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CONTENT

UNCLASSIFIED SEQUENCE VARIANTS (UVs) AND GENETIC PREDISPOSITION TO CANCER

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Keywords : *BRCA* genes, hereditary predisposition, breast/ovarian cancer, unclassified sequence variants, pathogenicity

Abstract : Hereditary breast and ovarian cancers are mainly attributable to predisposition genes whose germinal mutations are responsible for the disease. The most common genes associated with breast/ovarian cancer are *BRCA1* and *BRCA2* but at least 20 other genes of medium of high penetrance have been associated with these types of cancer. Lifetime risk of breast cancer for *BRCA* mutations carriers approaches 90%. Appropriate medical follow-up is therefore essential for women carrying mutations in these genes. *BRCA* mutational spectrum has not been entirely characterized but not all sequence variants are pathogenic. These are classified as benign polymorphisms or unclassified variants (UV) with unknown pathological potential. To date, 43,5% of over 3500 genetic variants *BRCA1* and *BRCA2* are reported as having uncertain clinical significance. Whether one sequence variant has or not a pathogenicity implication is often a hard decision to take, involving important consequences for diagnosis and medical follow-up. Here we present several cases of unclassified sequence variants detection and interpretation by *in-silico* analysis.

INTRODUCTION

Breast cancer is one of the most common diseases in Europe. It is estimated that the annual incidence in Romania is 14,000 [Ferlay et al., 2001]. Breast cancer can be divided into sporadic, familial and hereditary. In 30% of all cases there is a familial segregation while in at least 10% a clear Mendelian inheritance is usually shown in different genetic backgrounds/populations. In familial and hereditary breast cancer cases, other types of cancer may be present and there is a strong link with ovarian cancer. According to the literature [Easton et al., 2004; Ford et al, 1995; Antoniou et al, 2003], in cases where breast/ovarian cancer is present in a very young age (17-40yrs) or in cases where multiple breast and other types of cancer (prostate, colorectal, stomach etc) are present, a germ line mutation is most often the cause of the disease. Today, in most European countries genetic counselling is offered to most cancer patients (<60 years old) in order to define if there is a defect in a cancer predisposing gene (or cancer syndrome) underlying the presence of the disease. In cases of early onset breast cancer (<45yrs) or when an important family history is present, genetic testing is offered in order to determine the molecular basis of the disease. Appropriate medical follow-up, including early and more frequent mammography and pelvic examinations for the early detection of ovarian cancer, is therefore essential for women carrying mutations in these genes. At the moment, the emphasis is on early detection; preventive measures are mostly limited to prophylactic surgery, most notably annexectomy in post-reproductive women to reduce the risk of both ovarian and breast cancer.

The most common genes associated with breast/ovarian cancer are *BRCA1* and *BRCA2* but at least 20 other genes (*CHEK2, PALB2, ATM, PTEN, RAD51C, CDH1, STK11* and others) of medium of high penetrance have been associated with these types of cancer, genes that modify the genetic risk in carriers of mutations (modifier genes) and possibly other yet to be discovered genetic factors [McClellan et al., 2010; Walsh et al., 2007]. The contribution of these different genes in breast cancer in different populations remains unknown. Important steps have been made for familial/hereditary breast cancer after the initial identification of the major susceptibility genes (*BRCA1* in 1994 and *BRCA2* in 1995) [Narod et al., 2004]. During the last years, many contributions have been brought to knowledge of structure, functions and roles of the proteins coded by BRCA genes [Bertwistle et al., 2000 ; Eccles et al., 2006 ; Feunteun et al., 2001 ; Honrado et al., 2005 ; Mullan et al., 2006 ; Shivji et al., 2004 ; Sowter et al., 2005 ; Rosen et al., 2003]. Intense research showed that BRCA proteins were involved in crucially important cellular process as DNA repair, transcription and cell cycle regulation in response to DNA damage [Yoshida et al., 2004].

BRCA mutational spectrum has not been entirely characterized. Over one thousand small sequence variations have been reported in the Breast Cancer Information Core database [BIC]. More than half of these mutations (over 300 in BRCA1 and 200 in BRCA2) cause the lost of function by premature protein synthesis termination [46], and around 60% are unique to a family [BIC]. Other variations include mis-sense alterations and intronic variants with unknown disease relevance. These are classified as benign polymorphisms or unclassified variants (UV) with unknown pathological potential. To date, 43,5% of over 3500 genetic variants BRCA1 and BRCA2 are reported as having uncertain clinical significance [BIC].

The distribution of BRCA mutations and other sequence variants has been studied in many populations, though recently in Romania. The overall data obtained by now from 19 HBOC families allowed in 2010 the first description of

genetic factors in Romanian population [L. Negura et al., 2010a]. We observed a variety of *BRCA* mutations, which may place the North-Eastern Romanian population somewhere between western populations (few recurrent, many rare or unique mutations) and eastern ones (recurrent mutations responsible for the majority of predisposed families, very few novel or unique mutations). The overall mutation frequency was 41%. Meantime, complete BRCA1 and BRCA2 sequencing permitted the description of several unclassified sequence variants, with uncertain pathogenicity, as well as common SNPs which defined local BRCA1 haplotypes [L. Negura et al., 2010b; L. Negura et al., 2010c].

Whether one sequence variant has or not a pathogenicity implication, is often a hard decision to take, involving important consequences for diagnosis and medical follow-up. Variants with unknown clinical significance (unclassified variants) always need further examination to define their possible pathogenic role Here we present several cases of unclassified sequence variants detection and interpretation by *in-silico* analysis.

PATIENTS AND METHODS

The main recruitment criterion for HBOC families was three or more breast or ovarian cancer cases within the same family line. Situations with less then three cases were considered for cancer cases were diagnosed before age 40, for breast and ovarian cases in the same family, for breast cancers in men, for bilateral breast cancer cases and for any medullar breast cancer. We also compared these criteria with INSERM family scores [Eisinger et al., 2004]. All patients agreed by written informed consent. We analyzed 26 patients from 17 unrelated HBOC families..

Genomic DNA was extracted from 10 ml peripheral blood by optimization of the Wizard® Genomic DNA purification kit (Promega Inc, Madison, WI, USA). DNA amount was estimated by spectrophotometry. Multiplex-PCR, allele-specific PCR and PCR-RFLP were performed for detection of known *BRCA1* mutations, as shown elsewhere [Negura et al., 2010b; Negura et al., 2009a; Negura et al., 2008]. *BRCA1* was also screened for large deletions and duplications by MLPA [Negura et al, 2009b].

The entire coding sequence of both genes, including exon/intron boundaries, was analysed using amplification and Sanger sequencing. Polymerase chain reaction amplifying BRCA exons were performed in a final volume of 20 µl containing 0.4mM each dNTP, 0.8 µM of each primer (sequence available on demand), 100 ng genomic DNA, and one unit of either ApliTaq® or AmpliTaq®Gold Polymerase with appropriate 1X Buffer (Applied Biosystems Inc, Foster City, CA, USA). PCR cycling comprised an initial denaturation step at 94ºC for 5 min followed by 30 cycles of 94ºC for 20 sec, 54ºC for 20 sec and 72ºC for 30 sec, and a final extension of 7 min at 72ºC. Amplicons were verified by electrophoresis on a 1,3% agarose gel, then purified by ExoSap® enzymatic digestion (Affymetrix Inc, USA), following producer's instructions. The product was sequenced in forward and reverse reactions, using the BidDye® Terminator Cycle Sequencing Kit (Applied Biosystems™), according to the manufacturer's instructions. Cycle sequencing consisted of an initial denaturation step at 94ºC for 11 min, followed by 30 cycles of 94ºC for 10 sec, 52ºC for 5 sec and 30ºC for 3 min. Sequence analysis was performed using the Seqman (DNA Star Inc, Madison, WI, USA) and the CEQ8000 Investigator (Beckman Coulter™) softwares.

Mutation presence was systematically confirmed by forward and reverse sequencing on a second independent blood sample. All mutations and sequence variants are described according to HUGO approved systematic nomenclature [HGVS]. The nomenclature for BIC traditional mutations is also indicated. In-silico analysis, including Grantham scores, was performed using Alamut® (Interactive Biosoftware™), as well as freely available softwares as ESEfinder, GVGD alignment, SIFT (*Sorting Intolerant From Tolerant*) or PolyPhen (*Poly*morphism *Phen*otyping). BIC and NCBI (Entrez SNP) databases were used. NCBI reference sequences were U14680 and NP_009227.1 for *BRCA1*, respectively NM_000059.3 and NP_000050.2 for *BRCA2*.

RESULTS AND DISCUSSION

When completely sequencing *BRCA1* and *BRCA2* genes, beside deleterious mutations and known benign polymorphisms, a distinct sequence category can be identified, This include heterozygous nucleotide substitutions, very rare or unique within the population, with predictable effects on proteins, but with much less obvious involvement in hereditary predisposition to cancer. The incertitude of direct correlations with protein functions make us call these sequences variants with unknown clinical significance or "Unclassified variants" (UVs). In international databases, UVs are localized somewhere between deleterious mutations and benign SNPs, waiting for further research or bioinformatics analysis to confer them rather a pathogenic or SNP status. It is a very rude task to assign a consequence and to interpret identified UVs, and the

majority of explications often have just a speculative level. However, some clues could possibly allow an assignment within categories like “rather dangerous UVs” or “rather neutral UVs”.

Table I. Problematic sequence variants identified in BRCA genes

FAMILY	GENE	exon	BIC Nomenclature	HGVS nomenclature	Efect	BIC ?	TYPE	CLASS	AA conservation	grantham	domain	splice	ESE finder	GVGD alignment	SIFT	polyphen	NCBI
00101AN	B1	15	4763G>A	**c.4644G>A**	**p.Thr1548Thr**	no	silent	P		n/a		NO effect	no effect				
01601VH	B1	7	546G>C	**c.427G>C**	**p.Glu143Gln**	no	missens	UV	9 of 11 are E	29	brca1	NO effect	no effect	C0	tolerated	1.35 benign	
00701AC	B2	13	4486G>T	**c.4268G>T**	**p.Asp1420Tyr**	yes	missens	P	10 of 11 are E or D	160		NO effect	loss of 1	C15	not tolerated	2.17 probably	rs28897727
01301DT	B2	1109	4817A>G	**c.4589A>G**	**p.Lys1530Arg**	no	missens	UV	100%	26	brca2	NO effect	no effect		not tolerated	1.25 probably	
01902LM	B1	16	5075G>A	**c.4956G>A**	**p.Met1652Ile**	yes	missens	UV	100% hydrophobic	10	brct	NO effect	adds 1	C0	tolerated	1.11 benign	rs1799967

We identified five sequence variants other than mutations or common SNPs [Negura et al., 2010(a)]. In table I one can identify 3 variants for *BRCA1* and 2 for *BRCA2* gene. Two of these variants are already known and integrated in BIC database [BIC], while three others seem to be novel. All variants are mononucleotide substitutions. One of them has no consequence on amino acid sequence in the protein, due to the genetic code degenerescence, so it can be considered as silent. The other 3 variants generate amino acid substitutions, so we have to take in account Grantham substitution score and the amino acid position within the protein (conserved/not). We present below the 5 variants and their interpretation.

UV-1 (c.427G>C) – BRCA1

In figure 1 is presented (up), at a sequence level, the first sequence variant that we will call UV-1, in comparison with a wild-type sequence (down). The patient bearing this BRCA1 UV is also carrying the *BRCA2* c.8249_8251delAGA mutation within exon 18. We can see UV-1 is a heterozygous substitution of a G, within *BRCA1* exon 7, with a C nucleotide on modified allele.

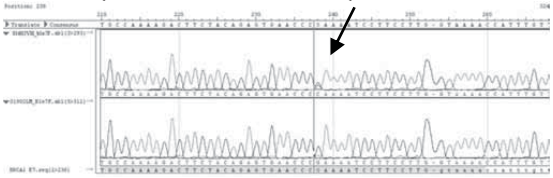
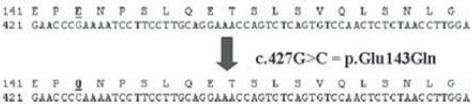


Figure 1. Identification of UV-1 (c.427G>C) in *BRCA1*-exon7

The substitued nucleotide has the position 427 in the coding *BRCA1* sequence (ref. U14680), so the variant will be called c.427G>C in HUGO nomenclature and 546G>C in BIC nomenclature. Affecting the first nucleotide of the codon 143 (GAA – glutamic acid), the substitution generates a CAA codon 143, coding for glutamine, so we will have a mismatch substitution causing p.143E>Q, as we can see below.



The Alamut software is considering that no splicing or ESE (*exonic splicing enhancer*) site is affected and is estimating that E143Q is rather a tolerated substitution (confirmed by SIFT). Polyphen is according a rather benign score of 1,35, while Grantham score for Glu>Gln is very low, 29. However, there is a 82% conservation of the Glu143 when comparing several species, and we should take this in account when assigning UV-1 as not a silent variant, even if tolerated. Still, since *BRCA1* c.427G>C occurred in a family with a deleterious *BRCA2* mutation, we believe it is unlikely to cause disease.

UV-2 (c.4644G>A) – BRCA1

Figure 2 presents, in forward and reverse sequencing, a sequence variant identified in two patients from the same HBOC family. Both are carrier of the deleterious *BRCA1* c.342_344delTC mutation. This UV-2 variants consists in a mismatch substitution of a G, within exon 15 of *BRCA1* gene, with an A on the modified allele, which appears heterozygous G/A for this site in the image below.

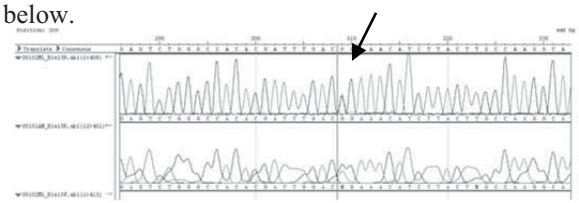


Figure 2. Identification of UV-2 (c.4644G>A) in *BRCA1*-exon15

The affected nucleotide is the 4644 in the coding *BRCA1* sequence (ref. U14680), so the variant will be called c.4644G>A in HUGO nomenclature and 4763G>A in BIC nomenclature. For the protein level, this nucleotide is the third of a ACG codon, coding for threonine 1584. The A for G substitution will have no consequence, since all codons starting with AC are coding for threonine. As we can see in the image below, the variation is translating in the protein as Thr1548Thr, so the UV-2 variant (c.4644G>A), although unidentified before, can clearly be considered as silent. Alamut software proved that neither splicing, nore ESE sites were affected by this sequence variation.

UV-3 (c.4956G>A) – BRCA1

A third sequence variation on *BRCA1* was observed within exon 16. As one can observe in figure 3, heterozygous substitution of a G nucleotide with an A appears in the upper sequence, in one patient's DNA (up), while the mother is wild-type (down)

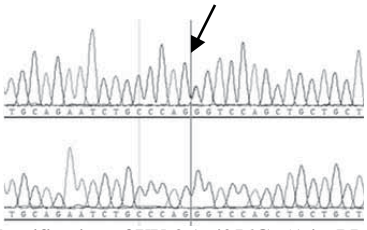
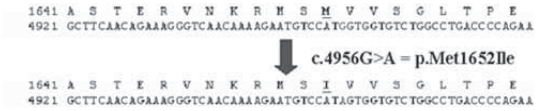


Figure 3. Identification of UV-3 (c.4956G>A) in *BRCA1*-exon16

The substituted G is situated in the position 4956 in coding *BRCA1* sequence (ref. U14680), so the variant will be called c. 4956G>A in HUGO nomenclature and 5057G>A in BIC nomenclature. It is the last nucleotide of the 1652 codon (ATG – coding for Methionine). Its replacement with an A will generate an ATA 1625 codon, coding for Isoleucine, so UV-3 is a mis-sense genetic variation with a p.Met1652Ile consequence, as we can see below.



This variant is already known in BIC database, where numbers of cases have been reported. The amino-acid in the 1625 position has a 100% hydrophobic conservation, which may be tolerated (Alamut-SIFT) as both Met and Ile are hydrophobic. Since the 10 Grantham score is one of the lowest possible in substitutions, and Polyphen is also calculating a 1,11 very low score, the benign level of the substitution seems quite certain. No effect is either estimated on splicing sites, although an additional ESE (*exonic splicing enhancer*) is estimated to appear within exon 16. Still, there is not enough argument to consider Met1652Ile a UV. Moreover, there is a SNP code for this variant in NCBI EntrezSNP database (rs1799967) and it happened to be used in H5 haplotype assignment in Judkins haplotype characterization [Judkins et al., 2005]. Overall, UV-3 is not a UV at all, it is just a simple benign common polymorphism.

UV-4 (c.4258G>T) – BRCA2

The first sequence variant identified in BRCA2 gene is a T for G substitution localized within exon 11. The patient bearing this UV-4 is carrying the BRCA1 deleterious c.5266dupC mutation (same as the recurrent 5382 eastern founder mutation). In figure 4, the heterozygous variation (G and T) can be observed in the upper sequence, comparing with the downer wild-type patient (G only).

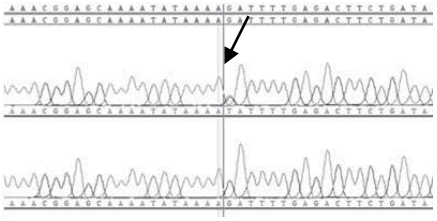
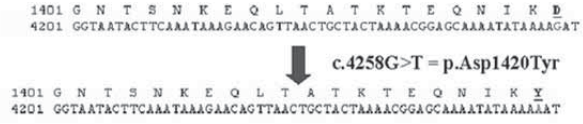


Figure 4. Identification of UV-4 (c.4258G>T) in *BRCA1*-exon11

In HUGO nomenclature, the variation is called c.4258G>T, while it is 4486G>T in the BIC nomenclature (ref. NM_000059.3). The affected nucleotide is the first G of the 1420 GAT codon coding for aspartic acid. Its substitution with a T brings a TAT Tyrosine coding 1420 codon, so the consequence will be p.Asp1420Tyr, as we can see below.



There is a lot to say about our UV-4, even if it already exists in BIC database and also in NCBI EntrezSNP as the rs28897727. Alamut software estimates a 90% conservation of the 1420 amino acid either as a aspartic (D) or a glutamic (E) acid form. SIFT doesn’t tolerate its substitution with a tyrosine, and the Grantham score of such substitution is huge (160). Polyphen is also estimating for Asp1420Tyr a probably damaging 2,17 score. An ESE site is generated

within exon 11 by the same substitution, even if splicing sites are not affected. GVGD gives a big C15 score of untolerance. All this should make c.4258G>T a damaging pathogenic substitution, but it is considered polymorphic by BIC, as common is certain populations and not affecting any known protein domain (why being so conserved then?). SNP frequency (0.26 calculated on 1000 Americans with European origin) makes homozygous context possible, although no such situation has ever been reported (here again, why?).

In our patient, this UV-4 has a special status, as it is making a genetic difference between two HBOC families bearing the same recurrent *BRCA1* 5382insC mutation [Negura et al., 2010a, Negura et al., 2010b]. Interestingly, the UV-4 bearing family shows an ovarian cancer history, whilst the non-bearing is rather a breast cancer family; this could open a discussion about wether *BRCA2* c.4258G>T could modifying the cancer risk and could influence cancer phenotype in *BRCA1* mutation carriers, even if not pathogenic by itself. The situation is far of being definitive and we believe *BRCA2* c.4258G>T is the typical situation of UV pending for a decision, with arguments for both being pathogenic or simply polymorphic.

UV-5 (c.4589A>G) – BRCA2

The last sequence variant we will discuss here is a G for A substitution within again BRCA2 exon 11. In figure 5, this variation clearly appears heterozygous in a breast cancer patient, with familial history and not carrying a BRCA mutation.

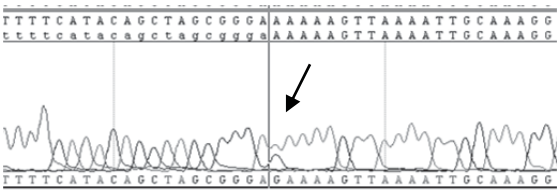
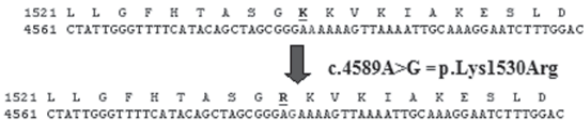


Figure 5. Identification of UV-5 (c.4589A>G) in *BRCA1*-exon11

As the substituted nucleotide is the 4589 within the coding sequence of BRCA2 gene (ref. NM_000059.3), the variation is called c.4589A>G in HUGO nomenclature and 4817A>Gin BIC nomenclature. It is the second nucleotide of a series of 6 adenines, ant it occupies the second position in a lysine coding AAA codon; when modified, the codon becomes AGA, coding for 1530 arginine, so the effect on the protein is p.Lys1530Arg, as shown below.



The conservation for the amino acid 1530 is estimated by Alamut at 100%, while SIFT does not tolerate substituting the concerning lysine with any other amino acid, although the low 26 Grantham score would allow it. No effect is estimated on splice sites or on ESE, and Polyphen is giving a low 1,25 score considering the substitution rather benign. Our UV-5 substitution was never identified before and doesn't exist in BIC database [Negura et al., 2010a]. The 100% conservation of the lysine and the lack of tolerance estimated for any substitution in this position make us consider c.4589A>G having an important pathogenic potential. In fact, is the most

probably pathogenic of all 5 UVs described here. Further investigations are necessary to determine whether this UV is responsible for the disease in the carrier patient.

