ANALELE ŞTIINŢIFICE
ALE
UNIVERSITĂŢII „ALEXANDRU IOAN CUZA”
DIN IAŞI

(SERIE NOUĂ)

SECŢIUNEA II

a. GENETICĂ ŞI BIOLOGIE MOLECULARĂ

TOMUL XVI, Fascicula 4 **2015**

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
www.gbm.bio.uaic.ro / gbmpapers@yahoo.com

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

CONTENT

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

SCARDINIUS GENUS IN MOLECULAR STUDIES – A REVIEW

OVIDIU POPESCUL[1], DUMITRU COJOCARU[1], MITICĂ CIORPAC[1*]

Received: 20 November 2015 / Revised: 21 November 2015 / Accepted: 23 November2015 /
Published: 14 December 2015

Keywords: *Scardinius*, molecular markers, phylogeny, phylogeography.
Abstract. *Scardinius* is a genus of ray-finned fish in the Cyprinidae family commonly called rudds. The common rudd (*Scardinius erithrophthalmus*) is a bentho-pelagic freshwater fish that occurs mainly in nutrient-rich, well vegetated lowland rivers, backwaters, oxbows, ponds and lakes and it is widespread in Europe and middle Asia. It has a medium length of 20-30 cm, but it can reach 50 cm. The classification of cyprinids has always been controversial the morphological traits have an unclear homology this led to the idea that the recognized monophyletic groups are surely misinterpreted. This paper aims to assess the current level of molecular data regarding *Scardinius* genera. Some of the molecular data obtained for *Scardinius* genus is from DNA barcoding studies on fresh water fishes, but studies regarding this genus and Cyprinidae family used mitochondrial genes like cytochrome b (*cyt b*) and cytochrome oxidase (*CO*), but nuclear genes or nuclear microsatellites were also used. We found that molecular data exists for both nuclear and mitochondrial genes, but this genus wasn’t studied separately and as many of the researchers suggest more taxonomic studies are required in order to solve the uncertainties within it.

INTRODUCTION

Scardinius is a genus of ray-finned fish in the Cyprinidae family commonly called rudds. Locally, the name "rudd" without any further qualifiers is also used for particular species, particularly the common rudd (*Scardinius erythrophthalmus*). The rudd can be distinguished from the very similar roach by way of the rudd's upturned mouth, allowing the rudd to pick food items such as aquatic insects from the surface of the water with minimal disturbance (Banarescu and Coad, 1991).

The common rudd (*Scardinius erithrophthalmus*) is a bentho-pelagic freshwater fish that occurs mainly in nutrient-rich, well vegetated lowland rivers, backwaters, oxbows, ponds and lakes. It is widespread in Europe and middle Asia: most European rivers north of Pyrenees and Alps, eastward to Ural and Eya drainages, Aral and White Sea basins; Black Sea basin in Europe and northern Asia Minor. Naturally absent from Iberian Peninsula, Adriatic basin, Italy, Greece south of Pinios drainage, Great Britain north of 54° N, Ireland and Scandinavia north of 62° N (Kottelat and Freyhof, 2007). Definitely absent in Siberia. It has been artificially introduced to Spain, Ireland, Norway, USA, Canada, Morocco, Madagascar, Tunisia. In New Zealand it has been illegally introduced in 1960s where they have spread around the country. They have the potential of adapting to unfavorable environmental condition (Kottelat and Freyhof, 2007) and they can irreversible damage indigenous ecosystems (Chadderton, 2010). The rudd is often confused with the roach (*Rutilus rutilus*), morphologically the two species appear very similar (Cihar, 1991). This paper’s purpose is to assess the level of molecular data obtained for the *Scardinius* genus until now.

The classification of cyprinids has always been controversial, the number of recognized families ranging from 2 to 12 depending on the author and the number of morphological traits considered (Arai, 1982; Chen et al., 1984; Howes, 1980; Cavender and Coburn, 1992) and even have been elevated to family level, being assigned European and North American leuciscins and phoxinins to the family called Leuciscidae (Chen and Mayden, 2009; Mayden and Chen, 2010). Because the morphological traits are usually subjected to homoplasy (Zardoya and Doadrio, 1999), the systematics based on them sometimes comes in contradiction with molecular data (Zardoya and Doadrio, 1999; Briolay et al., 1998; Gilles et al., 2001; Cunha et al., 2002; Liu et al. 2002; Saitoh et al., 2006; Rüber et al., 2007; Gorgan 2007, 2008, 2013) and also the morphological traits have an unclear homology (Bogutskaya, 1990; Howes, 1991). All these facts lead to the idea that the recognized monophyletic groups are surely misinterpreted (Perea et al., 2010).

Some of the molecular data obtained for *Scardinius* genus is from DNA barcoding studies on fresh water fishes with few exceptions where the species of this genus are treated separately. Another type of studies regarding this genus and Cyprinidae family used mitochondrial genes like cytochrome b (*cyt b*) and cytochrome oxidase (*CO*), but nuclear genes or nuclear microsatellites were also used.

PHYLOGENY RELATIONSHIPS AND LIFE HISTORY

One of the earlier studies regarding European Cyprinids, that used the *cyt b* gene (1140 bp) as marker for establishing the relationships and the radiation of this fishes in Europe, was conducted in 1999 by Zardoya and Doadrio who found that the first major radiation within leuciscins occurred in the mid-Miocene approximately 13.6 mya, and extant lineages such as those leading to, e.g., *Scardinius, Rutilus, Leuciscus,* and *Alburnus* were originated and that the fossil records of *Leuciscus* on the Iberian Peninsula that dates back to the middle Miocene is supporting these datings. Also the molecular data obtained in this study support the previous biogeographical hypothesis, stated by Bianco in 1990, Doadrio in 1990 and 1994, that points out the importance of the southern Mediterranean area in the evolution of some European cyprinid taxa.

Another study from 2004 conducted by Ketmaier et al., which is centered on the phylogeny of *Telestes* and *Scardinius* from the peri-Mediterranean area, based also on *cyt b* gene (786 bp), shows that in the *Scardinius* phylogeny the Greek species are situated basal on the tree. In the same paper is stated that the genetic data for *S. erythrophthalmus* in Italy shows an altered situation due to man-mediated fish transfer. Also these species populations do not appear to be clustered on monophyletic clade, *S. scardafa* being positioned between them. Regarding the biogeography of the *Scardinius* group the authors suggest that during the glaciations the dropping of the sea level determined confluences of water between rivers flowing into the epicontinental area of the Mediterranean Sea. Thus, this event has led to a major dispersal of this genus trough river confluences in low-lands.

An article published in 2005, which used the entire *cyt b* region of 1141 bp, by Freyhof et al., that aimed the introgression of mitochondrial DNA in Dalmatian cyprinids shows a case of a population of *S. dergle* that had mitochondrial DNA originating from *Squalius tenellus* in a clay-pit lake. Their findings are unclear regarding to the introgression hybridization events in the Leuciscini because this type of event could be a localized one or it could be more common than previously assumed. Nevertheless, this type of hybridization is common within close related cyprinids but in this case the *Scardinius* and *Squalius* are phylogenetically separated since the middle Miocene (~10 MYA) and further studies are required.

Scardinius erythrophthalmus species was included in a study published in 2008 regarding the molecular phylogeny of the Romanian cyprinids from the Danube River by Luca et al. In order to achieve this the authors used a fragment from the *COX2* gene (302 bp). Their findings show the positioning of *S. erythrophthalmus* species on the same clade with *Abramis sp.* and *Rutilus sp.* in the *Leuciscinae* group.

In 2010, a complex study was performed by Perea et al. regarding phylogenetic relationships and biogeographical patterns of Leuciscinae subfamily in the Mediterranean area. The research team used both mitochondrial (*cyt b* -1140 bp and *COI* – 646 bp genes) and nuclear (Recombination Activating Gene 1 (*RAG-1*) and Ribosomal Protein Gene S7 (*S7*) genes – 2647 bp) markers. Because the *Scardinius* genus was previously considered a sister group of *Tropidophoxinellus* (Zardoya and Doadrio, 1999; Bianco, 1988) and also the hypothesis that this genus and *Alburnus* genus are closely related (Briolay et al., 1988; Zardoya and Doadrio, 1999), the authors compared their findings considering this two cases. Their results regarding the phylogeny of this group strongly support the first theory. Also the phylogeny based on the nuclear markers showed a low support for the relationship between *Scardinius* and *Rutilus*, a fact already theorized using morphological characters (Cavender and Coburn, 1992). The analysis of cyt b gene grouped the lineages of *Scardinius, Tropidophoxinellus-Pseudophoxinus callensis-P.chaignoni*

and *Alburnus-Anaecypris-Leucaspius-Pseudophoxinus punicus*, but the basal relationships between these lineages could not be solved using *COI* gene, *RAG-1* and *S7* most probably because the different rate of evolution of these genes in comparison with *cyt b* gene. Still, the combined data matrix for all genes retrieved this relationship and inside the *Scardinius* genus the data obtained by the authors support some of the recognized species (Kottelat and Freyhof, 2007). So, according to their findings *S. erythrophthalmus* is a Central and Eastern European species, the specie *S. scardafa* is endemic to Lake Scanno in Central Italy as described by Bianco in 1994 and Ketmaier et al. in 2003 and it is a close relative of *S. hesperedicus* (Bianco, 1994; Bianco et al., 2001). Also the species *S. plotizza* formed an independent and well defined clade.

In the same study, the authors made a biogeographical approach. Their results showed that the Pleistocene glaciations were important in the European distribution of some taxa and that their origin is more recent because the colonization process from glacial refuges (for example Danube basin) happened after. Also the Danube basin probably was not the only refuge and other rivers of the Black Sea basin were refuges (Kotlik et al., 2004). The pattern of the widespread distribution of *S. erythrophthalmus* in Europe can be explained by the homogeneity conferred by the Danube basin refuge (Banarescu, 1992; Durand et al., 1999; Perdices et al., 2003; Culling et al., 2006).

A study made in 2013 regarding the phylogenetic relationship of two populations of *Scardinius* sp. from the Adriatic Sea drainage in Croatia was performed by Valić et al. who used as molecular markers the *cyt b* gene (1140 bp) and non-coding nuclear region *Cyfun P* (Cyprinid formerly unknown nuclear Polymorphism) and also sequences from GeneBank database. Analyzing the mitochondrial marker (*cyt b* gene) the researchers showed that the populations from the north and central Adriatic evolved recently, but that is difficult to analyze the relationship between them because probably they don’t reach monophyly yet due to too short time. The phylogeny based on the nuclear marker (*Cyfun P*) was different than the one using the mitochondrial marker. Although this nuclear marker is not adequate to compare the relationships between closely related species the authors found that *S. erythrophthalmus* from Germany, *S. graecus* from Greece, *S. dergle* from Krka River and Vrana Lake group together in one lineage in phylogenetic tree distant from other cyprinid species. Also they concluded that *S. dergle* is a close relative of *S. hesperidicus* but the two are not the same species.

PHYLOGEOGRAPHIC APPROACHES AND GENE DIVERSITY

In 1999, a study that analyzed the allozyme patterns of four common cyprinid species (one of the species was *S. erythrophthalmus*) for a comparison of intraspecific genetic variability was performed by Wolter, the study area being represented by Berlin and its surroundings. The results regarding *S. erythrophthamus* show that this specie had the lowest abundance in the studied area from the species considered in this study. Also the genetic distance between subpopulations is high supported by a high bootstrap value (92%), but this high genetic distance was expected for the rudd because of its type of habitat preferences. This specie relatively high level of specialization and its reduced abundance led to a low capability of dispersion and implicitly to a high genetic distance between subpopulations.

A phylogeographic study of *Scardinius erythrophthalmus* was performed in 2004, by Stefani et al., in order to determine the differentiation of Italian populations in relation to the presence of mountain barriers. Their results were based also on a portion of *cyt b* gene (409 bp) and lead to a plausible scenario of this specie phylogeography in the Italian peninsula that implies a former colonization of Adriatic regions by Balkan populations during glacial periods, and a first

colonization of Thyrrenian basins. This followed by a succession of isolation processes between populations caused divergences supported by restricted gene flows at single watershed scale. Also the authors concluded that because of the presence of a defined and natural phylogenetic structure of *S. erythrophthalmus* in the Italian peninsula no significant anthropogenic influences can be highlighted on its original distribution pattern.

Because of the unavailability of nuclear markers for *S. erythrophthalmus*, Holmen et al. tested in 2009 a number of 36 microsatellite loci developed for *Pimephales promelas* (Ardren et al., 2002), *Barbonymus gonionotus* (McConnell et al., 2001; Kamonrat et al., 2002), *Cyprinus carpio carpio* (Crooijmans et al., 1997), *Anaecypris hispanica* (Salgueiro et al., 2003) and *Carassius auratus auratus* (Zheng et al., 1995) on this species and *Phoxinus phoxinus*. Their results indicate that a number of 18 species-locus combinations were amplified for the rudd from which 9 are polymorphic and 15 for minnow (*Phoxinus phoxinus*) from witch 7 polymorphic. The authors conclude that this positive cross-species amplification could represent an important establishment of nuclear marker sets for population genetics studies on these two species.

In 2011 Triantafyllidis et al. conducted a study in which they analyzed the fish species diversity in four north Greek lakes (three natural eutrophic lakes and one dam-lake) by DNA barcoding. The marker used in this study was *COI* gene (655 bp) and the authors obtained 145 *COI* barcodes for 27 genera, including *Scardinius* genus. The results have shown a hybridization phenomenon between *S. dergle* and *Squalius* in the mitochondrial genome as previously demonstrated by Freyhof et al. in 2005. Also, for *S. erythrophthalmus*, high divergences between individuals in conspecific populations were found, this species having the highest divergence from all the species analyzed. According to the authors this is the first time when an analysis indicates such divergence between populations and more studies are needed as soon as possible.

A study that followed the identification of 89 commercially important fish species found in Turkey using DNA barcoding was conducted by Keskin and Atar in 2013. Although *S. erithrophthalmus* has little economic importance it was included in this study that used as marker *COI* gene (654 bp). The NJ tree obtained by the authors has shown no taxonomic deviation at the species level and they conclude that DNA barcoding is an efficient tool for identifying fish fauna.

A large scale study which followed the barcoding accuracy of freshwater fishes in Mediterranean Biodiversity Hotspot was published in 2014 by Geiger et al. and used *COI* gene as marker with a mean length of 646 bp. The *Scardinius* genus was taken in the study but it is not discussed because the purpose of the study was another. So the results of this study, according to the authors, support the expected divergence levels within the species, but not within genera, mainly because the scale of this study. Yet, the *Scardinius* species are mentioned as closely related but with the need for more research.

CONCLUSIONS

The *Scardinius* species are important freshwater fishes mainly because *S. erythrophthalmus* is widespread across Europe and western Asia, but also because more than half of this genera species have a status from near threatened to critically endangered on IUCN Red List.

Although for the *Scardinius* genera molecular data are available for both nuclear and mitochondrial genes, this genus wasn't studied separately and as many of the researchers suggest more taxonomic studies are required in order to solve the taxonomic, phylogenetic and phylogeographical uncertainties within it.

REFERENCES

Arai R., 1982. *A chromosome study on two Cyprinid Fishes Acrossocheilus labiatus and Pseudorasbora pumila pumila, with notes on Eurasian Cyprinids and their karyotypes*. Bull Natn Sci Mus, Tokyo Ser A 1982, 8:131-152.

Ardren W.R., Miller L.M., Kime J.A., Kvitrud M.A., 2002. *Microsatellite loci for fathead minnow (Pimephales promelas)*. Mol Ecol Notes 2002, 2(3):226-227.

Banarescu P., 1992. *Zoogeography of Fresh Waters. Distribution and dispersal of freshwater animals in North America and Europe*. Wiesbaden: Aula Verlag 1992, 2.

Banarescu P., Coad B.W., 1991. *Cyprinidae of Eurasia.* In Cyprinid fishes, Systematics, Biology and Explotation. Edited by: Winfield IJ, Nelson JE. London: Chapman and Hall; 1 1991:127-155.

Bianco P., 1990. *Potential role of the paleohistory of the Mediterranean and Paratethys basins on the early dispersal of Euro-Mediterranean freshwater fishes.* Ichthyiol Explor Freshwaters 1:167–184

Bianco P.G., 1988. *Leuciscus cephalus (Linnaeus), with record of fingerling adult males, Leuciscus pleurobipunctatus (Stephanidis) and their hybrids from western Greece*. J Fish Biol 1988, 32:1-16.

Bianco P.G., 1994. *L'ittiofauna continentale dell'Appennino umbromarchigiano, barriera semi permeabile allo scambio di componenti primarie tra fli opposite versanti dell'Italia centrale.* Biogeographia 1994, 17:427-485.

Bianco P.G., Ketmaier V., Bussato T., 2001. *Approccio multidisciplinare all'analisi tassonomica del genere Scardinius (Cyprinidae) en Europa.* Quaderni ETP 2001, 30:115-120.

Bogutskaya N.G., 1990. *Morphological basics in cyprinid fish of Leuciscinae subfamily (Leuciscinae, Cyprinidae).* Vop Ikthiol 1990, 30(6):920-933.

Briolay J., Galtier N., Brito R.M., Bouvet Y., 1998. *Molecular Phylogeny of Cyprinidae inferred from cytochrome b DNA sequences*. Mol Phylogenet Evol 1998, 9:100-108.

