of the biology of tooth eruption*. Crit Rev Oral Biol and Med 1992; 3:185-206.
Gulati A., Taneja JR, Chopra SL, and Madan S.: *Interrelationship between dental, skeletal and chronological ages in well nourished and malnourished children*. J. Indian Soc. Pedo. Prev. Dent. 1990; 8: 19-23.
Hernández, M., Espasa, E., Boj, J. R.: *Eruption Chronology of the Permanent Dentition in Spanish Children* Journal of Clinical Pediatric Dentistry Volume 32, Number 4 / Summer 2008
Johnsen, D.C.: Development and anomalies of the teeth. In: Nelson WE, Behrman RE, Kliegman RM, Arvin AM, eds. Nelson's Textbook of Pediatrics. Philadelphia, PA: WB Saunders Company; 1998:1037-1039
Kobayashi, T.Y. Gomide, M R; de Carvalho Carrara, C. F.: *Timing and sequence of primary tooth eruption in children with cleft lip and palate* Journal of Applied Oral Science vol.18 no.3 May/June 2010
Kochhar, R., Richardson, A.: *The chronology and sequence of eruption of human permanent teeth in Northern Ireland*. International Journal of Paediatric Dentistry: Volume 8, Issue 4, pages 243–252, December 1998
Lysell, L., Magnusson, B., Thilander, B.: *Relations between the times of eruption of primary and permanent teeth.* A longitudinal study. Acta Odontol Scand. 1969 Jun; 27(3):271-81.
Marks, **S., Mappes, Edward, F. Harris, Rolf, Behrents:** *An example of regional variation in the tempos of tooth mineralization and hand wrist ossification*. Am. J. Orthod. Dentofac. Orthop 1992; 101: 145-51.
Marks, S.C. Jr.: *The basic and applied biology of tooth eruption*. Connect Tissue Res 1995; 32:149-57.
Marks, S.C., Schroeder, H.E.: *Tooth eruption: theories and facts*. Anat Rec. 1996; 245:374-393.
Maxim, A., Păsăreanu, Marinela, Bălan A., Armencea, B.: *Paedodontics,* Ed. Contact internaţional, Iaşi, 1999.
Neville BW, Damm DD, Allen CM. *Oral and maxillofacial pathology*, Philade1phya: W.B. Saunders; 2002.
Rudolph, A.M.: *Growth and development of the teeth*. In Rudolph AM, Hoofman JIE, Rudolph CD, eds. Rudolph's Pediatrics. 20th ed. East Norwalk, CT: Appleton & Lange; 1998:978-979.
Suri, L., Gagari, E., Vastardis, H.: *Delayed tooth eruption: Pathogenesis, diagnosis, and treatment. A literature review* American Journal of Orthodontics and Dentofacial Orthopedics October 2004 Volume 126, Number 4
Zarnea, Livia: *Pedodonţie*. Ed. Didactică şi Pedagogică, Bucureşti, 1993.

[1] **"Gr. T. Popa" University of Medicine and Pharmacy of Iaşi**
[2] **"Apollonia" University of Iaşi**
[3] **"Alexandru Ioan Cuza" University, Iasi, Romania**
* **crauciuc@yahoo.com**

RHEOLOGICAL PROPERTIES AND SOLVENT STRUCTURE OF POLYSACCHARIDE HYDROGELS STUDIED BY MOLECULAR DYNAMICS SIMULATIONS

ANDREI NEAMTU[1*], ANA-MARIA OPREA[2], TUDOR PETREUS[3], LUCIAN GORGAN[4], CORNELIA VASILE[2]

Keywords: hydrogel, sulfated glycosaminoglycans, molecular dynamics simulations, rheology
Abstract: One important class of hydrogels based on natural polymers is the Glycosaminoglycan (GAG)-based hydrogels. In hydrogels biomaterial science, the mathematical modeling and computer simulation plays a complementary interpretative role in deciphering the complex physical/chemical and biological properties of this class of substances. **Aim:** the molecular modeling studies presented here aimed the information gathering regarding the particular molecular interactions responsible for rheological properties of this class of biomaterials. **Methods:** the methods included molecular dynamics simulations in the NPT ensemble for polysaccharidic matrices, radial function analysis for the solvent and viscosity calculations using periodic strain non-equilibrium molecular dynamics. All this methods were applied to models of 100%, 66% and 33% of maximum hydration compared to pure solvent simulations as control. **Results and conclusions:** decreasing the water content of the polymer matrix drastically affects the conformational flexibility of the polymer chains, the solvent percolation and viscosity coefficient of the biomaterials studied. The obtained viscosity coefficients were: $\eta_{H2O} = 0.982\times10^{-3}$ kg/(ms); $\eta_{100\%} = 1.520\times10^{-3}$ kg/(ms); $\eta_{66\%} = 1.862\times10^{-3}$ kg/(ms); $\eta_{33\%} = 2.602\times10^{-3}$ kg/(ms). The findings are useful for polysaccharidic hydrogel materials science as the rheological and solvent structuralisation can dramatically influence the physical stability of eventual macromolecular bioactive agents (e.g. therapeutic proteins) when they are loaded into such matrices for controlled delivery, especially during the storage period when the material is kept in lyophilised conditions.

INTRODUCTION

One of the most promissing class of polymers with adequate properties for drug or bioactive molecule/ macromolecule loading and release is represented by hydrogels. Hydrogels represent three-dimensional polymer networks that include chemically reticulated macromolecular chains (Hoffman 2002) embeeded in an aqueous environment. One important characteristic of hydrogels is that on the macroscopic scale they behave like solids while at molecular scale hydrogels have properties similar to solutions (Tanaka 2005). This is important especially for macromolecular active substances such as proteins because the mechanical properties of the reticulated polymer chains allow the immobilisation of the molecule inside the matrix while still keeping an aqueous medium similar to the natural one. Hydrogels are currently used in clinical practice and experimental medicine for: tissue engineering and regenerative medicine (Lee 2001); diagnosis (van der Linden 2003); controlled drug delivery (Lin 2006); surface cell immobilisations (Jen 1996); biomolecule and cell separations (electrophoresis) (Wang 1993); surface coatings for cell adhesion (Bennett 2003). Due to the increased water content and softness, similar to natural tissues, hydrogels may represent multicomponent systems that exhibit the characteristics of a natural material with an excellent biocompatibility (De Groot 2001). Regarding the chemical composition, the hydrogels are composed of 2%-80% polymer, 20%-98% water and 0.1%-5% additions. One important class of hydrogels based on natural polymers are the Glycosaminoglycan (GAG)-based hydrogels. Glycosaminoglycans are natural polymers made of specific repeating disaccharide units in which one sugar is uronic acid and the other is either N-acetylglucosamine or N-acetylgalactosamine. For obtaining succesfull hydrogel formulations GAGs have to be mixed with other polysaccharide types (ex. Cellulose or Xanthane) to increase the mechanical strength and to modulate the swelling degree (Oprea, 2010). In biomedical science these materials found their suitability in drug delivery (Soppimath 2002) (Byrne 2002), ophtalmology (Myers 1991; Compan 1998), tissue engineering (Darsov 1995; Draye 1998), urology (Di Tizio 1998), plastic and reconstructive surgery (San Roman 2001), orthopaedics (Broom 2000). Therewith, there are many important applications in pharmaceutics and biotechnologies. Also, GAG hydrogels (Hyaluronan, Chondroitin sulfate) and their derivatives were used for wound healing due to their potency of inducing re-epithelization (Luo 2000; Kirker 2004).

For obtaining an optimum hydrogel material with superior biomedical characteristics for a particular application the research has to be focused not only on the synthesis of the material but also on the physical and chemical characterzation of their properties which finally dictates the quality of the obtained therapeutic system. Along with the experimental techniques routinely used in the area of the biomaterials, in the recent years the computer simulation and modelling is playing an incresing role due to their ability to microscopically describe processes inaccessile or very difficult to asses by experimental means (Lam 2003, 2004). The tremendous progress in computing power and algorithmics makes now

possible to simulate systems at the mesoscale level (10-100nm and 10ns-10μs) (Rex 1998; Papisov 1998; Marrink 2003, 2004; Lee 2009a,b).

Although the Molecular Dynamics (MD) technique for simulating molecular systems is largely used in the material science there is still a lack of such studies for hydrogel based systems (Tamai 1996a,b; Oldiges 2002a,b,c; Jiang 2007; Lee 2009a,b).

The aim of the current study is to implement a methodological framework for glycosaminoglycan hydrogel simulations and to study the rheological properties and solvent organization in relation to the degree of hydration.

METHODS

Molecular dynamics technique was used to simulate pure cellulose (Figure 1) matrices in aqueous environment in order to evaluate the phenomena that occur on the microscopic scale in this class of materials. The GLYCAM06 forcefield (Kirschner 2008) was used for the description of the polysaccharidic chains while for the solvent the TIP3P model of Jorgensen et al. (1983) was selected. The initial structure of the hydrogel was constructed by generating with the aid of the xLeap program (AMBER Tools 10 suite, Cornell 1995) of a polymeric chain of 32 repeating units of β-D-Glucose linked in 1 – 4 positions.

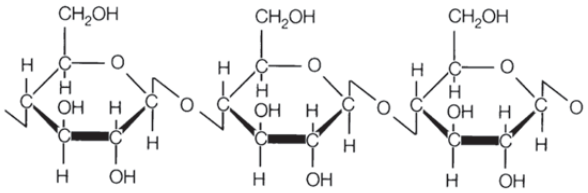


Figure 1. Cellulose structure

The resultant topology and coordinate files were then transformed to GROMACS (Berendsen, 1995) compatible input files with the aid of amb2gmx.pl script of Mobley (2006) corrected for negative dihedral potential barriers (GLYCAM forcefield in contrast with AMBER does not use phase shift for proper torsions and consequently it contains also negative values for some of the terms of the torsional energy barriers). In total there have been constructed 12 chains by replicating the original one and subsequently they were aligned to be perpendicular on the simulation cell faces as such there are 4 chains along each direction of the coordinate system (Figure 2). Due to the difficulties associated with the simulation of an infinite network (hydrogel) which can not make use of usual periodic boundary conditions, for each chain present in the system one of its was chemically connected to the other end of its corresponding periodic image using the „periodic molecules" option in GROMACS. Due to periodic boundary conditions the simulated system thus consists of infinite molecules arranged in a three dimensional network which is a good model for a hydrogel.

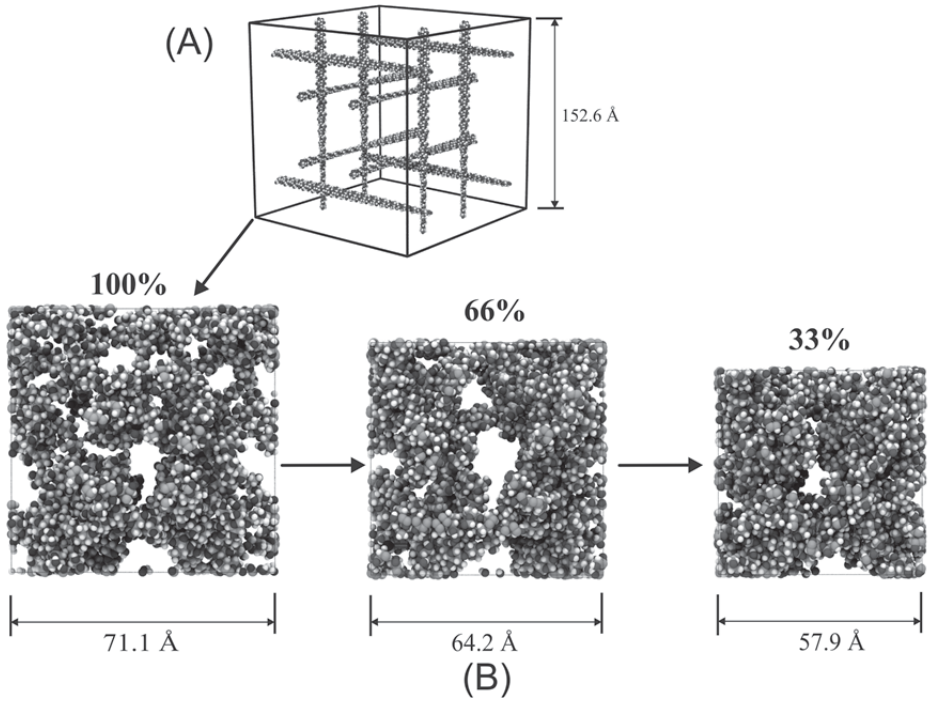


Figure 2. Depiction of the initial simulation cell constructed using the periodic molecules approach (A) and the compression of the cell to reach the necessary volume for 100%, 66% and 33% hydration (B).