CONCLUSIONS

BRCA sequence variants of unclear clinical significance (Table I) do not bring directly useful information. From five UVs described here, only one (*BRCA2* c.4589A>G) has a clear pathogenic potential, due to 100% conservation of the amino acid substituted. Two other UVs, *BRCA1* c.427G>C and less probably *BRCA1* c.4956G>A, need additional data to confirm a pathogenic potential. Two variants, *BRCA1* c.4644G>A and *BRCA2* c.4258G>T, are clearly defined as benign, either because being silent or quite common in the population. Three of four such UVs were found in patients also carrying a deleterious mutation, though this situation isn't much relevant since mutation and UV affect different genes in two of those cases. More interesting is rather the presence of the *BRCA2* c.4258G>T unclear UV in one patient harbouring the BRCA1 recurrent c.5266dupC, but not in other carrier patients from unrelated family. Still, its clinical involvement is not proven and whether c.4258G>T could be responsible for the difference of phenotypes between c.5266dupC carriers remains speculative.

Investigations continue in order to establish more clear involvement of those sequence variants into pathology.

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TRIPLE TEST ROLE IN IDENTIFYING CHROMOSOMAL DISORDERS IN THE SECOND TRIMESTER OF PREGNANCY

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Keywords: prenatal screening, biochemical markers, alpha-fetoprotein (AFP), human chorionic gonadotropin (HCG), unconjugated estriol (UE_3), trisomy, Down syndrome.

Abstract: The triple test plays a very important role in identifying chromosomal disorders, in the prenatal screening of the second pregnancy trimester. The scope of our research resides in investigating the level of human chorionic gonadotropin, alpha-fetoprotein and unconjugated estriol (markers that make-up the triple test), in the serum sampled and analysed from a group of 135 pregnant women. The observation of the above mentioned markers is made in order to identify the pregnancies that present a higher risk for the appearance of chromosomal disorders. We also, decided to associate the values gathered for human chorionic gonadotropin, alpha-fetoprotein and unconjugated estriol, with the maternal age. The interpretation of the data was made using the PRISCA 4.0 software, considering by default the gestational age, smoking, *in vitro* fertilization, diabetic status, medical history of the mother. We must say that the patients were pregnant in the second trimester, period specific for triple test survey and are not the same patients included in the double test survey.

Following the conducted biochemical analyses normal values were obtained, values that fit the ranges specified in the specific literature, but also values that were outside the normal ranges, indentifying in this way pregnancies with high risk for 21 and 18 trisomy.

INTRODUCTION

Finding some serum biochemical markers that can be determined in the second trimester of pregnancy and that are useful in the estimation of the risk for chromosomal aberrations of the fetus led to the development of the survey named triple test (Bogart et al., 1987; Cuckle et al., 1994).

The triple test includes determination of the serum markers: alpha-fetoprotein, human chorionic gonadotropin and unconjugated estriol.

Alpha-fetoprotein (AFP) is a plasma fetal protein dominant during the pregnancy. AFP is a glycoprotein with a unique polypeptidic chain, with a molecular weight of 65000-70000 daltons. From the whole molecule, the protein component represents 96%, while the carbohydrate one represents 4%. In the human embryo AFP synthesis takes place in the yolk sac, liver and gastro-intestinal tract. From the synthesis places, AFP in secreted in the fetal plasma, where in can be identified beginning with the 6^{th} week of pregnancy.

AFP concentration during the pregnancy grows progressively. AFP levels are higher in the pregnancies associated with open neural tube defects and in average lower in the presence of the Down syndrome and 18 trisomy (Cuckle, 2000 ; Heyl, 1990).

Human chorionic gonadotropin (HCG) is a complex sialoglycoprotein. This glycoprotein-hormone is produced during pregnancy. Initially, HCG is secreted by the trophoblastic cells in the blastocyst development, afterwards by the scitiotrophoblastic cells of the placenta. Its serum levels present the next aspect: rises from the moment of the fecundated ovule implantation, peaks in the 8-12 weeks interval, decreases gradually till in the 18-20 weeks interval, after which it stays in a plateau till delivery. HCG concentration is higher in the pregnancies associated with Down syndrome and lower in the presence of 18 trisomy (Laborator Synevo, 2006 ; Veduta et al, 2007).

Unconjugated estriol constitutes an important biochemical marker, in a prenatal screening for Down syndrome. Unconjugated estriol is produced by the feto-placentary unit; its level in the maternal serum rises progressively during pregnancy and is typically low in pregnancies associated with Down syndrome and 18 trisomy (Laborator Synevo, 2006).

The scope of the present study resides in investigating the levels of the markers that constitute the triple test (AFP, HCG, UE_3), in order to indentify pregnancies with high risk for development of chromosomal disorders.

MATERIALS AND METHODS

Researches were conducted on biological probes sampled from 135 women, pregnant in the second trimester (15-22 weeks), period optimal for the triple test investigation. Ultrasound and sampling of the biological probes were conducted on the same day (the pregnancy age being the same both during the ultrasound and during the biochemical

investigations). All of the biological probes of all the patients, with a pregnancy age of 15 to 22 weeks, were accepted, regardless of the fertilisation type (natural or *in vitro*), type of pregnancy (monofetal or twins), or of different fetal disorders found with the ultrasound. The probes that were unsuited for the determination of the biochemical markers necessary for the triple test were rejected, namely hemolysed and lipemic. Tests were conducted on the automate analyser Immulite 1000, of the medical analyses laboratory S.C. MEDICALTEST S.R.L. Bacău, Iaşi branch and interpreted through the means of the PRISCA 4.0 software, software known for his utility in the calculation of the multiple of median (MoM) corrected for the variable factors such as: gestational age, weight of the mother, race, smoking or not, diabetic status, monofetal or twin pregnancy, procedures for the *in vitro* fertilization. Once the MoM is calculated and corrected, the similarity ration is calculated for each of these values and the combination of all of the similarity rations with the risk presented by the maternal age (a priority risk) leads to the final risk (Muller et al., 1999).

Results of the biochemical tests are expressed in IU/mL for AFP, in MIU/mL for HCG and in ng/mL for unconjugated estriol. Statistical interpretation of the gathered values was made through the Student test (Văleanu, Hîncu,1990), revealing the values obtained from pregnant women from the second age group (26-29 years), third age group (30-35 years), respectively the fourth group (>35 years), dependent of the values obtained from the first age group.

RESULTS AND DISCUSSIONS

The 135 pregnant women included in the present study were divided in four groups depending of the maternal age: a first age group comprised the pregnant women with the ages between 21-25 years (n=18), a second age group comprised of pregnant women with ages between 26-29 years (n=53), a third age group comprised of pregnant women with ages between 30-35 years (n=50) and a fourth age group comprised of pregnant women with ages over 35 years (n=14).

In table 1 the results obtained after determination of the values for the three markers, AFG, HCG and UE_3, in the pregnant women serum are presented, the women being separated in the four age groups.

For AFP the median value calculated for the pregnant women from the first age group is 38.900 IU/mL, for those in the second age group is 34.881 IU/mL, for those in the third age group is 30.726 IU/mL, and for those in the fourth age group is 26.823 IU/mL (table 1).

A study conducted on pregnant women in the second pregnancy trimester, depending of the gestational age, presented values in the interval 10-300 IU/mL and different median values depending the gestational age as follows: at a number of 605 pregnant women, with a gestational age of 16 weeks, a median value of 28.5 IU/mL was calculated; at a number of 569 pregnant women, with a gestational age of 17 weeks, a median value of 32.6 IU/mL was calculated; at a number of 431 pregnant women, with a gestational age of 18 weeks, a median value of 37.3 IU/ml was calculated. These probes were all processed on Imulite 1000 the same type of analyser used in our present study (Siemens Medical Solution Diagnostics, Haddow *et al*, 1992).

Table 1. Median values calculated for AFP, HCG and UE_3 in pregnant women from the four age groups**.**

	AFP (IU/mL)		HCG(MIU/mL)		UE_3 (ng/mL)	
n=18 (21-25 years)	Median (M)	38.900	Median (M)	26541.667	Median (M)	3.352
	Standard Error (Es)	0.76461	Standard Error (Es)	940.05238	Standard Error (Es))	0.08352
	t	-	t	-	t	-
	p	-	p	-	p	-
n=53 (26-29 years)	Median (M)	34.881	Median (M)	28798.320	Median (M)	2.961
	Standard Error (Es)	0.23738	Standard Error (Es)	452.37045	Standard Error (Es)	0.03086
	t_1	5.01975	t_1	2.163	t_1	4.39261
	p_1	<0.001	p_1	0.05>p>0.01	p_1	<0.001

n=50 (30-35 years)	Median (M)	30.726	Median (M)	28505.890	Median (M)	2.534
	Standard Error (Es)	0.22850	Standard Error (Es)	349.51350	Standard Error (Es)	0.02907
	t_2	10.24281	t_2	1.95849	t_2	9.25408
	t_3	12.61092	t_3	0.51156	t_3	10.078
	p_2	<0.001	p_2	>0.05	p_2	<0.001
	p_3	<0.001	p_3	>0.05	p_3	<0.001
n=14 (>35 years)	Median (M)	26.823	Median (M)	32864.571	Median (M)	1.827
	Standard Error (Es)	0.76818	Standard Error (Es)	1601.97258	Standard Error (Es)	0.353
	t_4	9.33841	t_4	3.40413	t_4	12.10415
	t_5	7.59005	t_5	2.44275	t_5	11.42419
	t_6	2.42990	t_6	2.65829	t_6	7.15953
	p_4	<0.001	p_4	<0.001	p_4	p<0.001
	p_5	<0.001	p_5	0.05>p>0.01	p_5	p<0.001
	p_6	0.05>p>0.01	p_6	0.05>p>0.01	p_6	p<0.001
N=135						

The median values that we calculated for the pregnant women in the four age groups are close to those from the speciality literature, with little differences that can be explained through the fact that we did not analysed the pregnant women strictly after the same gestational age (for example: only for 16 weeks pregnant women, separated from 17 weeks pregnant women), but rather for the second trimester pregnancies depending on the maternal age. Considering the first group of pregnant women as our control group, for the patients in the second, third and fourth age groups the results are very significant, as follows: for the patients in the second age group $p<0.001$ (89.6%), for those in the third age group $p<0.001$ (78.96%) and as expected for those in the fourth age group the results were very significant (68.95%), as you can observe from the table 1 and figure 1.

In the case of the second marker analysed, from the component of the triple test and namely HCG, the following results were obtained: for pregnant women in the first age group a median value of 26541.667 MIU/mL was calculated, for those in the second age group a median value of 28798.32075 MIU/mL was calculated, for those in the third age group a median value of 28505.890 MIU/mL was calculated and for those in the fourth age group a median value of 32864.571 MIU/mL was calculated (table 1).

The reference values found in the speciality literature are comprised between 6140 and 103000 MIU/mL. The studies conducted by Siemens on a group of 593 pregnant women, of which 72 were in the second pregnancy trimester, reported on the 72 pregnancies a median value of 40989, specifying that each lab must establish its own reference values, their values serving only as guide (Siemens Medical Solution Diagnostics ; Haddow *et al,* 1992).

The data we gathered fit in the reference interval of 6140-103000 MIU/mL and are close to the literature median values. The differences between the median values calculated by us and those related by Siemens are explained through the fact that the interval we obtained for the pregnant women in each age group is much smaller then the one in the speciality literature, but the total number of analysed pregnant women is much bigger. In the pregnancies associated with the Down syndrome, the HCG levels are ≥ 1.97 MOM, and in those associated with 18 trisomy have a MoM value for HCG ≤ 0.55.

The MoM calculation for each marker consists in dividing the obtained value to the median correspondent to the gestational age. MoM correction is made by comparison of the obtained values for the specific patient with the general median value calculated for a population of pregnant women, these having normal pregnancies. In the present case, the highest values

were obtained for the pregnant women form the fourth age group (>35 years), recording a 123.82% increase from the results obtained for the pregnant women from the first age group, control group (21-25 years), the modifications being significant $0.005>p>0.001$(table 1 and figure 2). The results obtained for the pregnant women from the second and third age groups were slightly significant ($0.05>p>0.001$) and respectively unsignificant from a statistical point of view, $p>0.05$ (108.5%, respectively 107.4%).

For the third analysed marker, from the component of the triple test and namely free or unconjugated estriol(UE_3) the following median values were obtained: for the pregnant women from the first age group a median value of 3.352 ng/mL was calculated, for the pregnant women in the second age group a median value of 2.961 ng/mL was calculated, for the pregnant women in the third age group a median value of 2.534 ng/mL was calculated and for the pregnant women in the fourth age group a median value of 1.827 ng/mL was calculated (table 1).

The reference interval found in the speciality literature, for the second trimester of pregnancy in 0.46-7.41 ng/mL, according to Siemens studies, encountered median values being different for each laboratory. Siemens studies on 268 pregnant women, but in the third pregnancy trimester (after 23 weeks of pregnancy), revealed a reference interval comprised between 2.9 and >30 ng/mL with a median value from 6.5 to 23 (Siemens Medical Solution Diagnostics ; Haddow *et al,* 1992).

The data we obtained fit in the reference interval of 0.49 – 7.41 ng/mL and are closed to those found in the speciality literature. The differences between the median values we obtained and those related by Siemens are explained through the fact that the interval we calculated for pregnant women from each age group is much smaller than the one reported in the literature and mainly through the fact that Siemens conducted the survey on greater gestational ages, more than 23 weeks of pregnancy, where it is normal for the unconjugated estriol values to be higher.

As we mentioned earlier, unconjugated estriol is typically low in high risk of trisomy associated pregnancies. In this sense, we observed that the lowest values were obtained in pregnant women from the last age group (>35 years), recording a fall of 54.50% as to the results obtained for the pregnant women from the first age group, control group (21-25 years), modifications being significant $p<0.001$ (table 1 and figure 3). The results obtained for the pregnant women from the third and second age group are also significant from the statistical point of view ($p<0.001$), observing a diminishing of the values regarding the maternal age and namely: for the pregnant women from the second age group (26-29 years) a drop of 88.33% as to the first age group was recorded, and for the third age group (30-35 years) a 75.59% drop was recorded.

The final result of the triple test analysis, that represents the risk (high or low) for a trisomy appearance, is expressed regarding the MoM. The degree of risk for each pregnant woman is based on combining the obtained result with the maternal age by a complex mathematic algorithm using a software such as PRISCA 4.0, which is the one we used in our study.

The tests are interpreted as being with high or low risk, depending of the cut-off vale set for each trisomy. In the case of the 21 trisomy the cut-off value is 1/250, and in the case of the 18 trisomy the cut-off value is 1/100.

After the determination of the immunological markers, the obtained values were statistically worked by using PRISCA software edition 4.0 of DIAGNOSTIC PRODUCTS CORPORATION, USA. The PRISCA software 4.0 is an application that provides a statistical value to the risk for the Down syndrome (21 trisomy) and for the Edwards syndrome (18

trisomy), in the first and second pregnancy trimester and for the neural tubes defects in the second pregnancy trimester. The risk calculated through PRISCA, for a pregnant woman, is not a test for confirmation for chromosomal abnormalities, but has the scope, in the *in vitro* diagnosis, to be used as an additional support, in her decision to undertake or not the diagnosis procedures.

The biochemical risk for Down syndrome at birth is calculated based on corrected MoM for each of the three markers and maternal age at birth. The risk for the 18 trisomy at birth is calculated based on the corrected MoM for each of the three markers and maternal age. (Muller et al.,1999). PRISCA 4.0 compares the result obtained with the median specific for the gestational age in order to express the result as MoM, for each of the parameters: AFP, HCG, UE_3, during the second trimester of pregnancy.

Speciality literature offers data both on the triple test as an analysis in its self, but also on each marker individually. Hence Akalin et al. (2007), researching the biochemical screening in the second trimester of pregnancy, on 700 pregnant women, excluding the ones with twin pregnancies, using Immulite One analyser, after AFP analysis, gained a median equal with 32.5 and values comprised in the 14.5-95 IU/mL interval. Analysing HCG he gained a value equal to 20961 and values comprised in the 2260-60775 MIU/mL interval. For UE_3 he gained a value equal to 2.70 and values comprised in the 0.77-9.10 ng/mL interval. Cumulating the obtained data, he concluded that significant differences were given by the median value of alpha-fetoprotein ($p<0.001$) in the pregnancy period of 16-19 weeks. Following the analysis of these values we can see that they are similar if not even close to those obtained in our study (table 1).In the studies conducted by Johnson et al. (1984), the necessity of the maternal weight and race, in the utilisation of MoM, is related. Also, Reynolds et al. (2006), unveils the significant differences ($p<0.001$) between corrected MoM values depending on the maternal weight. Wald et al (2006) showed in its studies, the importance of the MoM calculus for the values obtained, resulting in a better screening and thus reducing the appearance rate of false positive results.

Analysing these information we can say that our results are in concordance with the ones in the speciality literature, which show that the patients with ages between 18 and 35 years, regarding the gestational age, smoking, *in vitro* fertilisation, diabetic status, medical history of the pregnant woman, have a lower rate of appearance of high risk pregnancy as do the ones with a maternal age of over 35 years.

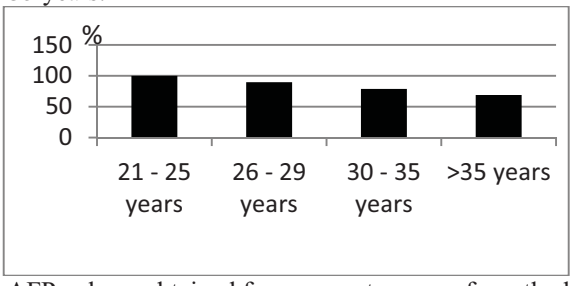

Figure 1. Relative AFP values, obtained for pregnant women from the last age group (>35 years), compared to the first, second and third age group

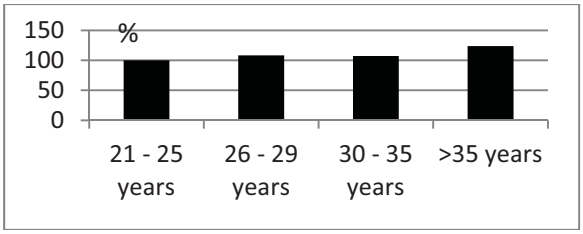

Figure 2. Graphic comparative representation of the relative values (%) for HCG, obtained after the analysis of the pregnant women serum from the four age groups

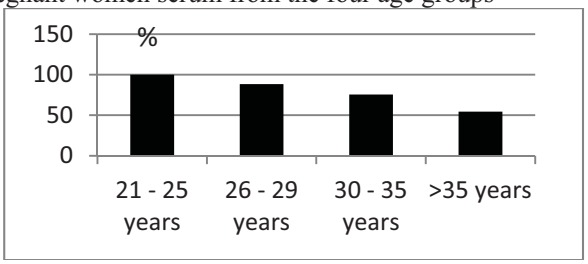

Figure 3. Representation of the relative values of unconjugated estriol obtained in patients from the four age groups

As we said earlier on the total of 135 pregnant women was divided in four age groups. The first group is taken as a control group in the statistical analysis of all of the age groups in this study, because no pathological values were recorded, which means that the pregnant women from the 21-25 years age group do not have risk pregnancies for chromosomal disorders and neural tubes defects.

General analysis of the results on groups shows us the following: the second age group presents a single case of a pregnancy with high risk, from the third age group, 30-35 years, formed from 50 patients, three of those gained a pathologic result, and the rest obtained normal results specific for low risk pregnancies. As the maternal ages progresses, so does the number of cases with risk pregnancies. Regarding the precedent age group, this third group has to more risk pregnancies, the total number of analysed cases being similar in the two groups.

Analysing the last age group where we expect to get more risk pregnancies, as the things evolved, we observe the following: from a total of 14 pregnant women, 4 recorded pathologic results, meaning they have pregnancies with high risk for the appearance of chromosomal disorders. Reported to the number of total pregnant women analysed, these situation is the most critical. To reveal the importance of maternal age in prenatal screening, in figure 4, we represented the percentage for the results obtained after conducting the triple test on patients in the fourth age group, the group with the most pathological results recorded.

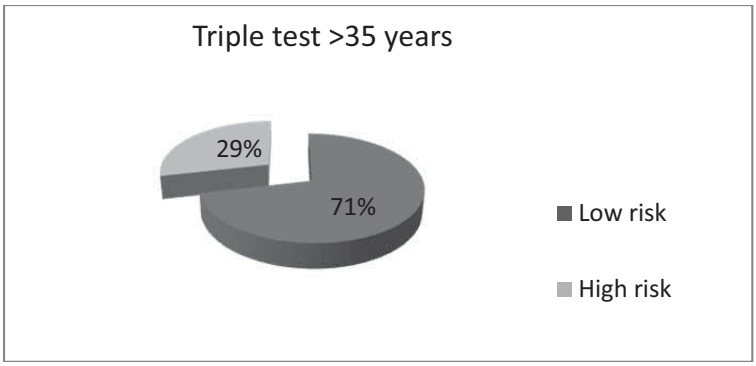

Figure 4. The percentage value of the results obtained after conducting the triple test on patients from the >35 years age group

CONCLUSIONS

The obtained results from the investigated pregnant women, on age groups, for AFP are in correlation with the data from the speciality literature, which demonstrates the importance of the maternal age in the prenatal screening. Also, according to the speciality literature, HCG is the most stable marker, being secreted by the placenta and correlated with the AFP values, it gives it a special importance in the gathering of the results. The association of these markers with UE_3, in the triple test, reveals a clear image in the final result in the prenatal screening with a low rate of false positive results, pointing out a good correlation between the biochemical markers and ultrasound.

The obtained values after testing the biochemical markers, in combination with the ultrasound data, maternal age and medical history of the mother, represents a feasible prenatal screening, a fact that is found both in our study but also in those in the speciality literature. The method of interpreting the triple test through the PRISCA software is exact and until now does not need adjusting. Because of the fact that in the first age group (21-25 years) for any of the pregnant women we got no pathological results specific for pregnancies with high risk of appearance of chromosomal disorders and the number of pregnant women with high risk pregnancies is higher to the patients in the fourth age group (>35 years), we can conclude that the obtained results depend largely on the maternal age.