Cavender T.M., Coburn M.M., 1992. *Phylogenetic relationship of North American Cyprinidae*. In Systematics, historical ecology and North American freshwater fishes. Edited by: Mayden RL. California: Standford University Press; 1992:293-327.

Chadderton W. L., 2010. *Management of invasive freshwater fish: striking the right balance!*, Hamilton, NZ: Department of Conservation.

Chen W-J., Mayden R.L., 2009. *Molecular systematics of the Cyprinoidea (Teleostei: Cypriniformes, the world's largest clade of freshwater fishes: Further evidence from six nuclear genes)*. Mol Phylogenet Evol 2009, 52:544-549.

Chen X.L., Yue P.Q., Lin R.D., 1984. *Major groups within the family Cyprinidae and their phylogenetic relationship.* Acta Zool Sinica 1984, 9:424-440.

Cihar, J., 1991. *Freshwater fish*. Silverdale Books, Leicester.

Crooijmans R.P.M.A., Poel J.J.Vd., Groenen M.A.M., Bierbooms V.A.F., Komen J., 1997. *Microsatellite markers in common carp (Cyprinus carpio L.).* Animal Genetics 1997, 28(2):129-134.

Culling M., Janko K., Boron A., Vasil'ev V., Coté I., Hewitt G., 2006. *European colonization by the spined loach (Cobitis taenia) from Ponto-Caspian refugia based on mitochondrial DNA variation.* Mol Ecol 2006, 15(1):173-190.

Cunha C., Mesquita N., Dowling T.E., Gilles A., Coelho M.M., 2002*: Phylogenetic relationships of Eurasian and American cyprinids using cytochrome b sequences.* J Fish Biol 2002, 61:929-944.

Doadrio I., 1990. *Phylogenetic relationships and classification of west palaearctic species of the genus Barbus (Osteichthyes, Cyprinidae).* Aquat Living Resource 3:265–282.

Doadrio I., 1994. *Freshwater fish fauna of North Africa and its biogeography.* Ann Mus R Afr Centr Zool 275:21–34.

Durand J.D., Persat H., Bouvet Y., 1999. *Phylogeography and postglacial dispersion of the chub (Leuciscus cephalus) in Europe.* Mol Ecol 1999, 8(6):989-997.

Freyhof J., Lieckfeldt D., Pitra C., Ludwig A., 2005. *Molecules and morphology: Evidence for introgression of mitochondrial DNA in Dalmatian cyprinids*. Molecular Phylogenetics and Evolution 37 (2005) 347–354.

Geiger M.F., Herder F., Monaghan M.T., Almada V., Barbieri R., Bariche M., Berrebi P., Bohlen J., Casal-Lopez M., Delmastro G.B., Denys G.P.J., Dettai A., Doadrio I., Kalogianni E., Karst H., Kottelat M., Kovacic M., Laporte M., Lorenzoni M., Marcic Z., Ozulug M., Perdices A., Perea S., Persat H., Porcelotti S., Puzzi C., Robalo J., Sanda R., Schneider M., Slechtova V., Stoumboudi M., Walter S. and Freyhof J., 2014. *Spatial heterogeneity in the Mediterranean Biodiversity Hotspot affects barcoding accuracy of its freshwater fishes.* Molecular Ecology Resources (2014).

Gilles A., Lecointre G., Miquelis A., Loerstcher M., Chappaz R., Brun G., 2001. *Partial combination applied to phylogeny of European cyprinids using the mitochondrial control region.* Mol Phylogenet Evol 2001, 19:22-23.

Gorgan D. L., 2007 – Filogenie Moleculară în cadrul genurilor Cyprinus şi Carassius, Editura Universităţii „Alexandru Ioan Cuza" Iaşi, 247-260.

Gorgan D. L., **Gherasim S. R.**, 2007 – The study of phylogenetic relationships and genetic diversity for Carassius' genera individuals from eastern part of Romania, Proceedings, Taal, 12th World Lake Conference, Jaipur, India, p. 578 – 584.

Gorgan D. L., 2008 – Introducere în studiul filogeniei şi filogeografiei moleculare, Editura Bioflux, Cluj-Napoca. Online, 96-158.

Gorgan L. D., **Ciorpac M.**, 2013 - Identification of Carassius gibelio migration patterns in Europe, AACL Bioflux, 6(2):92-96.

Howes G.J., 1980. *The anatomy, phylogeny, and classification of bariliine cyprinid fishes*. Bull Brit Mus Nat Hist Zoology 1980, 37:129-198.

Howes G.J., 1991. *Systematic and Biogeography: an overview.* In Cyprinid fishes: Systematic, biology and explotaition. Edited by: Winfield IJ, Nelson JE. London: Chapman and Hall; 1991:1-33.

Kamonrat W., McConnell S.K.J., Cook D.I., 2002. *Polymorphic microsatellite loci from the southeast Asian cyprinid, Barbodes gonionotus (Bleeker).* Mol Ecol Notes 2002, 2(2):89-90.

Keskin E. and Atar H.H., 2013. *DNA barcoding commercially important fish species of Turkey*. Molecular Ecology Resources (2013) 13, 788–797.

Ketmaier V., Bianco P. G., Cobolli M., Krivokapic M., Caniglia R., De Matthaeisa E., 2004. *Molecular phylogeny of two lineages of Leuciscinae cyprinids (Telestes and Scardinius) from the peri- Mediterranean area based on cytochrome b data*. Molecular Phylogenetics and Evolution 32 2004 1061–1071.

Ketmaier V., Bianco P.G., Cobolli M., De Matthaeis E., 2003. *Genetic differentiation and biogeography in southern European populations of the genus Scardinius (Pisces, Cyprinidae) based on allozyme data.* Zool Scr 2003, 32(1):13-22.

Kotlik P., Bogutskaya N.G., Ekmeckçi F.G., 2004. *Circum Black Sea phylogeography of Barbus freshwater fishes: divergence in the Pontic glacial refugium*. Mol Ecol 2004, 13(1):87-95.

Kottelat, M. and Freyhof J., 2007. *Handbook of European freshwater fishes*. Publications Kottelat, Cornol and Freyhof, Berlin, 646 pp.

Liu H., Tzeng C.S., Teng H.Y., 2002. *Sequence variation in the mtDNA control region and their implications for the phylogeny of Cypriniformes*. Can J Zool 2002, 81:1938-1946.

Luca C., Manea A., Luca A., Dinischiotu A., Costache M., 2008. *Molecular phylogeny of the Romanian cyprinids from the Danube River.* Roumanian Biotechnological Letters, Vol. 13, No. 5, 2008, pp. 3970-3975.

Mayden R.L, Chen W-J., 2010. The world's smallest vertebrae species of the genus Paedocypris: A new family of freshwater fishes and the sister group of the world's most diverse clade of freshwater fishes (Teleostei: Cypriniformes). Mol Phylogenet Evol 2010.

McConnell S.K.J., Skibinski D.O.F., Mair G.C., 2001. *Microsatellite markers from a commercially important South-east Asian cyprinid, the silver barb (Barbodes gonionotus Bleeker).* Mol Ecol Notes 2001, 1(3):135-136.

Perdices A., Doadrio I., Economidis P.S., Bohlen J., P. B., 2003. *Pleistocene effects on the European freshwater fish fauna: double origin of the cobitid genus Sabanejewia in the Danube basin (Osteichthyes: Cobitidae)*. Mol Phylogenet Evol 2003, 26(2):289-299.

Rüber L., Kottelat M., Tan H.H., Ng P.K.L., Britz R., 2007. *Evolution of miniaturization and the phylogenetic position of Paedocypris, comprising the world's smallest vertebrate.* BMC Evol Biol 2007, 7(38).

Saitoh K., Sado T., Mayden R.L., Hanzawa N., Nakamura K., Nishida M., Miya M., 2006. *Mitogenic evolution and relationships of the Cypriniformes (Actinopterygii, Ostariophysi): the first evidence toward resolution of higher-level relationships of the world's largest freshwater fish clade based on 59 whole mitogenome sequences.* J Mol Evol 2006, 63(6):826-841.

Salgueiro P., Carvalho G., Collares-Pereira M.J., Coelho M.M., 2003. *Microsatellite analysis of genetic population structure of the endangered cyprinid Anaecypris hispanica in Portugal: implications for conservation.* Biol Conserv 2003, 109(1):47-56.

Stefani F., Galli P., Crosa G., Zaccara S., Calamari D., 2004. *Alpine and Apennine barriers determining the differentiation of the rudd (Scardinius erythrophthalmus L.) in the Italian peninsula.* Ecology of Freshwater Fish 2004: 13: 168–175.

Triantafyllidis A., Bobori D., Koliamitra C., Gbandi E., Mpanti M., Petriki O., & Karaiskou N., 2011. *DNA barcoding analysis of fish species diversity in four north Greek lakes*. Mitochondrial DNA, October 2011; 22(S1): 37–42.

Valić D., Smrzlić I.V., Kapetanović D., Teskeredžić Z., Pleše B., Teskeredžić E., 2013. *Identification, phylogenetic relationships and a new maximum size of two rudd populations (Scardinius, Cyprinidae) from the Adriatic Sea drainage, Croatia*. Biologia 68/3: 539—545, 2013.

Wolter C., 1999. *Comparison of intraspecific genetic variability in four common cyprinids, Abramis brama, Abramis bjoerkna, Rutilus rutilus and Scardinius erythrophthalmus, within and between lowland river systems.* Hydrobiologia 364: 163–177, 1999.

Wolter C., 1999*. Comparison of intraspecific genetic variability in four common cyprinids, Abramis brama, Abramis bjoerkna, Rutilus rutilus and Scardinius erythrophthalmus, within and between lowland river systems*. Hydrobiologia 364: 163–177, 1999.

Zardoya R., Doadrio I., 1999. *Molecular evidence of the evolutionary and biogeographical patterns of European cyprinids*. J Mol Evol 1999, 49:227-237.

Zheng W., Stacey N.E., Coffin J., Strobeck C., 1995. *Isolation and characterization of microsatellite loci in the goldfish Carassius auratus.* Mol Ecol 1995, 4(6):791-792.

* mitica.ciorpac@uaic.ro
1 Faculty of Biology, University “Alexandru Ioan Cuza” Iaşi, Romania, Carol I 20 A, Iasi,

Acknowledgement: This work was co-founded by the European Social Found through Sectoral Operational Programme Human Resources Development 2007-2013, project number POSDRU187/1.5/S/155397, project title “Towards a New Generation of Elite Researchers through Doctoral Scholarships”

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

NEW INSIGHTS ON THE INVOLVING OF AMYLOID BETA PEPTIDE IN ALZHEIMER'S DISEASE PATHOLOGY

RADU IONITA[1]*, LUCIAN HRITCU[1]

Received: 13 November 2015 / Revised: 21 November 2015 / Accepted: 25 November 2015 /
Published: 14 December 2015

Keywords: amyloid beta peptides; oxidative stress; neuronal death; inflammation; Alzheimer's disease.
Abstract: Alzheimer's disease (AD) is the most common neurodegenerative disorder that affects millions of people worldwide causing massive economic burden and the number of cases is expected to rise dramatically. Currently, there is no treatment that can stop or reverse the effects of AD. This review attempts to present the current status of research, bio-pathological approach mechanisms, biomarkers and therapeutic interventions of AD.

INTRODUCTION

Alzheimer's disease (AD) is characterized by successive degradation and destructive neuro human brain structure and affect over 37 million people worldwide (Mount and Downton, 2006), with a loss of over $ 600 billion in 2010 (Wimo and Prince, 2010). Overall, 5 million new cases of AD are reported annually (Alzheimer's Association, 2015). The risk of developing AD is strongly age, ending in a deterioration in mood, behavior, in performance, knowledge and memory, therefore (Alzheimer et al., 1995), AD is becoming a crisis increasing social with growing life expectancy. Despite this, there is no current treatment that can stop or reverse the effects of AD (Citron, 2010). The disease is closely related to brain pathology involving (a) extracellular amyloid aggregates (known as senile plaques) (SP) formed by Aβ amyloid and (b) neurofibrillary tangles (NFTs) of tau protein (p-tau) (Belluti et al., 2013; Saido, 2013). Accordingly to amyloid cascade hypothesis, Aβ is primarily responsible for many of the pathological features of the disease, its oligomers representing the most toxic species (Sakono and Zako, 2010). Accumulation of Aβ (1-42) plaques inflammatory reactions starts by microglial activation due to pro-inflammatory cytokines in the brain areas that are the most representative: the right neocortex and hippocampus. Moreover, disturbances of the kinase and phosphatase which results in hyperphosphorylation of tau protein, which leads to deterioration and loss of neurons (Thota et al., 2007). Despite existing strong genetic links, including APP and PS-1 mutations, the PS-2 mutation (Bothwell and Giniger, 2000) is the dominant form of sporadic AD. In this regard, AD research deals with mechanisms for early onset of disease with a wider range of factors that lead to sporadic forms, which could be one reason for the failure of the majority of therapeutic trials and lack of preventive measures by more 20 years from proposal amyloid hypothesis (Hicks et al., 2012).

MEANS OF FORMING Aβ

The Aβ peptide, derived from the larger amyloid precursor protein (APP), was first isolated as the principal component of amyloid deposits in the brain and cerebrovasculature of AD (Glenner and Wong, 1984; Masters et al., 1985; Selkoe, 2001). Although the function of APP itself has not been resolved, extensive research has advanced our knowledge of how the Aβ peptide is produced, and how it is subsequently degraded within the brain, or transported out into the periphery. The final amount of Aβ that accumulates as amyloid deposits within the brain is determined by the interplay of these factors. Changes with disease progression could contribute to the age of disease onset and disease duration.

The enzymatic processes responsible for the metabolism of APP to Aβ are now reasonably well understood. APP is sequentially cleaved by two membrane-bound endoprotease activities, β- and γ-secretase. β-secretase first cleaves APP to release a large secreted derivative, sAPPβ. A fragment of 99 amino acids (CTFβ, which begins with the N-terminal aspartyl residue of Aβ) remains membrane bound, and is in turn rapidly cleaved by γ-secretase to generate Aβ. Cleavage by γ-secretase is somewhat imprecise, resulting in a C-terminal heterogeneity of the resulting peptide

population. Hence, numerous different Aβ species exist, but those ending at position 40 (Aβ40) are the most abundant (~80-90%), followed by 42 (Aβ42, ~5-10%). The slightly longer forms of Aβ, particularly Aβ42, are more hydrophobic and fibrillogenic, and are the principal species deposited in the brain (Giacobini, 2004; Rochette and Murphy, 2002; Selkoe, 2001).

β-Secretase activity is believed to be the rate-limiting step in the amyloidogenic pathway and processes ~10% of the total cellular APP. The remaining APP, close to 90%, is constitutively cleaved by α-secretase (a collection of metalloprotease enzymes), generating sAPPα and the 83 amino acid CTFα. The subsequent γ-secretase cleavage of CTFα produces the more benign p3 fragment instead of Aβ. γ-Secretase cleavage of either membrane bound CTF also generates a cytosolic element, AICD (APP intracellular domain, sometimes referred to as CTFγ), which may play a role in signal transduction. Because of their essential role in the generation of Aβ, both β- and γ-secretase are considered to be prime targets for the development of anti- AD pharmaceuticals (Murphy et al., 1999; Shah et al., 2005).

γ-Secretase is now known to be a multisubunit enzyme composed of the proteins APH1, PEN2, nicastrin, and presenilin (PS1 or PS2). The enzyme complex likely contains one copy of each subunit, and is responsible for the cleavage of multiple membrane proteins in addition to APP. Although the exact functional roles of each component have yet to be fully elucidated, presenilin is believed to form the active site of the aspartyl protease (Kessels et al., 2010; Querfurth and LaFerla, 2010), and nicastrin likely serves as a substrate docking subunit (Scheuner et al., 1996). All four components are necessary for γ-secretase to mature and function correctly (Kosicek and Hecimovic, 2013). γ-Secretase has a relatively novel mechanism in that it cleaves within the lipid bilayer and can only process substrates that are first cleaved by another protease to remove a large ectodomain region. The enzyme does not have identified specific sequence requirements for substrate recognition, and cleavage within the membrane is instead controlled by a variety of other factors, such as the length of the transmembrane domain (Bennett et al., 2000; Giacobini, 2004). Although the amount of γ-secretase activity does not appear to increase in AD, alterations in γ-secretase activity leading to the production of longer forms of Aβ are the major genetic cause of early-onset, familial AD (Hussain et al., 2000; Yan et al., 2001), an effect that can be mimicked with a variety of allosteric γ-secretase modulating agents (Basi et al., 2003).

β-Secretase is a membrane-bound aspartyl protease, but one that cleaves APP and its other substrates outside of the bilayer (Holsinger et al., 2002). There are two major forms of the enzyme, BACE1, and BACE2, which are >65% homologous. The major form of the enzyme responsible for Aβ production, BACE1, is highly expressed in brain, but is also found at lower levels in other organs (Fais et al., 2013; Shah et al., 2005). In contrast, the second form of the enzyme, BACE2, is low in the brain but is present in most peripheral tissues at higher levels (Rochette and Murphy, 2002). The knockout of BACE1 in mice leads to a massive reduction in the levels of the downstream products of the enzyme (Aβ and CTFβ) in brain. Although these studies indicate that BACE1 is the major β-secretase activity in brain, some residual activity might be attributable to BACE2 (Marcinkiewicz and Seidah, 2000), and both forms of BACE can compete for substrate (Hussain et al., 2000). β-Secretase activity and protein are both significantly increased in sporadic AD. This effect shows a brain regional selectivity that roughly parallels disease affected regions, and is related to both plaque burden and disease duration. β-Secretase activity has also been seen to increase with age in rodents and nonhuman primates, although these species do not develop AD. Recently, evidence has emerged that cathepsin B or cathepsin D may also be able to serve as β-secretase-like enzymes under some circumstances, although this view is controversial.