The system constructed in this way has all the chains fully extended which is far from equilibrium even for 100% hydration. To obtain the desired density at 100% hydration the initial expanded simulation cell was gradually compressed in an iterative manner until its volume reached the correct value. For each step the simulation cell was scaled down a geometry optimization simulation was performed to relax the compressed polymer chains. The 66% and 33% box geometries were obtained in a similar manner by further scale down the simulation boxes. The polymeric chains in each simulation box were hydrated with TIP3P water molecules as follows: 9458 molecules for 100% hydration, 6170 molecules for 66% hydration and 3498 molecules for 33% hydration. The simulations were performed in NPT ensemble (number, pressure and temperature constant) at 1 atm and 300K. Integration step was 0.001 ps, each simulations being 100ps long.

For the viscosity calculations the Hess (2002) nonequilibrium method was applied. This method apply an external acceleration cosine profile in one direction of the simulation box and it is based on the fact that the energy, which is fed into system by external forces, is dissipated through viscous friction. The generated heat is removed by coupling to a heat bath. The viscosity simulations used the same parameters as the equilibrium ones but they were two times longer (200ps). All the simulations were performed in parallel on a Dell Cluster with 64 computing cores (Dell PowerEdge 1950) with Infiniband interconnect in the Molecular Modeling Laboratory of the Center for The study and Therapy of Pain, UMF “Gr. T. Popa” Iasi.

RESULTS AND DISCUSSIONS

The radial distribution functions (or equivalently “pair correlation functions”) describes how the number of a certain species of atoms varies with the distance from one particular atom. They are very useful in determine which is the particular structural microenvironment in which certain type of atoms are located. The data resulted from the simulations were analysed in order to

compute the radial distribution functions between water oxygens and between water oxygen and the O5 atom of the pyranose ring. Some of the results are depicted in the Figure 3.

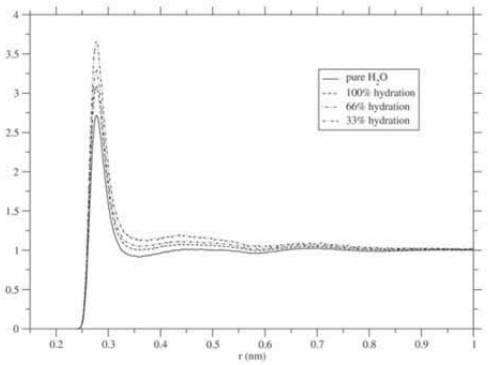


Figure 3. The radial distribution functions of OW (water) oxygen atoms for cellulose hydrogels at 100%, 66% and 33% hydration compared to the pure water .

It can be seen from the plots that the radial distribution function between water oxygens (OW) presents three peaks corresponding to the first three layers of hydration in all the three polymer matrices discussed here. This is comparable with the structuralization into a pure volume of water. The difference is that the peaks are increasing in amplitude when the water content of the hydrogel matrix is decreasing. In analysing these results we must state that there are two water populations in a hydrogel matrix: the bounded water compartment in which water molecules have strong interactions with the polymer chains and the bulk water compartment in which water molecules are located far from the chains and behave like in a pure volume of water. As the measurements were done on all the water molecules present in the system the increase in height of the first and subsequent peaks reflects that the bounded water compartment became more representative as the water content of the hydrogel lowers. This has an impact on the solvent mobility inside the polymer matrix as can be further analysed by computing the Mean Square Displacement (MSD) of water molecules.

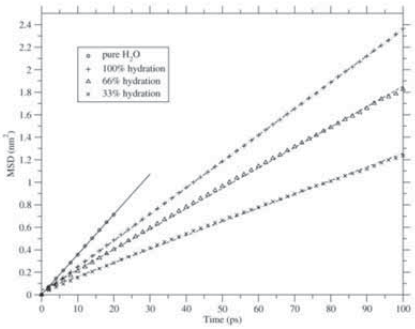


Figure 4. The Mean Square Displacement (MSD) of water molecules for cellulose hydrogels at 100%, 66% and 33% hydration compared to the pure water.

The MSD was computed from all the equilibrium simulations by following the motions of atoms from their initial positions. The results are presented in the Figure 4. The MSD, which is related to the diffusion coefficient of a molecule by the Einstein equation, is decreasing as the water content is decreasing. Again, as the MSD calculations were performed on the whole water population, and taking into account the results on pair correlation functions above, this decrease in the overall water mobility demonstrates that the bounded water compartment, beside being more ordered, is also far less mobile than the bulk water. These results sustain the experimental findings which correlates the degree of internal diffusion of different embeeded substances to the hydration level of hydrogels.

The solvent structure, along with the polymer chain packing, also influences the rheological properties of hydrogels. The percolation (filtration) of the solvent through the polymet network was evaluated using a non-equilibrium molecular dynamics technique as described in the *„Methods”* section.

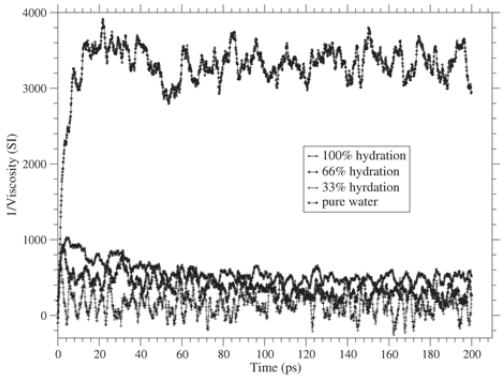


Figure 5. The viscosity of water flowing through the polymer network of cellulose hydrogels at 100%, 66% and 33% hydration compared to the pure water.

The results are expressed as an „average” viscosity coefficient which must not be identified with the usual shear viscosity coefficient of the entire hydrogel as the calculations performed here are subjected only to water molecules (which themselves further can be dinstinguished into two compartments as explained above). The „viscosity” coefficients computed qualitatively express the degree of water percolation through hydrogel matrices as a result of an applied external stress. The values obtained for the viscosity coefficients are: $\eta_{H2O} = 0.982\times10^{-3}$ kg/(ms); $\eta_{100\%} = 1.520\times10^{-3}$ kg/(ms); $\eta_{66\%} = 1.862\times10^{-3}$ kg/(ms); $\eta_{33\%} = 2.602\times10^{-3}$ kg/(ms). The results, presented in the Figure 5, demonstrate that the rheological properties of the solvent depend to a large extent on the hydration degree. This is due to either an increase of the bounded solvent population and also to a decrease of the pore dimensions of the polymer network as they become more tightly packed.

CONCLUSIONS

Molecular dynamics simulations were performed on cellulose hydrogel networks in order to establish a methodology suitable for the simulation of reticulated polysaccharidic biopolymers. The study also included the evaluation of the relationship between solvent organization,

rheological properties and the degree of hydration of the hydrogel matrices. While the first aspect is more general in its nature, providing methods of simulation not only for polysaccharidic polymers but for other reticulated structures too, the second one gives us more specific information about how water structure inside the hydrogel molecular microenvironment can influence its macroscopic properties. During the initial construction of the network model it clearly appeared that the usual periodic boundary conditions are not suitable for hydrogel simulations and further analysis proved that the 'periodic molecule' algorithm is the one to be chosen when three dimensional polymer networks are to be modeled. Regarding the solvent organization and dynamics the radial distribution functions and MSD suggest that as the water content decreases there is an increase of the bounded water fraction over the 'free' bulky water. The bounded water is more organized in the succesive layers around polymer chains than free water and also far less mobile which impose a limited diffusion of solvent (and of the dissolved substances) in matrices with low hydration. The degree of hydration in polysaccharidic hydrogels can be fine-tuned by inclusion in a mixed network of glycosaminoglycans (polyelectrolytes) on one hand and of cellulose on the other. Also, the reticulation density is the second major factor that can affect the degree of swelling of a hydrogel. The data presented here is important for the intimate understanding of the hydrogels behavior on molecular scale and to ease the correlations which can be made between the chemical structure of the reticulated polymers and the macroscopic properties (which can easily be measured experimentally).

REFERENCES

Bayly CI, Cieplak P, Cornell WD, Kollman PA *J. Phys. Chem.* 97:10269-10280, 1993

Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM Jr, Ferguson DM, Spellmeyer DC, Fox T, Caldwell JW, Kollman PA (1995) A Second Generation Force Field for the Simulation of Proteins, Nucleic Acids, and Organic Molecules. *J. Am. Chem. Soc.* 117: 5179–5197

D. L. Mobley, J. D. Chodera, K. A. Dill. (2006) On the use of orientational restraints and symmetry number corrections in alchemical free energy calculations, *Journal of Chemical Physics,* 125:084902.

Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys*, 79: 926-935

Soppimath, K. S., T. M. Aminabhavi, A. M. Dave, S. G. Kumbar, and W. E. Rudzinsky. *Drug Dev. Ind. Pharm.*, 28:957., 2002

Tanaka Y. et al. (2005), *Prog. Polym. Sci.,* 30:1-9

[1]Center for the Study and Therapy of Pain (CSTD) Iaşi, „Gr. T. Popa" University of Medicine and Pharmacy of Iaşi
[2]Laboratory for Polymer Physics and Chemistry, "P. Poni" Institute of Macromolecular Chemistry
[3]Department of Cell and Molecular Biology, „Gr. T. Popa" University of Medicine and Pharmacy of Iaşi
[4]Laboratory of Molecular Genetics, Faculty of Biology„Al. I. Cuza" University of Iaşi
* neamtuandrei@gmail.com

Acknowledgements: This paper was supported by funding from research Grant no. 2561/2008 CNCSIS

COMPARATIVE STUDY OF PHYSICAL INDICATORS AND THOSE OF THEIR REGIME OF OXYGEN, ON WATER QUALITY OF THE GREAT ŞOMUZU RIVER, IN THE YEAR 2009

CRISTINA MAXIM[1], CONSTANTIN FILOTE[2], DUMITRU C. COJOCARU[3*]

Keywords: Surface water, pollution, quality indicators, methods of analysis, monitoring.
Abstract: Water quality is an organic component of the system and economic management of water sources. The experimental data analyzed in this paper are part of a broader study on the assessment of water pollution in the upper basin of the Siret River and refers to the dynamics of physico-chemical and biochemical parameters, of Şomuzu Great River, a tributary of the Siret from Suceava County.

To do this, were collected every two months, samples of river water Şomuz from two different locations, from Dolhesti and Vorniceni, located 43 km apart. Water Sampling was done according to standards.

There were included in the main study physico-chemical quality, according to current regulations, PH - The concentration of dissolved oxygen, biochemical oxygen demand (BOD5) and chemical oxygen demand. Determinations and titrimetric methods are generally Spectophotometric. Determination of pH was done with a Seven Easy pH meter, which operates on the basis of electrochemical methods, and dissolved oxygen content was determined experimentally using electrochemical probe method

Based on data from water quality was monitored and were found to match the physical and chemical quality indicators.

The results allowed a characterization of water chemistry and establishing relationships with the natural factors, such as, fluid flow and temperature.

It can be concluded therefore that both the anthropogenic and natural factors can influence the general indicators describing the water chemistry.

INTRODUCTION

One of the major problems of the modern times is that of pollution of the soil, waters, air and of course of the aliments.

Romania as a member country of the EU, undertook that until November 2015 to reach a good ecological stage of the waters. The implementation of the nonpolluting, ecological technologies, must be accompanied by the constant monitoring of the surrounding environment in order to adopt optimal measures of stopping the pollution. Because of the fact that in Suceava are a multitude of industrial companies, mining complexes and animal farms with pollutant potential, in this work we have proposed to analyze some chemical and biochemical indicators of the chemical consumption and of the biochemical indicator of the oxygen (CBO, CCO-Mn and CCO –Cr) of the pollution degree of the waters of a branch of the river Siret from the territory of this county.

MATERIALS AND METHODS

To assess the dynamics of river water pollution Şomuzu Great water samples were collected from two different locations - Vorniceni section, the upstream location at 56 km from the mouth and Dolhesti section, downstream, 13 km away from the estuary - throughout the year 2008, every two months. There were included in the study as the main indicators related to the aeration system according to current regulations: the dissolved oxygen concentration was measured by electrochemical probe method, biochemical oxygen demand after five days (BOD5) and chemical oxygen demand, method potassium permanganate (COD-Mn) and potassium dichromate (COD-Cr).