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THE CERVICAL CANCER AND ITS OBSTETRICAL ANTECEDENTS

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Keywords: cervical cancer, parity, number of pregnancies, abortion, contraception

Abstract. Cervical cancer is an important public health issue, with a continuously increasing incidence. Identifying the main associated risk factors can lead to useful neutralizing measures, watching closely the risk factors: pregnancy, birth, sperm, genetic or hormone factors. The patients agreed to take part in the study by filling in a questionnaire sheet and after that they were investigated through clinic and paraclinic emainations. The screening was performed in “Elena Doamna” Third Clinic of Obstetrics-Gynecology Iaşi between 01.01.2001 and 31.12.2007 on a number of 145 women diagnosed with cervical cancer. The increase in the incidence of cervical cancer is associated with the increase of parity, abortions and the intensive use of contraceptive pills; the age when the sexual life begins also influences the incidence of cervical cancer.

INTRODUCTION

Cervical cancer is diagnosed mainly in its advanced stages, when the treatment applied is sometimes inefficient; improving the clinic and paraclinic means of investigation has the purpose of establishing an early diagnosis; the prevention measures are an important problem, both for each hospital unit and also for the decision factors in the network of medical assistance (4, 5, 11).

MATERIAL AND METHOD

The study was performed by a team of medical staff, in “Elena Doamna” Third Clinic of Obstetrics-Gynecology Iaşi, in the period of time between 01.01.2001-31.12.2007, on a number of 145 patients who had been diagnosed with cervical cancer in different stages.

The patients were questioned about their personal physiologic case history: the first menstruation, number of pregnancies and abortions.

They benefited from:

- genital examination and paraclinic examinations (hemoglobin-Hb, hematocrit-Ht),
- abdominal-pelvic ultrasound,
- colposcopy and biopsy,
- they were harvested smears for the bacteriologic and cytologic examination.

RESULTS AND DISCUSSIONS

The hereditary collateral antecedents show us that over half (64%) of the women with cervical cancer had an increased risk of gynecologic neoplasic disease in the family.

Number of pregnancies. Parity.

The increase of susceptibility can suggest the alteration of the nutritional status, the effect of the hormones on the cervix or on the expression HPV (human papilloma virus), or the effect of the trauma on the cervical epithelium during delivery.

Some studies suggest that the most important risk factor is the age when the woman has the first pregnancy and also the number of pregnancies. There is a very close relationship between invasive cancer and the young age of the first pregnancy and this relationship seems to be inter-dependent with the early age of the sexual debut, the presence of HPV and the subsequent births (3, 4, 7, 12, 15).

Pregnancy is a state of immunology depression state that favours the subclinic or clinic occurrence of HPV infection and also a physiologic situation that forces the patient to go for a gynecologic consult.

These parameters can have a distinct role in cervical cancer incidence, especially for teenagers. This statement is based on statistical analyses that showed that pregnancy and labour at young ages (14-20 year) represent a risk factor by the gravidic hormonal complex that is possible to be co-carcinogenic, but especially by viral infections and cervical trauma in an area of transformation that is still immature, of cylindro-pavimentous interference. The presence of a big number of pregnancies in the case history when compared with their absence could influence the risk status in cervical cancer (3, 4).

The study made revealed the fact that there is a difference among the number of patients with no pregnancies, one pregnancy or more than 4 pregnancies.

This fact can confirm that the bigger frequency of pregnancies can be an element of unfavorable prognosis in installing the risk of appearance and evolution of cervical cancer.

- no pregnancy - 6 patients-4,14%;
- one pregnancy -19 patients-13,10%;
- two pregnancies -36 patients-24,83 %;
- three pregnancies -11 patients -7,59 %;
- four pregnancies - 45 patients-31,03%;
- multiple pregnancies -28 patients -19,31%

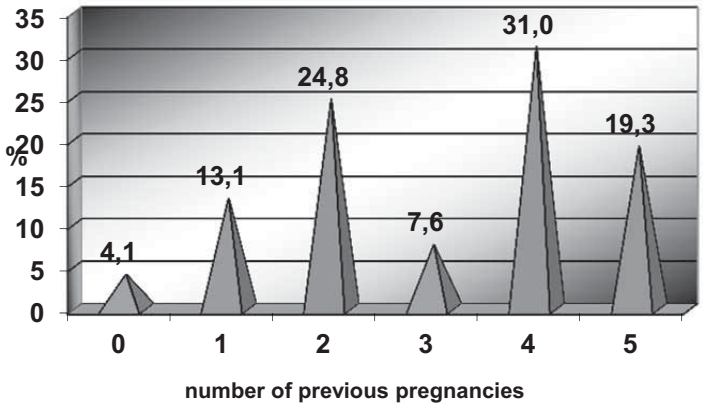

Fig. 1. The repartition of cases depending on the obstetrical history

The number of pregnancies seems to influence the incidence of the cervical cancer. A percentage of 57.93% is represented by the patients with a big number of pregnancies. This aspect can be considered as a risk factor both from the point of view of the endocrine changes induced by pregnancy and also from the point of view of an early start in the sexual life.

A bigger percentage of the mothers with twin pregnancies come from the rural area. Hence the conclusion that multiple pregnancies, if considered risk factors for the cervical cancer, act in a more powerful way in the rural area when compared to the urban area, probably because of the inferior quality assistance in child labour than in the case of the urban area.

The importance of the sexual life and of pregnancies in cervical cancer genesis, are explained by the presence of only 6 cases of women with no pregnancy.

Also in 39 cases -26.89% the women had child birth at a very young age and there were 3 cases -2.06% of cervical neoplasia associated with pregnancy.

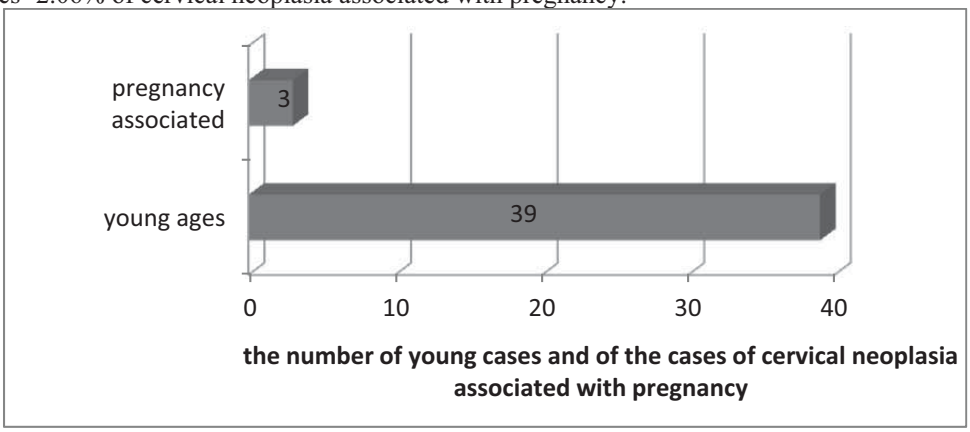

Fig. 2. The importance of sexual life and pregnancies in cancer genesis

Some studies made for detecting the HPV prove the increase of its incidence during pregnancy when compared with the non-puerperal period, thus reflecting a decrease of the cell mediated immunity (6, 8, 13).

A direct effect of pregnancy on the vulnerable cervical epithelium was suggested as a result of the instability of HPV oncogenes that was induced by progesteron. This instability of the viral genome can facilitate the viral DNA HPV integration in the genome of the host, promoting the progression towards malignancy (2, 6, 9).

The nutritional factors during pregnancy can be correlated with the vulnerability of the cervical epithelium, because pregnancy is known as a status with folate depletion. This combination between the increase of HPV expression and the increase of the vulnerability for the transformation zone can explain the increasing risk of cervical neoplasia associated with parity increment (8, 11).

OBSERVATION no 1.

Pregnancy + cervical cancer

The patient N.S., aged 33, is hospitalized for: metrorrhagia, abundant leucorrhoea, back pain.

Personal physiologic case history: first period= 14 years old, regular periods, pregnancies= 2; births= 1, abortions =0.

Vaginal examination with valves – increased volume of the cervix with the external orifice in transversal slot, half open; around the orifice there was an erosion of almost 1,5 cm, more extended towards the right edge; the liquid blood in the vagina was in moderate quantity and it was dark in colour, coming from the uterus.

Digital vaginal examination– increased volume of the cervix; increased volume of the uterine body, with the upper limit at 5-6 cm above the pubic symphysis, soft consistency; flexible vaginal cul-de-sacs; free annexes.

Paraclinic examinations:

Hb = 8,5 g%;

Ht = 32 %

- Peripheral blood smear – make anisocytosis with hypochromia, relatively frequent red cells into the target, granulocytes prone to hypersegmentation.
- CDS (cytodiagnostic smear) - type V
- Pelvic-abdominal ultrasound – increased volume of the uterus, 9 weeks pregnancy, annexes can't be visualized, free Douglas cul-de-sac.
- Colposcopy- polyp with a diameter of 8 mm that was ulcerated, situated in an area of rugous mucosa, hyperemic, hemorrhagic, with an aspect of superficial shuffle.
- Biopsy – unkeratinized epidermoid carcinoma with small cells, invasive;
- Lymph nodes- fibrosclerosis, histiomonocytosis; ovaries with stromal hyperplasia, luteinic cyst, atretic cysts; we could see changes in the pregnancy.
- Hystology- pT1N0Mx
- Performed treatment - enlarged Wertheim type colpo-hysterectomy + pelvic lymphadenectomy.

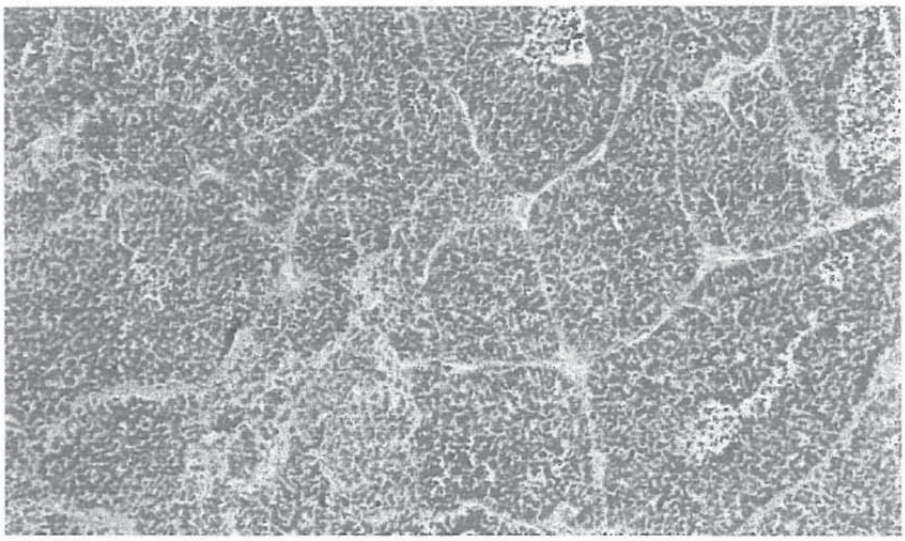

Fig. 3. Unkeratinized epidermoid carcinoma with small cells

OBSERVATION no. 2.

Pregnancy + cervical cancer

The patient M.A. aged 32 was hospitalized for assistance at birth.

Diagnosis when hospitalized: 40 weeks pregnancy, cervical neoplasia.

Personal physiologic antecedents: first menstruation =14 years old, regular menstruations, pregnancies= 1; births= 0, abortions =0..

The pregnancy evolved normally, she had metrorrhagia in in moderate quantity in the sixth month of pregnancy and she followed adequate treatment for it etc.

Personal pathologic antecedents: chronic cervicitis.

Genital examination:

Digital vaginal examination - increased volume of the cervix, of increased consistency;
Vaginal examination with valves - increased volume of the cervix with the external orifice slightly open.

Paraclinic examinations:

- Hb =11g%;
- Ht = 39%;
- FCD type V

Biopsy – unkeratinized epidermoid carcinoma with big cells, micro-invasive. The anatomo-pathological examination that was made postoperator showed: epidermoid carcinoma - 2mm; lymph nodes – with lympho-monocytary depletion; normal ovaries + salpingitis.

Hystologic – pTis – T1N0M0

Performed treatment – segmento-corporeal C-section surgery followed by enlarged Wertheim type colpo-hysterectomy + pelvic lymphadenectomy.

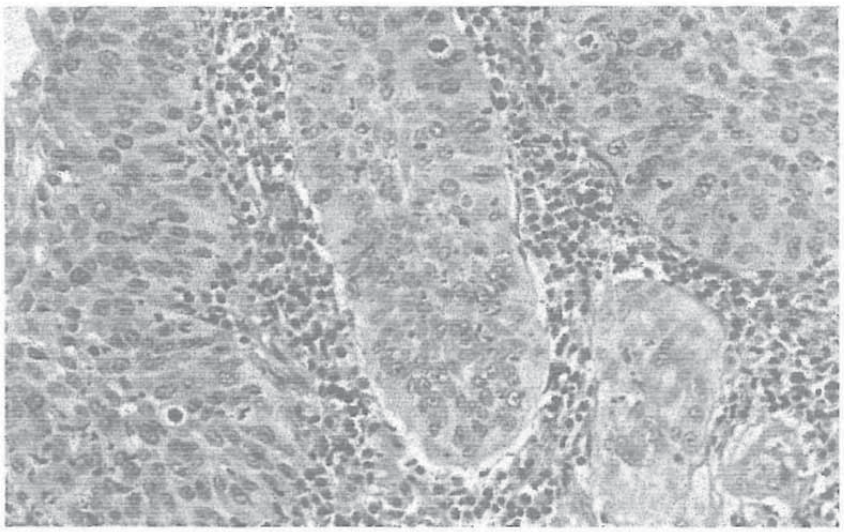

Fig. 4. Unkeratinized epidermoid carcinoma with big cells

Also speaking from the point of view of the significance of the sexual life, we could not find any case of virginity among the 145 cases of cervical cancer studied.

Abortions

There are significant differences between the sick women and the witnesses in this respect.

The number of abortions is difficult to be correlated with cervical neoplasia it is almost impossible to know for sure the exact number of abortions for the general population or even for the considered group because many of the women do not say the truth about it.

We studied pregnancies, births, quality of obstetrical assistance and abortions and we have found out that the obstetrical history of the sick women with cancer is significantly and under many aspects higher than in the case of the witnesses, but also that *it is not the number of births*

the one generating a high risk factor of getting sick, but rather the low quality of the assistance in delivery.

However, cervical neoplasia also appears in women who had a C-section when delivering the baby or in cases where the cervix remained malignant post-partum and where the role of the traumatic-cicatricial factor lacks. In these cases we will have to consider one or many of the outer factors we have mentioned before or eventually intrinsic factors.

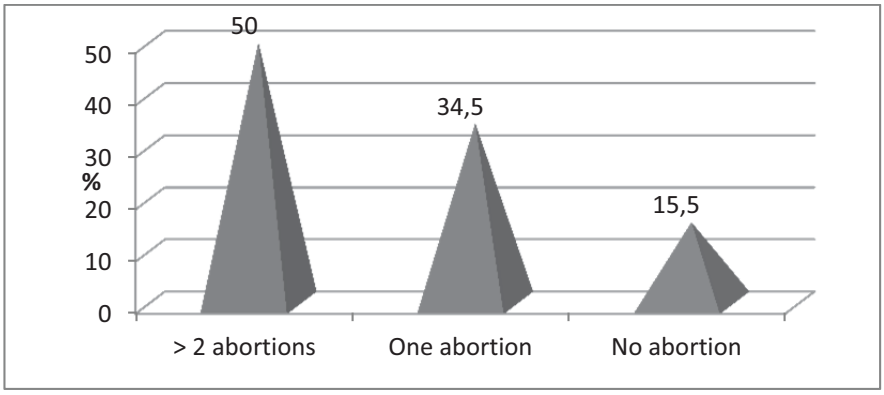

Fig. 5. Prevalence of obstetrical trauma by abortion patients with cervical cancer

Hormonal factors

The oncogenic risk of the long term use of oral contraceptive pills is very controversial. Many studies have shown an increased incidence of dysplasias in women who used oral contraceptive pills (1, 3).

This difference concerning the risk of getting cervical cancer was assumed to have been caused by the characteristics of the sexual behaviour and also to a possible direct effect of oral contraceptive pills (OC) on the cervical metaplasic epithelium.

Women who take oral contraceptive pills frequently are subjected to screening more often, thus increasing the possibility of detecting the pre-invasive cervical lesions. But they do not use blocking methods and increase the risk of being exposed to sexually, transmitted carcinogenic agents.

The possible effect of the oral contraceptive pills on the cervical epithelium could not be demonstrated and neither could the way in which it might influence the risk of cervical cancer. For some women the risk can be increased by the activity of OC on the immature metaplasic processes. Also the OC cause a decrease in the levels of blood folate, causing megaloblastice changes in the cervical epithelial cells, which would increase the incidence of cervical cancer.

The relatively poor association between OC and cervical cancer is not strong enough to recommend the ending of OC consumption in women diagnosed with pre-malignant diseases. It is possible for the contraceptive agents to interfere in the more advanced stages of the carcinogenesis.

The small number of cases that used a contraceptive method (18 cases-12.41% out of which 14 women – 9.65% used OC and 4-2.75% IUS) is not significant.

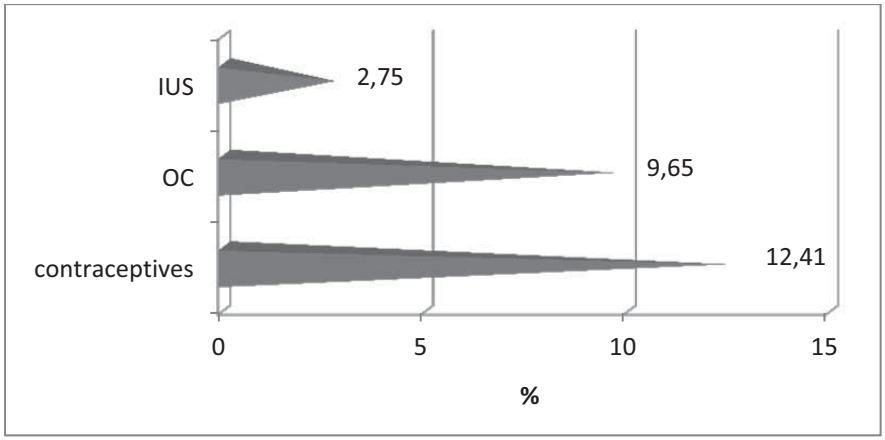

Fig. 6. Cases that used a contraceptive method

An explanation could be the fact that, until 1989, any contraceptive method was officially banned, and after that, after abortions were legalized, the population was educated gradually in order to accept a contraceptive method and for a long time women preferred abortion as a method of family planning instead of another contraceptive method.

The data in the specialized literature reveal a slightly increased risk of getting cervical cancer to the people who use hormonal contraceptives – either by changing the position of the squamo-cylindrical junction, or by changing the sexual behaviour (6, 14).

On the other hand, oral contraceptive pills (used for more than 5 years) would increase the risk of cervical cancer even after the statistic removal of other factors that might be convergent, for example the sexual behaviour, especially for adenocarcinoma. The mechanism could be to amplify the transcription of HPV and to transform the cells infected with HPV (the genome segments that control these mechanisms having hormonal receptors for estrogen and progesteron).

CONCLUSIONS

The obstetrical antecedents in patients with cervical cancer are more frequent than in witnesses.

The higher frequency of pregnancies is an unfavorable element of prognosis for installing the risk of appearance and evolution of cervical cancer.

The number of abortions is difficult to be correlated with cervical neoplasia because many of the women did not state the real number of abortions.

We noticed a reduced frequency of the patients who used a contraceptive method when studying the women in the study group (12.41%)

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MAP4K4 A POSSIBLE NEW BIOMARKER IN CANCER THERAPY

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Keywords: MAPkinases signaling pathway, MAP4K4, cell motility, inflammation, cancer
Abstract: MAP4K4 is a member of the germinal center kinase GCK-IV group and is involved in controlling cellular processes that include cell motility, rearrangement of the cytoskeleton and cell proliferation. MAP4K4 proved to be an upstream activator of the cJUN-n terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), and p38 SAP kinase. TNFα and p53 are two of the most important factors that have the capacity to increase the expression of MAP4K4. MAP4K4 is involved in a very complex network of signaling pathways and interactions that are involved in diseases like diabetes or cancer. The recent findings place MAP4K4 as a novel target that may provide insights into new therapies, in the effort to prevent or even treat these diseases.

INTRODUCTION

A protein kinase is an enzyme able to reassign a phosphate group from a donor molecule (usually ATP) to an amino acid residue of a protein. The mechanism used by protein kinases is involved in signal transduction for the modulation of enzymes. The activity of an enzyme can be activated or inhibited by the phosphorylation process.

Mitogen-activated protein kinases (MAPK) are components of a strictly conserved cascade of serine / threonine protein kinases that contain a Thr-x-Tyr motif within the activation loop in the kinase domain (Huangm *et al.*, 2004) and are involved in many signal transduction pathways (Johnson and Lapadat, 2002). They activate phosphorylation of transcription factors due to the signals received from the extracellular stimuli such as ultraviolet light, growth factors, cytokines and stress-inducing agents (Chang and Karin, 2001; Widmann *et al.*, 1999). There are a lot of essential cellular functions, such as differentiation (Aouadi *et al.*, 2006; Aouadi *et al.*, 2006; Bost *et al.,* 2005), proliferation (Roux and Blenis, 2004), apoptosis (Kyosseva, 2004; Willaime-Morawek *et al.,* 2003; Kolch *et al.,* 2005), that are regulated by MAPK pathways after their activation. It has been demonstrated that MAPKs play key roles in inflammation, stress responses and oncogenesis, (Huangm *et al.*, 2004), but there is recent evidence that this family is also determinant for the process of cell migration.