FACTORS INFLUENCING THE INITIATION AND PROGRESSION OF AD

The factors influencing the initiation and progression of the disease that have a role in the pathophysiology of AD are Aβ (1-42)/Aβ (1-40) oligomers, oxidative stress, proinflammatory cytokines produced by activated glial cells, changes in cholesterol homeostasis and changes in the cholinergic nervous system (Allam et al., 2008).

LIPID RAFT REDOX SIGNALING

Lipid raft microdomains are able to form membrane microdomains or platforms on different simulations, including redox signaling platforms, serving as a signaling mechanism critical for mediating or regulating cellular activities and functions. In particular, installation of NADPH oxidase subunit and binding to other receptors of related effector and control components, which serve to turn in the activation of NADPH oxidase and the redox regulation of downstream cellular functions (Jin et al., 2011).

OXIDATIVE STRESS AND NEURONAL DEATH

Increasing evidence highlights the role played by oxidative stress in AD (Cioanca et al., 2013; Hritcu et al., 2014). Central administration of Aβ induced learning and memory deficits in rats (Hritcu et al., 2015), cholinergic dysfunction (Olariu et al., 2001) and neuronal apoptosis in rats (Hritcu et al., 2014; Ruan et al., 2010), as well as increased the oxidative stress (Bagheri et al., 2011) and promoted neuroinflammation (Wang et al., 2012).
Elevated levels of reactive oxygen species (ROS) is one of the most important age-related harmful agents produced by normal mitochondrial activity, increased levels of glutamate, inducing acceleration characteristic neurodegenerative AD (Huber et al., 2006). Aβ stimulates the accumulation of hydrogen peroxide in cultured hippocampal neurons which causes oxidative damage to cellular phospholipid membranes thus suggesting the role of lipid peroxidation in the development of AD. The loss of membrane integrity due to Aβ and free radicals leading to cellular dysfunction, such as inhibition of the ionic motor of the ATPase, loss of calcium homeostasis, inhibition of glial glutamate-dependent Na^+ absorption system, loss of function of the carrier protein, disrupting signaling pathway and activation of nuclear transcription factors and apoptotic pathways (Allam et al., 2008).

INFLAMMATION AND NEURONAL DEATH

The significant increase in dose-dependent production of prointerleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), monocyte chemoattractant protein-1, macrophage inflammatory peptide-1, IL-8, mitogen activation of the pathway of protein kinase and macrophage colony stimulating factor as were observed after exposure to pre-aggregated Aβ (1-42) as foreign material, because during brain development in young nervous system (Allam et al., 2008). The involvement of the inflammatory process in the pathogenesis of AD is further supported by the observation that inhibiting or neutralizing TNF actions could be benefits for these patients with AD disease (Allam et al., 2008; Rosenberg, 2006).

CHOLINERGIC SYSTEM AND AD

A primary clinical symptom of Alzheimer's dementia is progressive deterioration of memory and learning capacity. There is a profound loss in the cholinergic system of the brain, including dramatic loss level acetylcholine, choline uptake and acetylcholine (ACh) from the neocortex and hippocampus and there is a small number of cholinergic neurons in the brain and core basal Meynert, which are closely linked to cognitive deficits in AD. Pharmacological actions that improve or block ACh levels fall through cholinergic neurotransmission therefore to improve the known improvements in learning and memory in AD (Allam et al., 2008; Giacobini, 2004). Aβ could increase the generation of free radicals and induce inflammation that may lead to profound loss of a cholinergic system of the brain (Allam et al., 2008). ACh also has anti-inflammatory action and, therefore, decreasing its level may further aggravate the inflammatory process and progression of AD. This cholinergic anti-inflammatory pathway" works by inhibiting the production of pro-inflammatory cytokines early TNF, IL-1, and suppresses the expression of NF - KB activation. Furthermore, systemic injection of IL-1 decreases extracellular ACh in the hippocampus. In addition, the IL-1 is APP mRNA positive cells and the ability to promote APP gene expression suggests that IL-1 plays an important role in AD (Pavlov and Tracey, 2004). Lipid raft location was recently linked to acetylcholinesterase (AChE), although its functional implications are still unclear (Hicks et al., 2011). AChE inhibition by compounds such as rivastigmine or galantamine is a major treatment option for the cure of cognitive impairment seen in early stages of AD. Also, inhalation of the juniper volatile oil presents both antiacetylcholinesterase and antioxidant activities in Aβ (1-42) rat model of AD and may contribute to increase the levels of acetylcholine in cholinergic neurons, while simultaneously helping to prevent further degradations caused by radical oxygen species (Cioanca et al., 2015). AChE exists in a number of different molecular forms (monomeric - G1, dimeric - G2, tetrameric - G4). The tetrameric G4 form is predominant in the brain. In AD, G4 AChE levels in the brain decrease as the disease progresses, while the G1 SG2 levels increase somewhat compared to normal brain. In some regions of the AD brain pathology, practically all the pain is located in the complex, which lead to the suggestion that AChE may promote Ap aggregation (García-Ayllón et al., 2010). It was proposed a direct interaction between Aβ and AChE, binding occurs at the peripheral anionic (PAS) enzyme. Those inhibitors of AChE holding PAS (e.g., propidium) showed the most significant reduction in fibril formation because the active site is not required for interaction with the Aβ. In addition, monoclonal antibodies directed against aspartyl proteinase (SAP) inhibit the formation of fibrils, which has led to the development of blocking PAS as well as compounds DUO, occupying also the active site. They show inhibitory activity on AChE and fibril formation and inhibition of Aβ 40 have been suggested as potential therapeutic agents targeting novel AD two facets of the disease (Alptüzün et al., 2010). AChE is a transmembrane protein, rather it is anchored to the plasma membrane rich in proline membrane anchor, which is a type I transmembrane protein, and may be acylated. The first contains the cholesterol recognition amino acid consensus motif that first seizes the lipid rafts and, therefore, also part of AChE is associated with plugs (Alptüzün et al., 2010).

EXOSOMES AND microRNAs

Plasma membrane-derived exosomes vesicles with a diameter 30-90 nm are secreted into the extracellular milieu (Vârna, Artenie, 2007; Fais et al., 2013). Besides containing different proteins

and the molecular components of cells reflective of origin, these vesicles contain microRNAs as their most abundant nucleic acids (Alexandrov et al., 2012). The body may be capable of paracrine transfer of genetic information between cells or in the local environment of the brain or spinal or systemically throughout the circulation, at least in part dependent on the plasma membrane mediated biological mechanisms (Alexandrov et al., 2012; Fais et al., 2013).

DIETARY AND ENVIRONMENTAL FACTORS

Environmental factors influencing food and plasma membrane effects such as flexibility and lipid raft may not be relevant to amyloidogenesis but also paracrine microRNA trafficking and the spread of these mobile genetic signals and soluble intercellular only. For example, biophysics of the plasma membrane, dynamic, and lipid raft field disturbance cholesterol statins would not be able to take only effect on cholesterol incorporation into the membranes and the formation of lipid raft, also on the exocytosis of exosomes (Fais et al., 2013; Kosicek and Hecimovic, 2013; Murphy and LeVine, 2010). More cholesterol can disrupt the structure of membrane biophysics and reorganize lipid raft domains, the protein, and protein-protein interactions lipids, contribute to membrane homeostatic dysfunction mediated APP neurobiology (Lukiw, 2013; Murphy and LeVine, 2010). The involvement of potential AD neurotrophic viral infection that involves processes that are plasma membrane-mediated proinflammatory and innate immune response elusive brain (Alexandrov et al., 2012; Kosicek and Hecimovic, 2013).

DIAGNOSTIC

Certain DNA diagnosis can only be done a postmortem. However, today, specialized clinics, using a combination of tools that include taking a history of the disease on patients and their families, as well as evaluating cognitive function by neuropsychological tests in combination with neuroimaging (CT, MRI and PET) to rule out other causes of dementia (Blennow et al., 2010) can diagnose AD with accuracy greater than 95%. Neurological tests, which are still the gold standard for diagnosis of AD are largely accurate in identifying people with dementia already developed. It provides structural MRI brain atrophy measures, reflecting the loss of dendrites, synapses, and neurons (Kosicek and Hecimovic, 2013).

CONCLUSIONS

AD is a devastating age-related neurodegenerative disease, which has a serious impact on an economic development system and healthcare worldwide. Although AD has been studied for over 100 years since the 1906, its exact pathogenity and mechanism remain to be clarified. Also, up to now there are no discovered treatments or diagnostic methods ideal for AD. The combination of a poor diet, unhealthy lifestyle, vascular problems, and genetic factors may increase the likelihood precursor of amyloid and the advanced rapid onset AD. Although the role of lipid rafts in the pathogenesis of AD is still controversial (lipid rafts that are controversial in itself), it is clear that specific membrane platforms are involved in APP, β- and γ- secretase co-location, in APP processing and formation of pathogen Aβ. Phospholipids provide an optimal environment for protein interactions.

REFERENCES

Alexandrov, P. N., Dua, P., Hill, J. M., Bhattacharjee, S., Zhao, Y., and Lukiw, W. J. (2012). *microRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF).* Int J Biochem Mol Biol **3**, 365-73.

Allam, A., Kiran, K., and Hanuman, T. (2008). *Bioinformatic analysis of Alzheimer's Disease using functional protein sequences.* J Proteomics Bioinform **1**, 36-42.

Alptüzün, V., Prinz, M., Hörr, V., Scheiber, J., Radacki, K., Fallarero, A., Vuorela, P., Engels, B., Braunschweig, H., Erciyas, E., and Holzgrabe, U. (2010). I*nteraction of (benzylidene-hydrazono)-1,4-dihydropyridines with β-amyloid, acetylcholine, and butyrylcholine esterases.* Bioorg Med Chem **18**, 2049-59.

Alzheimer, A., Stelzmann, R., Schnitzlein, H., and Murtagh, F. (1995). *An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde".* Clin Anat **8**, 429-31.

Alzheimer's Association (2015). *2015 Alzheimer's disease facts and figures.* Alzheimers Dement **11**, 332-84.

Bagheri, M., Joghataei, M.-T., Mohseni, S., and Roghani, M. (2011). *Genistein ameliorates learning and memory deficits in amyloid β(1–40) rat model of Alzheimer's disease.* Neurobiol Learn Mem **95**, 270-6.

Basi, G., Frigon, N., Barbour, R., Doan, T., Gordon, G., McConlogue, L., Sinha, S., and Zeller, M. (2003). *Antagonistic effects of β-site amyloid precursor protein-cleaving enzymes 1 and 2 on β-amyloid peptide production in cells.* J Biol Chem **278**, 31512-20.

Belluti, F., Rampa, A., Gobbi, S., and Bisi, A. (2013). *Small-molecule inhibitors/modulators of amyloid-β peptide aggregation and toxicity for the treatment of Alzheimer's disease: a patent review (2010 – 2012).* Expert Opin Ther Pat **23**, 581-96.

***Bennett, B. D., Babu-Khan, S., Loeloff, R., Louis, J.-C., Curran, E., Citron, M., and Vassar, R.* (2000).** *Expression analysis of BACE2 in brain and peripheral tissues.* J Biol Chem **275**, 20647-51.

Blennow, K., Hampel, H., Weiner, M., and Zetterberg, H. (2010). *Cerebrospinal fluid and plasma biomarkers in Alzheimer disease.* Nat Rev Neurol **6**, 131-44.

Bothwell, M., and Giniger, E. (2000). *Alzheimer's Disease: Neurodevelopment Converges with Neurodegeneration.* Cell **102**, 271-3.

Cioanca, O., Hancianu, M., Mihasan, M., and Hritcu, L. (2015). *Anti-acetylcholinesterase and antioxidant activities of inhaled juniper oil on amyloid beta (1–42)-induced oxidative stress in the rat hippocampus.* Neurochem Res **40**, 952-60.

Cioanca, O., Hritcu, L., Mihasan, M., and Hancianu, M. (2013). *Cognitive-enhancing and antioxidant activities of inhaled coriander volatile oil in amyloid β(1–42) rat model of Alzheimer's disease.* Physiol Behav **120**, 193-202.

Citron, M. (2010). *Alzheimer's disease: strategies for disease modification.* Nat Rev Drug Discov **9**, 387-98.

Fais, S., Logozzi, M., Lugini, L., Federici, C., Azzarito, T., Zarovni, N., and Chiesi, A., 2013. *Exosomes: the ideal nanovectors for biodelivery,* Biol Chem, p. 1.

***García-Ayllón, M.-S., Riba-Llena, I., Serra-Basante, C., Alom, J., Boopathy, R., and Sáez-Valero, J.* (2010).** *Altered levels of acetylcholinesterase in Alzheimer plasma.* PLoS ONE **5**, e8701.

Giacobini, E. (2004). *Cholinesterase inhibitors: new roles and therapeutic alternatives.* Pharmacol Res **50**, 433-40.

***Glenner, G. G., and Wong, C. W.* (1984).** *Alzheimer's disease and Down's syndrome: Sharing of a unique cerebrovascular amyloid fibril protein.* Biochem Biophys Res Commun **122**, 1131-5.

Hicks, D., John, D., Makova, N. Z., Henderson, Z., Nalivaeva, N. N., and Turner, A. J. (2011). *Membrane targeting, shedding and protein interactions of brain acetylcholinesterase.* J Neurochem **116**, 742-6.

Hicks, D. A., Nalivaeva, N. N., and Turner, A. J. (2012). *Lipid rafts and Alzheimer's disease: protein-lipid interactions and perturbation of signalling.* Front Physiol **3**, 1-18.

Holsinger, R. M. D., McLean, C. A., Beyreuther, K., Masters, C. L., and Evin, G. (2002). *Increased expression of the amyloid precursor β-secretase in Alzheimer's disease.* Ann Neurol **51**, 783-6.

Hritcu, L., Noumedem, J., Cioanca, O., Hancianu, M., Kuete, V., and Mihasan, M. (2014). *Methanolic extract of Piper nigrum fruits improves memory impairment by decreasing brain oxidative stress in amyloid beta(1–42) rat model of Alzheimer's Disease.* Cell Mol Neurobiol **34**, 437-49.

Hritcu, L., Noumedem, J. A., Cioanca, O., Hancianu, M., Postu, P., and Mihasan, M. (2015). *Anxiolytic and antidepressant profile of the methanolic extract of Piper nigrum fruits in beta-amyloid (1–42) rat model of Alzheimer's disease.* Behav Brain Funct **11**, 13.

Huber, A., Stuchbury, G., Burkle, A., Burnell, J., and Munch, G. (2006). *Neuroprotective therapies for Alzheimer's Disease.* Science **12**, 705-17.