RESULTS AND DISCUSSIONS

For a correct interpretation of values obtained in measurements of dissolved oxygen concentration, biochemical oxygen demand in 5 days (BOD5) chemical oxygen demand (COD and COD-Cr-Mn), we analyzed the results compared with the maximum permissible values for

the five classes of surface water quality, according to the Order 161/2006 on the approval of the standard benchmarks for surface water quality classification.

Permissible values of quality indicators of surface water according to the Order 161/2006 for approving the Norms on the benchmarks for quality classification

INDICATORS OF PHYSICAL AND CHEMICAL DYNAMICS OF THE GREAT RIVER SOMUZU , YEAR 2009

Table. 1.Dynamics of pH in The Great Somuzu River , locations : Voroniceni and Dolhesti in 2009

Collection Day	***03/02/2009***	***05/04/2009***	***07/06/2009***	***09/08/2009***	***10/10/2009***	***08/12/2009***
Dolheşti	**8.0**	**8.1**	**8.0**	**8.1**	**8.0**	**8.3**
Vorniceni	**8.1**	**8.2**	**8.0**	**8.1**	**8.1**	**8.2**

PH values, table 1, are almost constant, slightly alkaline water is determined by content of character appreciable alkali and alkaline earth carbonates. These waters will solubilize mineral acid rocks (sulphates, nitrates), resulting in increased concentration of anions SO_4^{2-} and NO_3^-in water. *(Daniela Cîrţînă, 2005)*

Apparently constant pH of natural waters suggest that they may be treated as steady state systems *(Mioara Surpăteanu, 2007)*

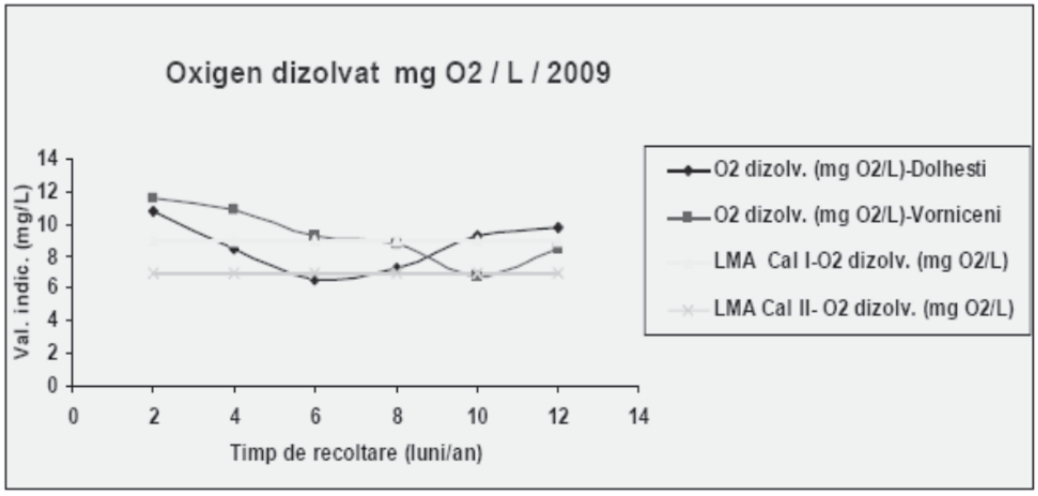


Figure 1. Evolution of dissolved O_2 concentration in The Somuzu Great River waters, location: Dolhesti and Vorniceni, during 2009

Dissolved oxygen content is the most important water quality parameter because the presence of oxygen is essential for aquatic life. The level of oxygen content can appreciate the

effect on aquatic organisms and oxidation residue of self-purification process development, *(Mioara Surpăţeanu, 2007).*

During 2009, the amount of dissolved oxygen is lower in downstream than upstream, until the eighth month and this month, with decreasing temperature increases the amount of dissolved oxygen downstream (Dolhesti) and decreases in the upstream (Vorniceni) Figure 1. This decrease in dissolved oxygen due to photosynthesis and improving processes to reduce oxidation of organic substances. Decrease the amount of oxygen in the water, reducing self-purification capacity of natural waters, favoring persistence of pollution. *(Mioara Surpăteanu,2002)*

In the spring-summer the river falls into the category Şomuz water quality II in Dolhesti location, and dissolved oxygen concentration Vorniceni exceeds the maximum permissible water quality I only in the tenth month, at a temperature of 140C (Fig. 1). Increasing the dissolved oxygen concentration may be due, first, contact with atmospheric air and secondly, UV radiation of sunlight, which in addition to their bactericidal role, allowing the aquatic plants photosynthesis reaction that results in the formation of glucose and oxygen:

$$6CO_2+6H_20 = C_6H_{12}0_6+6O_2$$

This explains the fact that daytime oxygen in water could double the quantity of oxygen during the night. Molecular oxygen, dissolved, promotes the destruction of anaerobic bacteria and at the same time contributes to the oxidation of organic matter. In addition, molecular oxygen dissolved aerobic bacteria help in their fight against anaerobic bacteria (much more dangerous to humans). Regarding the indicator changes BOD5 (Figure 1), which indicates the amount of oxygen used in a given time for the bacterial oxidation of organic matter present in water, the value of this parameter exceeds the maximum permissible water quality I, downstream (Dolhesti) throughout the year 2009, the sixth month reaching a maximum of 5.36 mgO2 / L, making in the River Şomuz in this case a third quality water. A high value of BOD5, water indicates the presence of large amounts of biodegradable organic compounds. Biochemical oxygen demand increases with the amount of organic substances in water *(Trufas Valer, 1980)* Upstream (Vorniceni), where the elevation is higher BOD5 parameter value does not exceed the maximum permissible water quality I, but very little at the end, probably due to low temperature.

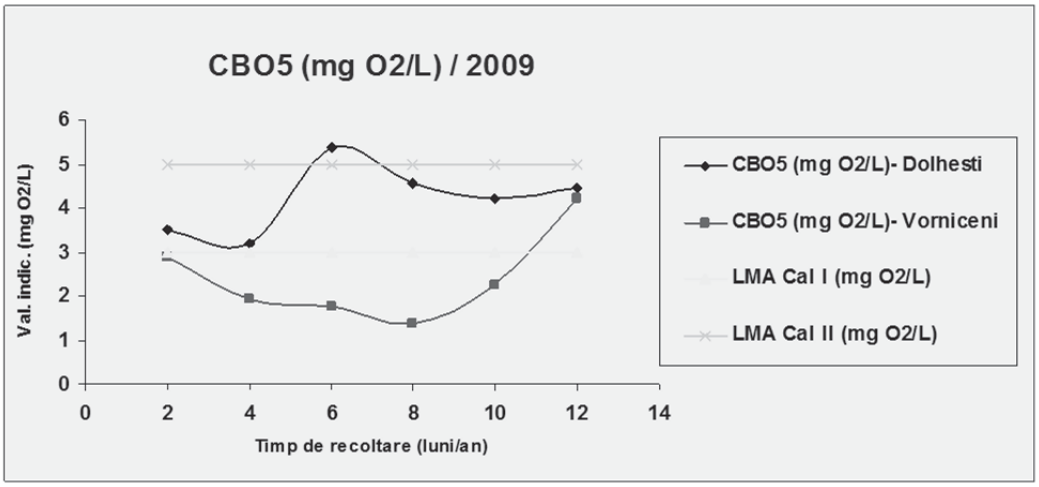


Fig 2. The Evolution of the BOD5 indicator in the Great River Somuzu , location : Dolhesti and Voroniceni, during 2009

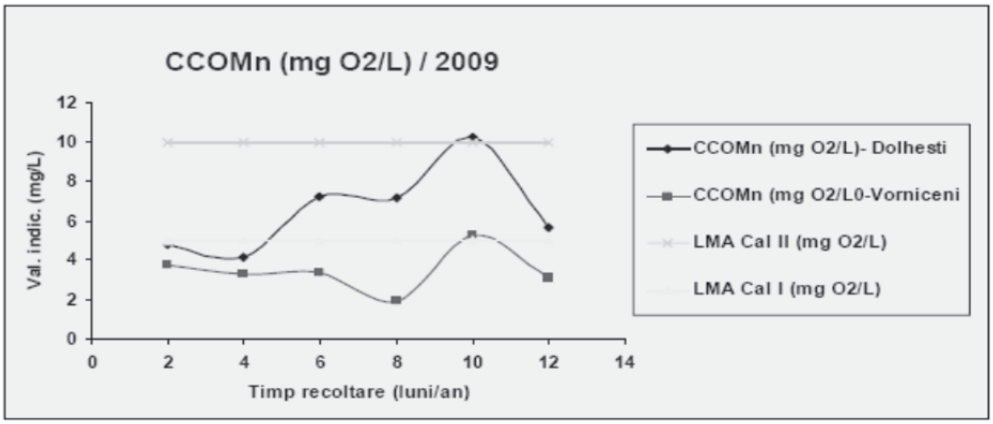


Figure 3. The evolution of the CCO-Mn indicator in the Great Somuzu River , locations : Dolhesti and Voroniceni , during 2009

CCOMn determination requires action on the excess KMnO4 oxidizable substances in water, in acid medium,at hot temperature

Subst. MnO_4 oxidizable $+ 8H^+ \rightarrow$ subst. oxidized $Mn^{2+} + 4\ H_2O$
(Trufas V., 2003).

COD-Mn dynamics indicator (fig.3) is similar to the BOD5 in the two locations. COD-Mn Downstream value increases during the spring-summer (high content of organic matter) and decreases at the end of the year, with decreasing temperature. During 2009, the river Şomuz in Dolhesti location is within the water quality category II, and the tenth month, less than the

maximum permissible limit for water quality II. Upstream (Vorniceni), CCO-Mn parameter value does not exceed the maximum permissible water quality I Şomuz river water quality is an I in terms of this parameter.

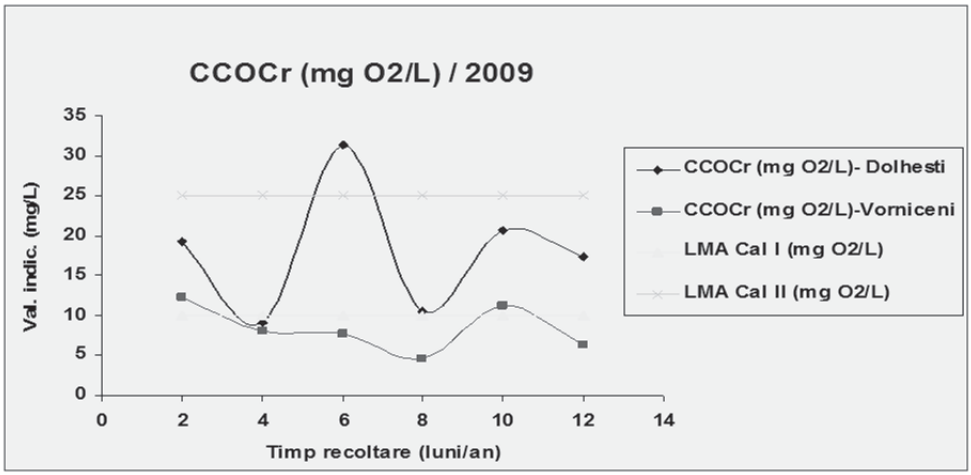


Figure 4. The evolution of the CCO-Cr indicator in the Great Somuzu River waters , location :Dolhesti and Vornoniceni , during 2009

Looking at Figure 4 reveals fluctuating parameter variation CCO-Cr Şomuz river water, downstream, the location Dolhesti, this indicator values exceeding the maximum permissible limit for water quality I. In the sixth, CCO-Cr value is 31, 25 mg O2 / L, river water is Şomuz this month, a water quality III a. The CCO-Cr causes 70% of non-biodegradable organic mass, so in the sixth month in Dolhesti location, the river water is rich in organic matter. Upstream, in terms of COD-Cr indicator, the river is the water quality Şomuz I, throughout the year 2009.