The main role of MAPK proteins in signal transduction, led to their involvement in the progression of cancer and autoimmune diseases. This is the reason for their election as new targets for drug development (Huangm *et al.*, 2004). The fact that MAPK pathways are very well conserved over the eukaryotic kingdom, made them eligible for the study of their function, structure and interconnectivity through genetic analysis of model organisms (Widmann *et al.*, 1999).

The complex protein interactions that involve MAPK signal transduction network has been supplemented by the recent work of Bandyopadhyay *et al*., (2010) who added new data as a resource for the future investigations. The main reaction through which all MAPK pathways act is the phosphorylation. During this cascade of events the phosphorylation of transcription factors leads to the regulation of gene expression and the phosphorylation of the cytosolic targets directs to the regulation of intracellular events. MAPKs are phosphorylated at the level of the threonine and tyrosine residues within the activation loop, through a kinase cascade, by MAPK kinases (MKKs), which in turn are phosphorylated and activated by MKK kinases (Raf and MKKK). The main purpose of these correlated cascades is the modulation of cellular proliferation and motility, cell cycle and differentiation, development, and transmission of oncogenic signals through gene transcription. This pathway is integrated in a rich net formed by correlations with a lot of other components, such as transcription factors, membrane receptors, and kinase scaffolds, and has many interactions with other activators and inhibitors of signaling (Kolch*.,* 2005).

Based on their type of activation loop, the MAPK family can be separated into three groups: extracellular signal - regulated protein kinase (Erk / MAPK), which has a Thr-Glu-Tyr motif; p38, which has a Thr-Ala-Tyr motif; and Jun N-terminus kinase (JNK), which has a Thr-Pro-Tyr motif (Johnson and Lapadat, 2002). Due to the diverse nature of the MAPK superfamily of enzymes, MAPK subfamilies ERK1 and ERK2 were the first group to be investigated with the purpose to understand MAPK signaling, but now the majority of studies are focusing on the role of the stress-activated kinases, especially p38 and JNK. There are many sequence similarities among constituents of each MAPK module used for stimulation of ERK1/2, JNKs and p38 but there is a very high fidelity and selective adaptation of each MAPK module in the processes of translation of the distinctive extracellular signals into physiological responses. Analyzing the specificity of all these processes and the importance of all interconnections inside each signaling cascade, is a detrimental problem that is now being investigated by specialists.

Lately, a lot of researchers have focused their interest on discovering new upstream kinases that modulate the downstream effector MAP kinases. Recently, a new class of MAP4Ks homologous to the Ste20 kinase (an upstream

constituent of the MAPK signaling pathway implicated in the pheromone response pathway in yeast - *Saccharomyces cerevisiae*) (Cowan and Storey, 2003), was identified analyzed and characterized.

This group of protein kinases can exert their action upstream of MAP3Ks. STE20p kinases can be separated into two groups, the germinal center protein kinases (GCK) and the p21 activated protein kinases (Cowan and Storey, 2003).

These group called MAP4K proteins bring a new level of modulation for the MAPK / JNK signaling cascade and maybe a connection to regulatory proteins that are located at the plasma membrane. The MAP4K group includes: HPK1 (Hematopoietic Progenitor Kinase-1), GCK (Germinal Center Kinase), GLK (GCK-Like Kinase), MAP4K4 (Mitogen Activated Protein Kinase Kinase Kinase Kinase 4), kinase homologous to Ste20/Sps1, GCKR (GCK-Related Kinase), (Cowan and Storey, 2003).

MAP4K4 – THE MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE KINASE 4

MAP4K4 (mitogen activated protein kinase kinase kinase kinase 4) is a member of the germinal center kinase GCK-IV group (Tang *et al*., 2006) of the sterile 20 protein (STE20p) kinases and is involved in controlling cellular processes that include cell motility, rearrangement of the cytoskeleton and cell proliferation (Collins *et al*., 2006; Zohn *et al*, 2006; Taira *et al*., 2004; Nishigaki *et al*., 2003; Hu *et al*., 2004; Wright *et al*. 2003).

The studies that investigate MAP4K4 have demonstrated that MAP4K4 is an upstream activator of the cJUN-n terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), and p38 SAP kinase (Collins *et al*., 2006; Zohn *et al*, 2006; Wright *et al*. 2003; Bouzakri and Zierath, 2007)

For the first time, MAP4K4 was identified in correlation with NCK adaptor protein 1 (NCK) which is a receptor tyrosine kinase adaptor protein (Su *et al.,* 1997). Su *et al*. demonstrated through their *in vitro* expression study that the MEKK1, MKK4 and JNK cellular signaling cascade is activated by MAP4K4. Later, the activation of JNK by MAP4K4 was confirmed in TNFα signaling in human cell lines.

TNFα is a cytokine involved in inflammation and in insulin resistance by decreasing the expression of PPARγand GLUT4 glucose transporter. This action has been demonstrated to be mediated also by MAP4K4.

Tesz *et al.* (2007) showed that TNFα stimulates the mRNA expression of MAP4K4 through a mechanism that involves its receptor TNFR1 and aims the transcription factors cJUN and ATF2.

In their study on cultured adypocites, Tesz *et al.* (2007) show that while TNFα up-regulates the expression of MAP4K4, it has little or no effect on the expression of the protein kinases MKK4, MKK7, p38 SAP kinase, ERK1/2, JNK1/2. They also prove that other cytokines such as LPS, IL-1β and IL-6 have no effect upon the mRNA expression level of MAP4K4 in the same conditions in which TNFα increases its mRNA expression level 3 times. By inhibition with siRNA of the two receptors of TNFα - TNFα receptor 1 (TNFR1) and TNFα receptor 2 (TNFR2), the authors showed that only TNFR1 acts as a mediator of the MAP4K4 gene expression up-regulation. This TNFR1 receptor is also involved in the stimulating effect that TNFα has upon the phosphorylation of JNK1/2 and p38 SAP kinase and their downstream transcription factor substrates cJUN and ATF2. In conclusion, TNFα which modulated the expression of MAP4K4 had a much stronger effect on the phosphorylation of c-JUN and ATF2, than the effect of the other citokine IL-1β, which did not modify the mRNA expression of MAP4K4. Tesz *et al*., proved that TNFα is the only cytokine among the ones they studied with the capacity to increase

the expression of MAP4K4 as a member of its signaling pathway and also MAP4K4 is the only MAP kinase they found to have this type of response to the action of TNFα.

Another key factor that was found to up-regulate MAP4K4 and to activate the JNK signaling pathway directing to apoptosis of the cells is p53 (Miled *et al.*, 2005). p53 is a tumor suppressor gene that modulates cell response to stress and is involved in cell progression in cancer. The activation of p53 stops cell cycle in G1 phase and can lead to senescence or apoptosis. That is why mutations of p53 are correlated with the majority types of cancer. Miled *et al.,* showed that MAP4K4 has four p53 binding sites. When p53 protein binds to these sites it up-regulates the mRNA expression of MAP4K4 gene 2 folds, and determines an increased level of the phospho-c-Jun protein through the activation of JNK signaling pathway. Also, the siRNA knockdown of MAP4K4 led to a reduction of p53 induced apoptosis. All these finding suggest that JNK signaling pathway is involved in p53 induced apoptosis through the modulator effect of MAP4K4.

JNK signal transduction pathway in which MAP4K4 is involved, is implicated in multiple physiological processes. JNKs were originally identified as the major kinases responsible for the phosphorylation of c-Jun, leading to increased activity of the AP1 transcription factor. Currently, there are new nuclear transcription factors that are also known to be targets: ATF2 Myc, Elk1, SMAD3, NFAT4, p53, MADD, DPC4.

All these transcription factors respond to different stimuli such as different types of stress, citokines or growth factors by modulating the expression of different genes. The activation of this signaling pathway can lead either to apoptosis of the cell or to tumorigenesis and inflammation.

Clarifying all the interactions and mechanisms that function inside this signaling pathway can lead to new therapies for the diseases in which this pathway is involved, including cancer and diabetes.

MAP4K4 A POSSIBLE TARGET FOR THERAPY

MAP4K4 is involved in a very complex network of signaling pathways, interactions and interconnectivities and its multiple implications in this net of mechanisms is yet to be discovered.

Yao *et al.* analyzed the MAP4K4 cDNA and they found two isoforms of the corresponding protein, one of the isoforms had a deletion in the region that contains two proline rich domains. The long isoform containing the two proline rich domains was predominantly expressed in the brain and the short isoform carrying the deletion was specific to other types of tissue such as human liver, skeletal muscle, and placenta (Yao *et al.,* 1999). The authors concluded that this variety of expression of different isoforms in different tissues could indicate a tissue or cell specificity of functions and mechanisms regulations.

MAP4K4 AND ITS ROLE IN CELL MIGRATION

Su *et al.,* (1998) have demonstrated that the gene MAP4K4 is required for *Drosophila* flies dorsal closure. Also in mice, the deletion of MAP4K4 determined the lack of migration of the mesodermal cells during gastrulation (Xue *et al.,* 2001). All these developmental processes are dominated by cell migration. In later studies, inhibition of MAP4K4 by siRNA transfection reduced the capacity of cancer cell lines to migrate (Collins *et al.,* 2006). However, the results about the activated pathway are controversy because some studies show that MAP4K4 acts

upstream of JNK, but others mention the activation of p38 in this process. Unfortunately the null MAP4K4 mice cannot survive, and because of that the role of MAP4K4 could not overcome the *in vivo* embryogenesis stage.

MAP4K4 AND THE METABOLIC REGULATION

Tang *et al.,* (2006) have recently discovered that the kinase MAP4K4 is a negative modulator of adipogenesis. They trasfected cultured adipocytes with siRNA corresponding to all the protein kinases expressed in adipocytes and they discovered that MAP4K4 is one of the four negative regulators of the insulin-responsive glucose transport. By attenuating MAP4K4 with siRNA, they increased the expression of PPARγ, the capacity of adipocytes to store triglycerides and the insulin stimulated transport of glucose. The experiments also proved that the silencing of MAP4K4 protected the inhibitory effects of TNFα on the expression of PPARγ and GLUT4 genes. This particular finding shows the involvement of MAP4k4 in mediating the effect of TNF α in adipocites.

The same function was noticed in human muscle explans (Bouzakri and Zierath, 2007). MAP4K4 activated ERK1/2 and silencing of MAP4K4 prevented the event of insulin resistance induced by the TNFα citokyne.

All these findings are also confirmed through genomic investigations. In a 5914 single nucleotide polimorphysm study on 1344 individuals Elbein *et al*., (2009) discovered MAP4K4 among 11 potential candidate genes correlated with type 2 diabetes in African American families.

MAP4K4 AS A MEDIATOR IN THE INFLAMMATORY PROCESSES

It has been demonstrated that MAP4K4 is a kinase involved in the activation of T-cells. Mack *et al.* (2005) could prevent the activation of primary mouse T-cells, by silencing MAP4K4. The authors showed that MAP4K4 is necessary for the control of the activation of TNFα promoter, but the mechanism of this control wasn't completely characterized.

Earlier, Yao and his collaborators have shown, using dominant-negative MAP4K4 mutants on 293T cells, that MAP4K4 mediates the TNFα-induced JNK activation (Yao *et al.,* 1999).

All these data show that MAP4K4 is involved in the regulation of TNFα signals in the immunological cells. Taking into account that TNFα cytokine has an important role in insulin resistance, MAP4K4 could be an interesting study target for metabolic diseases.

MAP4K4 IN CANCER

Metastasis is the biggest cause for death in cancer patients. Cell migration and motility are highly correlated with tumor invasion and metastasis. Later studies have revealed that many members of key signaling pathways involved in cell migration are also activated in cancer cells by overexpression or different types of mutations (Collins *et al.,* 2006). This is why, understanding the mechanisms behind tumor cell motility can lead to new clues in analyzing the insights of metastatic development.

MAP4K4 proved to be highly overexpressed in different types of cancers, such as ovarian cancer, hepatocellular carcinoma, lung cancer, pancreatic or prostate cancer (Collins *et*

al., 2006; Wright *et al.,* 2003; Han *et al*., 2010; Liang *et al.,* 2008). In these types of tumors MAP4K4 is associated with the processes of cell migration, invasiveness and adhesion.

The experiments on SKOV-3 ovarian carcinoma cell line (Collins *et al.,* 2006) show that the kinase MAP4K4 is involved in the motility of cancer cells by activation of JNK signaling pathway in this type of cancer. Collins *et al*. investigated the three possible transduction pathways on which MAP4K4 could direct its effect: c-Jun N-terminal kinase (JNK), p38, and Erk (1/2). The knockdown of MAP4K4 by specific siRNA did not have any effect on the phosphorylation of p38 or ERK, but a highly significant decrease of the phosphorylation of JNK could be noticed. The authors could not determine the intermediate kinases implicated in the activation of JNK by MAP4K4 and the downstream factors through which MAP4K4-JNK mediates its effects.

Recombinant retroviruses based experiments on HepG2 cultured cells show that knockdown of MAP4K4 in this type of hepatocarcinoma cells inhibits the adhesion and cell growth, compared with the control cultures that had no inhibition of MAP4K4 expression (Han *et al*., 2010). MAP4K4 was also found to be highly overexpressed in tumoral versus nontumoral adjacent tissue in hepatocellular carcinoma patients (Liu *et al.,* 2011). MAP4K4 overexpression could be correlated with worse overall survival and high recurrence rate, larger tumor size, metastasis and advanced tumor stage. The *in vitro* knockdown experiments on HepG2 and Hep3B HCC cell lines highlight the role of MAP4K4 in activating proliferation of the cells, stimulating cell cycle and inhibiting apoptosis and repressing multiple signaling pathways such as JNK and NFκB. *In vivo* silencing of MAP4K4 determined a retarded tumor xenograft growth (Liu *et al.,* 2011).

MAP4K4 was found to be among the 42 genes up-regulated in nonmelanoma skin cancer, in a microarray based study verified through quantitative real-time PCR, on normal versus tumoral tissue biopsies (Nindl *et al.,* 2006).

Microarrays validated through quantitative real-time PCR and immunohistochemical analysis of the protein expression in formalin-fixed, paraffin-embedded colorectal tumour samples, found MAP4K4 to be a component of the five-gene signature as a potential predictor of lymph node metastasis and overall survival in colorectal cancer patients (Hao *et al.,* 2010).

MAP kinases pathway was recently discovered to be involved in the progresion and metastatic process of prostate cancer (Chandran *et al.,* 2007). Comparing the expression of certain genes in primary tumors and metastatic prostate cancer samples, Chandran and his collaborators found MAP4K4 among the 3 fold overexpressed genes, correlated with the progression and metastasis of the tumors.

Little is known about the involvement of MAP4K4 in the progression of pancreatic cancer. Recent studies show that members of the MAP kinases family such as MAP4K4 and MAPK9 have an aberrant expression in pancreatic ductal adenocarcinoma (PDAC), (Ammerpohl and Kalthoff, 2007). The microarray based study on a group of 36 romanian patients with PDAC revealed MAP4K4 to be one of the overexpressed genes correlated with survival in tumor compared with normal pancreatic tissue (Badea *et al*., 2008).

After discovering the TNFα – MAP4K4 interaction pattern in human skeletal muscle, Bouzakri *et al* (2009) investigated the effect of TNFα treatment on primary beta pancreatic cells. They showed that by treating mouse primary beta cells with TNFα, MAP4K4 gene had a correlated increased mRNA expression. Also, the knockdown of MAP4K4 by siRNA transfection, inhibited the TNFα induced peripheral insulin resistance on beta cells (Bouzakri *et al.*, 2009).

Liang *et al.* (2008), investigated the expression of MAP4K4 protein in 66 stage II PDAC, and their pair benign tissue, 48 chronic pancreatitis and 14 normal tissue samples. They found that MAP4K4 was overexperssed in 46% of the PDAC samples, but also in 23 % of chronic pancreatitis tissue samples, these results indicating that MAP4K4 could be correlated not only with the stage II PDAC but also with chronic pancreatitis. The authors could also correlate the MAP4K4 protein overexpression in stage II PDAC with poor overall survival, metastasis and high rate of tumor recurrence, larger tumor size and higher number of positive lymph nodes. They also suggested MAP4K4 as a prognosis marker for stage II pancreatic ductal adenocarcinoma (Liang *et al.,* 2008; Liang *et al.,* 2009).

CONCLUSIONS

MAP4K4 was found to be involved in cell migration, proliferation and adhesion but also in the inflammatory processes and metabolic regulation signaling pathways. Malignancies require events like cell motility and migration, and these processes are activated by TNFα through MAP4K4. These findings could be the premises that lead to a possible role of MAP4K4 in regulating tumor invasion (Collins *et al*., 2006; Wright *et al*., 2003). MAP4K4 could regulate the cytoskeletal elements involved in cell migration and adhesion, so the role of this kinase could extend not only into cancer cells but in modulating the normal function of moving cells like myeloid lineages.

The recent findings place MAP4K4 as a novel target that may provide insight into new therapies, in the effort to prevent or even treat many metabolic diseases like diabetes or even multiple types of cancer such as colon, prostate, breast, ovarian, pancreatic or hepatic cancer.

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METHODS OF DIAGNOSIS IN CERVICAL NEOPLASIA

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Keywords: cervical cancer, cytology, colposcopy, biopsy

Abstract. The early screening of a precancerous lesion that can develop spontaneously into a cancerous lesion that is first non-invasive and then invasive. **Materials and methods**. The study group was made of 1485 patients who were hospitalized between 2001 and 2009 in the Section of Obstetrics and Gynecology of „Sf. Apostol Andrei” Emergency Hospital in Galaţi. The patients filled in a questionnaire on a sheet of paper in a freely consented way and they were diagnosed with cervical cancer by a cytologic and colposcopic examination, biopsy and other complementary examinations. **Results and discussions**. It is obvious that, even if cervical carcinoma benefits now from a remarkable methodology of detecting the early forms and even the precursor lesions, in our country this lesion has an important frequency and even represents a public health issue with important social-economic implications; in most cases it affects people in full physical and procreative activity; it implies a laborious therapy, long time hospitalization and high costs. The risk of getting infected with HPV(human papilloma virus) is at least 50% for the sexually active people for the rest of their lives. Although most infections eliminate with the help of our own immunity, the people who are infected are not aware of HPV presence and they can spread the virus. When our own immune system cannot eliminate the infection, the persistence of the viral oncogenic strains from the cervical mucosa can lead to the appearance of precancerous lesions. The cytological examination is a simple and fast method that is also cheap and reliable being considered the most effective technique for preventing and detecting the precancerous conditions of the cervix, which, treated correctly, can offer a primary prevention of cervical cancer. The diagnostic certainty was accomplished exclusively by histopathology of the material obtained during biopsy. **Conclusions.** We consider that the patients with cervical cancer, no matter what their condition stage is when they come to see the doctor must never be abandoned because in many cases they have a satisfactory evolution after following a complex and well led treatment.

INTRODUCTION

The early detection of cancer consists of the discovery of an asymptomatic cancerous lesion in a person who is considered to be in good health. The asymptomatic cervical cancer is generally small in dimensions and the right treatment can cure it in high percentages - 80-90% of the cases (the patients survive without signs of the illness and get to be over 72.5 years old) (10).

It is also very important to discover precancerous lesions that represent the secondary prevention of cancer if diagnosed and treated.

Screening is a medical examination that is practiced on asymptomatic people with the purpose of establishing the suspicion if the disease exists there or not (14).

MATERIAL AND METHODS

The study group was made of 1485 patients who were hospitalized between 2001 and 2009 in the Department of Obstetrics and Gynecology of „Sf. Apostol Andrei” Emergency Hospital in Galaţi.

We used the following examinations for establishing the diagnosis of cervical cancer: cytologic smear (5, 7, 13, 20); colposcopic examination (15, 21); biopsy (9); complementary examination (11, 12).

RESULTS AND DISCUSSIONS

When talking about cervical cancer we know there are no symptoms that are characteristic to a certain form or stage, so there can be some forms with no symptoms or forms that are dominated by a certain symptom or having an association of signs and symptoms that are more or less important (10, 14). In about 29.0% of the patients the cervical cancer is detected following a routine medical check up.

The haemorrhage at sexual intercourse appeared in 305 patients (20.5%) or during the local cleaning (289 cases – 19.5%); this is the most important symptom that suggests a cervical neoplasia (especially in menopause) and it has a double role:

- it triggers alarm for the woman in question;
- it is supposed to draw the doctors' attention and to lead the case towards specialized examinations.

According to the cases we studied, we can positively predict that the patients who bleed during the sexual intercourse are over 40 years old in a percentage of 61.3% (p=0.0004). Statistically speaking, bleeding during the sexual intercourse for the women over 40 means that the risk of cervical neoplasia is 1.35 times higher for them than in the case of women under 40 (RR=1.35; IC95%: 1.14÷1.59).

The positive predictive value that the patients bleeding during sexual intercourse come from the urban area is of 72.8%, which is a significant difference, statistically speaking (p=0.027). These patients who come from the urban area have a relative risk of developing cervical neoplasia 1.23 times higher if they bleed during sexual intercourse (RR=1.23; IC95%: 1.02÷1.28).

After examining the women in our study group we could find no significant differences between the married patients and the single ones in terms of bleeding during sexual intercourse (p=0.113).

Table 1. The structure of the group depending on bleeding on age groups, environment and marital status

Epidemiologic characteristic	haemorrhage				Statistic significance
	during sexual intercourse		during local cleaning		
	n	%	n	%	
Age group					
≤ 40 years old	118	7.9	155	10.4	χ^2=12.75; GL=1; **p=0.0004**
> 40 years old	187	12.6	134	9.0	
Environment					
Urban	222	14.9	185	12.5	χ^2=4.90; GL=1; **p=0.027**
Rural	83	5.6	104	7.0	
Marital status					
Married	178	12.0	149	10.0	χ^2=2.21; GL=1; p=0.113
Single	127	8.6	140	9.4	

The early symptom appeared was the bleeding, especially during sexual intercourse and more rarely between menses.