Hussain, I., Powell, D. J., Howlett, D. R., Chapman, G. A., Gilmour, L., Murdock, P. R., Tew, D. G., Meek, T. D., Chapman, C., Schneider, K., Ratcliffe, S. J., Tattersall, D., Testa, T. T., Southan, C., Ryan, D. M., Simmons, D. L.,

Walsh, F. S., Dingwall, C., and Christie, G. (2000). *ASP1 (BACE2) cleaves the Amyloid Precursor Protein at the β-secretase site.* Mol Cell Neurosci **16**, 609-19.
Jin, S., Zhou, F., Katirai, F., and Li, P.-L. (2011). *Lipid raft redox signaling: molecular mechanisms in health and disease.* Antioxid Redox Signal **15**, 1043-83.
Kessels, H. W., Nguyen, L. N., Nabavi, S., and Malinow, R. (2010). *The prion protein as a receptor for amyloid-β.* Nature **466**, E3-E5.
Kosicek, M., and Hecimovic, S. (2013). *Phospholipids and Alzheimer's Disease: alterations, mechanisms and potential biomarkers.* Int J Mol Sci **14**, 1310-22.
Lukiw, W. J. (2013). *Alzheimer's disease (AD) as a disorder of the plasma membrane.* Front Physiol **4**, 1-3.
Marcinkiewicz, M., and Seidah, N. G. (2000). *Coordinated Expression of β-Amyloid Precursor Protein and the Putative β-Secretase BACE and α-Secretase ADAM10 in Mouse and Human Brain.* J Neurochem **75**, 2133-43.
Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., and Beyreuther, K. (1985). *Amyloid plaque core protein in Alzheimer disease and Down syndrome.* Proc Natl Acad Sci USA **82**, 4245-9.
Mount, C., and Downton, C. (2006). *Alzheimer disease: progress or profit?* Nat Med **12**, 780-4.
Murphy, M. P., Hickman, L. J., Eckman, C. B., Uljon, S. N., Wang, R., and Golde, T. E. (1999). *γ-Secretase, evidence for multiple proteolytic activities and influence of membrane positioning of substrate on generation of amyloid β peptides of varying length.* J Biol Chem **274**, 11914-23.
Murphy, M. P., and LeVine, H. (2010). *Alzheimer's Disease and the β-amyloid peptide.* J Alzheimers Dis **19**, 311.
Olariu, A., Tran, M., Yamada, K., Mizuno, M., Hefco, V., and T., N. (2001). *Memory deficits and increased emotionality induced by beta-amyloid (25-35) are correlated with the reduced acetylcholine release and altered phorbol dibutyrate binding in the hippocampus.* J Neural Transm (Vienna) **108**, 1065-79.
Pavlov, V. A., and Tracey, K. J. (2004). *Neural regulators of innate immune responses and inflammation.* Cell Mol Life Sci **61**, 2322-31.
Querfurth, H. W., and LaFerla, F. M. (2010). *Alzheimer's Disease.* N Engl J Med **362**, 329-44.
Rochette, M., and Murphy, M. P. (2002). *γ-Secretase.* Mol Neurobiol **26**, 81-95.
Rosenberg, P. (2006). *Cytokine inhibition for the treatment of Alzheimer's disease.* Med Gen Med **8**, 24.
Ruan, C.-J., Zhang, L., Chen, D.-H., Li, Z., Du, G.-H., and Sun, L. (2010). *Effects of trans-2,4-dimethoxystibene against the neurotoxicity induced by Aβ25–35 both in vitro and in vivo.* Neurosci Res **67**, 209-14.
Saido, T. C. (2013). *Metabolism of amyloid beta; peptide and pathogenesis of Alzheimer's disease.* Proc Jpn Acad Ser B Phys Biol Sci **89**, 321-39.
Sakono, M., and Zako, T. (2010). *Amyloid oligomers: formation and toxicity of Aβ oligomers.* FEBS J **277**, 1348-58.
Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, L., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996). *Secreted amyloid [beta]-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease.* Nat Med **2**, 864-70.
Selkoe, D. J. (**2001**). Alzheimer's Disease: genes, proteins, and therapy. *Physiol Rev* **81**, 741-66.
Shah, S., Lee, S.-F., Tabuchi, K., Hao, Y.-H., Yu, C., LaPlant, Q., Ball, H., Dann Iii, C. E., Südhof, T., and Yu, G. (**2005**). *Nicastrin Functions as a γ-Secretase-Substrate Receptor.* Cell **122**, 435-47.
Thota, H., Appa Rao, A., Kumar Reddi, K., Akula, S., Babu Changalasetty, S., and Srinubabu, G. (2007). *Alzheimer's disease care and management: Role of information technology.* Bioinformation **2**, 91-5.
Vârna, M., **and Artenie, V. (2007).** *siRNA et microRNA : biogenese, mecanisme et fonctions.* Analele Ştiinţifice ale Universităţii " Al.I.Cuza" Iaşi (Serie nouă). Secţiunea Genetică şi Biologie Moleculară, Tom VIII, **fasc.2**, 15-24.
Wang, C., Yang, X.-M., Zhuo, Y.-Y., Zhou, H., Lin, H.-B., Cheng, Y.-F., Xu, J.-P., and Zhang, H.-T. (2012). *The phosphodiesterase-4 inhibitor rolipram reverses Aβ-induced cognitive impairment and neuroinflammatory and apoptotic responses in rats.* Int J Neuropsychopharmacol **15**, 749-66.
Wimo, A., and Prince, M., 2010. *World Alzheimer Report 2010-the global economic impact of dementia* edn.) Alzheimer's Disease International: London.
Yan, R., Munzner, J. B., Shuck, M. E., and Bienkowski, M. J. (2001). *BACE2 functions as an alternative α-secretase in cells.* J Biol Chem **276**, 34019-27.

1 - Department of Biology, Alexandru Ioan Cuza University of Iasi, Romania
* radu.ionita09@gmail.com
CONFLICT OF INTEREST DISCLOSURE: The authors declare that they have no potential conflicts of interest to disclose.

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

OVARIAN DRILLING IN INFERTILITY ASSOCIATED WITH POLYCYSTIC OVARY SYNDROME

EDUARD CRAUCIUC[1*], RALUCA GRIGORIU[2], DIANA POPOVICI[1], MARIANA BRATU[3], ALLIA SINDILAR[1], OVIDIU TOMA[4], DRAGOŞ CRAUCIUC[1]

Received: 20 November 2015 / Revised: 21 November 2015 / Accepted: 28 November 2015 /
Published: 10 December 2015

Keywords: Polycystic Ovary Syndrome, drilling, infertility, clomiphene

Abstract. About 7-8% of the women of reproductive age are affected by the polycystic ovary syndrome (PCOS), and this is considered to be the main cause of infertility and also the most frequent endocrine illness in women. The study offers an open perspective for the physician who treats the polycystic ovary syndrome, regarding the judicious use f investigations in order to analyse the amplitude of the syndrome objectively, so as the treatment of infertility can become the major objective for the woman diagnosed with this syndrome. The study was a retrospective one, both analytical and descriptive and included 156 patients (28.41%) who addressed the hospital for primary/secondary infertility issues, of ovulatory cause, with previous medical treatment and who accepted ovarian drilling as a method of treatment in the Clinic of Obstetrics-Gynaecology of the Emergency County Hospital Bacău. The values of LH and the ratio LH/FSH are increased for the patients with polycystic ovary syndrome, but the sensitivity and specificity of gonadotropins in defining the syndrome are not certain. There is no correlation between the level of FSH and LH that can have statistical significance, even if the slope of the regression line has an ascending character. In reality, what is important for diagnosing the illness is the ratio between the two pituitary tropes and not the absolute value of each of them. If you consider the pharmaceutical and economical point of view, the laparoscopic ovarian drilling could be the first choice when deciding what treatment to use for the patients with PCOS that are resistant to clomiphene, as it is cheaper than the treatment with gonadotropins. The laparoscopic ovarian drilling, followed by an appropriate stimulation with clomiphene is an efficient method of treatment for the patients with anovulatory infertility.

INTRODUCTION

The cuneiform ovary resection is the first therapy introduced for PCOS and it represents the excision of a half up to three quarters of the ovarian medulla through laparotomy. Because of the risks of postsurgical adhesions that transformed endocrinologic infertility into mechanical infertility, it was abandoned little by little and replaced with medical therapy. Stein and Leventhal described the surgical treatment for the first time in the year 1935 – cuneiform resection – and consequently the menstrual cycle came back to normal for about 80% of the patients and also the pregnancy rate increased to 50% (Stein I. & Leventhal M., 1935). This method was abandoned because it caused pelvic adherences to appear.
Gjonnaess introduced the technique of laparoscopic ovarian drilling in 1984 for the patients with polycystic ovary syndrome who were resistant to clomiphene citrate (Gjonnaess H., 1984).
Fernandez et al. published a retrospective study in 2011 and it was used to create an overview of the laparoscopic ovarian drilling (Fernandez H. et al, 2011). The laparoscopic ovarian drilling (LOD) is the new laparoscopic method, an alternative for the anovulatory patients that are also resistant to the medicine treatment. It consists of electrocoagulation- puncture and laser vaporisation, in order to create multiple perforations on the ovarian surface and the stroma.
The method can reduce the use of ovulation stimulators, expensive drugs that need a long time of treatment. Many authors state that this method obtained very good results on the short term, regarding the percentage of ovulations and pregnancies and re-establishing the normal menstrual cycles (Rossmanith W.G., 1991; Farquhar C.M., 2002; Bayram N., et al, 2004; Kaya H., 2005; Madnani N., et al, 2013).

PURPOSE AND OBJECTIVES

The study intends to determine:

- the efficiency of laparoscopic ovarian drilling for the patients with polycystic ovary syndrome (PCOS) that are resistant to the therapy with clomiphene over the improvement of the endocrine and clinical parameters;
- the factors that influence the results of the laparoscopic ovarian drilling (LOD), as a method of treatment for the women with polycystic ovary syndrome;
- the efficiency of the laparoscopic ovarian drilling in comparison with other methods of treatment;

- how to identify a protocol that will lead to an increase in the pregnancy rate for the patients with PCOS that are resistant to the treatment with citrate clomiphene.

MATERIALS AND METHODS

The retrospective study took place in the period of time between January 2010 - December 2014 and it was performed on 156 patients, with a mean age of 30 years old, who were investigated for a mean period of time of 28 months after the procedure.
Selection criteria: age between 20 and 30 years old - 115 patients (73.71%); 30-40 years old - 41 patients (26.29%).
The reasons for seeing the doctor: Oligo-spaniomenorrhea and infertility - 103 patients (66.03%); infertility - 30 patients (19.23%); secondary amenorrhea and infertility - 21 patients (13.46%); primary amenorrhea - 2 patients (1.28%).
Indications: anovulatory infertility caused by PCOS; patients with a persistent hypersecretion of luteinizing hormone (LH); trspeonding to citrate clomiphene, because LOD reduces the secretion of LH; patients with no ovulations who have PCOS and who need surgery in order to assess the pelvis, permanent monitoring during the treatment with gonadotropins.
Principles to follow up when treating infertility. The laparoscopic ovarian drilling can be performed through transvaginal hydrolaparoscopy, culdoscopy or fertiloscopy. In the first instance doctors always use citrate clomiphene. In case it doesn't work they associate gonadotropins with follicle-stimulating hormone (FSH) and LH. The last option is the laparoscopic electrocautery of cysts. Sometimes the drilling can improve receptivity to exogenous gonadotropins and the doctor can be tempted to start a new treatment with gonadotropins post operatory (Onofriescu A., et al, 2012).
The diagnostic of PCOS was established based on the Rotterdam criteria (2 out of 3 present): oligo/anovulation, clinical/biological signs of hyperandrogenism, polycystic ovaries (the presence of 12 or more follicles of 2-9 mm in each ovary and/or an increased ovarian volume >10 mL) (The Rotterdam ESHRE/ASRM, 2004).
The ratio LH/FSH was established in the third day of the menstrual cycle ≥ 2 and/or high levels of the androgenic hormones (testosterone ≥ 0.7 ng/ml, free testosterone ≥ 2pg/ml) for the patients with oligomenorrhea or amenorrhea; there was also an image of ovarian stromal hypertrophia and multiple follicles (≥ 10 follicles) of small dimensions (6-8 mm) that appeared during the ultrasound endovaginal examination.
The normal values of the biochemical parameters tested were as follows: glucose - 76-110 mg/dl; FSH – 3.5-12.5 mUI/ml; LH – 2.4-12.6 mUI/ml; total testosterone – 0.06-0.82 ng/ml; free testosterone – 0.1-4.1 pg/ml.
Citrate clomiphene (CC) remains the first choice treatment for inducing ovulation in the case of the women with anovulatory cycles and PCOS. They are taken orally, with few side effects.

RESULTS AND DISCUSSION

From the paraclinical point of view, out of the156 patients:
- 141 patients (90.4%) showed suggestive ultrasound aspects for polycystic ovary;
- 15 patients (9.6%) had normal ultrasound scans, but associated suggestive clinical signs of PCOS: hirsutism, menstrual disorders and hormonal changes;
- an increased testosterone value for 98 patients (62.8%);
- 103 patients (66.0%) had an increased BMI above the normal values, the rest being of normal weight;
- a changed ratio FSH/LH for 85 patients (54.5%).

Intraoperative, 47 patients (30.12%) from the total of 156 showed associated pathologies:
- ovarian cysts - 28 patients (59.6%) representing 17.9% from the total;
- adherences - 12 patients (25.5%) representing 7.7% from the total;
- unilateral tubal obstruction - 5 patients (10.6%) representing 3.2% from the total;
- bilateral tubal obstruction - 2 patients (4.3%) representing 1.3% from the total.

The postoperative monitoring, lasted for an interval of 2-24 months:
- 2 months - 41 patients - 26.28%;
- 2-6 months - 53 patients - 33.97%;
- 6 months - 12 months - 18 patients - 11.53%;
- over 12 months - 9 patients - 5.76.

Reevaluation

✓35 from the total did not come back;
✓91 patients had ovulatory cycles - 58.34%;
✓34 patients had pregnancies - 21.79%;
✓31 patients did not answer - 19.87%

The clinical and hormonal profiles of the patients were recorded before and after the procedure. There were significant differences in the serum values of FSH (p=0.048), LH (p=0.059), LH/FSH ratio (p=0.05) and testosterone (p=0.025).

Table I. ***Hormonal profiles of the patients with PCOS before and after treatment***

Hormonal profile	Before drilling	After drilling	p
FSH (mUI /ml)	3.87	4.61	**0.048**
LH (mUI /ml)	5.63	4.43	**0.049**
LH /FSH	1.59	1.01	**0.050**
Testosterone (ng/ml)	0.71	0.63	**0.025**
Free testosterone (pg /ml)	2.03	1.70	**0.049**
Glucose (mg /dl)	83.1	83.8	0.999
Regular cycles (%)	16%	80%	**0.001**

Ovarian laparoscopic drilling, followed by an appropriate stimulation with clomiphene is an efficient method of treatment for the patients with anovulatory infertility. The stimulation of follicular maturation by using Citrate clomiphene, which increases the endogenous secretion of FSH, or exogenous FSH administration, resulted in a complete follicular maturation and ovulation (Onofriescu A., et al, 2013).

In our study, the ovulation rate was 80% and the pregnancy rate 36.6%.

The distribution of pregnancies depending on the clomiphene dose used was as follows: for 50 mg there were 10 pregnancies (41.66%) out of the total of 24 patients; for the dose of 100 mg there were 18 pregnancies (51.42%) out of the 35 patients; and for the dose of 150 mg there were 9 pregnancies (47.36%) out of the 19 patients.

Hormonal and ultrasound monitoring are compulsory during the protocols of ovarian stimulation in order to control the risk of ovarian hyper stimulation syndrome.

Based on the cases studied, ROC curve is drawn in order to highlight the efficiency of the treatment method used for infertility and it shows a better accuracy for the patients who had ovarian drilling, in comparison with the group of patients for which they used citrate clomiphene (CC).

Table II. LOD efficiency in comparison with the classical treatment of infertility

Method	**VPP (%)**	**VPN (%)**	**Sensibility (%)**	**Specificity (%)**	**Accuracy**	**p**
Drilling	84.6	0	85.0	75.0	80.0	**0.002**
Classic	86.7	0	65.0	47.5	56.3	**0.007**

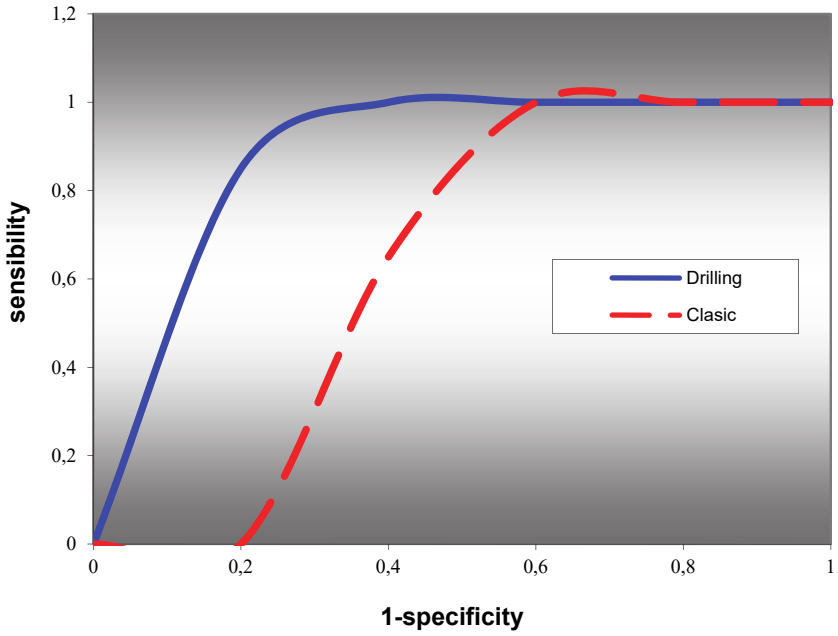

Fig. 1. *ROC curve for the effectiveness of infertility treatment*

Laparoscopic ovarian drilling through diathermy or laser is actually another option of treatment for women with anovulatory infertility associated with PCOS. This laparoscopic version of the ovarian resection uses electric monopolar coagulation or ovarian puncture with laser in 4-6 places, at a depth of 4-10 mm on each ovary (Liguori G., 1996; Mencaglia L., 2003).
After the laparoscopic ovarian drilling, the ovary volume increases transiently, followed by a decrease in the ovary volume (Rossmanith W.G., 1991).
It is not known exactly why some patients with PCOS do not respond to this treatment. A plausible explanation could be the fact that the destroyed ovarian tissue is not enough to produce the expected effect for some patients or the presence of an inherent resistance of the ovary to the drilling effect (Amer S., et al, 2007).
Efficiency. For about 50% of the women treated with laparoscopic ovarian drilling (LOD) an additional therapy was also necessary. For these women, the additional CC can be considered after 12 weeks, if ovulation did not appear. The additional FSH must be considered after 6 months (Bayram N., et al, 2004).
Five random controlled clinical studies, that compared the efficiency of laparoscopic ovarian drilling (LOD) with that of gonadotropins in women with PCOS who are resistant to CC, did not show any difference regarding the rate for keeping the pregnancy or the birth rate. In one of the clinical studies, if there was no ovulatory cycle 8 weeks after surgery or if the patient became anovulatory again, then she was administered CC in increasing doses. The rate of the multiple pregnancies was significantly higher for the group who were administered gonadotropins during the five clinical trials in comparison with LOS (relative risk [RR] 0.13; confidence interval [CI] of 95%, 0.03-0.98). On the other hand, there were no differences regarding the abortion rate between

the group with LOD and the group of women treated with gonadotropins (RR 0.61; CI de 95%, 0.17-2.16). There were no cases of SHSO in either of the recent studies (Farquhar C.M., 2001; Kaya H., 2005).