Comparative study of the chemical and physical indicators on the quality of the Great Somuzu River waters, location Dolhesti, year 2009

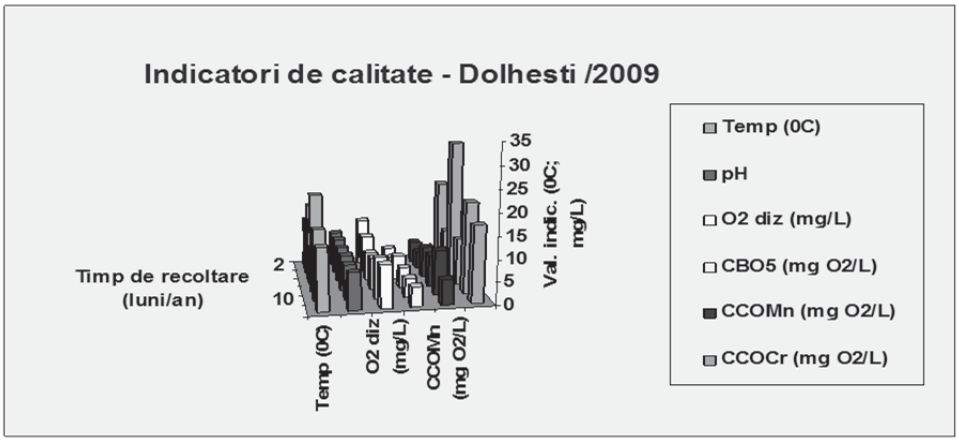


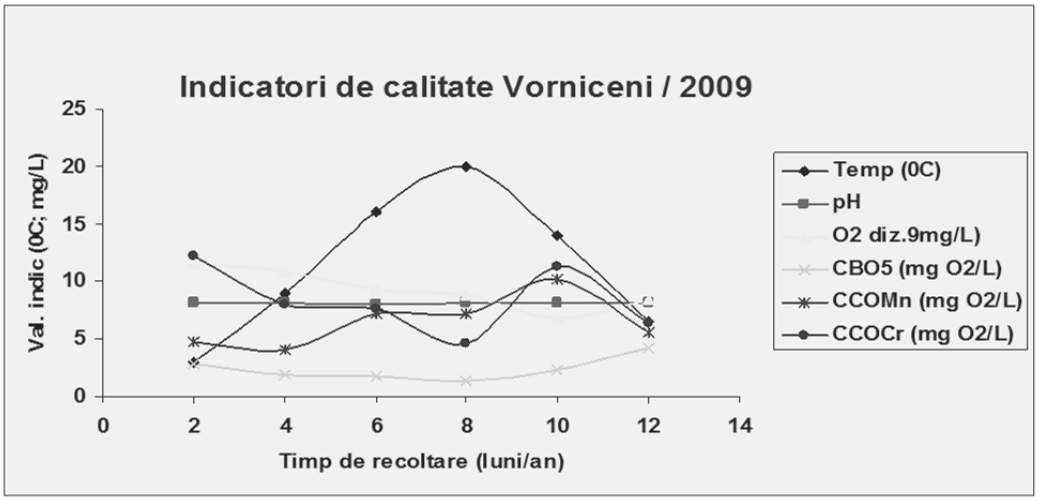
Figure 5. Comparative study of the physical indicators(temperature,pH,dissolved O2)with the chemical indicators(CBO5, CCO-Mn, CCO-Cr),location Dolhesti , year 2009.

The comparative study of physical indicators (temperature, pH, dissolved O2) with chi-small and biochemical indicators (BOD5, COD-Mn, CCO-Cr), the location Dolhesti, 2009 (Figure 5 shows the following:

- ✓ At the lower temperature regime indicators increased concentrations of oxygen, O2 dissolved due to the reduction of oxidation processes of riverbeds, and increase opportunities to dissolve a larger amount of oxygen in the atmosphere;
- ✓ At low dissolved oxygen concentrations correspond to high levels of COD-Cr indicator which confirms the presence of biodegradable organic matter in water; In water devoid of oxygen, decompose organic matter by anaerobic processes,produce the production of hydrogen sulphide and other toxic gases with bad smelling *(Gavrilescu Elena, 2007*)
- ✓ Once the heating water,⎫ there is a decrease of oxygen in hipolimnion due to oxidation processes, bacterial decomposition or fermentation of organic matter, and breathing creatures, processes that occur with the consumption of oxygen;
- ✓ At high concentrations of dissolved oxygen indicators correspond to small values of BOD5 and COD-Mn. These indicators correlate best: report CBO5/CCO-Mn having values*;(Daniela Cîrţînă,2005)*
- ✓ Biological self-purification ≥ 0.6 indicates, there is a synergistic context (cumulative) of the determinants of self-purification capacity of water;

- ✓ BOD5 parameter values relatively low, indicating reduced amount of biodegradable compounds in water, so the amount of oxygen consumed by microorganisms to decompose, the biochemical pathway, existing organic substances in water is low.

Comparative study of the physical and chemical indicators on the quality of the Great Somuz River waters, location Vorniceni, year 2009



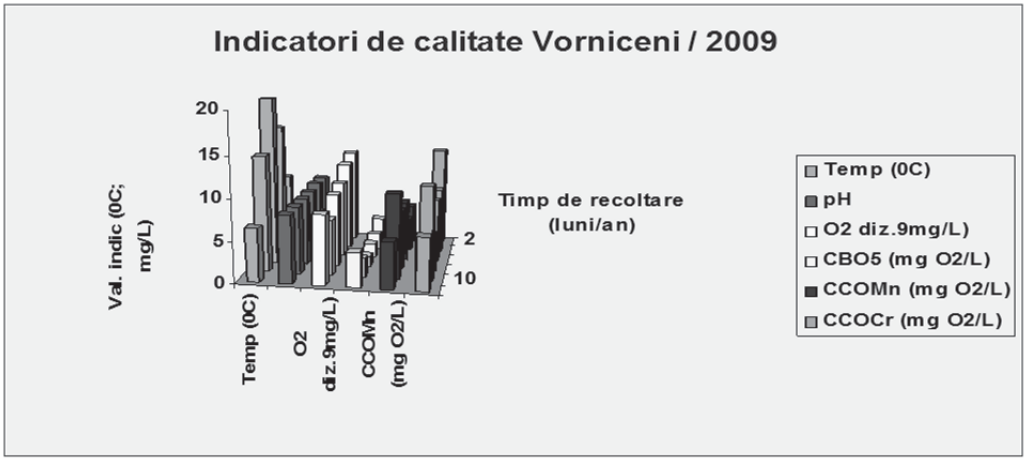


Figure 6. Comparative study of the physical indicators(temperature ,pH, dissolved O2) and chemical indicators(CBO5, CCO-Mn, CCO-Cr),location Vorniceni,year 2009.

From the data analysis presented in Fig. 6, we deduce:

- At high temperatures, corresponding to small amounts of oxygen dissolved in water and low levels of BOD5 indicators, and CCOMn CCOCr, so small amounts of biodegradable and non-biodegradable organic matter;
- BOD5 parameter correlates better with CCOMn parameter, the report has value ≥ 0.6 CBO5/CCO-Mn which confirms the existence of a synergistic context (cumulative) of the factors that caused a slight biological self-purification**;**
- The presence of large amounts of water causes oxygen levels low BOD5. The BOD5 value is less than the amount of biodegradable compounds in water is less;

In oxygen-rich waters, biological mineralization of organic matter is a complex process that takes place in two phases: first phase oxidizes carbon in organic substrate, and the second phase, nitrogen (nitrification). The difference between the chemical oxygen demand COD and BOD5 water is due to substances that cannot be decomposed microbiologically.

CONCLUSIONS

By studying the evolution of some physical and chemical parameters of river Great Şomuz over a period of two years, as it considers its status in the locations studied. Observe how the river responds to the elements disrupters, such regains balance through a process of self-purification.
Water quality does not remain constant over time, it may vary due to many factors, either man-made (anthropogenic factors) are of natural origin (of which, obviously, some man has a contribution).
The Report BOD5 / COD-Mn gives information about biological self-purification capacity: ≥ 0.6 if self-cleaning will be easier if the range of 0.2 to 0.4 will produce only self-cleaning thermal conditions favorable, and the report below 0.2 there is no biological self-purification. The experimental data shows that for all locations studied, the report CBO5/CCOMn is ≥ 0.6, which indicates that it has the ability to neutralize the natural water impurities falling into it and restore the ecological balance previously existing contamination: physical processes : dilution, mixing, diffusion, sedimentation, flocculation, dissolved oxygen, releasing gas into the air, influenced by IR and UV solar radiation, water temperature - chemical processes: neutralization, oxidation, reduction, floculaţie, precipitation, adsorption, absorption , photochemical degradation - biological processes: the biocoenosis own competing foreign elements, either directly, by action lithic (bacteriophages), filtering (scallops), consumption or secretion of toxic substances for intruders - biochemical processes - in the nitrogen cycle, sulfur and carbon, based on specific microorganisms (bacteria, fungi). They are more influenced by various factors such as pH, sunshine, oxygen saturation, temperature. The latter acts as Van `t Hoff's law: increase doubles decompositions 10oC.
In 2009, the situation is worsening in location Dolhesti, over 2008,indicator values CCOCr, CCOMn, nitrates, phosphates,phosphorus, water quality remains at the level II, but concentrations of CBO5, ammonia, nitrates value indicates the quality of water III, and nitrates indicates the quality IV, for Somuz rever water.
In 2009, water quality at Vorniceni Section is kept at I for all indicators studied, except nitrogen indicator, which places the Great Somuz River waters at grade II quality.
During one year, the average monthly dissolved oxygen in river water has an opposite variation of temperature. In summer high temperatures acting on the lower oxygen solubility, and promote

the growth of bacteria that consume it, winter supply of groundwater reduces oxygen saturation water *(Valer Trufas, 2003*)

All the conclusions presented above show that the assessment of water quality (and hence the possibility of using it for different purposes) is a task of great complexity. The mere existence of accurate results of a large variety of organoleptic analysis, physical, chemical, biological and bacteriological etc. proves insufficient for a correct interpretation, determining causality, the prediction of evolutionary trends and other elements necessary to establish an appropriate management accordingly. It requires interdisciplinary collaboration between biologists, chemists, physicists, geographers / hydrologists, geologists, meteorologists, physicians, computer, etc..

REFERENCES

Cîrţină D., (2005). *Water pollution. Siteck Publishing House, Craiova, p. 45,53,60-62*

Gavrilescu E., Oltean I., (2003). *Environmental quality. vol.II, water quality monitoring. University of Craiova, p. 39-51, 108 -115.*

Gavrilescu E., (2007). *Sources of pollution and environmental pollutants. Sitech Publishing House, Craiova, Pg185-187*

Pantazica M, Scrham M (1982). *Analysis of water quality indices of the Siret River and Prut. Geographic Seminar,""Dimitrie Cantemir, No.3, Al. I Cuza "Iasi p. 65-80.*

Surpăţeanu M., Zaharia C., (2002). *ABC Analysis Methods of quality environmental factors. Publishing T, Science, p. 72-85*

Surpăţeanu M., (2007). *Hydrochemistry and water quality analysis. Performance Publishing Iasi, p. 76,133,134,139*

Trufas V., (2003). *Hydrochemistry. Agora Publishing, Calarasi, pg.201*

Trufas V. (1980). *River water chemistry of the Siret River Basin. Ed.Univ. Bucharest, pg.108-110, 160*

1 The National College Of “Mihai Eminescu “ Suceava
2 The University”Ştefan Cel Mare’’ from Suceava
3 The University “ Al. I. Cuza” from Iasi , The Faculty of Biology
* cdumitru@uaic.ro

HEMATOLOGICAL CHANGES IN MULTIPLE MYELOMA

MIHAI BULARDA MOROZAN [1]*, COSTICĂ MISĂILĂ [2]

Keywords: blood, multiple myeloma, bone marrow, red cells, white cells,
Abstract: This paper presents the results of some hematological investigations which were done in the laboratory of the Municipal Emergency Hospital, Barlad on a number of 10 patients diagnosed with multiple myeloma. This type of blood cancer, also called plasmocytoma or Kahler’s disease, is characterized by a malignant proliferation of the plasmocytes in the bone marrow with serious hematopoietic perturbations, dramatic increase of ESR, bones pain, hypercalcemia, renal affection, etc.

Our results confirm both the grouping of the red cells into scrolls and the presence of the big myeloma plasmocytes, grouped into nests (foci), and sometimes can take unusual shapes (dumb bells). The hemoglobin and hematocryte decrease untill 63-65% compared to the normal physiological limit, the number of red cells reaches up to 20-23% from the normal limit, both for men and women, and the ESR amplifies compared to the normal as it follows: up to 12 times for men (65-158 mm/h) and up to 13,2 times for women (33-156 mm/h). The white cells and thrombocytes present some restricted numerical changes compared to the red series.