For the present cases haemorrhage (with different characters and having a variable intensity) was dominant as unique symptom or associated with other signs and symptoms: *leucorrhoea; diffuse pain in the abdomen, loss of appetite; loss of weight; pallor etc.*

In the case history the patients had the following conditions:

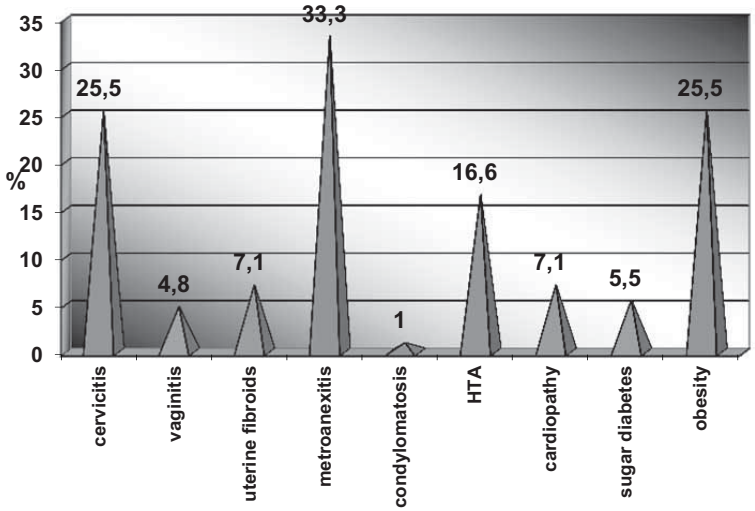

Fig. 1. The share of the cases depending on the pathological antecedents

We can notice an increased frequency of *genital inflammatory diseases.* The most frequent etiological agents involved are *Trichomonas*, *E.coli*, staphylococcus, *Chlamidia.*

All this data suggests that in the county of Galaţi the frequency of genital inflammatory diseases is very high and it exposes the population in this area to an increased risk of cervical cancer.

Obesity*,* of all associated diseases, has the highest share (25.5%).

HBP *(high blood pressure)* has a pretty high share, too (16.6%), and we have met it both in women with cervical neoplasia who were about to be treated, but also in women who had gone through surgical procedure and radiotherapy.

All the other diseases met have their role, as they lead to a decrease in the general resistance of the body and to a change in the hormone status.

Diagnosis by cytologic smear

Cervical exfoliative cytology is the first examination in the series of examinations for cervical cancer and it is the most accessible one from the point of view of the simplicity of the method and the economical advantage, having unlimited addressability for the feminine population. A cytological-diagnostic smear that is rhythmically made to all the feminine population at risk and not only, decreases considerably the incidence of cervical neoplasia and mortality by this disease, being in fact the principle at the base of cytological-screening. The statement is based on the studies made in countries where the introducing of screening determined a decrease of the incidence of cervical cancer to half (USA, Norway, Sweden).

In order to accomplish the active surveillance of the entire feminine population at risk you need to collaborate with public health organizers, general practitioners and especially with gynecologists, because automatic sampling of a cytological smear from every sick woman does not satisfy the principles of a rhythmic surveillance (10, 16, 17).

This is intended in the near future for our country, too.

Considering the cases we studied, the cytological examination was performed to all patients, except the ones sent by other medical centres for a direct biopsy examination, when they were sampled Pap smear.

Colposcopic diagnosis

The colposcopic examination was performed on 168 patients (11.3%) and confirmed the diagnosis in 99.4% of the studied cases. From the statistic point of view we did not find any significant differences in the distribution of the patients who had a colposcopic examination on age groups (p=0.888).

The positive predictive value that the patients with colposcopic examination would come from the urban area is 94%, which is a significant difference from the statistic point of view (p<0.001). The colposcopic examination reveals the fact that the patients coming from the urban area have a relative risk of having cervical neoplasia which is five times higher when compared to the ones coming from the rural area (RR=5.02; IC95%: 2.68÷9.40).

We could not find any significant differences for the group studies between the married patients and the single ones regarding their distribution depending on the performing of the colposcopic examination (p=0.358).

Table2. The structure of the group in accordance with the colposcopic examination on age groups, environment and marital status

Epidemiologic characteristic	Colposcopic Examination				Statistic significance
	performed		Not performed		
	n	%	n	%	
Age group					
≤ 40 years old	66	4.4	510	34.3	χ^2=0.02; GL=1; p=0.888
> 40 years old	102	6.9	807	54.3	
Environment					
Urban	158	10.6	969	65.3	χ^2=32.82; GL=1; **p<0.001**
Rural	10	0.7	348	23.4	
Marital status					
Married	120	8.1	890	59.9	χ^2=0.85; GL=1; p=0.358
Single	48	3.2	427	28.8	

Diagnosis by biopsy

The positive predictive value that the age of the patients with biopsy would be over 40 was of 88.2%, which is a significant difference, statistically speaking, when compared to the one for the patients under 40 (p<0.001). Biopsy shows that the patients over 40 years old have a relative risk of having cervical neoplasm 4.75 times higher than the ones under 40 (RR=4.75; IC95%: 3.82÷5.92).

The positive predictive value that the patients with biopsy would come from the urban area is 65.8%, which is a significant difference from the statistic point of view (p<0.001). The biopsy shows that the patients who come from the urban area have a relative risk 1.63 times higher of getting cervical neoplasia when compared to the ones coming from the rural area (RR=1.63; IC95%: 1.46÷1.83).

When we observed the study group we noticed significant differences for the distribution of married and single women depending on their performing of the biopsy or not (p<0.001), with

a positive predictive value of 90.6%, so the relative risk of getting cervical neoplasia is 4.54 times higher in married patients when compared to the single ones (RR=4.54; IC95%: 3.56÷5.80).

Table 3. The structure of the group depending on biopsy on age groups, environment and marital status

Epidemiologic characteristic	Biopsy				Statistic significance
	performed		Not performed		
	n	%	n	%	
Age group					
≤ 40 years old	74	5.0	502	33.8	χ^2=333.64; GL=1; **p<0.001**
> 40years old	555	37.4	354	23.8	
Environment					
Urban	414	27.9	713	48.0	χ^2=59.57; GL=1; **p<0.001**
Rural	215	14.5	143	9.6	
Marital status					
Married	570	38.4	440	29.6	χ^2=254.54; GL=1; **p<0.001**
Single	59	4.0	416	28.0	

Histopathology examination

The histological examination was performed on 614 patients (41.3%).

The histological examination of the excision part showed the following anatomic-pathological forms:

Table 4. The distribution of cases depending on the microscopic shape of the tumour

Histological type	No cases	Percentage
Unkeratinized epidermoid carcinoma with big cells	412	27.7
Unkeratinized carcinoma with small cells	2	0.1
Undifferentiated carcinoma	3	0.2
Adenocarcinoma with clear cells	55	3.7
Fibroma	6	0.4
Dysplasia	43	2.9
Hyperplasia	73	4.9
Mixed Tumours	10	0.8
Previously stated	871	58.7

The 417 carcinomas were divided like this:

- 241 cases of epidermoid carcinoma (16.2%)
- 6 patients with endometrium carcinoma (0.4%)

- 89 patients with pavimentous carcinoma (6.0%)
- 57 patients with squamous carcinoma (3.8%)
- 12 patients with spinocellular carcinoma (0.8%)
- 1 case of papilloma carcinoma (0.07%).

Staging cervical neoplasia

Staging appeared because of the need to systematize the data of the objective examination of a given case, which is situated in a certain stage of the disease evolution, with the purpose of getting an optimum therapeutic instruction and also for comparing the results of the different types of treatment or the results of the different centres of oncologic therapy.

The staging of cervical cancer reflects the degree of extension of the tumour. There have been a few trials in the last years, which were statistically founded, that intended to represent elements of prognostic appreciation, depending on the dimensions of the lesion – when it is strictly limited to the cervix- or by the direction and degree of neighbouring extension in the cases where the tumour goes beyond the limits of the cervix.

Staging cervical cancer serves to attain the following objectives:

1. Establishing the plan of treatment.
2. Indications on the prognosis.
3. Assessment of the treatment result
4. Scientific and research purpose.
5. Exchange of information between the treatment centres.

When staging you must consider two major elements: staging must be done before starting the treatment; in case of hesitation between two stages you will always choose the less advanced stage in order not to increase the number of healings in advanced stages without reason.

When dealing with the distribution of neoplasic lesions depending on the anatomo-clinical classification, we noticed a worrying thing: we could not find any case in stage 0 and we found only three cases in stage Ia.

This is due on the one hand to the lack of an appropriate health education (the woman does not come to the doctor for the annual check up, but only when there are some symptoms and when they are annoying), but also to the lack of an efficient sanitation in Romania, where the women at risk in special can have periodic and systemized check ups in order to identify the disease in its early stages.

Based on the cases we studied, we remark the following aspects (fig. 2):

- 21.4% of the cases were identified in stage Ia; - 19.3% of the patients in stage Ib, with obvious clinical lesions of the cervix or a pre-clinic lesion;
- 38.6% of the patients were in stage II and in 26.9% of them there was an obvious invasion of the parameters;
- 6.9% were in stage III of the disease and in 4.1% of them the lesion was extended to the pelvic wall, hydro-nephrosis or non functional kidney;
- 17.2% of the cases were in stage IV and the carcinoma was extended beyond the pelvic wall or clinically invaded the bladder mucosa and/or that of the rectum, all of the cases having a tumour invasion towards the adjacent organs.

Staging has a relative character, as there is a percentage of errors in the clinical assessment of the extension of the cancer process (14, 18).

Sometimes it is very difficult to stage a case correctly. We are referring to the case of infiltration of parameters which can't be always appreciated exactly (18).

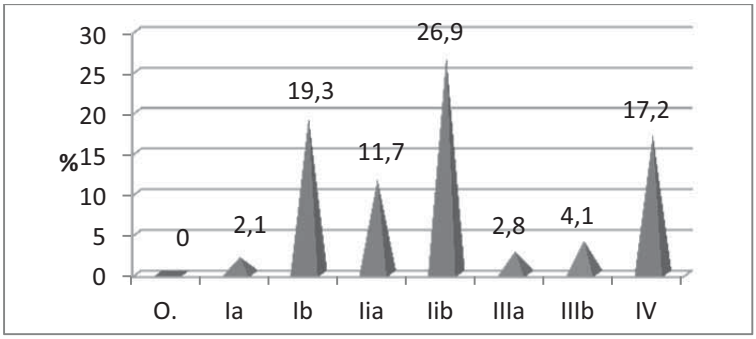

Fig. 2.The distribution of cases on lesional stages

The fact that a parameter is infiltrated does not necessarily mean a certain neoplasic invasion; it is difficult to establish precisely just how much of it is neoplasic infiltration and how much inflammatory, whether pre-existent or happening at the same time with an exofitic cervical neoplasic lesion, usually very infected.

In these cases palpation is sometimes extremely painful, so it is recommended to perform it under narcosis in order to avoid the reflex contraction of the abdominal muscles.

There is no doubt that present staging is far from being perfect. In fact, the study of the disease evolution at a certain point is reflected by the degree in which the neoplasia extended, the way it is determined by using the means of investigation that are presently used for a diagnostic purpose, but also by a series of other factors that are not integrated in the above-mentioned stages, for example the histological type of the tumour, the relationship between the tumour and its host, the interval of time between the first sign perceived by the patient and the moment of the diagnosis etc.

The present stage of knowledge creates objective premises for selecting the cases of cervical lesions with an irreversible potential of evolving towards malignancy, from the ones with a benign evolution.

The infections with the oncogenic HPV types would need a more aggressive treatment, in order to block the progression towards cervical neoplasias with high malignancy (1, 2, 3, 4).

CONCLUSIONS

Cervical cancer is an important public health issue, as it is the second in Romania after breast cancer, in terms of incidence and mortality by malignant tumours in women. Its incidence is continuously increasing and the therapeutic results are not satisfactory, in spite of the progress made in the last few years in getting to know the natural evolution of the disease, in improving the diagnose methods and also in improving the treatment quality. It is a chronic disease with a severe evolution, especially when it is identified in its advanced stages.

The increasing incidence of the disease in the last years, especially in young people requires an increase in the level of health education of the population and in starting some efficient screening programmes that will be able to cover all the feminine population at risk.

The efficiency of the screening by cervical-vaginal cytology was proved in the countries where it was repetitive and extended, by reducing the mortality rate with up to 80%.

We consider that the patients suffering from cervical cancer, no matter what their stage is when they come to the doctor, should not be abandoned because they sometimes have a satisfactory evolution after a complex and well conducted treatment.

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NEW POTENTIAL ANTITUMORAL AGENTS OF POLYPHENOLIC NATURE OBTAINED FROM *HELLEBORUS PURPURASCENS* BY MEMBRANARY MICRO- AND ULTRAFILTRATION TECHNIQUES

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Keywords: polyphenolic biopreparations, HeLa cell cultures, cytophysiological processes, cell cultures development, cytostatic and/or cytotoxic effect.

Abstract: The *in vitro* action of some total aqueous and hydroalcoholic extracts and of their concentrate or permeate fractions, separated by membranary micro- and ultrafiltrations of the primary homogenates, obtained from *Helleborus purpurascens* ethnomedicinal plant, upon the cell protein biosynthesis, proliferation, viability and development of the HeLa cancerous cells cultures was investigated. The significant proteinsynthesis alteration, protein dynamics modification, decrease of total cell number, cell viability diminution, inhibitory impact upon the cell cultures development, during studied evolution period, suggest the behaviour of these polyphenolic hellebore extracts as *in vitro* active cytostatic and cytotoxic agents. Our preliminary characterization of these vegetal biopreparations as protein, mitotic, growth inhibitors offers the informational background for further investigations, on many other cancerous and normal cell lines and adequate experimental models to *in vitro* prescreening, as well as for their introduction in the in *vivo* antitumoral screening program on different experimental tumoral systems.

INTRODUCTION

From the ancient times, using plants as a source of remedies to treat many diseases has captured the attention of the people. With the progress in chemistry, systematic studies have been conducted to identify bioactive compounds in plant extracts and to evaluate their biological activity. Polyphenols, as bioactive constituents, show numerous pharmacological effects (anti-inflammatory, antioxidant, antibacterial, anti-estrogenic, immunomodulatory, neurogenic etc.).

The genus *Helleborus* (Ranunculaceae) includes approximately 20 species of herbaceous, perennial rhizomatous plants, widely spreaded in Europe and Asia (Ardelean and Mohan, 2008; Watanabe et al., 2003). Hellebore extracts have been extensively investigated for their biological activities (Rosselli et al., 2009), conferred by the biologically active constituents (bufadienolides, saponosides; ecdysteroids, protoanemonin) (Stanescu et al., 2004) which allow their use in medicine, in the treatment of urologic and rheumatic diseases, several tumor types and hematological malignancies (Horstmann et al., 2008; Lupu et al., 2009; Yang et al., 2010). An original medicine utilizing purified *Helleborus* extracts, prepared in Romania and registered as BOICIL in USA (Kerek, 1981, US Patent) proved antalgic and antirrheumatic activity, and MCS-18 compound, isolated from *H. purpurascens*, has beneficial effects in inflammatory and autoimmune disorders by attenuating antibody production (Kerek et al., 2008; Littmann et al., 2008). The antiinflammatory action is attributed to steroidal constituents (Lacaille-Dubois and Wagner, 2000).

Although continuous progresses are registered in cancer prophylaxis, diagnosis and treatment, the neoplastic disease still holds pride of place in contemporary pathology. The antineoplastic chemotherapy is still characterized, by a relatively low effectiveness (De Vita, 2004; Stroescu, 1998), fact which explains the major significance given to the oncobiologic research, oriented towards optimizing its efficiency, by discovery of new oncochemotherapeutic medicines with preferential action upon cancerous cells and lower impact upon health ones (Miron, 2000; Owens, 2001). In recent years a lot of studies are focused on anticancer, immunomodulating, cytotoxic and antioxidant activities of *Helleborus* extracts (Rosselli et al., 2009; Paun-Roman, 2010), but there are some contradictions and debates relative to this issue.

An attractive source of new cytostatic agents is *H. purpurascens*, which have a high content of biologically active polyphenolic compounds. Therefore, we have considered opportune to obtain some total polyphenolic extracts or their fractions from *H. purpurascens*, as well as to investigate their antineoplastic capacity. The aim of present paper is to emphasize on the modern technology’s applications for the *H. purpurascens* extracts processing by a multi-stages membranary micro- and ultrafiltrations cascade, to obtain some natural biopreparations and to investigate, in an *in vitro* preliminary screening on the HeLa neoplastic cells, their impact upon the cell proteinsynthesis, proliferation, viability and development degree of cell cultures, in view of highlighting and evaluating their cytostatic and cytotoxic properties.

MATERIALS AND METHODS

16 natural polyphenolic biopreparations of vegetal origin were performed from diverse anatomical components (roots and rhizomes) of *Helleborus purpurascens* medicinal plant. The roots and rhizomes were dried, homogenized and grounded to a fine powder, using the GRINDOMIX GM200 mill; the extracts were prepared by maceration in cold distillate water or in aqueous: alcoholic (ethanol) mixtures 50% v/v , as solvents. The contact time between the plant and the solvent was maintained of 24 h for aqueous extracts and 7 days for hydro-alcoholic extracts, extracts have been sporadic, mechanically stirring, working temperature (20°C). The herbal's mass concentration in the solvent was of 6% (w/v) for aqueous and hydroalcoholic extracts. After filtering the initial extracts through Isolab quantitative filter paper "medium" for removing the coarse suspensions and sterilization, each filtrate was processed by microfiltration (MF), using Millipore membranes with 0.45 μm pores, to remove the impurities. Microfiltration (MF) process is meant to perform feed clarification and sterilization. These steps were followed by a four-stages ultrafiltration cascade, using four ultrafiltration plane membrane types from regenerated cellulose (Millipore) with cut-off 30,000 MWCO (UF1), 10,000 MWCO (UF2), 3,000 MWCO (UF3) and 1,000 MWCO (UF4). The permeate obtained from UF1 was introduced into the cross-flow circuits for UF2 and then the resulted permeate from UF2 or UF3 was eventually introduced into the cross-flow circuits for UF3 or UF4. A KMS Laboratory Cell CF-1 installation purchased from Koch Membrane (Germany) firm was used for both MF and UF.

The concentration ratio in ultrafiltration processes (expressed as permeate and concentrate volume ratio) were of 2:1. All ultrafiltration experiments – meant for concentration of rejected solutes and fractionation of solutes – were carried out at room temperature (cca. 23 C).

The total polyphenols (PT) were assessed in the hydrous and alcoholic total vegetal extracts or in the separated concentrate and permeate fractions through the Singleton and Rossi' s spectrophotomerical method (36, 40), the concentrations being expressed in equivalents galic acid/ L (GAE/L) compared to the standard curve with galic acid.

Cell oncobiology experimental protocol.

In vitro testing of the cytostatic and cytotoxic actions, on cancerous cell cultures, has included a series of total and fractionated, hydrous and hydroalcoholic polyphenolic extracts: **HphE** (*H. purpurascens* total hydrous extract); **0.45 μm MF-HphE (**microfiltrate of **HphE**); **30,000Da UF1C-HpE** (concentrate of first ultrafiltrate); **30,000Da UF1P-HphE** (permeate of first ultrafiltrate); **10,000Da UF2C-HphE** (concentrate of second ultrafiltrate); **10,000Da UF2P-HphE** (permeate of second ultrafiltrate); **3,000Da UF3C- HphE** (concentrate of third ultrafiltrate); **3,000Da UF3P-HphE** (permeate of third ultrafiltrate); **1,000DaUF4C- HphE** (concentrate of fourth ultrafiltrate); **1,000Da UF4P-HphE** (permeate of fourth ultrafiltrate); **HphaE** (*H. purpurascens* total hydroalcoholic extract); **0.45 μm MF-HphaE (**microfiltrate of **HphaE**); **30.000Da UF1C-HphaE** (concentrate of first ultrafiltrate); **30,000Da UF1P-HphaE** (permeate of first ultrafiltrate); **3,000Da UF2C-HphaE** (concentrate of second ultrafiltrate); 3**,000Da UF2P-HphaE** (permeate of second ultrafiltrate).

The biological material used in the *in vitro* experiments, was represented by mycoplasm-negative negroid human cervix epitheloid carcinoma HeLa cells, which were cultured in DMEM medium (Dulbeco's Modified Essential Medium, Biochrom AG, Germany) supplemented with 10% fetal bovine serum, (Sigma, Germany), 100 μg/mL streptomycin (Biochrom AG, Germany), 100 IU/mL penicillin (Biochrom AG, Germany) and 50 μg/mL amphotericin B (Biochrom AG, Germany), at a density of 5 x 10^5 cells in 75 cm^2 flasks, in a humidified 5% CO2 atmosphere at 37° C.

When the cells reached confluence, they were detached from the flask with 0.25% trypsin + 0.02% EDTA (ethylenediaminetetraacetic acid, Biochrom AG, Germany) in the normal medium and then centrifuged at 1800 rpm for 2 min. The cells, at a density of 1 x 10^5 cells/mL, were seeded in the tubes containing growth DMEM medium and were introduced at 37° C. The culture medium of the 24 h cell cultures was changed either with a normal one (control) or with one containing the hydrous or hydroalcoholic polyphenolic extracts, in a dose of 5 μg/mL (treated cultures).