Safety. The immediate complications of the surgical intervention are rare. From the total number of 778 cases of LOD, only two cases of haemorrhage were reported as they needed laparotomy and also one case showed an intestine lesion. The long term incidents can include formation of adhesions and early menopause. Premature ovarian failure is a risk of ovarian drilling, especially when it is used with a big number of punctions (Amer S., et al 2002). The risks of surgery are those of anaesthesia, of damage to some abdominal organs, the occurrence of postoperative adhesions.

The adherence rate is considered to be 25%. Some people proposed a laparoscopic second-look to lyse any adhesions formed. Apparently when a part of the ovarian tissue is destroyed that does not cause a more rapid start of menopause; but there is a risk of some premature ovarian disorders, although this risk is still being estimated (Hamilton-Fairley D. and Taylor A., 2003).

In comparison, ovarian drilling has the same success rate as the use of gonadotropins. But the multiple pregnancy rates seem to be more reduced. Followed on a period of time of 25 years, the partial ovary resection continues to get the best results. The cumulative rate of pregnancies was 76%, but if we add those induced by medication it goes up to 88%. The cumulative rate of pregnancies was 78%. The only method used in the present that is comparative as results is the laparoscopic cauterise (Hashim H., et al, 2013).

CONCLUSIONS

Laparoscopic ovarian drilling, followed by an appropriate stimulation with clomiphene associated with metformin is an effective method of treatment for the patients with anovulatory infertility.

In our study, the ovulation rate was 80%, and the pregnancy rate was 36.6%.

When talking about LOD safety, there are few complications recorded (haemorrhage during surgery, intestinal perforation), postoperative adhesions and early menopause.

The results are more favourable when the biochemical profile of the patients before surgery is less deteriorated.

The costs of the procedure can recommend it as second-line therapy for the cases that do not respond for a period of 6 months to citrate clomiphene therapy alone, being able to successfully replace the therapy with gonadotropins.

When performing LOD many types of intervention can be used: cuneiform resection, electrocautery, laser vaporization, multiple ovarian biopsies, procedures that lead to an endocrine profile change.

REFERENCES

Bayram, N. et al. (2004) *Using an electrocauters strategy or recombinant follicle stimulating hormone to induce ovulation în polycystic ovary syndrome:randomised controlled trial.* BMJ*,* 328(7433): 192.

Dalton ME. (2000) *Sindromul ovarului polichistic*, Medical update, p. 218-220.

Farquhar, C., Vandekerckhove, P., Lilford R. (2001) *Laparoscopic drilling by diathermy or laser for ovulation induction în anovulatory polycystic ovary syndrome.* Cochrane Database Syst Rev. 4: CD001122.

Farquhar, C., Williamson, K., Gudex, G., Jonson, N., Garland, J., Sadler, L. (2002) *A randomized controlled trial of laparoscopic ovarian drilling diathermy versus gonadotropi therapy for women with clomiphene citrate- resistant polycystic ovary syndrome.* Fertility and Sterility, 78(2): 404-411.

Fernandez, H., Morin-Surrica, M., Torre, A., Faivre, E., Deffieux, X., Gervaise, A. (2011) Ovarian drilling for surgical treatment of polycystic ovarian syndrome: a comprehensive review, *Reproductive Bio Medicine* Online, 22(6): 556-568.

Gjonnaess, H. (1984) *Polycystic ovarian syndrome treated by ovarian electro cautery through the laparoscope.* Fertil Steril, 49: 956-960.
Hamilton –Fairley, A. and Taylor, A. (2003) *ABC of subfertility. Anovulation,* BMJ, 327:546-549.
Hashim, H., Al- Inany, H., De Vos, M, Tournaye, H. (2013) *Three decades after Gjönnaess's laparoscopic ovarian drilling for treatment of PCOS; what do we know ?An evidence- based approach*, Arch Gynecol.Obstet, 288(2): 409-422.
Kaya, H., Sezik, M., Ozkaya, O. (2005) *Evaluation of a new surgical approach for the treatment of clomiphene citrate-resistent infertility în polycystic ovary syndrome:laparoscopic ovarian multi-needle intervention*.J Minim Iinvasive Gynecol, 12(4): 355-8.
Liguori, G., Tolino, A., Moccia, G., Scognamiglio, G., Nappi, C. (1996) *Laparoscopic ovarian treatment în infertile patients with polycystic ovarian syndrome (PCOS):endocrine changes and clinical outcome.*Gynecol endocrinol. 10: 257-264.
Madnani, N., Khan, K., Chauhan, P., Parmar, G. (2013) *Polycystic ovarian syndrome.* Indian J Dermatol Venereol Leprol, 79(3): 310-321.
Mencaglia, L., Wattiez, A. (2003) Manual of Gynaecological Laparoscopic Surgery. Tuttlingen:EndoPress, 120-123.
Onofriescu, A., Nemescu, D., Onofriescu, M., Vulpoi, C. (2012) *Laparoscopic ovarian drilling for polycystic ovarian syndrome,* Temporomandib Joint, 62(1-2): 41-44.
Onofriescu, A., Nemescu, D., Onofriescu, M., Vulpoi, C. (2013) *Clinical outcome and hormone profile of laparoscopic ovarian drilling în women with polycystic ovarian syndrome,* Gineco, 4: 170-173.
Rossmanith, W.G., Keckstein, J., Spatzier, K., Lauritzen, C. (1991) *The impact of ovarian laser surgery on the gonadotrophin secretion în women with polycystic ovarian disease.* Clin Endocrinol, 34: 23-30.
Stein, I., Leventhal, M. (1935) *Amenorrhea associated with bilateral polycystic ovaries*. Am J Obstet Gynecol; 29: 181-5.
The Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. *Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS)* Hum Reprod; 2004; 19: 41-7.

[1] "Gr.T.Popa" University of Medicine and Pharmacy, Iasi, Romania, „Elena Doamna" Iaşi Clinical Hospital
2 Clinic of Obstetrics-Gynaecology of the Emergency County Hospital Bacău
[3] County Emergency Hospital Galati
[4] "Alexandru Ioan Cuza" University, Iasi, Romania

crauciuc@yahoo.com

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

THE IMPACT OF $CoFeO_4$ NANOPARTICLES ON SOLUBLE PROTEIN CONTENT AT WHITE ROT FUNGUS *Phanerochaete chrysosporium*

LĂCRĂMIOARA OPRICĂ[1*], EUGEN UNGUREANU[1]

Received: 23 November 2015 / Revised: 26 November 2015 / Accepted: 1 December 2015 /
Published: 10 December 2015

Keywords: *Phanerochaete chrysosporium*, electrophoresis, soluble protein content, $CoFeO_4$ nanoparticles
Abstract: Experimental investigation focused on influence of different CoFeO4 nanoparticles concentrations on soluble protein and electrophoretic pattern of *Phanerochaete chrysosporium* fungus. Number of electrophoretic fraction is higher in older mycelium *P. chrysosporium* 14-day-old compared to that 7-day-old. Moreover, there is an increase in staining intensity of polypeptides of mycelium 14-day-old fact confirmed also by the higher amount of soluble protein. On the other hand, there are no visible differences as regards the different concentrations of nanoparticles effect on the polypeptides pattern.

INTRODUCTION

Nanotechnology, in general and nanomaterials, in particular, have the potential to revolutionize different sectors such as pharmacy and medicine, electronics and agriculture and allied sectors such as food processing, packaging and storage with modern tools (Rai and Ingle, 2012).

Much research has been focused on nanoparticles (dimension < 100nm) over the last decade due to their bulk counterparts. The advancement of research on the synthesis of NPs from natural living sources has attracted considerable attention in the field of nanobiotechnology and its application. It is also known that the shape, morphology and size of nanoparticles play an important role in controlling their special properties (Kamat, 2002; El-Sayed, 2001).

Metal NPs have as possible uses and applications various fields such as electronics, cosmetics, medicine (medical treatment) and biotechnology (Shah and Tokeer, 2010) or solar energy conversion and water treatment (Dubchak et al., 2010). A number of nanoparticles / nanomaterials have an enormous potential for medical applications, such as clinical diagnosis and / or treatment of cancer. The biomedical use of magnetic nanoparticles in diluted suspensions includes nanosized iron oxides as contrast agents in magnetic resonance imaging, in experimental cancer treatment through hyperthermia, in magnetically targeted drug delivery, magnetic separation of biomolecules and cells etc. (Oprica et al., 2015).

On the other hand, nanotechnology may be a better alternative for environmental remediation (Zang Wei-xian, 2003). For transformation and detoxification of pollutants, the nanoremediation methods involve the use of nanomaterials which have properties that allow the chemical reduction and catalyze, lowering the concentration of pollutants (Karn et al., 2009).

Microorganisms have the potential to synthesize NPs intracellulary or extracellularly under ambient conditions without toxic chemically and stringent conditions; even the properties of such nanoparticles to chemically synthesized materials (Baűerlein, 2003). The role of microorganisms in bioremediation, biotransformation, biosorption and biomineralization are well known; however, the microbial-based biogenesis of nanoparticles is a relatively new and largely unexplored area of research. The biogenic potential of different prokaryotes (bacteria and actinomycetes), eukaryotes (fungi and yeast) and viruses have been explored for the synthesis of nanoparticles.

White rot basidiomycetes make an essential contribution to global carbon cycling by efficiently degrading the recalcitrant aromatic biopolymer lignin, which encloses the cellulose and hemicelluloses of vascular plants and is second only to these polysaccharides as a repository of terrestrial biomass (Shary S et al., 2008). The most widely studied white rot organism, *Phanerochaete chrysosporium*, belongs to the homobasidiomycetes. White-rot fungi have the apparently unique ability to degrade lignin to the level of CO_2 (Kirk, Farrell, 1987). Due to the heterogeneity of the substrate, white-rot degradation of lignocellulosic material involves an ensemble of extracellular enzymes.

In order to emphasize the response reaction and particularities detection of P. *chrysosporium* protein spectrum it was analyzed the polypeptide pattern of intracellular protein from fungus grown on media with various concentration of CoFeO4 nanoparticles.

MATERIALS AND METHODS

1. Phanerochaete chrysosporium cultivation.

P. chrysosporium white rot fungus was purchased from the Institute Sciéntifique de Santé Publique, Belgium (HEM no. 5772). This fungus was cultivated in Petri dishes on agarized Sabouraud medium (peptone 10 g/l, glucose 35g/l, agar 2g/l, distilled water up to 1.0 l).

The Erlenmeyer flasks with liquid Sabouraud medium was inoculated with 0,8 cm diameter disks from 7 days' culture of *P. chrysosporium*. In addition, in the medium was added the different concentration of CoFeO4 nanoparticles resulting four variants: V1- 15mg/l, V2- 20mg/l, V3- 30mg/l, V4- 35mg/l and control which not present nanoparticles. All samples were incubated at 28°C in INCUCELL room. The determination of proteins content was assayed in fungus mycelium at 7 days and 14 days after inoculation. All reagents for the culture medium preparation were purchased from Merck.

2. Nanoparticle preparation

Metal salt precursors were Merck chemicals at molar ratio 2:1, each dissolved in deionized water (Kim et al., 2003). In all steps of magnetic nanoparticle suspension synthesis was used deionized water (18.2 MΩ/cm, Barnstead EASYPureII ultrapure water system). Cobalt ferrite coprecipitation was produced by stirring the two stock solutions at 75 °C and by slowly pouring of 2M NaOH (150 mL). To ensure ferrite particles uniform dispersion in deionized water, 12 mL perchloric acid aqueous solution (25%) was added (under continuous stirring at 75 °C - thus modifying the MNPs surface in order to prevent their agglomeration in the presence of ubiquitous gravitational and magnetic fields (Laurent et al., 2008). The final product was a magnetizable nanofluid based on electrostatic stabilization (Gazova et al., 2012) that presented good stability over time at pH close to biological one.

3. Protein assay

The determination of soluble protein content was done according to Bradford method (Bradford, 1976) using 50 mM Tris–HCl buffer, pH 7, with bovine serum albumin as standard. The assay is based on the binding of Coomassie Brilliant Blue G-250 (from Fluka) at aromatic amino acid radicals with the measure of light intensity at 595 nm. The result was expressed in mg protein per g of mycelium.

4. Electrophoresis

Protein electrophoresis was performed in the system of Laemmli (1970) tampons, in the polyacrylamide vertical plates with 0,75 mm thickness, under denaturing conditions and post electrophoresis operations were carried out according to the standard method.

Sample preparation involved protein extraction in Tris-HCl buffer solution, 50 mM pH=7. Protein precipitates were dissolved in Tris-HCl buffer solution (pH=7), contained SDS – 4.25 %, sucrose about 20%, β-mercaptoetanol – 6% and bromophenol blue – 0.004%. Prior to electrophoresis samples were thermally treated at 95°C with a denaturing buffer solution containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol. Aliquots of 10 µl of protein extract were loaded onto the gel and electrophoresed with Tris-glycine buffer (pH=8.3). For the determination of relative molecular mass of separated polypeptide fractions were used protein markers Sigma Wide Range with known molecular weight.

Electrophoresis was performed at 30mA until the bromophenol blue tracking dye entered the resolving gel. The power was decreased to 10mA until the tracking dye reached the bottom of the resolving gel (~ 4 h). After the bromophenol blue reached the bottom of the gel briefly washing with deionized water was carried out and then dying with Coomasie Brilliant Blue R250 was applied. Colored gels were photographed with a high resolution camera (Canon 550D). Images were analyzed with GE Image Quant 8.1 software. Reagents came from Carl Roth and/or Sigma.

5. Statistical analysis

All experiments were carried out with four independent repetitions and the results were expressed as the mean values ± standard deviation (SD). Statistical significance was ensured by four replies of each $CoFeO_4$ nanoparticles concentration and by *t*-test application.

RESULTS AND DISCUSSIONS

Changes of the intracellular soluble protein content of *Phanerochaete chrysosporium* grown on media with different concentrations of $CoFeO_4$ nanoparticles are shown in figure 1. The results obtained evidenced that soluble protein amount increased at 14 days than the 7 days after inoculation.

At 7-day-old culture, the introduction of nanoparticles in the medium determined a slightly increased protein content with increasing the concentration of $CoFeO_4$ nanoparticles. In

contrast, on 14 days it noticed a slight decrease in protein content 14-day-old culture on nanoparticles culture.

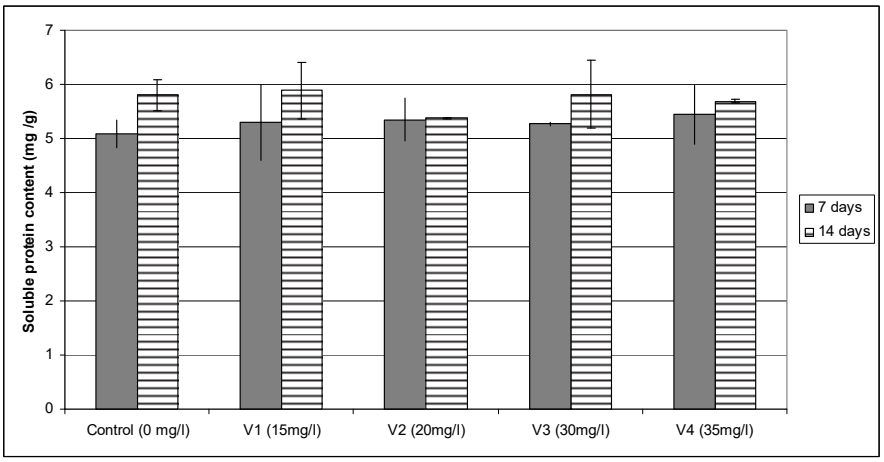

Figure 1. Protein content in mycelium of *Phanerochaete chrysosporium* grown on media with different concentrations of $CoFeO_4$ nanoparticles

The SDS electrophoresis showed distinct protein bands for both ages of *P. chrysosporium* mycelium, namely at 7 and 14 days after inoculation (Figure 2, Figure 3). Using homogeneous gels, proteins in mycelium extract were detected in the entire range detectable by the method.

Comparatively with control, there were no significant differences between mycelium grown on media with different concentrations of $CoFeO_4$ nanoparticles or between the nanoparticles variants. Nevertheless, the electrophoretic pattern showed consistent differences regarding the number of polypeptides and the staining intensity of polypeptides between mycelium 7-day-old and 14-day-old. The overall intensity of the bands of *P. chrysosporium* mycelium at 14 days was stronger both in control and at low nanoparticles concentration (40µl and 60µl) than high nanoparticles concentration (80µl and 100µl).

At 7 days after inoculation, the intensity of the polypeptides from the fungal mycelium was very low and did not show any differences between the various concentrations of nanoparticles or compared to control. At 14 days after inoculation a group of nine polypeptides (with molecular weights between 15-25KDa) it has been emphasized in all variants which have been treated with nanoparticles including the in the control.