INTRODUCTION

The multiple myeloma, also called plasmocytoma or Kahler’s disease is a neoplastic disease of the blood tissue, characterized by a malign proliferation of the plasmocytes within the bone marrow and a production of an abnormal quantity of immunoglobulins (BUTOIANU and STANICA, 1973). The plasmocytes represent a class of B cells which produce the humoral factors of immunity, that means the antibody.

This type of blood cancer is produced by an unknown cause. It rarely strikes individuals aged under 40; it’s more common among those older than 60 years, especially when we talk about individuals exposed to radiations or chemical subtances. It is not a contagious disease, not even a hereditary one,even though the first-degree relatives (parents, sisters, brithers) of those patients diagnosed with multiple myeloma present a higher risk to develop neoplasia.

Men are more frequently affected than women are, and the incidence at the black people is double compared to that of the white people. MM is a severe neoplasm which represents about 1% from all the malignant disorders on white people and about 2% on black people, which means an average of 13% from the hematologic cancers at white people and 33% at black people. The life expectancy from the diagnosis is from 3 to 5 years.

The data from the scholarly literature records tha fact that the malignant proliferation of the plasmocytes within the multiple myeloma interferes with the normal production of the blood cells, causing anaemia, leukopenia, and even thrombocytopenia. ESR- the red cells sedimentation rate – has high values- most often over 100 mm/h, (MUT POPESCU, 2003)and, together with the normochromic anaemia, is an important sign in diagnosing a patient with bone pain.

The main objective of this report is to study the hematological changes collateral to the malignant proliferation of plasmocytes, according to the age and gender of the patients suffering of multiple myeloma. This study was done between 2004-2005, and the surveyed patients were from the Municipal Emergency Hospital in Barlad.

MATERIALS AND METHODS

The investigations were developed on a casuistry composed of a number of 10 patients diagnosed with multiple myeloma (6 women and 4 men), aged between 47 and 70 years. The samples of venous blodd were gathered on an anticoagulant (Na2-EDTA), the morphological examination and the achievement of the haemoleucogram were realized with the help of the hematological analyser called „CELLTAC MEK – 6318K”. Within the comparative study of the cases surveyed, the age and the gender of the patients were correlated with the following hematologic values: the ESR, the strength of hemoglobin (g/dl), hematocryte (%),the number of red cells, (x 106/µl), the number of thrombocytes (x 103/µl) ,eosinophils (%), basophils (%), and monocytes (%). The peripheral blood smear and the bone marrow smear were realized according to the classic methods (TANASESCU, 1974; MISAILA nad COMANESCU, 1999), and for colouring it was applied the May-Gründwald Giemsa method (ŢIŢEICA and MARINESCU, 1984). The red cells sedimentation rate was measured through the Westergreen technique (KONDI, 1981). The realization and colouring of these smears were made in Hematology Laboratory from the Municipal Emergency Hospital, Barlad, and the pictures were taken in the Genetics Laboratory of the Biology Department from „Al.I.Cuza””University, Iasi, by using a MC 5A microscope.

RESULTS AND DISCUSSIONS

The complexity of the interferences between the malignant proliferation of the plasmocytes within the multiple myeloma and other collateral functional perturbations goes beyond the sphere of the blood tissue, taking the shape of some various complications. The most severe complications dwell on the bone pain, hypercalcemia, renal insufficiency, bone marrow compression. Over 50% of the patients reported these issues of renal pathology, and an average of 80% of the patients with multiple myeloma showed signs of anaemia. Most of the times, this is normochrome and normocytic, as a result of both replacing the hematogenous marrow through the expansion of the tumoral cells, and also due to the hematopoiesis' inhibition by the tumoral factors. Besides that, a moderate hemolysis also contributes to the anaemia.The microscopic examination of the bone marrow smears (fig.1 and fig. 2) has shown the fact that, from a mmmooorphological point of view, the aspect of the plasmocytes (myeloma cells) is unstable from a patient to another, remaining relatively constant on the same patient, during the development of the disease. In most of the cases, the big cells with prominent nucleoli are prevalent, while the small cells with characters similar to those of the normal cells are more rare.

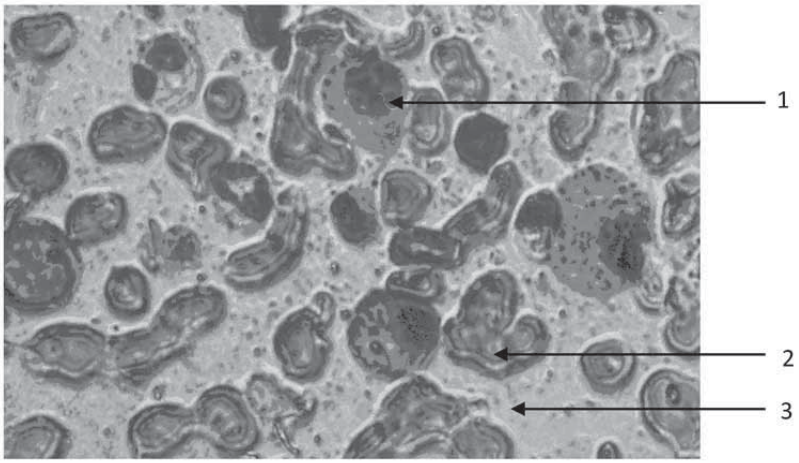


Figure 1: Bone marrow smear (multiple myeloma) – original (20x40)
1 – plasmocytes; 2 – thrombocytes; 3 – red cells grouped in scrolls

On the surface of these smears it could be noticed both the red cells tendency to be grouped in scrolls and the presence of the myeloma plasmocytes. Also the presence of some uncommon shapes of plasmocytes could be seen ((RĂILEANU and MOŢOIU, 1974), such as those taking the shape of dumb bells (BERCEANU , 1977). The data from the literature specifies that the appearance and proliferation of the myeloma cells lead to two types of basic perturbations, as it follows: infiltrations of tissues and organs, and protean anomalies. These are directly pr indirectly responsible for almost all the other simptoms and signs of the disease.
The distribution of these cells can be diffused or insular and this explains the variability of the cytological paintings that can been observed on different bone punctures.

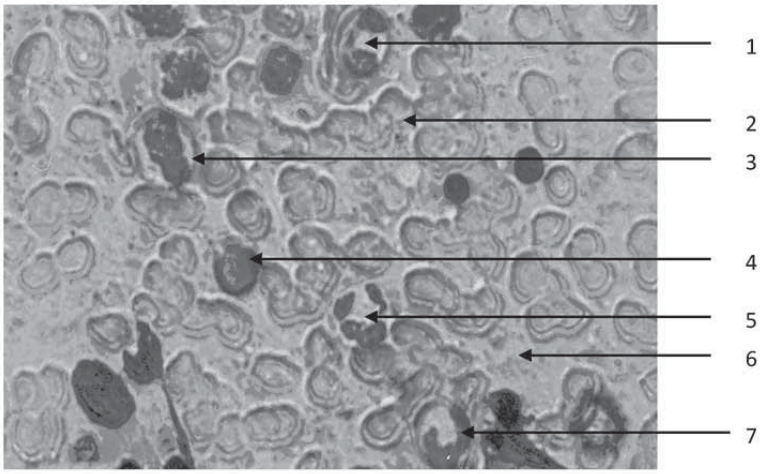


Figure 2: : Bone marrow smear (multiple myeloma) – original (20x40)
1 –eosinophil; 2 - erythrocytes arranged into scrolls; 3 – basophil;
4 – lymphocyte; 5 – neutrophil; 6 – blood platelet; 7 – monocyte

Our observation confirm the way of grouping of the red cells, and also the presence of the big myeloma plasmocytes and the aspect of the nucleus which is, obvious, disposed toward to cell periphery. The proliferated plasmocytes present modified morphological features compared to normal plasmocytes, some of them being able to be grouped into nests (foci) or to take various shapes (dumb bells).
It is very important to state precisely that these observations have been made on a heterogeneous casuistry, on patients of both genders, aged between 47 and 70 years and seen in different stages of the disease. As a result, even the degree of irregularity of the studied hematological values from the normal physiologic extreme limit is extremely varied from a patient to another.

Table 1: The distribution of the studied casuistry on genders and ages

Case No	Name’s initial letter	Gender	Age (years)
1	C.M.	F	47
2	N.H.	F	50
3	I.O.	B	56
4	S.G.	F	56
5	S.L.	B	57
6	E.R.	B	60
7	N.M.	F	67
8	F.H.	F	69
9	T.O.	F	70
10	N.A.	B	70

The hemoglobin. According to the data from the scholarly literature concerning the normal physiological limits (LOTREANU, 2000) the surveyed patients suffering of multiple myeloma from this report show obvious tendencies to anaemia. For every surveyed individual, the personal values of hemoglobin concentration are constantly situated under the normal physiologic level, representing an average of 64, 4% of the normal physiologic extreme limit in men (fig.3) and 63,3 % in women (fig. 4).

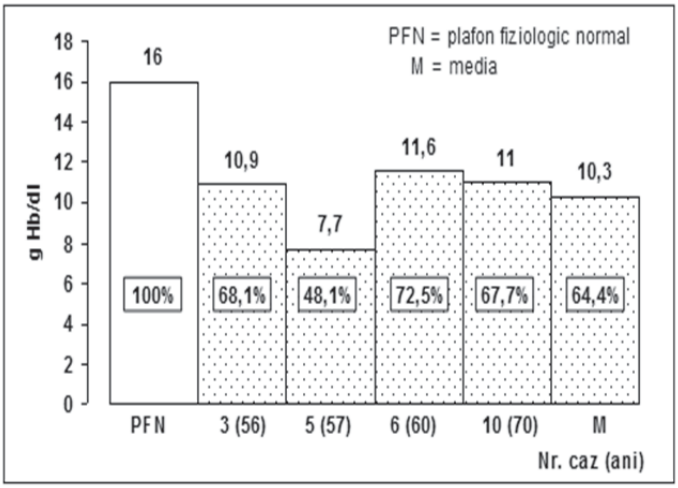


Fig. 3 The variation of the Hb in men with multiple myeloma

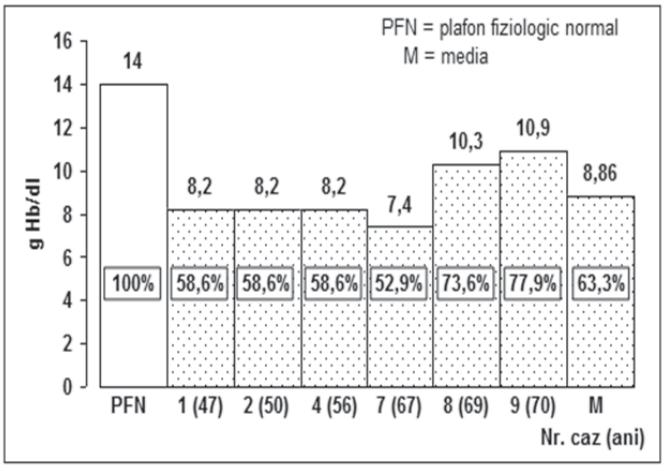


Fig. 4 The variation of Hb in women with multiple myeloma

The Hematocryte. The normal values of the hematocryte present the same tendency to decrease compared to the normal physiological limit, with a more homogeneous distribution between the cases analized on men (fig.5) and more irregular on women (fig.6). On average, the values of the hematocryte decrease when we talk about the male patients untill 65,9% of the normal physiologic extreme limit, but when we talk about women, the interindividual variation extends

from 43,6 to 112% of the normal extreme limit. So the average is situated at 82,1% of the normal physiological limit.