After 24 and 48 h of *in vitro* treatment, the medium was discarded from the test tubes, the layer of cells was washed with PBS (salin phosphate buffer) and then subjected to the analysis methods for: the evaluation of the total protein content using Lowry method modified by Oyama (Oyama et al., 1956) and tracing of the protein dynamics; the cytometrical assessment of the total cell number with Türk haemocytometer on the basis of the formula: N = n x d . 104, where, N= total cellular number; n = number cells from a square of 1/25; d = dilution of 2 the mathematical estimation of the cell proliferation inhibition: % mitoinhibitory impact = Nt / Nm x 100, where: Nt = treated sample cells number; Nm = control sample cells number; the cytometrical assessment of the alive and dead cells number by trypan blue exclusion test (Doyle et al., 1998); the mathematical estimation of the % cytotoxicity = Ncmt / Nctt x 100,where: Ncmt = living cells number of the treated sample; Nctt = total cells number of the treated sample (Doyle et al., 1998); mathematical evaluation of cell cultures degree after the action of the hellebore extracts, the inhibition of this last process representing their cytostatic effect upon cell protein biosynthesis and cell mitosis, as well as their cytotoxic action upon cell viability.

The cytostatic property signification of the studied biopreparations was appreciated on the basis of the American prescreening program, which imposed a minimum induced inhibitory impact of 50% for the *in vitro* selection of the

potential antitumoral agents (Leiter et al., 1965). For each culture type and time interval, five culture tubes were used and the results were evaluated statistically by Student's test (Cann, 2002).

RESULTS AND DISCUSSIONS

The *in vitro* action of some total aqueous and hydroalcoholic polyphenolic extracts and of their concentrate or permeate fractions, separated by membranary micro- and ultrafiltrations of the primary homogenates, obtained from *Helleborus purpurascens* ethnomedicinal plant, upon the cell protein biosynthesis, proliferation, viability and development of the HeLa cancerous cells was investigated in the present work.

Thus, in a first experimental model, the reactivity profile of the cell proteinsynthesis process of the HeLa tumoral cells cultures was outlined by the registered total protein concentrations and dynamics during their evolution period in the presence of the tested biopreparations

It can be seen, in figure 1, that the control group presents a 50% progressive augmentation of the total protein content in untreated HeLa neoplastic cells cultures, from 24 hours age up to 72 hours age. The protein dynamics of the untreated tumoral cell cultures has presented an ascendant route with increasing amplitude These characteristics of the untreated cell cultures are the expression of an inherent proteinsynthesis enhancement, conditioned by the cell proliferation process, which assures a double number of functional cells and, respectively, the normal development of the control cultures, appreciated by us as reference percentage value (100%).

In all ten variants, the cultures incubated with the total hydrous polyphenolic extract or with its derivated fractions have presented lower protein concentrations than control values, which attended the statistical and cytostatical significance both at 48 hours and at 72 hours, after 24 and 48 hours treatments with the bioactive preparations. Therefore, we assist at decline of the cell proteinbiosynthesis, induced by the tested agents. The proteinsynthesis inhibitory impact of the diverse bioproducts is characterized by different manifestation degrees, its intensity depending both on the separation mode (micro- or ultrafiltration) of samples and the fractionated type (concentrate or permeate).

The UF1P-HphE polyphenolic fraction has determined the biggest reductions of the total protein concentrations (19.79 and 10.91 μg protein/culture, respectively) to the 48 and 72 hours old HeLa cultures, treated 24 and 48 hours with this agent. The impact of the others polyphenolic samples (HphE, MF-HphE, UF1C- HphE, UF2C- HphE, UF2P- HphE, UF3 C and P- HphE, UF4 C and P- HphE) upon the protein biogenesis of the HeLa cultures was more attenuated, but still significant. The successive graphical transposition of the total proteins value, obtained at different time intervals of treated cell cultures evolution, traces their proteinsynthesis dynamics. Contrary to the controls, in the case of the treated cancerous cultures, it is observed that the protein dynamics is characterized by a descendent route and by a decrease amplitude, the most visible modifications being cronologically registered in the case of UF1P-HphE, UF2C- HphE, UF2P, MF-HphE, UF4 C and P- HphE, UF3 C and P- HphE and UF1C- HphE biopreparations.

The negative changes of the cell protein contents and dynamics, have demonstrated the induction of a very significant proteinsynthesis inhibitory impact, which confirms the cytostatic property of these bioproducts. The total protein content in the HeLa cells incubated with total hydrous extract (HphE variant) was similar both after 24 and 48 hours treatment indicating a blocking of protein biosynthesis process in a short period after incubation. This result is consistent with data from literature according to which 2 hours of contact between cell cultures and polyphenols, such as gallic acid, are sufficient to induce apoptosis (Inoue et al., 1994).

Significant alterations of the protein biosynthesis were also registered during the 48 and 72

hours evolution of the HeLa cultures treated either with a total hydroalcoholic extract or with its permeate and concentrate derivated fractions. These modifications are suggested by the negative quantitative variations, which have different degrees of expression, the amplitude of the cytostatic potential being dependently on the bioproduct type used in cell treatments.

During the entire investigated time interval (72 h), the cell cultures treated with different hydroalcoholic polyphenolic preparations, as compared to the control values, were characterized by significantly smaller protein contents (μg protein/culture), confirming the perturbation of the tumoral cells protein biogenesis.

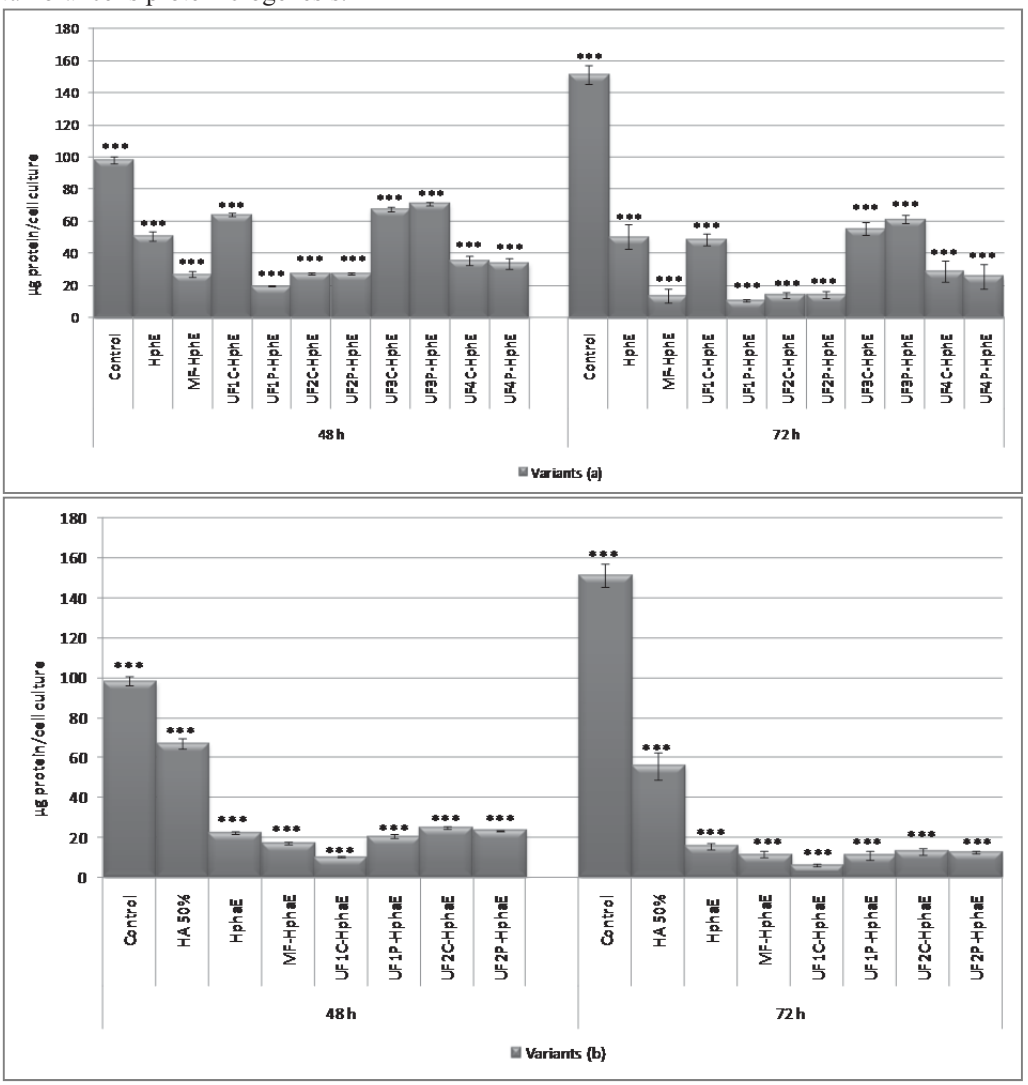

Fig. 1. The total protein content (μg/culture) and proteinsynthesis dynamics of the HeLa cancerous cell cultures treated with total hydrous (a) and hydroalcoholic (b) polyphenolic extracts from *Helleborus purpurascens* and their concentrate or permeate fractions (15μg/mL), during a 72 hours of evolution. Significantly different from control: *p<0.05, **p<0.01, ***p<0.001

This profound negative impact of hydroalcoholic bioproducts upon intracellular protein biogenesis, with maximum expression at UF1C-HphaE extract – and not UF1P-HphE – is also outlined by the descendent route and very low amplitude of the proteinsynthesis dynamics. The significant cytostatic potential of all hydroalcoholic polyphenolic products seems to be somewhat higher than that of hydrous samples, this difference being attributed to the extractive agent (HA 50%), which has himself a cytotoxic impact.

In a new series of tests, we have investigated the reactivity of HeLa cells' proliferation process, expressed by the total cell number evolution during the development time of treated cultures, which has revealed the sense and extent of the bioactive extracts' interference with this cellular process. The mean values of the evaluation index of the cell mitosis, registered at different ages of the control and treated cell cultures, are included in Table 1.

In the case of control HeLa cell cultures, it can be observed a progressive augmentation of the total cell number from 24 hours age up to 48 hours age, which assures the normal development of this culture type. Instead, the 48 hours old HeLa cell cultures, treated 24 hours with the hydrous hellebore extracts, are characterized by a decrease of the total cells number, comparatively with the corresponding control value. This numerical decline signals a regression of the cellular proliferation rate – due to the hellebore extracts – which expresses the mitoinhibitory property of these bioproducts, their potential being variable from one preparation to another. Although all treated HeLa cultures, with different hydrous extracts, have presented statistic and cytostatic significant decreases of total cells number, six extracts (MF- HphE, UF1P-HphE, UF2C-HphE, UF2P-HphE, UF4C-HphE and UF4P-HphE) have been characterized by an antiproliferiative effect of 61.05 - 76.29%, these values being superior to the 50% minimum reference level imposed by the American prescreening program for the appreciation of a new drug as potential antitumoral agent.

Table 1. The total cells number variation and cellular proliferation reactivity of the HeLa neoplastic cells incubated with the hydrous and hydroalcoholic biopreparations (in a dose of 15 μg/mL), obtained from *Helleborus purpurascens*. In brackets is indicated the number of experimental cultures for each type

Variant	24 h	48 h		% Proliferation rate	% Antiproliferative degree
	X●10^3 ± ES	X●10^3 ± ES	p		
Hydrous extracts					
Control	152.34±2.3(5)	358.98±11.23 (5)	–	100	-
HphE	152.34±2.35 (5)	194.25±10.10 (5)	<0.001	54.11	45.89
MF-HphE	152.34±2.35 (5)	117.51±5.6 (5)	<0.001	32.73	67.27
UF1C-HphE	152.34±2.35 (5)	240.22±4.12 (5)	<0.001	66.92	33.08
UF1P-HphE	152.34±2.35 (5)	85.10±5.10 (5)	<0.001	23.71	76.29
UF2C-HphE	152.34±2.35 (5)	118.92±6.03 (5)	<0.001	33.13	66.87
UF2P-HphE	152.34±2.35 (5)	120.04±4.12 (5)	<0.001	33.44	66.56
UF3C-HphE	152.34±2.35 (5)	251.95±4.32 (5)	<0.001	70.18	29.82
UF3P-HphE	152.34±2.35 (5)	263.83±6.23 (5)	<0.001	73.49	26.51
UF4C-HphE	152.34±2.35 (5)	139.83±5.00 (5)	<0.001	38.95	61.05
UF4P-HphE	152.34±2.35 (5)	133.78±6.00 (5)	<0.001	37.27	62.73
Hydroalcoholic extracts					
Control	152.34±2.35 (5)	358.98±11.23 (5)	–	100	-
HA 50%	152.34±2.35 (5)	250.02±7.89 (5)	<0.001	69.65	30.35
HphaE	152.34±2.35 (5)	93.95±3.69 (5)	<0.001	26.17	73.83
MF-HphaE	152.34±2.35 (5)	75.44±4.36 (5)	<0.001	21.02	78.98
UF1C-HphaE	152.34±2.35 (5)	51.58±2.14 (5)	<0.001	14.37	85.63

Variant	24 h	48 h		% Proliferation rate	% Antiproliferative degree
	X●10^3 ± ES	X●10^3 ± ES	p		
UF1P-HphaE	152.34±2.35 (5)	87.83±4.21 (5)	<0.001	24.47	75.53
UF2C-HphaE	152.34±2.35 (5)	103.74±3.89 (5)	<0.001	28.90	71.10
UF2P-HphaE	152.34±2.35 (5)	97.91±6.21 (5)	<0.001	27.27	72.73

The total number of the tumoral HeLa cells, from the cultures' componence incubated with the hydroalcoholic bioproducts, is much lower than control, demonstrating a strong mitoinhibitory potential of these extracts. The mitoinhibitory impact of the 50% HA was of about 30%. In the light of this reference value, the antiproliferative level of the total and fractionated hydroalcoholic products was ranged between 51% (UF2C-HphaE) and 79.37% (UF1C-HphaE), both limits being superior to the 50% minimum standard level, imposed by the American prescreening program.

Some experimental studies have highlighted that the mitoinhibitory effect of various total polyphenolic extracts, upon cancerous cell lines, could be induced by polyphenolic glycosides of bufadienolide type, which were also isolated from underground vegetal organs of the *Helleborus* sp (Bassarello et al., 2008; Kemertelidze, 2008; Muzashvili et al., 2006).

In an another *in vitro* experimental model, was followed the action of the total polyphenolic hellebore extracts and of their filtrated fractions upon cell viability, expressed by numerical differences between alive and dead cells. Using this parameter we assessed the cytotoxic potential of all investigated biopreparations upon HeLa cell cultures.

As illustrated in the below Figure 2, the control cultures have been characterized by a major number of alive cells and very few dead ones, their cell viability being in around of 98.23%. Contrary, the treated cultures have presented preponderantly death cells, the significant descreases of cell viability ranging from one type of hellebore extract to another.

Thus, the total hydrous polyphenolic extracts as well as the concentrate and permeate derived fractions have induced either a nonsignificant low cytotoxic impact – in the case of HphE (10.2%), UF1C-HphE (2.23%), UF3C-HphE (13.71%) and UF3P-HphE (9.64%) extracts– or a significant strong cytotoxic effect, which was reported in the case of MF- HphE (54.14), UF1P-HphE (65.40%), UF2C- HphE (63.89) and UF2P- HphE (50.95%) extracts, between these limits enrolling UF4C and P – HphE samples, which generated a moderate declin of the cell viability (43.15 and 45.61%).

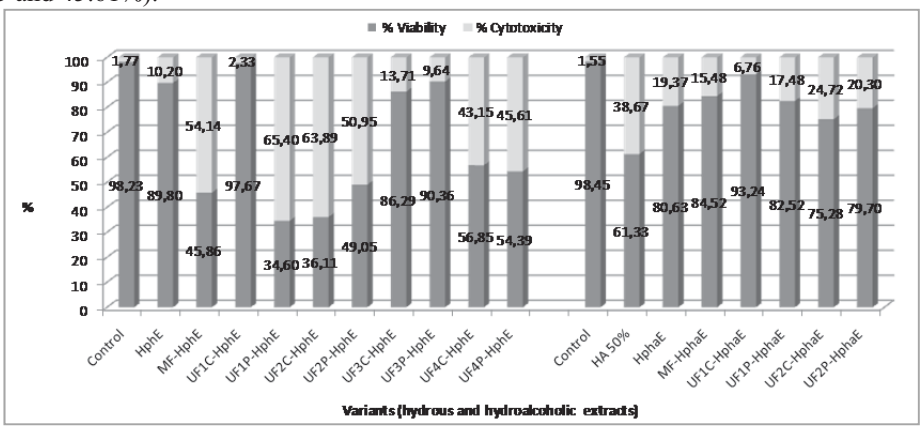

Fig. 2. HeLa cancerous cell cultures viability and the cytotoxic impact of the total and fractionated, hydrous and hydroalcoholic hellebore biopreparations (15 μg/mL).

The level of the viability regression of the HeLa cell cultures subjected to the hydroalcoholic hellebore treatment has reached values ranging between 6.76% (UF1C-HphaE) - 24.72% (UF2C-HphaE), all revealing a moderate cytotoxic effect. In the light of the cytotoxic impact of the HA 50% vehiculating agent (38,67%), we can suppose that the normal cytotoxic potential of the compounds is attenuated by the presence of diluted ethanol, which was used as extractant solvent.

Like in the case of the proliferation process, it can assume similar action patterns of the studied agents. In a same context, Watanabe et al., 2003 revealed that some glucosidal bufadienolides were cytotoxic both upon the several neoplastic cell lines and on healty cell cultures, while the rhamnosidal bufadienolides had higher specificity on cancerous cells than on normal ones. On the other hand, in vitro administration of the aqueous extracts from Helleborus niger has been correlated with induction of sister chromatid exchanges, which is the molecular mechanism of their property to destabilize the DNA and to cause apoptotic cell death in various cancerous cell lines (Büssing. and Schweitzer, 1988, Jesse et al., 2009).

A last *in vitro* experimental model was used for investigation of the consequences of hellebore’s interaction with the HeLa neoplastic cells upon the cell cultures development degree, the percentage values of this index being included in Figures 3 and 4.

The neoplastic cell cultures incubated with the total or fractionated hydrous hellebore extracts have reached various levels of cultures development process. In comparison with the development degree of control cell cultures, considered as the 100% reference value, we observed significantly diminished percentage values of this process, which allowed highlighting and evaluation of an inhibitory impact of the polyphenolic samples upon cell cultures development In relation to their inhibitory potentials on HeLa cell cultures development, the ten extracts can be grouped as follows:

- the biopreparations which exert a very strong cytostatic effect – over 90% (MF-HphE, UF1P- HphE, UF2C-HphE and UF2P-HphE), with very low limits of variation (from 90.57% - UF2P-HphE, to 90.81% - MF-HphE);
- the concentrate and permeate fractions, obtained after the fourth ultrafiltration, which have an significant inhibitory rate of 80.91% (U43C HphE) and 82.96% (UF4P- HphE), respectively;
- the biopreparations which induce an inhibition of tumor cell development level smaller than 70%: HphE, UF1P- HphE, UF3C HphE and UF3P- HphE, the limits of variation ranging from 59.46% (UF3P- HphE) to 67.80% (UF1P- HphE).

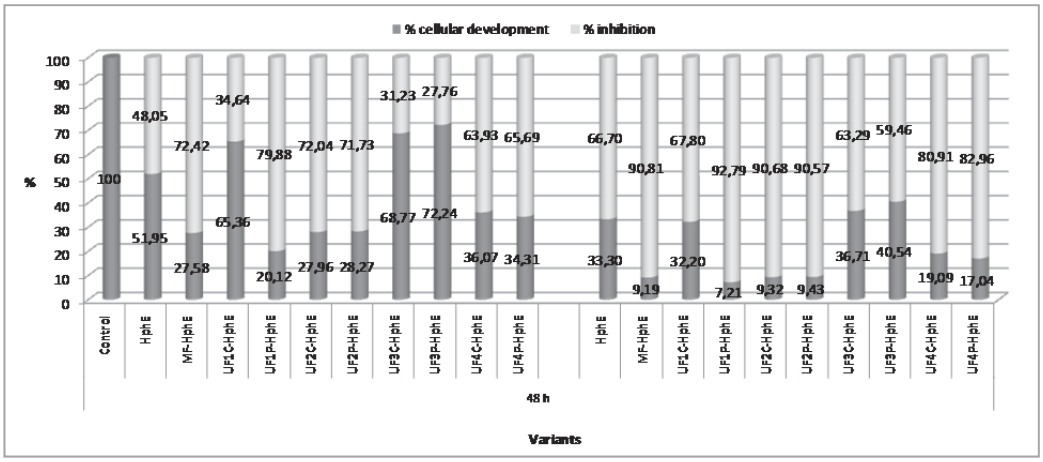

Fig. 3. The development degree of HeLa tumor cell cultures during their evolution in presence of *Helleborus purpurascens* total and fractionated hydrous extracts (in a dose of 15 μg/mL) and their corresponding inhibitory impact, comparatively to the control ones

Therefore, indifferently of the used hydrous extract, we have emphasized the capacity of all the tested hydrous polyphenolic extracts to inhibit the development of HeLa cell cultures, their action levels being statistically and cytostatically significant.