In previous research (Oprica et al., 2014) conducted by us regarding influence of electromagnetic fields on protein synthesis of *P. chrysosporium* it was established that there no qualitative modification in protein fraction pattern but the soluble protein amount was diminished with 25%.

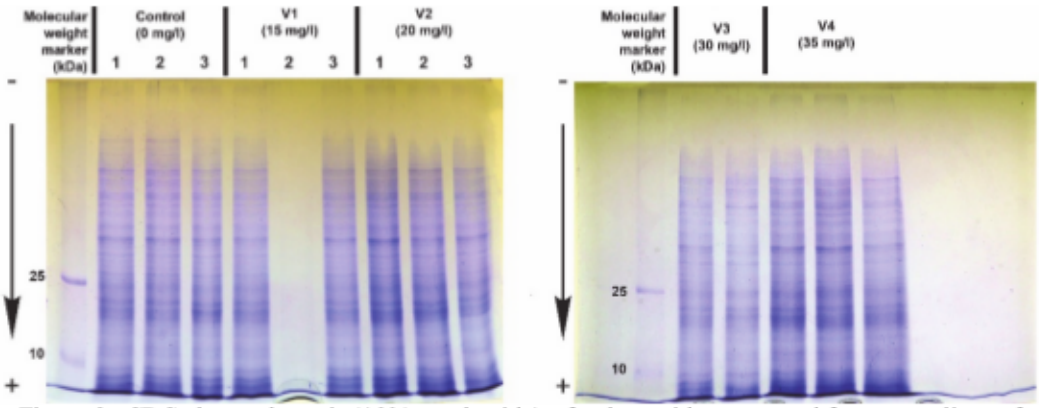

Figure 2. SDS electrophoresis (10% acrylamide) of polypeptides extracted from mycelium of *Phanerochaete chrysosporium* 7-day-old grown on media with different concentration of $CoFeO_4$ nanoparticles (Sigma Wide Range = Molecular weight standards indicated by number on the left of the gel/KDa)

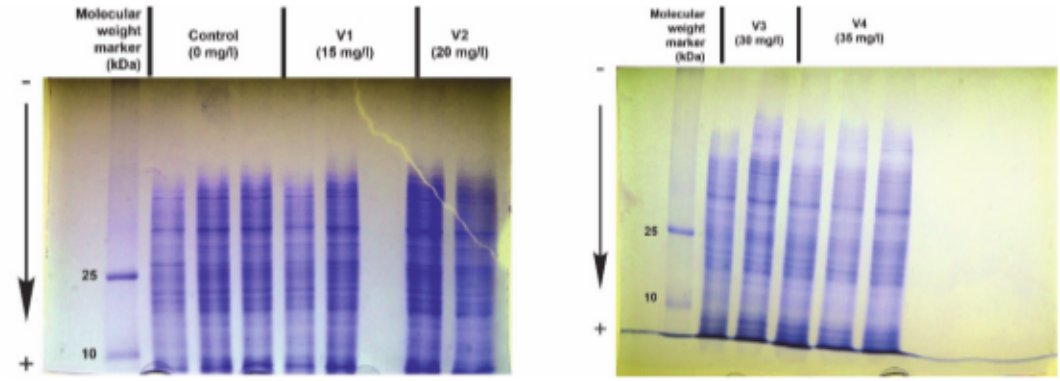

Figure 3. SDS electrophoresis (10% acrylamide) of polypeptides extracted from mycelium of *Phanerochaete chrysosporium* 14-day-old grown on media with different concentration of $CoFeO_4$ nanoparticles (Sigma Wide Range = Molecular weight standards indicated by number on the left of the gel/KDa)

CONCLUSIONS

The comparative analysis of soluble protein pattern from *P. chrysosporium* revealed the presence of common polypeptides both in control and variants with $CoFeO_4$ nanoparticles however, varying by color intensity as well as the bands dimension at 14-day-old mycelium comparatively with 7-day-old mycelium.

REFERENCES

Abbas, A., Koc, H., Liu F., Tien, M., (2005): *Fungal degradation of wood: initial proteomic analysis of extracellular proteins of Phanerochaete chrysosporium grown on oak substrate.* Curr Genet, 47(1):49-56.

Bauerlein, E., (2003): *Biomineralization of unicellular organisms: an unusual membrane biochemistry for the production of inorganic nano- and microstructures.* Angew. Chem. Int. Ed., 42 (6),614-641.
Bradford, M.M., (1976): A rapid and sensitive method for microgram quantities of protein utilizing the principle of protein-dye binding, An. Biochem., 72, 248–254.
Dubchak, S., Ogar, A., Mietelski, J.W., Turnau, K., (2010): *Influence of silver and titanium nanoparticles on arbuscular mycorrhiza colonization and accumulation of radiocaesium in Helianthus annuus*. Spanish J. Agric. Res., 8: 103-108.
El-Sayed, M.A., (2001): Some interesting properties of metals confined in time and nanometer space of different shapes. Acc Chem Res 34:257–264.
Gazova, Z., Siposova, K., Koneracka, M., Antosova, A., Zavisova, V., Ubovcikova, M., Fedunova, D., Bagelova, J., Tomasovicova, N., Daxnerova, Z., Kopcansky, P., (2012): *Presence of magnetic fluids leads to the inhibition of insulin amyloid aggregation*, Acta Physica Polonica A, **121**, 5-6.
Kamat, P.V., (2002): *Photophysical, photochemical and photocatalytic aspects of metal nanoparticles*. J Phys Chem B 106:7729–7744.
Karn, B., Kuiken, T., Otto, M., (2009): *Nanotechnology and in situ remediation: a review of the benefits and potential risks*. Environ Health Perspect, 117:1823–31.
Kim, Y., Kim, D., Lee, C.S., (2003): *Synthesis and characterization of CoFe2O4 magnetic nanoparticles prepared by temperature controlled coprecipitation method*, Physica B: Condensed Matter, **337**, 42-51.
Kirk, TK, Farrell, RL, (1987): *Enzymatic "combustion": the microbial degradation of lignin*, Annu Rev Microbiol, 41:465-505.
Laemmli U.K., (1970): Nature (London), 227, 680–685.
Laurent, S., Forge, D., Port, M., Roch, A., Robic, C., Elst, L.V., Muller, R.N., (2008): *Magnetic iron oxide nanoparticles: synthesis, stabilization, vectorization, physicochemical characterizations, and biological applications*, Chemical Reviews, **108**, 2064-2110.
Oprica, L., Ungureanu, E., Vochita, G., Creanga, D., Miclaus, S., (2014): *Electromagnetic exposure influence on protein synthesis in cellulolytic fungus – an environmental Issue*, Rom. Journ. Phys., 59, (7–8), 817–825.
Oprica, L., Nadejde, C., Andries, M., Puscasu, E., Creanga, D., Balasoiu, M., (2015): *Magnetic contamination of environment – laboratory simulation of mixed iron oxides impact on microorganism cells*, Environmental Engineering and Management Journal, *14* (3), 581-586.
Rai, M., Yadav, A., Gade, A., (2009): *Silver nanoparticles as a new generation of antimicrobials.* Biotechnol Adv 27:76–83.
Shary, S., **Kapich, A.N.**, **Panisko, E.A.**, **Magnuson, J.K.**, **Cullen, D.**, **Hammel, K.E.**, 2008, *Differential Expression in* Phanerochaete chrysosporium *of Membrane-Associated Proteins Relevant to Lignin Degradation*, Appl. Environ. Microbiol.**,** 74(23), **7252-7257**.
Shin, S., Liu, F., Koc, H., Tien, M., (2007): *Expression analysis of extracellular proteins from* Phanerochaete chrysosporium *grown on different liquid and solid substrates*, Microbiology, 153(Pt 9): 3023–3033.
Shah, V., Nerud, F., (2002): *Lignin degrading system of white-rot fungi and exploitation for dye decolorization*, Can. J. Microbiol., 48, pp. 857–870.
Zang, Wei-xian, (2003): *Nanoscale iron particles for environmental remediation:An overview*, Journal of Nanoparticle Research 5: 323-332.

1 – University Alexandru Ioan Cuza Iași, Romania
* - lacramioara.oprica@uaic.ro

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

THE CHEMICAL COMPOSITION ASSESSMENT OF THE FETEASCĂ NEAGRĂ GRAPE POMACE AND ITS FRACTIONS OBTAINED FROM WINE INDUSTRY IN DIFFERENT YEARS

SILVIA MARIANA PASCARIU[1] *, IOAN MIRCEA POP[1]

Keywords: grape pomace, fractions, chemical content, polyphenols, tannins.
Abstract: The aim of the hereby study was to analyze and to compare the chemical content of the grape pomace and its fractions: skins and seeds from the red grape variety Feteasca neagra (from Iași area), obtained in different years 2013 and 2014 respectively, from the winemaking process. Measurements targeted the dry matter content (DM%), organic matter (OM%), crude ash (CA%), crude protein (CP%), crude fat (EE%), crude fiber (CF%), nitrogen-free extractive substances (NFES%), total polyphenols (TP%) and tannins (Ta%). The results obtained showed significant differences in the chemical composition in favour of the grape pomace obtained in the 2014 climatic conditions: in the case of the seed for the content of DM%, SEN%, TP% and Ta%, in the case of the skins for the content of DM%, OM%, CF%, TP%, Ta% and in the case of the grape pomace for the content of DM%, OM%, CF%, SEN%, TP%, and Ta%. Comparative analysis of the chemical composition showed an annual variation of the chemical components, which may be due to climatic conditions and winemaking process. Therefore, an annual chemical quality assessment of the grape pomace is necessary, for the efficient use in the animal feed.

INTRODUCTION

The grape pomace is a by-product generated during the winemaking process, in a very large amounts which contains a variable proportion of seeds, skins and stalks fragments.
Studies on the chemical composition of the grape pomace revealed that in addition to its content in proteins, fats, sugars and minerals (Pop I.M., *et al.,* 2014; Baumgartel T., 2007; Zalikarenab L.R., *et al.,* 2007) it also contains high amounts of polyphenols with antioxidant properties (Alonso A., *et al.,* 2002). Polyphenols accumulate in the solid parts of the grapes, in skins (28-35%) seeds (60-70%) and stalks (less than 10%) which pass, after the maceration-fermentation process into the grape juice and wine, up to 50% of them, the rest remaining in the grape pomace (Bișboacă S.E., 2012).
The skins from the red grapes are rich in anthocyanins, the hydroxy acids and flavonol glycosides, while the seeds contain mainly flavanols and gallic acid. Flavonoids are distributed in the seeds and stalks that mainly contain catechins, epicatechin and procyanidins (Xia E.Q., *et al.,* 2010).
Several studies have shown the beneficial effects of polyphenols including the antioxidant, antibacterial, anti-inflammatory and anti-methanogenic ones, as well as a protective role against the degenerative diseases thus being able to present a great interest in animal health and nutrition (Brenes A., *et al.,* 2008).
Various studies indicate that the differences in the grape pomace chemical composition are due to both the grape variety and species and to their growing conditions (Rondeau P., *et al,* 2013; Zalikarenab L.R., *et al.,* 2007), but also due to the way the grape pomace is dried and stored (Pirmohammadi R., *et al.,* 2007).
The objective of the hereby study was the comparative assessment of the chemical content of the grape pomace and its fractions: skins and seeds from the red grape variety Feteasca neagra, obtained in different years 2013 and 2014 respectively, from the winemaking process.

MATERIALS AND METHODS

The fresh grape pomace (FGP) resulted from the red (Feteasca neagra of Iași area) vinification process was naturally dried, in a clean and well ventilated space at a constant temperature of 20°C. Following the drying process, the vegetal fractions (seeds, skins) from the red grape pomace were separated to determine the chemical content of each fraction in part.
In order to determine the chemical composition, the samples were milled up to particles of 1 mm diameter, according to the standard (SR ISO 6498:2001). The chemical composition intended to determine the dry matter (DM%), crude ash (CA%), crude protein (CP%), crude fat (EE%), crude fibre (CF%) using standardized methods (SR EN ISO 5983-1:2006/AC:2009; SR EN ISO 2171:2010; SR EN ISO 6865:2002; SR ISO 6496:2001; SR ISO 6492:2001); total polyphenols (TP%) and tannins (Ta%) of the alcohol extracts (50%) of the pomace obtained from the grape pomace samples according to the patent (Bișboacă S.E., 2012) were determined by the Folin- Ciocâlteu method (Singleton and Rossi, 1965).
Determination of chemical components (DM%, Ash%, CP%, EE%, CF%) helped to obtaining through mathematical

calculation of organic matter (OM%) and nitrogen-free extractive substances (NFES%). Three determinations were made for each sample of fraction and grape pomace analysed.
Means and standard deviations were calculated for each parameter. The expression of the chemical composition of the analysed grape pomace and grape pomace fractions was made by reporting all the compounds analysed to the DM. The results obtained were statistically processed using the single factor ANOVA test ($p<0.05$) to highlight significant differences between the calculated values.

RESULTS AND DISCUSSIONS

Comparative analysis of the chemical components of seeds from the red grape pomace of the Fetească neagră obtained in different years, 2013 and 2014 respectively, is shown in the table 1.

Table 1 Chemical composition of seeds

Parameter		Results (Mean ±SD)		Statistical significance (Anova)	
		Seeds-2013	Seeds- 2014	p-value	Significance threshold
DM		93.09±0.04	94.25±0.006	9.80E-07	p<0.001
% of DM	CA	3.08±0.16	3.27±0.06	0.11	p>0.05
	OM	96.92±0.16	96.53±0.06	0.014	p<0.05
	CP	14.47±0.25	11.38±0.31	0.0002	p<0.001
	EE	14.82±0.6	13.21±0.07	0.0097	p<0.01
	CF	45.57±1.62	38.19±0.42	0.0016	p<0.01
	NFES	22.07±1.54	33.74±0.56	0.00025	p<0.001
	TP	4.22±0.08	5.23±0.09	5.95E-24	p<0.001
	Ta	3.63±0.09	4.47±0.1	9.55E-21	p<0.001

The results revealed that the seeds from the grape pomace obtained in 2013 had a significantly higher content of OM (96.92±0.16%), CP (14.47±0.25%), EE (14.82±0.6%), CF (45.57±1.62%), compared to the one obtained in 2014. Instead, the seeds from the grape pomace obtained in 2014 showed a significantly higher content in DM (94.25±0.006%), TP (5.23±0.09%), Ta (4.47±0.10%) and NFES (33.74±0.56%).

There is insufficient data on proteins content in grape seeds, in the published literature (Kamel S., *et al.,* 1985; Ohnishi M., *et al.,* 1990). The seeds are not considered to be an important source of protein, their average content can vary between 11-13% (Fantozzi P., 1981; Goni I., *et al.,* 2005), and it depends on the grape variety, fertilization and climate conditions.

The oil content of the seeds may vary from 11.6 to 19.6%, being dependent on the variety and maturity of the grapes (Rao P.U., *et al.,* 1994).

Seeds total polyphenols content differs depending on the grape varieties, climate and environmental conditions, soil type, degree of ripeness and processing method of grapes in winemaking process (Fuleki T., *et al.,* 1997).

Our results on the crude chemical content and seed polyphenolic compounds are comparable to those in the published literature (Kamel S., *et al.,* 1985; Ohnishi M., *et al.,* 1990, Fuleki T., *et al.,* 1997).

The differences of chemical composition of seeds from the grape pomace of the same grape variety but obtained in different years may be due to pedoclimatic conditions, grape processing technology, maceration-fermentation time on the pomace and preservation period of grape pomace.

Almost the entire fraction of soluble carbohydrates is fermented by the wine specific microbial flora, when the maceration-fermentation period is longer (Baumgartel T., *et al.,* 2007). A higher quantity of polyphenolic compounds can be extracted in wine from the solid fractions of pomace, when the maceration period is prolonged, which may lead to decreased of these compounds in the grape pomace.

In table 4 is presented the comparative analysis of the chemical composition of skins and stalks from the grapes pomace obtained in different years.

Table 2 Chemical composition of skins

Parameter		Results (Mean ±SD)		Statistical significance (Anova)	
		Skins + stalks (6,77%)- 2013	Skins + stalks (9,65%)- 2014	p-value	Significance threshold
DM		93.04±0.01	93.21±0.02	0.0001	p<0.001
% of DM	CA	11.60±0.23	7.40±0.21	2.06E-05	p<0.001
	OM	88.40±0.23	92.60±0.21	2.06E-05	p<0.001
	CP	19.08±0.66	15.26±0.12	0.0006	p<0.001
	EE	3.65±0.22	3.36±0.12	0.11	p>0.05
	CF	12.13±0.09	19.31±0.35	4.29E-06	p<0.001
	NFES	53.54±0.72	54.67±0.26	0.06	p>0.05
	TP	1.95±0.05	2.92±0.03	2.81E-34	p<0.001
	Ta	1.30±0.01	2.36±0.06	1.26E-32	p<0.001

The results obtained revealed that the seeds from the grape pomace of 2014 had a significantly higher content in DM (93.21±0.02%), OM (92.60±0.21%), CF (19.31±0.35%), TP (2.92±0.03%) and Ta (2.36±0.06%), appreciation supported by the statistical calculation ($p<0.001$). Instead, the skins from the grape pomace obtained in 2013 showed a significantly higher content in CP (19.08±0.66%), CA (11.60±0.23%), compared to the one obtained in 2014.