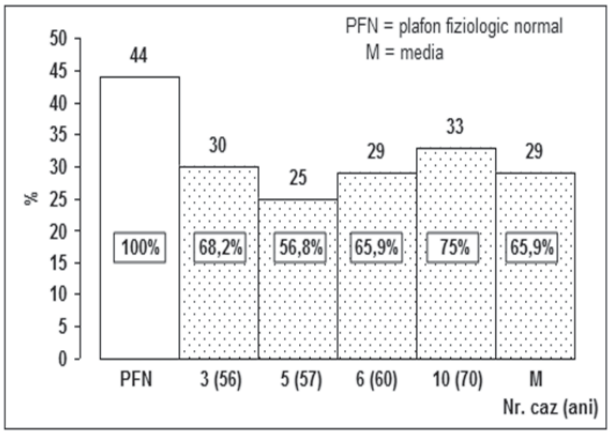


Fig. 5 The Ht variation in men with multiple myeloma

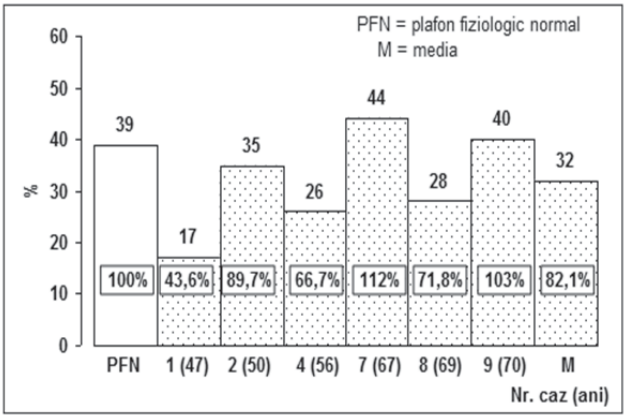


Fig.6 The Ht variation in women with multiple myeloma

The number of red cells. This hematological indicator claims one of the most dramatic levels of insufficiency distinguished on this category of patients. As the data from fig. 7 and fig. 8 have suggested, the number of red cells significantly decreases in all the surveyed patients, reaching to 20,6% of the normal physiological extreme limit in men and 21,6% in women.

Taking account of the much stronger depletion of the red cells number compared to the levels of concentration of hemoglobin, it is easily to understand the fact that anaemia is not hypochromically marked, each of the few left red cells being loaded with normal, even over normal quantaties of hemoglobin. The data that we got confirm the fact that the malign proliferation of the plasmocytes in multiple myeloma interferes with the normal production of blood cells, and the reported hemoglobin deficiency is rather a consequence of the erythropoiesis’s perturbation than of the hemoglobin synthesis’perturbation.

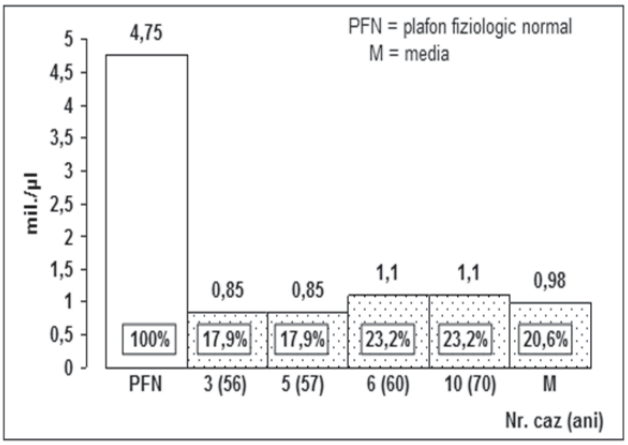


Fig.7 The red cells number variation in men with multiple myeloma

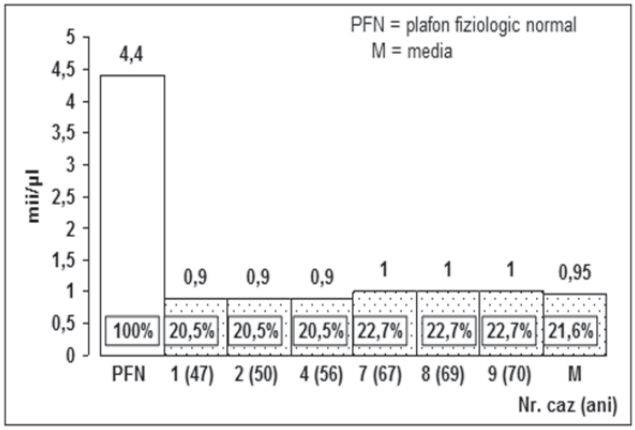


Fig.8 The red cells number variation in women with multiple myeloma

The erythrocyte sedimentation rate. As it results from the studies of other authors, all the patients with multiple myeloma present serious perturbations of the ESR, with values that increase from 5-12 mm/h to more than 100mm/h. Our data (fig.7 and fig.8) certify the extremely pronounced seriousness of the disease in the studied casuistry also through the fact that the values of ESR are amplified, compared to the normal, to 12 times in men (65-158 mm/h) and to 13,2 times in women (33-156 mm/h).

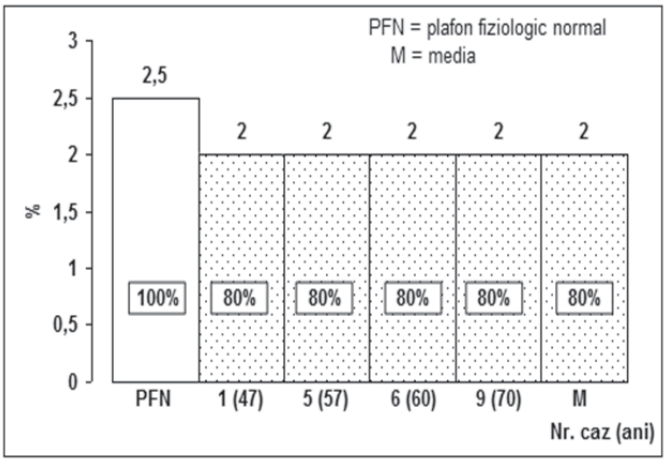


Fig.9 ESR variation in men with multiple myeloma

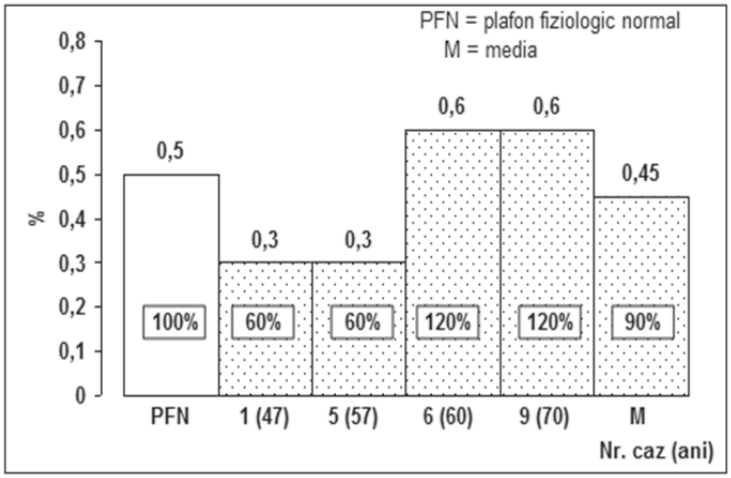


Fig.10 The ESR variation in women with multiple myeloma

Besides the study related to the variation of the hemoglobin concentration, of the hematocryte and of the number of red cells, inclusively the evolution of the ESR values, the investigation on the hematological changes in patients with multiple myeloma have also taken into account the white cells and the thrombocytes.

The white cells. The analysis of the eosinophils number in the cases taken to be studied have emphasized, on the one hand, a small uniformity of the values in the surveyed patients (fid.11), and on the other hand, a light tendency of eosinophilia. The individual values were constantly situated with 20% under the normal physiological extreme limit.

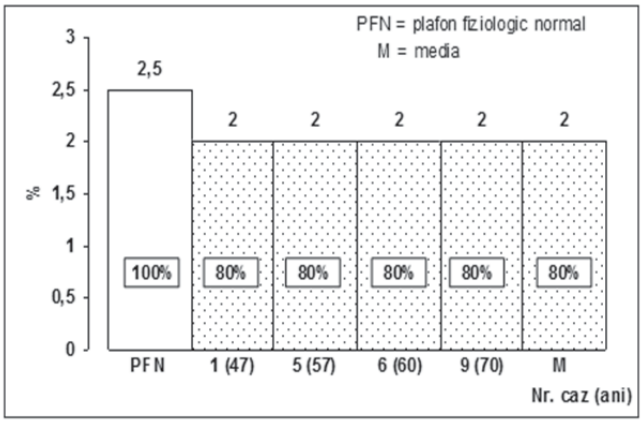


Fig. 11 The variation of the eosinophils percentage in some of the studied cases

When we refer to the basophils (fig.12) and the monocytes in men (fig. 13), the individual values are distributed both under the normal physiological extreme limit, and over this medium value, so that in the invastigated patients, the average of these individual values remains similar and lightly smaller (-10% on basophils and -17% on monocytes) than the normal physiological limit.

The number of monocytes in female patients describes a variation different from that of men's, meaning that at almost all the patients, the individual values are superior in relation to the normal physiological limit, resulting an avergae higher with 25 % compared to this limit. (fig.14).

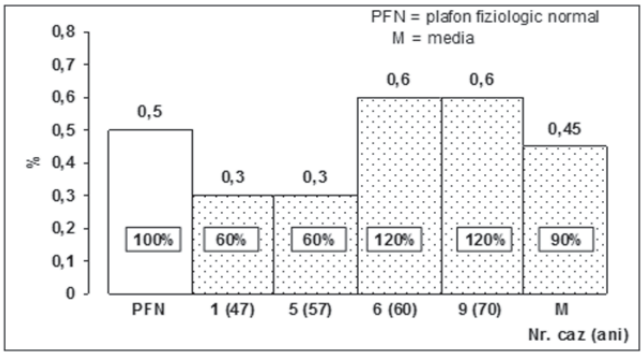


Fig.12 The variation of the basophils percentage in some of the studied cases

The thrombocytes. The analysis of the changes that occured in the level of the number of blood platelets,in the case of the patients suffering of multiple myeloma, confirms the informations from the scholarly literature related to thrombocytopenia. Indeed , as the data from fig. 15 have shown, the individual values of thrombocytes'number in the surveyed patients are constantly situated under the normal physiological limit, the medium value representing only 56% of this limit.

CONCLUSIONS

The patients with multiple myeloma from the casuistry of the Municipal Hospital Barlad were between 47 and 70 years old, and related to the genre, the incidence was higher on women than on men;

The homogeneous bone marrow smears have emphasized both the way in which the red cells were grouping into scrolls and that of the proliferated plasmocytes grouping into foci, some of them having uncommon shapes (dumb bells);

The patients have shown real hemoglobin deficiency, the medium values of the Hb concentration being up to 35,6% smaller than those of the normal physiological limits on men and to 36,7% on women;

The hematocryte of the patients suffering from nmyeloma has medium values under the normal physiological limits with 34,1% on men, and 17,9 % on women;

The number of red cells significantly decrease in all the surveyed patients, reaching 20,6% of the normal physiological limit on men and 21,6% on women;

The ESR values are amplified compared to the normal, by 12 times in men (65-158 mm/h) and by 13,2 times in women (33-156mm/h)

The white cells and the blood platelets in patients with multiple myeloma have values which can be compared to or even inferior to the normal physiological extreme limit.

REFERENCES

Berceanu, S., (1977): ***Hematologie clinică***, Editura Medicală Bucureşti.
Butoianu, E., Stănică, T., (1973): ***Vademecum hematologic***, Editura Medicală Bucureşti.
Kondi, V., (1981): ***Hematologie – laboratorul clinic***, Editura Medicală Bucureşti.
Lotreanu, V., (2000): ***Analize medicale biochimice, hematologice, imunologice***, Editura Coresi Bucureşti.
Mailat, F., Ivanciu, L., ***Hematologie partea I.***
Misăilă, C., Comănescu, **G.,** (1999): ***Elemente de hematologie generală,*** Editura Corson
Mut Popescu Delia, (2003): *Hematologie clinică, ediţia a II –a,* Editura Medicală Bucureşti
Răileanu, C., Moţoiu, I., (1974): ***Atlas de hematologie clinică***, Editura Academiei Române.
Tănăsescu, R., ***Diagosticul hematologic volumul I-II***, Editura Medicală Bucureşti;
Ţiţeica, M., Marinescu, S.,H., (1984): ***Practica laboratorului clinic (hematologie-microscopie clinică-hemostază-imunologie clinică*****)** Editura Academiei Române.

1 Liceul Teoretic ,, Mihai Eminescu” Bârlad
2 Universitatea Alexandru Ioan Cuza Iaşi
* mihaibularda_licemin@yahoo.com

Aknowledgements I would like to express my gratitude to Mrs Gica Purnavel, laboratory doctor, and also to all of those who directly or indirectly have given me suggestions and in this way they have contributed to the achievement of this scientific work. "This work was supported by the the European Social Fund in Romania, under the responsibility of the Managing Authority for the Sectoral Operational Programme for Human Resources Development 2007-2013 [grant POSDRU/CPP 107/DMI 1.5/S/78342]".

IN MEMORIAM

Profesor universitar dr.
Ion V. NEACŞU

(1939 - 2010)

Pentru întreaga colectivitate academică de la Facultatea de Biologie a Universităţii "Alexandru Ioan Cuza" din Iaşi, zilele de la mijlocul lunii august 2010 au adus una din cele mai neaşteptate, mai cutremurătoare şi mai triste veşti, trecerea în eternitate a bunului nostru coleg şi prieten, Profesor dr. Ion V. Neacşu.