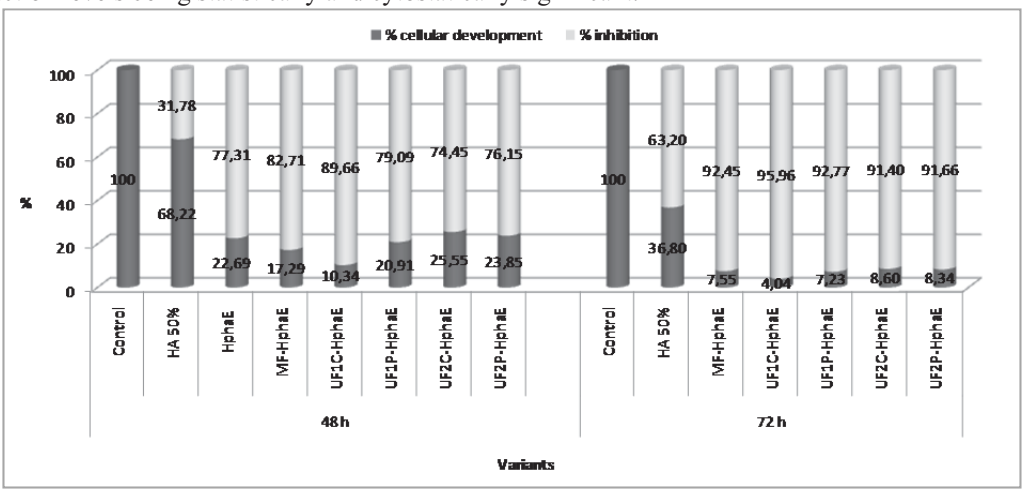

Fig. 4. Variations of the Hela cell cultures development level and the corresponding inhibition degrees, after the polyphenolic treatment with 15 μg/mL from total and fractionated hydroalcoholic extracts, as compared with untreated tumoral cultures

In vitro treatment of HeLa cancerous cell cultures, with the total and fractionated hydroalcoholic Helleborus extracts, has conditioned a more pronounced decrease of cell cultures development after 24 and 48 hours incubation, this potentiation of effect being, probably, due to the presence of HA 50% in the componence of the samples, himself inducing a perturbation of cell cultures development of 31.78 and 63.2%. Thus, the maximum inhibition rates, above 90%,

were registered at the 72 hours old treated cultures (Fig. 4) and have characterized MF- HphaE, UF1C- HphaE, UF1P- HphaE, UF2C- HphaE and UF2P- HphaE.

The inhibitory impact upon HeLa cancerous cell cultures development – this last aspect expressing *in vitro* antitumoral effect, which remember us the *in vivo* tumoral regression – which has characterized both total hydrous or hydroalcoholic hellebore extracts and their permeate or concentrate fractions, is consequence of cell proteinosynthesis and proliferation alterations, as well as of aggression upon cellular viability induced by the tested polyphenolic samples.

The inhibitory potential of the bioactive extracts upon HeLa cultures development is dependent on the used extract - the most active being UF1P- HphE and UF1C- HphaE– this can be probably amplified by the prolongation of the *in vitro* treatment period.

The cytostatic potentials of the total hydrous or hydroalcoholic hellebore extracts are inferiorly that ones of microfiltrates and some ultrafiltrates (UF 1, 2, 4- permeates). These observations confirm the opportunity of utilization of the membranary micro- and ultrafiltration techniques of the total extracts, which assure a specific repartition of the bioactive polyphenolic compounds in the different fractions and an *in vitro* various cytostatic effectiveness.

The analysis of our experimental results has highlighted the indubitable interference of the hydrous and hydroalcoholic, total and fractionated polyphenolic biopreparations, extracted from *Helleborus purpurascens*, with cell proteinbiosynthesis, mitosis processes and cell viability of the HeLa neoplastic cells. The inhibitory impact upon cell proteinsynthesis, the antiproliferative effect, the decrease of cell viability have conditioned the regression of the cell cultures development, this *in vitro* antitumoral action expressing the *in vitro* strong cytostatic and moderate cytotoxic properties of these bioactive extracts.

The action pattern of the bioactive preparations is similarly, but the *amplitudes of the in vitro* cytostatic and cytotoxic potential are different from a sample to another, the polyphenolic agents being characterized by a superior cytostatic impact rather than a cytotoxic one. The complementarity of these actions suggested that the vegetal polyphenolic extracts had a major *in vitro* antitumoral effectiveness. This conclusion is not hazardous if we take into account that *in vitro* American prescreening program imposes the induction of a minimum inhibitory impact upon the cell cultures developmentof at least 50%.

Our results, corroborating with the specialty literature (Cunha-Filho et al., 2010), have argued that the autochthonous total or fractionated, hydroalcoholic or hydrous polyphenolic hellebore extracts may be included in the category of cytostatic and cytotoxic agents, which, finally, could enrich the oncochemotherapeutic arsenal.

CONCLUSIONS

In vitro investigation of the interaction of some total and fractionated polyphenolic preparations, extracted from roots and rhizomes of *Helleborus purpurascens* perennial medicinal plant with HeLa cancerous cells has highlighted a decrease of cell proteic content, a regression of total cell number during evolution of the cultures, a moderate increase of dead cells number, a negative perturbation of cell cultures development.

All these observations have suggested a cytostatic and cytotoxic impact expressed by the inhibition of cellular proteinsynthesis, by alterations of protein dynamics, by mitostatic effect, by cell viability decrease and by the cell cultures regression.

The negative repercussions upon some cytophysiological processes of HeLa neoplastic cells allow us to characterize some polyphenolic biopreparations as potential cytostatic and cytotoxic agents.

Preliminary characterization of our total or fractionated polyphenolic samples as potential cytostatic agents imposes the extending and thoroughgoing of the *in vitro* research, on other human malignant and normal cell lines, in order to: prove their cytostatic effect reproducibility and the dependence of cytostatic effectiveness on the treatment doses; highlight another cell, subcell and molecular effects of these natural biopreparations and their probably action mechanism; evaluate the reactivity degree of the normal cells to the action of these new cytostatic products.

The elucidation of cytostatic property of some autochthonous polyphenolic biopreparations will impose their introduction in the *in vivo* screening circuit, on tumor-bearing animals, in order to qualitative and quantitative pharmacological evaluation of their antineoplastic pharmacodynamic effect. The final pharmacological characterization of a polyphenolic bioproduct as antineoplastic agent with possible clinical significance will provide a new pathway of superior capitalization of *Helleborus purpurascens* medicinal plant.

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SOME COMORBIDITIES OF BENZODIAZEPINE ADDICTED PATIENTS

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Keywords: benzodiazepine, alcohol, anxiety disorders.

Abstract: Comorbidity represents the diagnosis of one or more disorders in addition to the primary disease in a person for a certain period of time. In other words, comorbidity refers to a simultaneous process not related to the main pathological process. However, patients with addiction to alcohol or other drugs have anxiety comorbidity which requires an efficient and appropriate pharmacotherapy.

INTRODUCTION

Benzodiazepine addiction and abuse may be often associated with the abuse of other substances (such as alcohol, cannabis, cocaine, heroine, methadone, amphetamines). Sedatives are frequently used for relieving the adverse effects of these substances (Midmer et al. 2006). Contrary to their wide margin of safety when used alone, if benzodiazepines are taken with alcohol they are extremely dangerous, and accidental overdose were also described (Skurtveit et al. 2008).

By repeated use looking for the euphoric effect, patients develop tolerance to the sedative effect, and progressively, bigger doses are used. However, tolerance to the depressing effects on the brain stem is developed much slower and the patient consumes increasingly more substance to become euphoric which may trigger respiratory depression and low blood pressure that might threaten the life of the patient (Mohammadi et al. 2006).

In most cultures, alcohol is the most frequently used brain depressant and the cause of a significant morbidity and mortality. It is believed that 90% of adults from USA have had some experience with alcohol at a certain time of their life and a large number of them had one or several adverse life events related to alcohol (Chirita et al. 2002, Klein-Schwartz et al. 1991).

MATERIALS AND METHODS

The group study was composed of 134 patients (ages ranging between 20 and 66 years), 49 male and 85 female, selected among benzodiazepine addicted patients, admitted in Clinic V of Socola Psychiatric Hospital from Iasi, during the period January 2009 – December 2010. Before their initial evaluation, patients were explained the structure of the study. The patients included in the study agreed and gave an informed consent, which was obtained before performing any procedure of the study.

In the case of our study, the consumption of other substances was pointed through the interview of the patients and caregivers. Also, the data obtained had a more limited frequency than what it is described in literature.

RESULTS AND DISCUSSION

Alcohol, as associated risk factor in our study, shows no statistically significant differences ($\chi^2 = 0,03$; GL = 1, p = 0,853).

In the studied cases, the statistical processing confirmed the significant association between anxiety disorder patients and alcohol consumption (**p=0,0002**), the relative risk of anxiety being nearly 6 times higher in the case of alcohol consumption (RR=5,85; IC95%: 2,21 – 15,49).

Table no. 1. Statistical differences in the distribution of patients with anxiety disorders according to vicious habits

Characteristic	Anxiety disorder		None		Test χ^2
	n	%	n	%	
Alcohol consumption					
Yes	12	9,0	27	20,1	$\chi^2 = 14,02$; GL = 1; **p = 0,0002**
No	5	3,7	90	67,2	
Smoking					
Yes	7	5,2	23	17,2	$\chi^2 = 2,81$; GL = 1; p = 0,093
No	10	7,5	94	70,1	

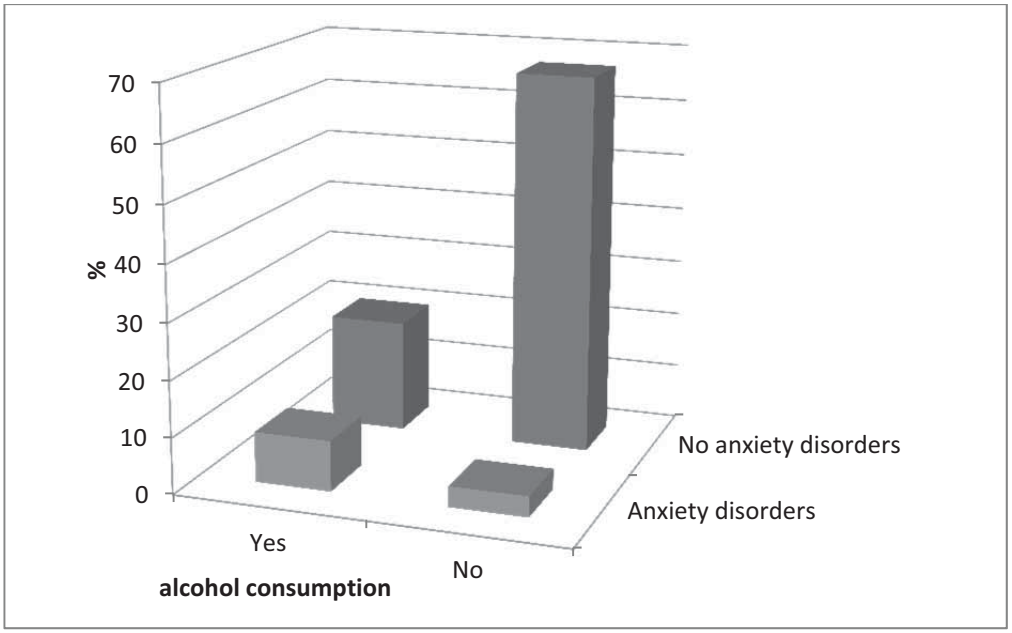

Fig. no. 1. Distribution of patients with anxiety disorders according to alcohol consumption

Smoking patients with anxiety disorders showed no statistically significant distributions. (p=0,093).

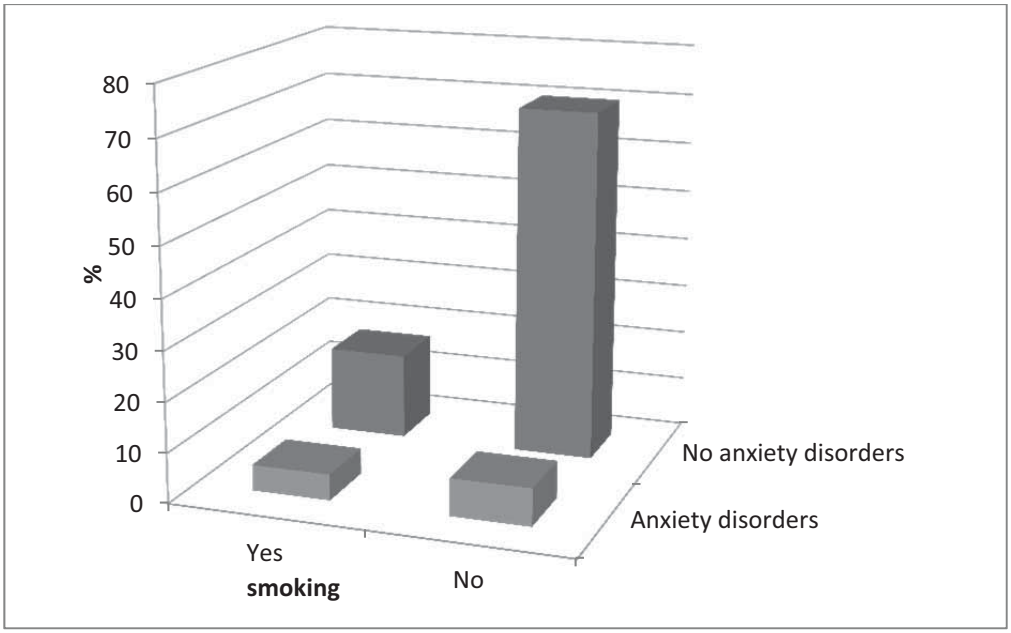

Fig. no. 2. Distribution of patients with anxiety disorders according to smoking

Smokers or those who acknowledged alcohol consumption represent approximately equal shares in patients with or without major significant depression (p=0,579 *vs* p =0,874).

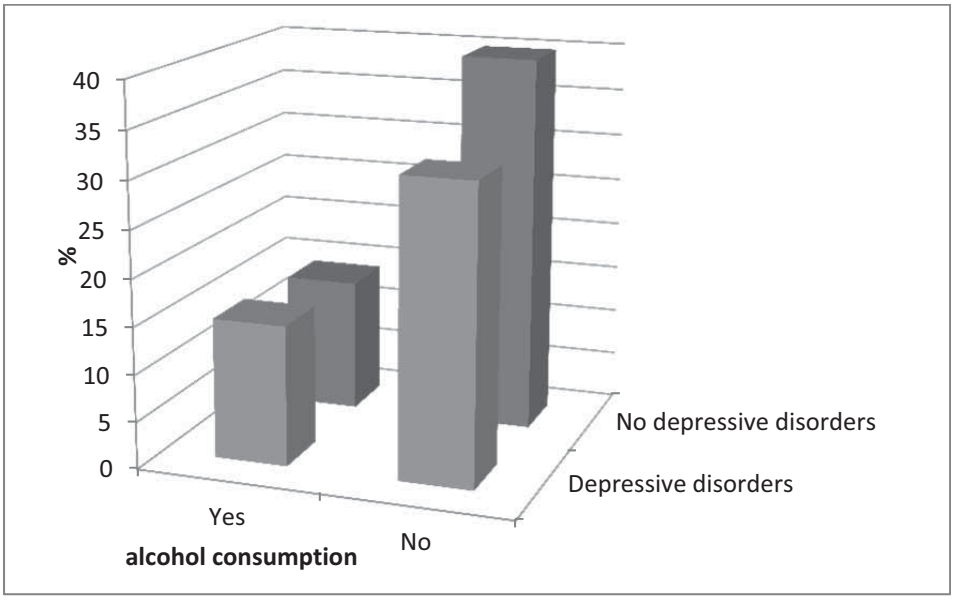

Fig. no. 3. Distribution of patients with major depressive disorder according to alcohol consumption

Smoking patients with anxiety disorders showed no statistically significant distributions (p=0,093).

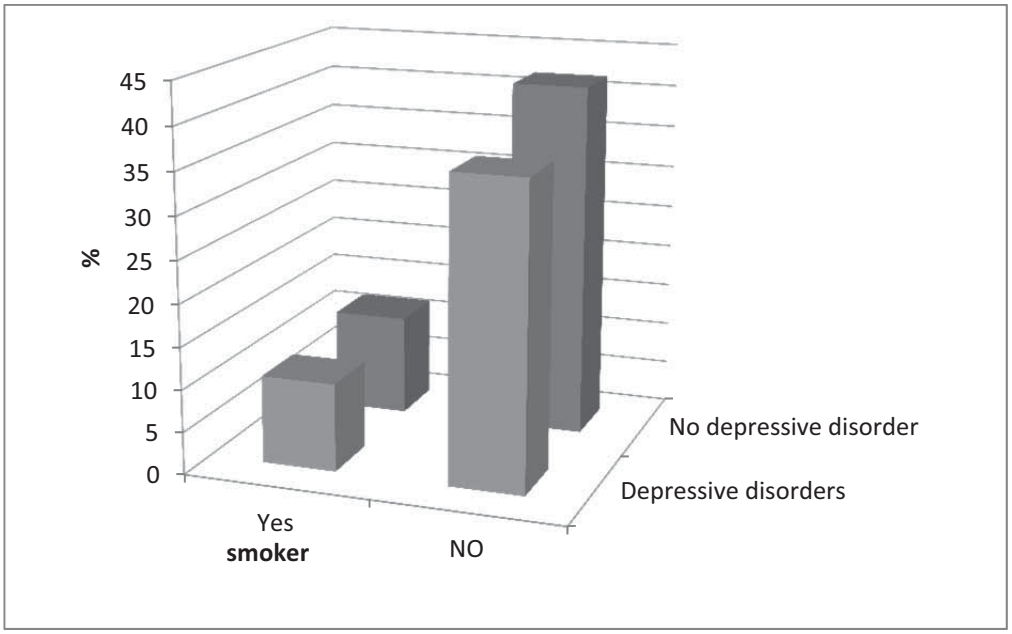

Fig. no. 4. Distribution of patients with major depressive disorder according to smoking

Table no. 2. Statistical differences in the distribution of patients with major depressive disorder according to their vicious habits

Characteristic	Depressive disorders		None		Test χ^2
	n	%	n	%	
Alcohol consumption					
Yes	20	14,9	19	14,2	$\chi^2 = 0,31$; GL = 1; p = 0,579
No	42	31,3	53	39,9	
Smoking					
Yes	14	10,4	16	11,9	$\chi^2 = 03$; GL = 1; p = 0,874
No	48	35,8	56	41,8	

In the studied cases, the statistical analyses confirmed the significant association between personality disorder patients and alcohol consumption (**p=0,0002**), the relative risk of anxiety being 3 times higher in the case of alcohol consumption (RR=3,23; IC95%: 1,57 – 6,63).

Smoking patients with personality disorders showed no statistically significant distributions in comparison with those not having this vicious habit (p=0,067).

Table no. 3. Statistical differences in the distribution of patients with personality disorders according to their vicious habits

Characteristic	Anxiety disorder		None		Test χ^2
	n	%	n	%	
Alcohol consumption					
Yes	14	10,4	25	18,7	$\chi^2 = 3,23$; GL = 1; **p = 0,002**
No	10	7,5	80	59,7	
Smoking					
Yes	9	6,7	21	15,7	$\chi^2 = 3,37$; GL = 1; p = 0,067
No	15	11,2	95	70,9	

The statistical processing of behavioral parameters in the case of patients with sleep disorders showed that a significantly higher number of patients associate sleep disorders with smoking (**p<0,001**), and not with alcohol (p=0,761) which was also pointed out in specialty studies.

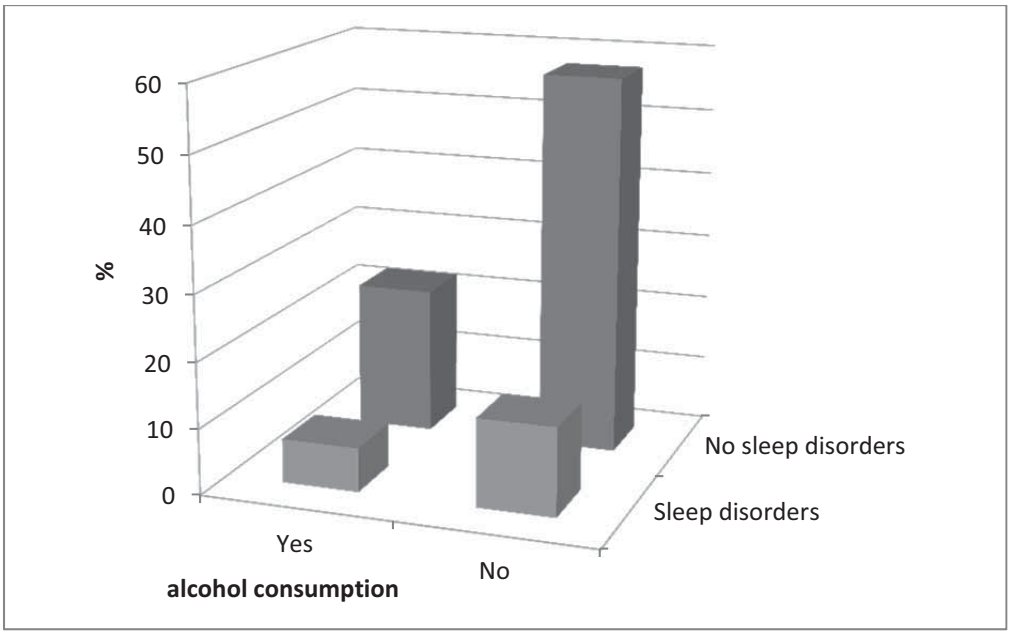

Fig. no. 5. Distribution of patients with sleep disorders according to alcohol consumption

CONCLUSIONS

Patients with addiction to alcohol or other drugs have anxiety comorbidity which requires an efficient and appropriate pharmacotherapy. Consequently, for such patients, cautiousness should override the prohibition of anxiolytic prescription. Nevertheless, although the incidence of benzodiazepine non-medical use is quite low in comparison to their legitimate medical use, which is very widespread, the issue of addiction and the risk of benzodiazepine addiction remains of utmost importance in the psychiatric, hospital and ambulatory practice, as well as its appropriate treatment.

The motivation for choosing this study was the fact that this type of studies may be used for making analogies with other populations or regions (by series of comparable studies performed on various populations) or for documenting evolution in time by means of a number of comparable studies conducted on the same population.

Moreover, this kind of study may be useful for: drawing public and political attention to the extent of a community issue, for service planning, for finding existing as well as necessary needs, describing the impact of a disease in a population, the level of disabilities associated with the disease, service demand and economic costs.