No significant differences ($p>0.05$) were observed regarding the content of crude fat and nitrogen-free extractive substances, of the skins from 2013 and 2014 grape pomace.

The increase of the skins chemical components from the grape pomace obtained in 2014 may be due to a higher proportions of stalks: 9.65 g stalk from 100 g of skins and stalks compared to the one obtained in 2013, namely 6.77 g stalk from 100 g.

The differences of chemical composition of skins and stalks from the grape pomace obtained in different years may also be due to pedoclimatic conditions of those years and grape processing technology.

The comparative analysis of the chemical composition of the grape pomace obtained in different years, is presented in Table 3.

Table 3 Chemical composition of grape pomace

Parameter		Results (Mean ±SD)		Statistical significance (Anova)	
		Grape pomace-2013	Grape pomace -2014	p-value	Significance threshold
DM		92.30±0.01	94.06±0.02	2.89E-08	p<0.001
% of DM	CA	7.12±0.05	5.53±0.05	8.96E-06	p<0.001
	OM	92.29±0.09	94.12±0.05	6.87E-06	p<0.001
	CP	16.60±0.12	13.55±0.04	1.89E-06	p<0.001
	EE	10.19±0.09	9.24±0.08	0.00016	p<0.001
	CF	28.54±0.52	30.27±0.7	0.027	p<0.05
	NFES	36.95±0.33	41.05±0.64	0.00059	p<0.001
	TP	2.20±0.08	4.00±0.06	8.79E-06	p<0.001
	Ta	1.44±0.06	3.34±0.07	5.94E-06	p<0.001

The results obtained showed a significantly higher content in the case of 2014 grape pomace for the following components: DM (94.06±0.02%), OM (94.22±0.05%), CF (30.27±0.70%), TP (4.00±0.06%), Ta (3.34±0.07%) and NFES (41.05±0.64%). Instead, the grape pomace obtained in 2014 showed a significantly higher content in CA (7.12±0.05%), CP (16.60±0.12%) and EE (10.19±0.09%) compared to the one obtained in 2014.

The increase of the grape pomace chemical components obtained in 2013 (CA%, CP%, EE%) may be due to higher proportions of seeds (59,47%) compared to the grape pomace obtained in 2014 which had a lower proportion of seeds (50 %).

The higher content of CF% and NFES% in the 2014 grape pomace may be due to higher proportions of skins (41%) compared to the one obtained in 2013 (33,76%).

In the case of red winemaking, the skins are crushed and crumbled by the pressing process, which can decrease the proportion of these fractions in the grape pomace biomass.

A longer contact time between juice and solid parts of the grapes lead to lower content of polyphenols, including tannins from the grape pomace (Gomez-Plaza E., *et al.,* 2006).

In addition to proportion of seeds, skins and stalks from the grape pomace biomass, the chemical composition differences between the two types of grape pomace may also be due to pedoclimatic conditions of those years, grape processing technology, fermentation - maceration time on the pomace and preservation period of grape pomace.

CONCLUSIONS

Our research revealed a higher content of nutritional substances in the fractions of grape pomace obtained in 2014 climatic conditions: DM%, NFES%, TP% and Ta%, in the seeds, DM%, OM%, CF%, TP%, Ta% in the skins; DM%, OM%, CF%, NFES%, TP% and Ta% in the grape pomace. Comparative analysis of the chemical composition showed an annual variation of the chemical components, which may be due to climatic conditions and winemaking process. Therefore, an annual chemical quality assessment of the grape pomace is necessary, for the efficient use in the animal feed. Also, our data supports the possibility of the grape pomace use or its fractions in the farm animals' feed, in compliance with the maximum permitted levels of crude fiber for the animal category and species concerned.

REFERENCES

Alonso, A., Guillean, D., Barroso, C., Puertas, B., Garcia, A., (2002): *Determination of antioxidant activity of wine byproducts and its correlation with polyphenolic content*. 50 (21), 5832–5836 .J Agric Food Chem.
Baumgartel, T., Kluth, H., Epperlein, K., Rodehutscord, M., (2007) – *A note on digestibility and energy value for sheep of different grape pomace.* Vol. 67, 302-306, Small Rumin. Res.
Bișboacă Simona Elena, (2012): *A process to obtain the polyphenols from grape pomace*. Patent RO125704 B1, pp.1-5
Brenes, A., Viveros, A, Goni, I, Centeno, C, Sayago-Ayerdy, S.G., Arija, I., Saura-Calixto, F., (2008): *Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens*. 87 (2), 307–316 Poult Sci.
Fantozzi P., (1981): *Grape seed: a potential source of protein*. 58, 1027-1032, J Am Oil Chem Soc.
Fuleki, T., Ricardo da Silva, J.M., (1997): *Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario*. 45, 1156-1160, J Agric Food Chem.
Gomez-Plaza, E., Minano, A., Lopez-Roca, J.M., (2006) - *Comparison of chromatic properties, stability and antioxidant capacity of anthocyanin-based aqueous extracts from grape pomace obtained from different vinification methods*. 97, 87-94, Food Chem.
Goni, I., Martin, N., Saura-Calixto, F., (2005): *In vitro digestibility and intestinal fermentation of grape seed and peel*. 90, 281-286, Food Chem.
Kamel, S., Dawson, H., Kakuda, Y., (1985): *Characteristics and composition of melon and grape seed oils and cakes*". 62 (5), 881–883. J Am Oil Chem Soc
Ohnishi, M., Hirose, S., Kawaguchi, M., Ito S., Fujino, Y., (1990): *Chemical Composition of Lipids, Especially Triacylglycerol, in Grape Seeds*, 54(4):1035-1042, Agric. Biol. Chem.
Pirmohammadi, R., Golgasemgarebagh, A. and Mohsenpur, Azari A., (2007): *Effects of ensiling and drying of white grape pomace on chemical composition, degradability and digestibility for ruminants*. 6, 1079–1082, J. Anim. Vet. Adv.
Pop, I.M., Pascariu S.M., Simeanu D., Radu-Rusu C., Albu A., (2014): *Determination of the chemical composition of the grape pomace of different varieties of grapes*. Vol. 63 (20), 76-80, Scientific Papers Journal, Animal Science Series, U.A.S.V.M., Iași
Rao P.U., (1994): *Nutrient composition of some less-familiar oil seeds*. 50, 378-382 Food Chem.
Rondeau, P., Gambier, F., Jolibert, F, Brosse, N., (2013): *Compositions and chemical variability of grape pomaces from French vineyard*. 43, 251-254, Ind Crops and Prod.
Singleton, V.L. and Rossi, J.A., (1965): *Colorimetry of total phenolics with phosphomolybdic – phosphotungstic acid reagents*. 16, 144-158, Am. J. Enol. Vitic.
Xia, E.Q., Gui-Fang Deng, Ya-Jun Guo, Hua-Bin Li, (2010): *Biological Activities of Polyphenols from Grapes*, 11 (2), 622-646, Int J Mol Sci.
Zalikarenab, L.R., Pirmohammadi, Teimuriyansari, A., (2007): *Chemical composition and digestibility of dried white and red grape pomace for ruminants*. 6(9), 1107-1111, J. Anim. Vet. Adv.
***- SR EN ISO 5983-1:2006/AC:2009: Animal feeding stuffs – Determination of total nitrogen content and calculation of crude protein content First Part : Kjeldahl Method
*** SR EN ISO 2171:2010: Cereals, Legumes and Derived Products - Determination of Ash content via calcination
*** SR EN ISO 6865:2002: Animal feeding stuffs – Determination of crude fibre content Method with intermediate filtration
*** SR ISO 6498:2001: Animal feeding stuffs. Preparation of Samples for Analysis
*** SR ISO 6496:2001: Animal feeding stuffs – Determination of moisture and other volatile matter content
*** SR ISO 6492:2001: Animal feeding stuffs – Determination of fat content

[1] Faculty of Animal Sciences, “Ion Ionescu de la Brad” University of Agricultural Sciences and Veterinary Medicine of Iasi, Romania
* pascariu_silvia@yahoo.com

Acad. Professor PhD Constantin TOMA – the 80-th autumn of his life

In this season of chrysanthemums, we are living few celebration moments, 80 years of life for a distinguished colleague, Acad. Professor PhD Constantin Toma.

Nothing can express better these pink melancholy moments full of some sweet regrets, looking back at the years which have flown. What could I say in few words? I will try to evoke some emotions, some parts of nearly six decades that have elapsed on the slide of time, since I met this man.

In 1956 when I came in Iasi as a student, this man who is celebrated today (19 November 2015) was student in the III[rd] academic year. Since then, we stepped constantly, shoulder to shoulder, through the faculty doors, during working days and holidays. In that year, long time ago, the new students from the first academic year, me, Mihai Mititiuc and Toader Chifu watched full of admiration and respect the older students from the third year: Constantin Toma, Ionel Miron, Geanina Comanescu, Octaviţa Ailiesei, Mircea Varvara, Ionel Petcu and many others.

From the deepest parts of our memory, as much as it is, with its whimsical game, I will tell you several events which define this special person as man of books, partner and, I hope I am not wrong, if I say as a friend.

I know Constantin Toma well. I knew his bitterness, even his disillusionments and hopes. If I had to define him in a few words, beyond what has conceived, I would choose without hesitation – his bigotry for work. We already know that human value is stated by effort and his actions.

It is said that there are two ways to live: to burn and languish. Professor PhD Constantin Toma burned like a living torch along his 80 years of life. That is his nature. He knew that a successful outcome is not easy and every effort has its own reward.

Now, when I try to do a small evocation of what happened in those passed years, I feel that delicate scent, full of warm, sweet, which melt inside of me, after all these years, together with that vision of youth drowned in a white silence.

Although, he left his home when he was very young, but those places have remained very special for him, his native village, Gugesti, near of Huşi (Vaslui District). Long time ago, we were there together, I as a new driver who couldn’t climb a small slope at the first attempt and Professor PhD

Academician, Profesor Doctor Constantin TOMA în a 80-a toamnă

În acest anotimp al crizantemelor trăim un moment de sărbătoare, împlinirea a 80 ani de viaţă, a unui distins coleg al nostru, profesorul academician Constantin Toma.

Nimic nu poate exprima mai bine starea de roză melancolie şi dulcele regret, privind înapoi, la anii ce au zburat. Ce aş putea spune în câteva cuvinte? Voi încerca să evoc câteva trăiri, din cele aproape şase decenii, care s-au scurs pe vertiginosul topogan al timpului, de când l-am cunoscut pe acest om.

În 1956 când am venit student la Iaşi, sărbătoritul nostru de astăzi (19 noiembrie 2015) era în anul III de studii. De atunci am păşit constant, umăr la umăr, pe porţile facultăţii atât în zile de lucru cât şi în zile de sărbătoare. În acel an îndepărtat, noi, bobocii – subsemnatul, Mititiuc Mihai, Chifu Toader, priveam cu admiraţie şi respect la studenţii din anii mai mari: Constantin Toma, Ionel Miron, Geanina Comănescu, Octăviţa Ailiesei, Mircea Varvara, regretatul Ionel Petcu şi mulţi alţii.

Din sihla memoriei, cât a mai rămas, cu jocul ei capricios, voi selecta câteva întâmplări care îl definesc pe sărbătorit ca om de carte, coleg de facultate şi sper că nu greşesc să spun şi ca prieten.

L-am cunoscut bine pe Constantin Toma. I-am cunoscut amărăciunile, chiar şi deziluziile şi speranţele lui. Dacă ar fi să-l definesc în câteva vorbe, dincolo de ce a zămislit, aş alege fără ezitare – habotnicia cu care s-a dăruit muncii. Ştim că valoarea omului se afirmă prin efort, prin faptele sale.

Se spune că sunt două moduri de a trăi: a arde şi a lâncezi. Profesorul Constantin Toma a ars ca o torţă vie de-a lungul celor 80 de ani de viaţă. Asta este firea lui. A ştiut că obţinerea unui succes nu se face cu uşurinţă şi orice efort îşi are răsplata sa.

Acum când încerc să fac o mică evocare a ce s-a întâmplat, în anii care au trecut, simt că o mireasmă fină, călduţă, dulce, se topeşte în mine, după ani şi ani odată cu viziunea aceea din tinereţe înecată într-o albă linişte.

Deşi plecat de mic de acasă, cele mai dragi locuri i-au rămas cele natale, satul său, Gugeşti de Huşi. Aici l-am însoţit cu mulţi ani în urmă, când eram proaspăt conducător auto şi nu am reuşit să urc o pantă, spre sat, din prima încercare. Pe atunci profesorul Constantin Toma era director

Constantin Toma as a Director of the Botanical Garden. Finally, we arrived at his parents' house guarded in front of by some white cedar bushes. There, he takes off his coat but keep the tie on, and started to work hardly in the garden with hoe. I felt as he forgot about tired and all worries disappeared. He was glad as a stork which returns to its own nest in springtime. I tend to think that the smell of earth from where he was born was deep imprinted in his being and it hold fast on any other place where he traveled around the world.

His special city, Huşi remained inside of his soul, although he was a child of regiment, he had lots of troubles, emptiness and hungry. All these passions cannot be forgotten, but they are linked to the childhood memories and all of them remain in the chest of soul.

Once, here in Huşi, we scheduled some special practical applications with our students, as we use to do few years ago. Professor Constantin Toma, as a real host, invited us to visit the old wine cellars with. In the same place, we organized academic methodical meetings with biology teachers from all around the country. Here, the academic staff from our faculty presented new information, analyzed the content of textbooks and, and, I think that those meetings have been the most successfully in our domain. All those meetings were sponsored by Professor PhD Constantin Toma.

I remember about those methodical visits which we made together. Once, at a small school, the teacher was struggling to explain the water circuit in nature. And, as a rule, the more you want to be scientist, the more you confuse notions, so she did the same. Then, the Professor went to the blackboard, full of calm, take chalk and made a simple diagram with: producers - consumers - decomposers, their relationships, showing clearly and precisely that well-known principle: nothing is lost, everything is transformed.

The Professor wrote a lot and it is an excellent scientific work. Other time, at a meeting with pupils from "C. Negruzzi" College, a pupil came in and he took in hand the huge Plant Anatomy Handbook and he was very confused. He just could not believe that the man in front of him could achieve something so complex and large.

In Northern of The Bucovina District, during the similar activities, I felt that he steps into the mysterious kingdom of another world, with other habits and other concerns. I saw him there and in some other circumstances. I saw that his actions la Grădina Botanică. Ajunşi la casa părintească străjuită în faţă de tufe de tuia, şi-a dat hainele jos, iar cravata a lăsat-o la gât, s-a apucat la lucru în grădină, cu sapa. L-am simţit cum capătă puteri. Era bucuros ca barza ce se întoarce primăvara la cuib. Înclin să cred că acel miros al pământului unde s-a născut s-a imprimat adânc în fiinţa sa şi l-a păstrat pe orice meridian al lumii a călătorit.

Oraşul său drag Huşi i-a rămas în suflet, deşi aici a fost copil de trupă la regiment, a trăit multe necazuri, goliciuni şi flămânzire. Toate aceste patimi nu se pot uita, dar ele sunt legate de anii copilăriei şi rămân în scrinul cu amintiri.

Aici, la Huşi, noi am programat anume – aplicaţii practice cu studenţii, cum se făceau cu ani în urmă, iar profesorul Constantin Toma, fiind de-al casei, am avut acces în hrubele cu vin vechi. Tot aici, s-au organizat întâlniri metodice cu profesorii de biologie din ţară, iar cei veniţi de la universitate aduceau informaţii noi, discutau conţinutul manualelor şi mai cred că au fost cele mai reuşite acţiuni în specialitate. Toate întâlnirile erau patronate de profesorul Constantin Toma.

Îmi aduc aminte de inspecţiile de grad făcute împreună. La o şcoală, profesoara se chinuia să explice circuitul materiei în natură, şi ca regulă, cu cât vrei să fii mai savant, cu atât încurci lucrurile. Profesorul, calm, a ieşit la tablă, a făcut cu creta o schemă simplă cu: producători – consumatori – descompunători, relaţiile lor, ilustrând clar şi precis acel principiu cunoscut: nimic nu se pierde, totul se transformă.

Profesorul a scris mult şi bine. La o întâlnire cu elevii de la Liceul „C. Negruzzi” din Iaşi, un elev din clasa a V-a, a luat în mână voluminosul manual de Anatomia plantelor şi era extrem de nedumerit , nu-i venea să creadă că omul din faţa lui a putut realiza aşa ceva.

În nordul Bucovinei, la alte acţiuni asemănătoare, l-am simţit că păşeşte în domnia misterioasă a unei alte lumi, cu alte obiceiuri şi alte preocupări. L-am văzut de aproape acolo dar şi în alte împrejurări că este guvernat în actele sale de sacra lege a omeniei, care este în firea neamului acesta românesc. Omenia la el este o modalitate de viaţă firească. Nu a adunat averi şi cred că a ştiut că ceea ce contează nu e să te simţi bogat, ci să ai darul de a capta şi de a transmite mai departe o frumuseţe care vine din afară. Câtă frumuseţe am văzut la imaginile cu lumea orhideelor!

La dumnealui am văzut, pentru prima dată, fantastica trudenie a secţiunilor histologice şi

are governed by humanity laws which are something very common for the Romanian nation. Humanity is a natural part of his own life style. He has no treasures or fortunes, and I think he knew that what matters is not to feel rich, but to have the gift to capture and forward the beauty that comes from outside. How much beauty we could saw through those images with orchids world.