Trecuseră doar două luni şi jumătate de la împlinirea celor 71 de ani, vârstă care-l găsise în plină activitate creatoare, participant activ la proiecte de cercetare ştiinţifică, cu articole ştiinţifice în diferite faze de finalizare, multe planuri în derulare şi altele care îşi aşteptau rândul, când moartea necruţătoare l-a smuls pentru totdeauna dintre noi.

Deşi devenise moldovean autentic, atât prin locul de muncă şi domiciliu, dar mai ales prin admiraţia pentru plaiurile şi vocaţia cultural-ştiinţifică a Iaşului, soarta a vrut ca în ultimile zile ale vieţii să revină la obârşie, la locurile natale, de care îşi amintea şi ne vorbea adesea cu nestăpânită admiraţie şi vie emoţie.

Firul vieţii s-a curmat pe neaşteptate, trecerea în nefiinţă survenind în ziua de miercuri 18 august 2010 în jurul orei 10, în timp ce se întorcea singur de la casa părintească din Gruieri către locuinţa uneia din surorile sale din Dobriceni, în urma unui stupid accident, constând din dezechilibrare şi cădere în albia betonată a pârâului Sarata, care traversează aşezarea sa natală. Înmormântarea a avut loc la Cimitirul Eternitatea din Iaşi, în ziua de sâmbătă, 21 august 2010.

Văzuse lumina zilei pe meleaguri vâlcene, la 28 mai 1939, în aşezarea numită Gruieri, care aparţinea comunei Dobriceni din raionul Horezu, regiunea Argeş, astăzi satul Dobriceni, comuna Stoeneşti, judeţul Vâlcea.

Părinţii. Victor şi Elena Neacşu l-au înscris la şcoala din Dobriceni pentru clasele primare, iar clasele V-VII le-a absolvit la şcoala din Bărbăteşti, raionul Horezu. Au urmat studiile medii, terminând în mod strălucit Liceul nr. 1 din Râmnicu Vâlcea, (1956), iar desăvârşirea pregătirii de naturalist s-a făcut în capitala culturală a Moldovei, absolvind cursurile Facultăţii de Biologie-Geografie, de la Universitatea "Al.I.Cuza" Iaşi, (1964). Format sub bagheta iluştrilor profesori ai vremii, care slujeau biologia românească ieşeană (Petre Jitariu, Olga Necrasov, Constantin Papp, Mihai Constantineanu, Zicman Feider ş.a.), Profesorul Ion Neacşu a devenit la rândul său un urmaş destoinic, care prin faptele sale a cinstit cum se cuvine memoria înaintaşilor.

Având o temeinică pregătire biologică, şi o cultură generală de remarcat, în faţa proaspătului absolvent al primei universităţi moderne din România se deschidea o frumoasă perspectivă de cercetător ştiinţific şi dascăl apreciat. Într-adevăr, după absolvire

colegul Ion Neacşu, funcţionează doar câteva luni (noiembrie 1964 – februarie1965) ca profesor de biologie la Şcoala generală din Agigea – Constanţa, după care îşi începe prodigioasa carieră de cercetător ştiinţific, în urma transferului la Staţiunea de Cercetări Marine "Prof. Ioan Borcea" Agigea Constanţa, Laboratorul de fiziologia animalelor marine, unde va funcţiona până la desfiinţarea unităţii, la 1.09.1970.

Acest eveniment avea să-l marcheze în mod dureros, ca de altfel pe toţi colegii de la Agigea. Cu toate acestea, tăria de caracter, pregătirea profesională de excepţie şi harul de cercetător cu care era înzestrat l-au ajutat şi nu a cedat. Distinsul Profesor Gheorghe Mustaţă, unul din decanii facultăţii de Biologie în vremurile de după 1989 îl caracteriza pe Ion Neacşu astfel: *"avea sădit în sufletul său focul sacru al cercetării ştiinţifice şi spiritul de la Agigea, întronat de ctitorul Staţiunii, profesorul Ioan Borcea"*

Revenit în Iaşi la recomandarea Profesorului Petre Jitariu, magistru care îi va fi şi conducător de doctorat, colegul Neacşu Ion regăseşte cu adâncă emoţie locurile şi mireasma de tei din studenţie şi devine pentru mai bine de două decenii cercetător ştiinţific gradul III la Centrul de Cercetări Biologice Iaşi, Laboratorul de Biologia membranelor celulare (1.08.1970 – 12.10.1990) şi apoi cercetător ştiinţific principal gradul II la Institutul de Cercetări Biologice Iaşi, Laboratorul de Biologie celulară, în perioada 12.10.1990-1.10.1994. Este perioada în care devine Doctor în Biologie (1984), la Universitatea "Al.I.Cuza" din Iaşi, cu teza *"Acţiunea unor ioni şi a unor agenţi organici asupra proprietăţilor electrice ale membranei celulare"*, sub conducerea ştiinţifică a Acad. Prof. Petre Jitariu. Această lucrare-eveniment, cât şi numeroasele articole ştiinţifice publicate în domeniul biologiei membranelor celulare, prin profunzimea concluziilor şi originalitatea rezultatelor îi motivau admiraţia adesea exprimată a Profesorului îndrumător, dar şi a colaboratorilor, din care amintim cercetătorii: Margareta şi Valer Crăciun, Pincu Rotinberg, Ştefan Agrigoroaei, precum şi profesorii Gh. Dimitriu, Vasile Hefco ş.a.

Anul 1990 marchează debutul activităţii didactice a colegului Ion Neacşu, pentru început pe un post de conferenţiar asociat la Facultatea de Biologie a Universităţii "Alexandru Ioan Cuza" Iaşi, disciplina de Biologie celulară şi moleculară (oct.1990 –oct.1994), apoi conferenţiar şi Profesor universitar la aceeaşi facultate, titular atât la disciplina de Biologie celulară şi moleculară, cât şi la cea de Biofizică: 18.02.2000 – 1.10.2005. Şi de această dată, colegul Neacşu a impresionat la modul real prin maniera cu care a reuşit să se integreze în cadrul învăţământului academic. Profesorul Gheorghe Mustaţă notează în legătură cu acest aspect următoarele: *„numai un om cu o vastă cultură biologică, numai un cercetător format şi ancorat în domeniu putea să preia din mers cursul de Biologie celulară şi să-l predea la o înaltă ţinută academică. A fost specialistul care a salvat Facultatea de Biologie în momentul de criză provocat de dispariţia regretatului Profesor Gheorghe Dimitriu. Înzestrat fiind cu un har didactic bine nuanţat, colegul Ion Neacşu a reuşit să preia apoi şi cursul de Biofizică, rămas descoperit după dispariţia prematură a colegului Profesor Mihai Isac. Din nou colegul Ion Neacşu a făcut servicii Facultăţii de Biologie, care trebuie să-i fie recunoscătoare".* Deşi arăta uscăţiv şi adesea lipsit de energie, avea o vitalitate ieşită din comun. Nu era simplu să elaborezi şi să predai două cursuri foarte importante şi să participi nemijlocit la pregătirea lucrărilor practice la serii mari de studenţi, concomitent cu efectuarea de cercetări contractuale, finalizate cu zeci de articole ştiinţifice publicate. La toate acestea se adaugă
implicarea Profesorului în elaborarea în premieră şi predarea unor cursuri la secţiile de Master ale Facultăţii de Biologie: *Mecanismele moleculare ale comunicării celulare; Culturi de celule animale în biotehnologii; Biologia moleculară a ciclului celular ş.a.*

Şi-ar fi dorit mult să dispună de răgazul necesar pentru a împărtăşi aceste cunoştinţe moderne la cât mai multe serii de studenţi. Din păcate, scadenţa care ne urmăreşte pe toţi a venit şi începând cu luna octombrie 2005, Profesorul Ion Neacşu a devenit pensionar, statut la care refuza cu înverşunare să se acomodeze. Simţea că mai poate fi de folos cercetărilor de biologie, în special cele privind substanţele biologic active. De aceea nu a ezitat nici un moment atunci când i s-a oferit un post de cercetător ştiinţific gr. I la Centrul de Cercetări pentru Oenologie al Filialei Iaşi a Academiei Române (1.01.2006 – 18 august 2010).

Această nouă provocare a vieţii sale i-a conturat şi mai pregnant personalitatea. Fiind un bun coleg, cu har de la Dumnezeu pentru cercetare, Profesorul Ion Neacşu stăpânea tainele lucrului în echipă, astfel încât să poată depăşi şi cele mai grele încercări. S-a acomodat rapid şi în noul colectiv de cercetare. Regăsirea şi colaborarea cu vechiul său coleg şi prieten Cristinel Zănoagă, cât şi cunoştinţele sale de autentic „om de laborator", i-au oferit Profesorului Ion Neacşu posibilitatea de a-şi pune în valoare calităţile de iscusit „căutător în cifre", pentru a scoate la iveală surprinzătoare interpretări şi teoretizări originale. Aşa se face că în nici cinci ani de activitate, colegul Neacşu se regăseşte în calitate de participant în peste şapte proiecte de cercetare din planul Centrului de Cercetări pentru Oenologie Iaşi, iar în acest răstimp, lista sa de lucrări ştiinţifice publicate s-a îmbogăţit cu încă 52 de titluri, cu cinci dintre acestea prezentându-se la Congresele Internaţionale ale Viei şi Vinului, ţinute la Budapesta, Ungaria în 2007 şi Verona, Italia în 2008.

Un mare profesor spunea că, „*după timp omul este muritor, dar după suflet este nemuritor*. *Noi citim în sufletul său prin intermediul operei sale, pentru că în opera sa el pune o bună parte din sufletul său*". Din acest punct de vedere, profesorul Ion Neacşu a produs o operă ştiinţifică şi didactică la care poate mulţi se vor opri să o înţeleagă şi să o valorifice. Un bilanţ făcut cu doi ani înaintea zilei fatidice de 18 august 2010 arăta un număr total de 375 articole publicate, din care 80 în revistele Academiei Române şi 27 articole publicate în străinătate. Se mai adăugau 7 cărţi (monografii şi manuale), din care una publicată în străinătate şi 7 brevete de invenţie.

O analiză tematică a acestei opere dezvăluie dorinţa vie a autorului de a-şi lărgi în permanenţă aria de cunoaştere, maniera iscoditoare în dialogul său cu viul şi disponibilitatea sa neobosită de a se poziţiona în slujba binelui. Dovadă stau lucrările sale din domenii ca: biologie celulară şi moleculară, biofizică, procese de permeabilitate membranară (transport pasiv şi activ, pompe ionice); potenţial de membrană, potenţial redox, procese ale ciclului celular, procese de metabolism intermediar şi energetic, fiziologia celulară a animalelor acvatice, osmoreglare, procese celulare implicate în acvacultură, efecte citostatice, antitumorale, hepatoprotectoare, hipocolesterolemiante şi antialcoolice ale unor extracte vegetale şi produşi biologic activi.

Ca recunoaştere a unor asemenea merite ştiinţifice, profesorul Ion Neacşu a fost membru în 9 societăţi ştiinţifice din România şi 4 societăţi ştiinţifice internaţionale şi membru în colectivul de redacţie la 4 reviste ştiinţifice din România şi o revistă din străinătate. Toate aceste semne de recunoaştere sunt merite care îi luminau în primul rând
chipul Profesorului, dar revărsau fără îndoială o aură de mândrie şi asupra întregii comunităţi academice ieşene şi în ultimă instanţă asupra biologiei româneşti în ansamblu.

El a plecat fizic dintre noi, dar a rămas cu sufletul său nobil aici, prin tot ce a creat.

Colegul nostru Ion Neacşu – omul nu era o fiinţă clădită pentru a atrage atenţia de la distanţă. Profesorul Gheorghe Mustaţă îl vede ca pe: „*un om discret, liniştit şi plăcut, cu un suflet deosebit, care putea trece pe lângă tine tiptil, neobservat, dar dacă te opreai şi îl întrerupeai şi pe el din gândurile şi visele sale simţeai cum se aprinde în el o flacără vie şi*

începea să-şi etaleze trăirile şi preocupările, iar dialogul se transforma într-o confesiune de toată lauda". Adesea, zăbovea cu „bună dimineaţa" pentru câteva momente lăsând să transpară nevoia de a-şi descreţi un pic fruntea cu o „vorbă de duh". Ştia că nu-l las mult să aştepte şi după ce-i îndeplineam dorinţa, pleca mulţumindu-mi că l-am ajutat să-şi încarce bateriile pentru tot restul zilei.