I saw at him, for the first time, a fantastic and huge work made for few histological sections and I understood that it is for man power to reveal many mysteries. Unfortunately, the mystery of life cannot be achieved. It is something divine there. In fact, if we knew everything that mean we would deprive the nature of its own charm.

The curiosity of his mind for the intimacy of fabulous structures I will compare it with that which man has, generally, for learn and explore, following the example of the first couple - Adam and Eve – from The Eden Garden – who walking naked, with no worries, they taste fruits of Knowledge Tree. After that, they opened their eyes and saw their bodies. The price was their immortality. The knowledge is infinite; human beings tends towards knowledge. Why we not reach the end of road? Because the breath is too short. However, we have to thank to Lord who gave us air to breath and clay to return to the stars!... From this man I learned collegial behavior, academic, respect for the effort of others. I learned a lot from him without giving me lessons.

For all those teaching-methodical papers or doctoral theses, he awarded full credit to the scientific coordinators and their teams. This complete confidence seems to be a real act of trust for all his colleagues. We all know him as a man with book, meticulous, scrupulous, maybe too formalist for some of us, a man of order and cleanliness. All these are evidenced from his personal clothing style to handwriting, from his calm walking on street to the manner of how to place a text on a white paper. The studies made by him are clearly, they do not need comments or explanations. The persons who knew him well, could appreciate sensibility, efforts honesty and vibration of his deep feelings.

I saw at this man a special care to talk about his teachers. Immortality of his teachers is much more real than books, cathedrals or sculptures. As long as we mention our teachers it means they did not succumb definitively. Professor PhD Constantin Toma was a real leader for the am înţeles că stă în puterea omului să dezvăluie multe mistere. Din păcate misterul vieţii nu poate fi atins. Este ceva de natură divină. De fapt, dacă am şti totul ar însemna să lipsim natura de farmecul ei.

Curiozitatea minţii sale de a intra în intimitatea fabuloaselor structuri a-şi asemui-o cu cea pe care o are omul, în general, de a fi dornic de a cunoaşte şi cerceta, urmând pilda primei perechi – Adam şi Eva – din Grădina Edenului – care umblând goi, fără nici o grijă au stăruit să guste din fructul cunoaşterii. Li s-au deschis ochii, au văzut cum sunt. Preţul a fost pierderea nemuririi. Cunoaşterea este infinită, omul tinde spre cunoaştere. De ce nu ajungem la capăt de drum? Pentru că respiraţia este prea scurtă. Totuşi, să mulţumim Domnului Ceresc, care ne-a dat aerul ca să respirăm şi lutul spre întoarcerea în stele! De la acest om am învăţat comportamentul colegial, academic, respectul pentru efortul celor din jur. De la dumnealui am învăţat multe fără să-mi dea lecţii.

La toate lucrările de grad sau de doctorat acorda credit deplin conducătorilor ştiinţifici. Această încredere deplină mi s-a părut un act de încredere faţă de colegii de alături. Îl ştim cu toţii ca pe un om cu carte, meticulos, scrupulos, pentru unii tipicar, un om al ordinii şi curăţeniei; pornind de la modul cum se îmbracă pâna la modul cum scrie pe foaia de hârtie. Analizele făcute de dumnealui sunt limpezi, nu au nevoie de comentarii. Cine l-a cunoscut bine nu poate să nu-l preţuiască pentru sensibilitatea, ostenelile, cinstea şi vibraţia trăirilor sale.

Am văzut la acest om grija deosebită de a vorbi de dascălii ce i-a avut. Nemurirea dascălilor este mai reală decât a cărţilor, catedraleor, sculpturilor. Atât timp cât ne pomenim dascălii înseamnă că ei n-au pierit definitiv. Profesorul Constantin Toma a condus destinele facultăţii în timpurile grele. După revoluţie a fost ţinta unor găşti care vroiau cu orice preţ să dea jos conducătorii. De ce? Nici ei nu ştiau. Aşa era valul. Au fost momente care le-a trăit cu stoicism şi e bine să se afunde tot mai mult în hăul uitării.

Pentru unele situaţii de viaţă cred că este omul ce ilustrează clar că: un destin se poate juca, decisiv şi ireparabil, între un „Da” nefericit sau un „Nu” oportun şi viceversa. L-am văzut vibrând cu mânie la nedreptăţi şi măsuri demente. Acest om care a descoperit frumosul în arhitectura histologică a plantelor a iubit şi iubeşte frumosul în general şi totdeauna a ştiut cum să vâslească încet şi calm spre un ostrov al păcii şi mulţumirii de sine. De fapt cu

Faculty of Biology during some hard times. After The Revolution it was a target by gangs who wanted to take down all leaders with all costs. Why? Even they did not know. It was the wave of history. There were times that we lived with stoicism and it is good to sink more and more into the abyss of oblivion.

For some life situations, I think that man illustrating that: a fate can play decisive and irreparable between an unhappy "yes" or an appropriate "not" and vice-versa. I saw him quivering with anger at injustice and crazy measures. This man discovered the beautiful architecture of plant pathology, he loved and loves beauty and always knew how to paddle slowly and calmly to an island of peace and self-gratification. In fact, we all love life because it's fragile and uncertain. We don’t have to despised voluptuousness of love, is only void beyond, before which any contrition is superfluous.

When some health problems tested his life, and they were enough, I watched him stepping hesitantly, burdened by grief, together with his sadness walking to an unknown destination. I tend to believe that it was an order from God that this man who was baptized with a name of a saint (Constantin), this son of a peasant, to became academician. No one helped him to be one. Huge quantity of knowledge was gained through efforts and with few books. By his own work and stringency, this man climbed, step by step, the ladder of scientific hierarchy and become a personality of our faculty, a real reason for our pride.

What did he done for himself and what did he done for other persons from around mean a lot to me and I want to give him all my appreciation and respect. I am proud that I have met a REAL MAN.

Now, let me be the echo of all honest feelings of your special guests. We want to thank you for this true life lesson that he has given us and wish him good health and many years.

Professor Iordache ION, PhD
University ”Alexandru Ioan Cuza” Iaşi,
Faculty of Biology
19th of November 2015
Iaşi - Gaudeamus

toţii iubim viaţa pentru că-i fragilă şi nesigură. Nu trebuie dispreţuită voluptatea iubirii căci dincolo este doar neantul, în faţa căruia orice căinţă este de prisos.

Când unele probleme de sănătate l-au pus la încercare şi au fost destule, l-am urmărit păşind şovăielnic, împovărat de mâhnire, cum îşi duce tristeţea către necunoscut. Înclin să cred că a fost o rânduială de la Dumnezeu că cel ce a primit botezul de Constantin Toma – din fiu de ţăran să ajungă academician. Nu l-a ajutat nimeni. Cultura acumulată a făcut-o cu efort şi cu puţinele materiale de care a dispus. Prin forţe proprii şi cu o îndârjire, cum poate rar se poate vedea la un om, a urcat treaptă cu treaptă pe scara ştiinţifică şi reprezintă astăzi mândria facultăţii noastre.

Ce a făcut singur şi cu cei apropiaţi îmi stârneşte admiraţia şi respectul, bucuria de a fi cunoscut un OM.

Să fie îngăduit a mă face ecoul tuturor sentimentelor celor de aici de care este firesc să fim cuprinşi, să-i mulţumim pentru lecţia de viaţă ce ne-a dat-o şi să-i urăm ani mulţi şi buni în sănătate.

Profesor Dr. Iordache ION,
Universitatea „Alexandru Ioan Cuza” Iaşi,
Facultatea de Biologie
19 Noiembrie 2015
Iaşi - Gaudeamus

Analele Ştiinţifice ale Universităţii „Alexandru Ioan Cuza”, Secţiunea Genetică şi Biologie Moleculară
TOM XVI, Fascicula 4, 2015

At the 80th ANIVERSARY OF THE ACADEMICIAN PROFESSOR PhD TOMA CONSTANTIN

Dear Mr. Academician Professor PhD TOMA CONSTANTIN,
Dear Mr. Rector,
Dear Mrs. Vice-Rectors,
Ladies and gentlemen and honoured guests

We all live today the sincere, friendly and collegial joy occasioned by the beautiful 80th anniversary of Mr. Academician Professor TOMA CONSTANTIN. Indeed, it is beautiful but also full of results, the honourable anniversary of our colleague, honourably both for us and especially for the institutions that he served and has collaborated with.

For all of us, nature bestowed us the destined talent. However, few knew how to revaluate the invaluable gift. This one has been realized at the highest degree by the Man, TOMA CONSTANTIN, in all stages of his life: student, university specialist with all its hierarchy, researcher, creator of school and trainer of destinies, organizer and leader-every time.

All those who have been close to him and have known him in all stages of his life and activities have practically exhausted all the superlatives of all honourable adjectives, even the associations between them in order to characterize Professor TOMA - and also to characterize his work as a total master.

Thus, it emerges the general consensus that his scientific, didactic work of absolutely impressive creator and organizer – but also the social achievements of the Academician Professor TOMA CONSTANTIN through a work beyond the ordinary limits, characterized by passion, perseverance, total dedication, thoroughness in all, in the laboratory and in the field, his amiability and dedication in the guidance of young people, made him the ideal collaborator and fore runner as a Professor and researcher, visionary and school trainer, ”Primus inter pares" or better said, Primus inter primi!

And the appreciations that come up down, the recognition “in his country, "as they say, has come close on the measure. Thus, since the last anniversary, the 75th, Professor TOMA CONSTANTIN was awarded the title of full Academician of the Romanian Academy and that of Honorary member of the Academy of Sciences of the Republic of

La a 80 a aniversare a Acad. Prof. Dr. Constantin Toma

Stimate Domnule Acad. Prof. Constantin Toma,
Stimate Domnule Rector,
Stimaţi Domnilor Prorectori,
Stimate doamne, Stimati Domni si invitati.

Trăim astăzi cu toţii bucuria sinceră, prietenească şi colegială prilejuită de frumoasa aniversare de 80 de ani a D-lui Acad. Prof. CONSTANTIN TOMA. În adevăr este frumoasă dar şi plină de roade, aniversarea onorantului nostru coleg. Onorant, atât pentru noi, cât mai ales pentru instituţiile pe care le-a slujit şi cu care a colaborat.

Tuturor, natura ne-a hărăzit talantul destinat. Puţini însă au ştiut să valorifice nepreţuitul dar. Acest lucru l-a realizat însă cu asupra de măsură, Omul CONSTANTIN TOMA, în toate ipostazele vieţii sale: student, cadru universitar cu toată ierarhia, cercetător, ctitor de şcoală şi formator de destine, organizator şi lider de fiecare dată.

Toţi acei care i-au fost aproape şi l-au cunoscut, în toate ipostazele vieţii şi activităţilor sale, au epuizat practic toate superlativele tuturor adjectivelor onorante, ba chiar şi asocierile dintre acestea, pentru a-l caracteriza pe Profesorul TOMA şi pentru a-i caracteriza opera de magistru total

Se desprinde astfel, părerea generală că opera absolut impresionantă, ştiinţifică, didactică de ctitor şi organizatorică dar şi socială, a realizat-o Academicianul Prof. CONSTANTIN TOMA, printr-o muncă peste limitele obişnuite, cu pasiune, perseverenţă, totală dăruire, meticulozitate în toate, în laborator şi teren, probitate şi sobrietate, omenie şi dăruire în pregătirea şi îndrumarea tinerilor, ideal colaborator şi înainte-mergător ca didact şi cercetător, vizionar ca formator de şcoală. „Primus inter pares” sau mai bine zis Primus inter primi! Iar aprecierile care vin de sus în jos, recunoaşterea „în ţara lui”, cum s-ar spune, a venit aproape pe măsură. Astfel, de la precedenta aniversare, aceea de a 75 - a, Profesorului C. TOMA i s-a acordat titlul de Academician plin de către Academia Română şi cel de Membru de onoare al Academiei de Ştiinţe a Rep. Moldova. Academicianul Prof. C. TOMA şi-a urmat drumul creaţiei cu aceeaşi forţă, cu acelaşi elan tineresc şi mobilizator, dovedindu-se încă o dată aserţiunea sau mai modest, zicerea ce îmi aparţine şi anume că, în domeniul muncii de creaţie nu trebuie să vorbim de senectute. În acest domeniu

Moldova. The Academician Professor TOMA CONSTANTIN has followed the road of creation with the same force, with the same youthful and mobilizer enthusiasm proving once again the assertion, or more modest the saying that belongs to me, namely, that in the field of work and creation we should not talk about old age. In this field of science there are not old people, but young ones of different ages. And so it is, if even now, with the impulse and youthful satisfaction that always characterized him, Professor TOMA CONSTANTIN together with his collaborators GRIGORE MARIUS and IVANESCU LACRAMIOARA published in the famous Publish House Springer, at Heidelberg, New-York, London, the valuable monograph, ”Halophites: an integrative anatomical study, 548 pages, a prestigious achievement that crowns the creation of the school of morphology and vegetal anatomy developed by Professor TOMA CONSTANTIN and enriches the world scientific heritage.

With the sincerest admiration, with the most sincere congratulations to all the authors and the most friendly wishes of health and new professional successes to the distinguished colleague and master, Academician and Professor DR. TOMA CONSTANTIN

Happy Years, beloved Professor and colleague!

ANDRIESCU IONEL, University Professor PhD emeritus,

Iasi, the 19th of November, 2015

nu sunt bătrâni ci tineri de diferite vârste! Şi aşa este dacă, tot acum, cu elanul şi satisfacţia tinerească ce l-a caracterizat totdeauna, Prof. C. Toma, împreună cu colaboratorii săi MARIUS GRIGORE şi LĂCRĂMIOARA IVĂNESCU, publică în celebra Editură Springer, Heidelberg, New-York, London, „Halophytes: an integrative anatomical study” 548 pp, o realizare de prestigiu care încununează creaţia şcolii de morfologie şi anatomie vegetală dezvoltată de Prof. CONSTANTIN TOMA, şi care îmbogăţeşte patrimoniul ştiinţific mondial.

Cea mai sinceră admiraţie, cele mai sincere felicitări pentru toţi autorii şi cele mai prieteneşti urări de sănătate şi noi succese profesionale, distinsului coleg şi magistru Acad. Prof. Dr. CONSTANTIN TOMA.

La Mulţi Ani! iubite profesor şi coleg

Prof. Dr. Ionel Andriescu 19.11.2015

INSTRUCTIONS FOR AUTHORS

SUBMISION OF THE PAPER(S)

The author(s) have to register using the on-line form available at **http://www.gbm.bio.uaic.ro/index.php/gbm/user/register**
and then submit the paper(s) using the ***On-line submission tool*** available in her/his/their account(s) at **http://www.gbm.bio.uaic.ro/index.php/gbm/login .**

ENGLISH **in the only language accepted for all manuscripts.**

The papers are no longer accepted via e-mail at **gbmpapers@yahoo.com .**

TECHNICAL INSTRUCTIONS

Please check the journal's web site (**http://www.gbm.bio.uaic.ro**) for the extended / updated version of the **Instructions for Authors** (**http://www.gbm.bio.uaic.ro/index.php/gbm/information/authors**) and / or send your inquiries to **gbmpapers@yahoo.com** to find latest news concerning the volumes.

Manuscripts are accepted all year round, but they will be included in one of the 4 volumes according to the accepting date pf the paper and with the publishing date for each volume.

Be aware that manuscripts may be editorially rejected, without review, on the basis of poor language, lack of conformity to the standards set forth in the instructions or inappropriate subject compared with the scientific field of the journal.

ORGANIZATION AND FORMAT OF THE MANUSCRIPT

Manuscripts should be submitted in Microsoft® Word© document format (.docx) - and we suggests using the latest version of the program - and should be written using Times New Roman typeface (font). For non-European languages please check the compatibility of the local characters subset with the European subset before submission. We also accept RTF file format. Do not send the paper(s) as PDF file format because it will be rejected.

The manuscript should contain an even number of pages. It is recommended as a general guideline a maximum of 8 pages for the original papers and 6 up to 10 pages for a review.

Paper format is ***Academic (if missing use Custom with the following dimensions 17 cm × 24 cm)***; mirror margins; up/down margins 2.5 cm; exterior margin 1.2 cm and interior margin 2 cm. No text is allowed in header/footer. ***The entire document should be in one section***.

Authors should avoid using excessively long sentences and are also encouraged to have instead shorter paragraphs, for easy reading.

The manuscript should contain the following sections:
Title / Authors / Keywords / Abstract / Introduction / Materials and Methods / Results and discussion / Conclusions / References / Author(s) affiliation / Acknowledgments.

In some cases, presentation will be clearer and more effective if the author combines some of these sections.

See on the publication website the details about how to format the text for each section of the paper.

The graphical elements in the paper (all figures, graphics and images) must be submitted also in their original form (for vector images use WMF or SVG and for bitmap images use TIFF or JPG). Recommended resolutions are as follows:
300 dpi for grayscale and color,
600 dpi for combination art (lettering and images),
1200 dpi for line art.

Include only the significant portion of an illustration.

All the required materials will be submitted together with the paper. We strongly suggest archiving the required files using 7Zip software before sending them to us.

Our journal is printed in **black and white** so all the color elements will be converted to **gray scale**. Have this in mind when choose the graphic elements!