Structura sa sufletească îl făcea un bun partener în toate împrejurările. Nu l-am auzit vreodată pe colegul Ion Neacşu folosind vorbe mai „tari" la adresa cuiva. Este greu să nu-l preferi ca exemplu pentru modul cum se exprima la adresa colaboratorilor, cum suferea la cel mai mic insucces al acestora şi cât de bucuros era dacă reuşea să-i ajute de fiecare dată, atât pe Cristi Câmpeanu, cât şi pe Călin Maniu sau Lucian Fusu.

Şi-a iubit nemăsurat şi până la suferinţă familia, căreia i-a sacrificat atât cât a putut şi a avut. Vorbea cu vădită emoţie şi compasiune despre surorile sale, despre soţie, doamna Livia, precum şi despre cele două nepoate, ambele profesoare de Biologie, Carmen de la Liceul „Dimitrie Cantemir" din Iaşi şi Adela de la Şcoala din Dobriceni.

Cred în final că ne-ar onora efortul de a-i păstra în inimi imaginea de bonom şi pilda luminoasă a Profesorului Ion Neacşu, pe care el ni le-a dăruit cât a fost alături de noi şi care poate ne zâmbeşte acum dintr-o altă lume mai bună poate, încercând să ne alinte cu o zicală sau cu o glumă de pe meleagurile vâlcene.

Prof. univ. dr. Costică MISĂILĂ
Facultatea de Biologie
Universitatea „Alexandru Ioan Cuza"
Iaşi

20.01.2011

INSTRUCTIONS FOR AUTHORS

GENERAL NOTES

All manuscripts should be submitted at the following e-mail address: gbmpapers@yahoo.com

Look on the paper's web site at

http://www.bio.uaic.ro/publicatii/anale_biochimie/anale_gbm_index.html

or send your inquiries to gbmpapers@yahoo.com / aeu@uaic.ro to find latest news concerning the volumes.

Manuscripts are accepted all year round, but they will be included in one of the volumes according to the closing date for each volume.

Manuscripts will be submitted in English or French. Be aware that manuscripts may be editorially rejected, without re-view, on the basis of poor language, lack of conformity to the standards set forth in these Instructions or impropriate subject compared with the scientific field of the journal.

The sole responsibility on the content and graphic form of the published paper(s) is on the author(s) only.

ORGANIZATION AND FORMAT OF THE MANUSCRIPT

1. Manuscripts should be submitted in **Microsoft® Word**© document format (.doc(x)). From all other editors the document should be exported as RichTextFormat (.rtf).

The documents have to be written using Times New Roman (**TNR**) typeface (font).

Using other fonts (like Arial) or non-standard fonts (such as Times R or Times New Roman R) is not allowed. For non-European languages please check the compatibility of the characters subset with the European subset before submission.

The manuscript should contain an even number of pages else your paper will be returned to the author(s).

It is recommended as a general guideline a maximum of 8 pages for the original papers and 6, 8 or 10 pages for a review.

2. Paper format is ***Academic (if missing use Custom 17 cm × 24 cm)***;

mirror margins; up/down margins 2.5 cm; exterior margin 1.2 cm and interior margin 2 cm.

Don't use sections in your document (one document means one single section).

Don't put any text in header or footer (these spaces are reserved for the editor).

3. Latin words should be italicized (for example*: Gleditsia triacanthos, in vitro*, *et al*., *per se*). Authors should avoid using excessively long sentences and are also encouraged to have shorter paragraphs, for easy reading.

4. The manuscript should contain the following sections: (a) **Title** (b) **Authors** (c) **Keywords** (d) **Abstract** (e) **Introduction** (f) **Materials and Methods**, (g) **Results and discussion**, (h) **Conclusions** (i) **References** (j) **Acknowledgments (**if any), **affiliation of authors and address for e-mail correspondence**. In some particular cases, presentation will be clearer and more effective if the author combines some of these sections.

5. **The title**: TNR, Uppercase, 12p, bold, centered. **The title** should be informative and as short as is consistent with clarity.

6. **Authors:** TNR, Uppercase, 10p, bold, centered. List full names of all authors, first name then family name.

7. **Keywords**: for title TNR, low caps, 8p, bold, left; for body TNR, low caps, 8p, left, immediately after the title. Authors should supply a maximum of five keywords descriptive of the research carried out

8. **Abstract**: for title TNR, low caps, 8p, bold, left; for body TNR, low caps, 8p, left, immediately after the title. Every paper must begin with a brief **abstract** (up to 8 rows) presenting the plan, procedures, and significant results of the investigation. The abstract should be intelligible to the general public as well as the specialists in the field and, hence, should avoid specialized terms and abbreviations.

9. **Introduction**: for title TNR, Uppercase, 10p, bold, centered; for body TNR, low caps, 8p, justified. **The introduction** should state the purpose of the investigation and its relation to other works in the same field, but should not include an extensive review of the literature. A phrase at the end of the introduction will describe clearly the purpose or the objective of the study.

10. **Materials and Methods**: for title TNR, Uppercase, 10p, bold, centered; for body TNR, low caps, 8p, justified. Description of **Material and methods** should be brief, but adequate for repetition of the work by a qualified operator. Refer to previously published procedures employed in the work by citation of both the original description and pertinent published modifications. If several alternative methods are commonly used, it is helpful to identify the method briefly as well as to cite the reference / example. It is preferable to state “cells were broken by ultrasonic treatment as previously described (Gupta et al., 2005)” rather than to state “cells were broken as previously described (Gupta et al., 2005).” This allows the reader to assess the method without constant reference to previous publications. Do not include extensive write-ups unless they present substantially new modifications. Manufacturers cited in the text should be styled, for example, as *Sigma Chemical Co.* and give sources of unusual chemicals, equipment, or microbial strains.

11. **Results and discussions**: for title TNR, Uppercase, 10p, bold, centered; for body TNR, low caps, 10p, justified.

The Results and discussions section should include the results of the experiments and their extensive interpretation in relation with the previously published work. Present the results as concisely as possible in one of the following: text, table(s), or figure(s). Presenting the data both as graphs and as values in text, or presenting the same data both as table and graph is not allowed. Also, avoid extensive use of graphs to present data that might be more concisely presented in the text or tables. For example, except in unusual cases, double-reciprocal plots used to determine apparent Km values should not be presented as graphs; instead, the values should be stated in the text. Similarly, graphs illustrating other methods commonly used (e.g., calibration plots for molecular weight by gel filtration or electrophoresis) need not be shown except in unusual circumstances. Limit photographs (particularly photomicrographs and electron micrographs) to those that are absolutely necessary to show the experimental findings. Number figures and tables in the order in which they are cited in the text, and be sure to cite all figures and tables.

Numbering of figures is independent of that from tables and both should be made using Arabic numbers. (Note: If a table(s) or/and a figure(s) is too big and could cause a major break in the text it must be moved to the end of paper as an appendix and referred as such in text).

12. **Conclusions**: for title TNR, Uppercase, 10p, bold, centered; for body low caps, 10p, left. The conclusions should be unnumbered.

13. **References**: for title TNR, Uppercase, 10p, bold, centered; for body low caps, 8p, left.

References cited in the text. References inside the text should be cited as follows:

. . . similar results (Layton and Weathers, 2009)...
. . . as described previously (Gordon et al, 2006)...
References to unpublished data, manuscripts submitted for publication, unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings), personal communications, patent applications and patents pending, computer software, databases, and websites (home pages) should be made parenthetically in the text as follows.
. . . similar results (Layton and Weathers, unpublished data).
. . . system was used (McInerney, J. L., Holden, A. F. And Brighton, P. N. submitted for publication).
. . . as described previously (Gordon, M. G. And Rattner, F. L. presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {For nonpublished abstracts and posters etc .}
. . . available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html)
..using ABC software (version 2.2; Department of Microbiology, State University [http://www.state.micro.edu])

References are to be collected in alphabetical order and not in citation order at the end of the manuscript under the heading “References”. This must include all journal articles (both print and online), books and book chapters (both print and online), theses and dissertations, published conference proceedings, meeting abstracts from published abstract books or journal supplements, as well as in-press journal articles, book chapters, and books (publication title and page numbers must be given). References should be collected in alphabetical order (letter by letter, ignoring spaces and punctuation) by first-author surname and number consecutively. In the case of papers with multiple authors all of them should be listed. Where there are more than five authors, the first four should be listed, followed by et al. All listed references must be cited in the text. Abbreviate journal names according to the PubMed Journals Database (National Library of Medicine, National Institutes of Health which is available at http://www.ncbi.nlm.nih.gov/sites/entrez?db6journals).
Follow the styles shown in the examples below for print references:

Schwartz, D. C., Cantor C. R., (1984): *Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis*. Cell, 37(1), 67-75 {Journal Article}

Cox, C. S., Brown, B. R., Smith, J. C.: J. Gen. Genet., in press.* {Article title is optional; journal title is mandatory.}

Perlin, M. H. (2003). The Subcellular Entities a.k.a. Plasmids in Y. E. Streips N.U. (Ed.), *Modern microbial genetics* (Vol. 1, 464-482). John Willey and sons. {Chapter in a Book}

Westermeier, R. (2005): *Electrophoresis in Practice: A Guide to Methods and Applications of DNA and Protein Separations*, Wiley-VCH. {Book}

Fitzgerald, G., and D. Shaw. In A. E. Waters (ed.), Clinical microbiology, in press. EFH Publishing Co., Boston, MA.* {Chapter title is optional.}

Bradford, J., Langsdorf, C., Hicks, J., Buller, G., Luke, T., Davies D., (2009): *Non-cytotoxic, near-ir DNA stain for live cell cycle analysis.* Cytometry part B-Clinical Cytometry, 76B(6), 396, {Meeting abstract published in journal supplement.}

Smith, D., C. Johnson, M. Maier, and J. J. Maurer. 2005. *Distribution of fimbrial, phage and plasmid associated virulence genes among poultry Salmonella enterica serovars*, abstr. P-038, p. 445. Abstr. 105th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. {Poster with abstract published, abstract title is optional.}

Green, P. N., D. Hood, and C. S. Dow. 1984. Taxonomic status of some methylotrophic bacteria, p. 251–254. In R. L. Crawford and R. S. Hanson (ed.), *Microbial growth on C1 compounds*. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.

O'Malley, D. R. 1998. Ph.D. thesis. University of California, Los Angeles, CA. {PhD Thesis, Title is optional.}

Online references must provide essentially the same information that print references do. For online journal articles, posting or revision dates may replace the year of publication, and a DOI or URL may be provided in addition to or in place of volume and page numbers. Some examples follow.

Dionne, M. S., and D. S. Schneider. 2002. Screening the fruitfly immune system. Genome Biol. 3: REVIEWS1010. http://genomebiology.com/2002/3/4/reviews/1010

Smith, F. X., H. J. Merianos, A. T. Brunger, and D. M. Engelman. 2001. Polar residues drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. USA 98: 2250–2255. DOI: 10.1073/pnas.041593698.

Winnick, S., D. O. Lucas, A. L. Hartman, and D. Toll. 2005. How do you improve compliance?, Pediatrics 115: e718–e724.

URLs for companies that produce any of the products mentioned in your study or for products being sold may not be included in the article. However, companies URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

14. **Acknowledgements**: for title TNR, low caps, 8p, bold, left; for body TNR, low caps, 8p, left, immediately after the title. No more than 3 rows.
15. **The institutional affiliation** of author(s) will be placed after acknowledgements using TNR, 8p, left List the institutions in which the work was carried out and identify the affiliations of all authors and their institutions, departments, or organizations by use of superscript lower case numbers (1, 2 etc.). The asterisk symbol * in superscript should be reserved for the author to whom correspondence should be addressed by e-mail.

As final notes: The graphical elements in the paper (all figures, graphics and images) must be submitted also in their original form (we prefer vector images such as WMF or bitmap images such as TIFF or JPG). Minimum resolutions are as follows:
300 dpi for grayscale and color,
600 dpi for combination art (lettering and images),
1200 dpi for line art.

All graphics must be submitted at their intended publication size. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration.
Be aware that our journal will be printed in black and white only so all existing colored elements will be converted by the editor to gray scale and could lose some of the intended appearance.

All the required materials will be submitted together with the paper. We strongly suggest archiving the required files using 7Zip software before sending it to us.