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PRELIMINARY STUDY OF MOLECULAR VARIABILITY FOR NEOLITHIC PIG (*SUS SCROFA DOMESTICUS*) FROM ROMANIA USING THE CYTOCHROME B

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Keywords: mitochondrial DNA, cytochrome b, *Sus scrofa domesticus*, Neolithic

Abstract: The aim of the present study is to reveal molecular differences between old and actual populations of pig (*Sus scrofa domesticus*), based on the analysis of hyper variable first part of cytochrome b. Neolithic pig remains (bone fragments) are sampled from Poduri-Dealul Ghindaru *Tell* (Bacau County). The *Tell* of Poduri-Dealul Ghindaru (positioned in the Eastern part of Romania) has a complex stratigraphy and the archaeologists have carried out analyses on Chalcolithic (Precucuteni and Cucuteni) and Bronze Age levels.

INTRODUCTION

The Neolithic represents one of the most important times of Prehistory, when mankind achieved spiritual and physical progress, whose legacies can be traced up to modern times. One of the most important changes in human behaviour, during the Neolithic, is the transition from the way of predator and consumer, to the way of producer of food resources and other goods, thus making the new age mean more than just a “food revolution”. During the transition from the Mesolithic to the Neolithic, an important element in the evolution of human communities was, besides the beginnings of plant cultivation, the ability of taming wild animals, which would later be domesticated and raised. This domestication is a fundamental acquisition of the Neolithic way of life, whose premises are found in the final stage of the Upper Palaeolithic when human communities achieved an advanced form of hunting. The beginnings and the ways of domestication are still debateable because the morphological differences between the domestic and wild animals are not clear but the step forward achieved by the process of domestication is obvious (Boghian, 2003). In recent times, based on DNA studies, a phylogenetic tree of modern domestic species can be constructed. Analyses of DNA extracted from fragmentary remains of skeletons and teeth provide an important insight into the ancestry of domesticates (Moritz *et al*., 1987; Nei & Kumar, 2000; Reitz & Wing, 2008; Haile *et al*., 2010).

Our most important domesticates originated from south-western Asia in the area known as the Fertile Crescent and then spread following a northwest or northeast direction. Because of the environmental conditions, a series of modifications occurred in the conformation, variability and behaviour of domestic animals (Reitz & Wing, 2008). In the domestic fauna of Europe, cattle (*Bos taurus*) and pig (*Sus scrofa domesticus*) were predominant and were also abundant in the wild form, some researchers even assuming the possibility of local domestication and re-domestication of aurochs (*Bos primigenius*), as in the case of the wetland, forest and steppe Neolithic, and wild boar (*Sus scrofa ferus*). The eventual domestications would have contributed to the increase of local livestock or to its regeneration following battles, natural disasters and plagues (Boghian, 2003). Pigs also suffered some domestication adaptations even if some archaeozoologists have shown that they could have been raised in a state of semi-domestication. It is important to show that the domestication of animals did not stop at the Neolithic stage and, after a period of the previously acquired human knowledge consolidation, this process continued.

Cucuteni Culture appeared, evolved and spread in eastern part of Romania during a millennium, 4600-3500 Cal B.C. (Mantu, 1998), and it is divided in three chronological phases: *A*, *AB*, and *B*. Cucuteni Culture developed in the Chalcolithic period, representing the end of the Neolithic and the transition to Bronze Age. The Cucuteni culture is one of the most representative for the Romanian Chalcolithic, known especially for its decorative and figurative art. The Cucuteni culture still is an important topic in prehistory research because of the complexity of the uncovered artefacts and the spatial and social organization elements. This is one of the reasons why a synthetic archaeozoological analysis was necessary (Cavaleriu & Bejenaru, 2009).

The *Tell* of Poduri-Dealul Ghindaru (Bacau County) has the following position: 46º47’’ North latitude and 26º53’’ East longitude, with an absolute altitude of 429 m. The *Tell* is situated on a fragment of the terrace of 30 m on the right bank of the Tazlau Sarat River and it currently has a surface of around 1.2 ha. The high complexity of the stratigraphy was emphasized along over 27 archaeological excavations campaigns that took place so far. There were reported levels belonging to the Precucuteni and Cucuteni Chalcolithic cultures and to Bronze Age.

Using the morphometric differentiation in *S. scrofa*, 16 different subspecies have been described in the past (Groves, 1981). Groves and Grubb (1993) classified the European wild boar into a group of ‘western races’, including the subspecies *S. s. scrofa* (Central-Western Europe), *S. s. meridionalis* (Sardinia and Corsica), *S. s. attila* (Eastern Europe)

and *S. s. lybicus* (Southern Balkans). Nonetheless, multivariate morphometric of skull measurements suggested the existence of a single subspecies (Genov, 1999) with a possible north-east to south-west dimensional cline due to environmental variation and its effect on food availability and growth rate (Randi *et al*., 1989). In addition, the wild boar is the ancestor of the domestic pig, with which it shares a close genetic affinity and can hybridize. Thus, genetic introgression from the domestic pig may represent a further source of differentiation throughout the natural range of the wild boar, and may represent a major threat to its genetic integrity (Scandura *et al*., 2011).

Many researchers have sampled European wild boars, for studies with various purposes, and so, most of the data are scattered. No molecular survey has yet been carried out to evaluate the overall genetic variation throughout Europe, on the basis of wide and comprehensive sampling. Our goal is to focus on the relationship between pigs from different historical periods, by evaluating the results obtained with first part of cytochrome b as molecular marker.

MATERIALS AND METHODS

Samples' description

For this study we analysed pig remains (bone fragments) sampled in 2007 from the Cucuteni *A* level within the *Tell* in Poduri-Dealul Ghindaru, dated between 4460-4050 Cal B.C. (Mantu, 1998; Monah *et al*., 2003). With a view to comparing, we also analysed bone fragments from Middle Ages sites and muscle tissue from modern specimens (Table 1). Anatomical and taxonomical primary identification was made according to archaeozoological methodology (Udrescu *et al*., 1999).

Mitochondrial DNA isolation and purification

The bone fragments have been previously washed with a solution of sodium hypochlorite and exposed to UV rays for 12 hours. The DNA template purification was performed using the DNA IQ Kit (Promega) for forensic analysis and quantified by agarose gel electrophoresis and by spectrophotometry.

DNA amplification and Sequencing

According to complete the fragments containing the first part of cytochrome b (approximately 500 bp) two primers L14724 (5′-CGAAGCTTGATATGAAAACCATCGTTG-3′) (Pääbo, 1990) and H15149 (5′-AACTGCAGCCCTGAATATTTGTCCTCA-3′) (Kocher *et al*., 1989) were used for this study. The PCR protocol was carried out in a 25 µl volume using 1XGoTaq Green Master Mix (Promega), 2 mM $MgCl_2$, 0.2 mM each dNTP, 10 mM of each primer, GoTaqDNA polymerase 1.25U. The amplification process was performed in an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles at 95°C for 1 minute, 45°C for 1 minute and 72°C for 1 minute. The amplicons were tested by 1% agarose gel electrophoresis, purified from the gel through Wizard SV Gel and PCR Clean-up System columns (Promega) commercial kit, following the producer's protocol. Purified PCR products were sequenced with the both PCR primer, due to the fragments length, following the Methods Development Kit (Beckman Coulter) protocol and the ethanol precipitation. Samples were sequenced with 8 capillaries CEQ 8000 Beckman Coulter analyser.

Table 1. *Sus scrofa domesticus* samples' type and origin.

Sample ID	Samples' age	Archaeological site	Tissues type
01S1	2009	-	muscle
01S2	2009	-	muscle
31S1	2009	-	muscle
31S2	2009	-	muscle
SR	Middle Ages (IV-VI century A.D.)	Slava Rusa (Tulcea County)	bone fragment
2667	Neolithic (4665-4050 B.C.)	Poduri-Dealul Ghindaru (Bacau County)	bone fragment

Sequence alignment and phylogenetic analysis

For phylogenetic inference, one alignment corresponding to the concatenation of all gene sequences was created. Sequences of each fragment were automatically aligned using the Clustal W method (Thompson *et al*., 1994) and MegAlign software (Lasergene v.7; DNASTAR Inc., Madison, WI).

Phylogenetic analysis including neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) trees of DNA sequence alignments analysis were conducted using Paup 4.0b10 (Swofford, 2003) using PaupUp graphical interface (Calendini & Martin, 2005). Genetic distances used in NJ trees are Kimura two-parameter model distances with a transition: transversion ratio of 2:1. Non-synonymous/synonymous substitutions distances (dN/dS) ratios, as described by Nei & Gojobori (1986), were calculated using MEGA4 software (Tamura *et al*., 2007). Bootstrap analysis was made with 1000 replicates except in ML where only 100 replicates were generated. Hierarchical likelihood ratio tests were conducted using a batch file supplied with MODEL TEST 3.7 (Posada & Crandall, 1998) to provide the evolutionary models used in ML and Bayesian analysis. In total, the data set were analysed under three criteria, described below.

In Maximum parsimony (MP) analysis characters were unordered and equally weighted and gaps were treated as missing data (“?”). Analyses started with a stepwise addition tree with taxa randomly added in 5000 replicates. During searches branches were collapsed when minimum branch length was zero. Trees of MP analyses resulting in more than one most parsimonious tree were summarized in strict consensus trees. Branch support values for combined data sets were estimated with nonparametric bootstrap values (BP; Felsenstein, 1985) (5000 replicates).

For Maximum likelihood (ML) we used the Akaike Information Criterion (AIC) as implemented in Model Test (Posada & Crandall, 1998) to choose substitution models for the entire partition. ML analysis for the data set was run using HKY (Hasegawa *et al*. 1985) model.

RESULTS AND DISCUSSIONS

The first part of mitochondrial cytochrome b (cyt b) was amplified by PCR and sequenced in both directions in all tested individuals. The amplicons were tested by agarose gel electrophoresis (Figure 1). The electrophoregram analysis reveals fragments of approximately 500 base pairs (bp) for all analysed individuals. The amplicons resulted from the PCR reaction after purification process, presents a sequencing optimal amount of DNA only for 6 individuals: 01S1, 01S2, 31S1, 31S2, 2667 and SR. All others individuals have a very low amount of total DNA due to fragmentation process present in old bones fragments, unusable for sequencing.

The 300bp sequences were analysed and aligned for haplotypes identification. Based on the alignment process 6 different haplotypes were analysed and characterized. The haplotypes were compared and the similarity and divergence matrix was achieved (Table 2). Comparing the similarity percentage for the pig individuals they vary between 99.7% for present specimens, 69.9% comparing present specimens with the Middle Ages individuals and 67.2% for the Neolithic pig compared with present ones.

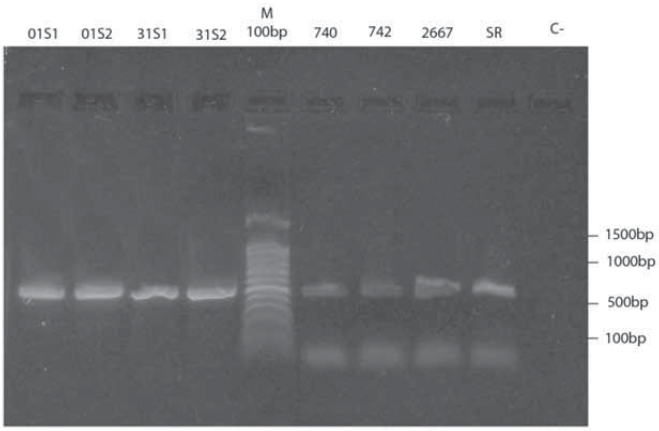

Figure 1. The first part of cytochrome b amplicons.

Table 2. The similarity and divergence percentage for the analysed haplotypes.

	Similarity Percentage								
Divergence Percentage		01S1	01S2	31S1	31S2	HM1047	2667	SR	
	01S1		100.0	99.7	99.7	100.0	76.7	69.9	01S1
	01S2	0.0		99.7	99.7	100.0	76.7	69.9	01S2
	31S1	0.3	0.3		99.3	99.7	77.0	69.6	31S1
	31S2	0.3	0.3	0.7		99.7	76.3	69.6	31S2
	HM1047	0.0	0.0	0.3	0.3		76.7	69.9	HM1047
	2667	28.1	28.1	27.6	28.7	28.1		67.2	2667
	SR	39.1	39.1	39.7	39.7	39.1	43.9		SR
		01S1	01S2	31S1	31S2	HM1047	2667	SR	

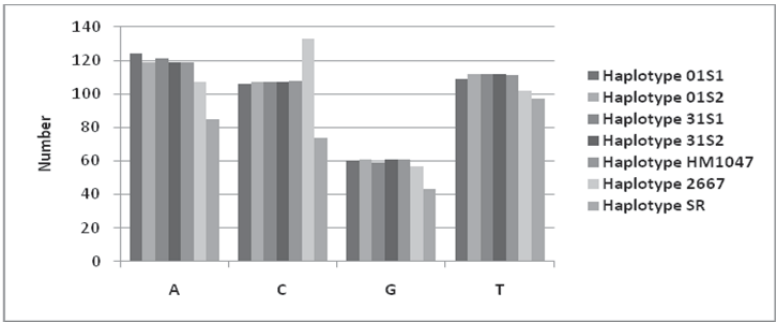

Figure 2. The nucleotides frequencies for the analysed haplotypes.

The statistical analysis of all identified haplotypes based on Clustal W alignment report was performed for the conserved, variable and parsim-informative sites (Table 3).

Table 3. Sequences statistics.

	Conserved sites	Variable sites	Parsim-info sites	Singleton sites	0-fold degenerate sites	2-fold degenerate sites	4-fold degenerate sites
Number	37	262	55	207	180	10	14
Frequency	12.3	87.6	18.3	69.2	60.2	3.3	4.6
Total	299						

Table 4. Estimates of codon-based evolutionary divergence between sequences.

	01S1	01S2	31S1	31S2	HM1047	2667	SR
01S1							
01S2	0.0						
31S1	0.0	0.0					
31S2	0.0	0.0	0.0				
HM1047	0.0	0.0	0.0	0.0			
2667	45.6	45.6	45.6	45.6	45.6		
SR	38.5	38.5	38.5	38.5	38.5	46.0	

The number of synonymous differences per sequence from analysis between sequences is shown (Table 4). All results are based on the pair wise analysis of 7 sequences. Analyses were conducted using the Nei-Gojobori method in MEGA4. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 84 positions in the final dataset.

Also, from the haplotypes comparison, the nucleotides number and frequencies have been analysed (Figure 2). The adenine is the most frequent in all present haplotypes, followed by thymine and cytosine. For the Neolithic specimens, the cytosine has highest frequency followed by adenine and thymine. The similarity dendrogram (Figure 3) was build using the MegAlign module of Lasergene software and conclude about the origin of present pig (*Sus scrofa domesticus*), in Neolithic forms of the same genera.

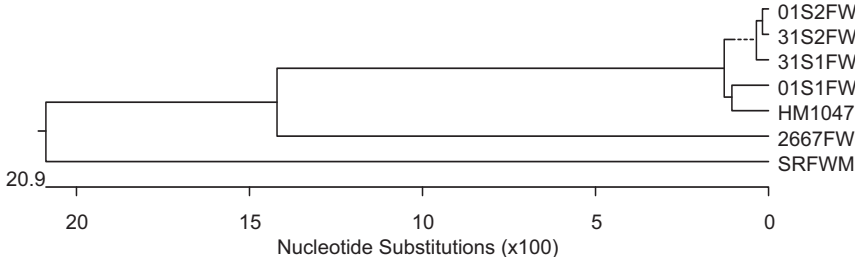

Figure 3. Phylogenetic tree of the analysed haplotypes

The evolutionary history was inferred using the Maximum Parsimony method (Figure 4) (Eck *et al*., 1966). Tree #1 out of 25 most parsimonious trees (length = 346) is shown. The consistency index is (0.982456), the retention index is (0.982143), and the composite index is 0.979304 (0.964912) for all sites and parsimony-informative sites (in parentheses). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (5000 replicates) are shown next to the branches (Felsestein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei & Kumar, 2000) with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). The codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 299 positions in the final dataset, out of which 55 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

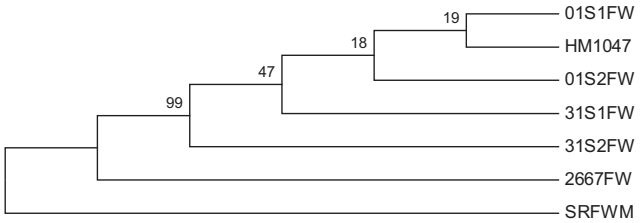

Figure 4. Evolutionary relationships of 7 taxa.

CONCLUSIONS

The results confirm the potential and the applicability of molecular techniques for inferring the phylogenies based on mitochondrial DNA sequences even from ancient bone fragments and the ability to identify the major lineages and the evaluation of homology degree between different species and subspecies of the same genera.

The sequence alignment and the phylogeny show high differentiation between individuals of *Sus scrofa* species from different historical periods of time.

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CONSIDERATIONS ON THE RELATIONSHIP BETWEEN CHROMOSOME CONSTITUTION AND BIOCHEMICAL PHENOTYPE IN FIVE ECOTYPES OF SEABUCKTHORN

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Keywords. *Hippophae rhamnoides* L., karyotype constitution, phenotype variability
Abstract. Seabuckthorn is a small tree showing pronounced morphological, physiological, biochemical and genetic variability, high ecological plasticity and large limits of resistance to unfavourable factors and to phytopathogens. It is largely exploited in biotechnological, nutritional, and pharmaceutical purposes, cosmetics domain and in environmental protective field. The possibility that some karyotype traits of five seabuckthorn ecotypes to be used as markers in relation with some specific biochemical features was discussed in this paper. There is intraspecific chromosome variability; the formula of haploid complement is different concerning the preponderance of chromosome morphotypes. Also a marked chemical heterogeneity was evidenced. At this research stage, the results not allow us to establish a direct relationship between some chromosome characteristics and certain morphological and biochemical parameters.

INTRODUCTION

Hippophaë rhamnoides L. is a dioecious species, with a marked morpho-physiological polymorphism and with the possibility to fix atmospheric nitrogen due to symbiotic mycorrhizal *Frankia* fungus (Kato *et al*., 2007; Kanayama et al., 2008). The seabuckthorn capacity to fix nitrogen is twice higher than that of soybean (Lu, 1992). The average amount of nitrogen fixed in seabuckthorn forest is 30 - 60 kg/ha/year (Zike *et al*., 1999) or 180 kg/ha/year, according to Stobdan *et al.* (2008). Seabuckthorn has been largely used to improve eco-environments.

The special characteristics of this small tree are exploitable in biotechnological, nutritional, and pharmaceutical purposes or in cosmetics domain and in environmental protective field (Raţi and Raţi, 2003). Besides pronounced morphological, physiological, biochemical and genetical variability, seabuckthorn shows a high ecological plasticity and large limits concerning resistance to unfavourable factors and to phytopathogens. This plant was mentioned as medicinal plant in traditional Tibetan pharmacopoeia (Persson, 2001). More than 100 phytonutrients and bioactive substances have been evidenced in seabuckthorn leaves and berries (Ahmad and Kamal, 2002), these organs being rich in carbohydrates, organic acids, carotenoids, tocopherols, sterols and other lipids, tannins, amino acids, vitamins (C, E, B1, B2, F, K, P, provitamin A) etc. A protein content of 30% and the presence of polyphenols, fatty acids, alkaloids, cellulose and microelements (P, Ca, Mg, K, Fe, Na) also amplify the value of seabuckthorn preparations. Vitamin C content largely ranges in berries (Yao and Tigerstedt, 1992; Li and McLoughlin, 1997), it exceeding more times the content of dog rose hips, orange, kiwi, hawthorn, tomato and other berries like strawberry, raspberry and blackberry. Leaves contain 11 to 22% crude protein, 3 to 6% of crude fat and some flavonoids (Lu, 1996).

The complex composition results in utilization of seabuckthorn preparations in prevention and treatment of some diseases such as flu, cardiovascular troubles, gingivitis, mucosa injuries, skin problems (Negi *et al*., 2005; Chauhan *et al.*, 2007). The fruits, leaves and juices have protective actions against hypertension and coronary heart disease; also hyperinsulinemia and dyslipidemia can be ameliorated or modulated by seed purified flavones (Pang et al., 2008). The flavonols and triterpenoids have as effect the inhibition of proliferation of some tumour cells (Hibasami et al., 2005; Yasukawa *et al*., 2009; Grey *et al.,* 2010).

It seems that the most of the pharmacological effects of seabuckthorn and its health benefits may be partly attributed to their high content of phenolic compounds, as phenols possess a wide spectrum of properties such as antioxidant, antimicrobial, antimutagenic and anticarcinogenic potential (Negi *et al*., 2005; Ercisli *et al*., 2007; Pang *et al.*, 2008). Phenolic fractions are responsible for free-radical scavenging activity and for DNA protection (Goel *et al*., 2005), the predominate polyphenols being represented by flavonols. More probably, the numerous health benefits are the result of synergy among many different bioactive components in the plant parts (Yang, 2009).

Additionally to these effects and uses, high quality wines, jams, jelly’s, squash, powder juice, butter, ferments, tea and other healthful foods and syrups are prepared form the fruits of seabuckthorn (Lu, 1992; Shigri, 2001).

The aim of this paper is to discuss on the possibility that some karyotype characteristics of five seabuckthorn ecotypes to be used as markers and to be correlated in a reliable manner with some specific chemical phenotypes.

MATERIAL AND METHODS

Seeds from individuals of five *Hippophaë rhamnoides* Romanian ecotypes (noted as HR-L3; HR-L4; HR-B8; HR-Bu2; HR-S16) were used as biological material for cytogenetic investigations. The germination was carried out at 22°C, in dark. At 10-15 mm length, the root tips were pretreated with 8-hydroxyquinoline (0.002 mol/L), for 4 h and were fixed in ethanol-acetic acid mixture (3:l) for 24 h at room temperature. The plant material was stored in refrigerator, at 4^0C, in 70 % alcohol.

In view of analysis, the root tips were hydrolyzed in 50 % hydrochloric acid for 8 minutes. A modified carbol fuchsin solution (Gamborg and Wetter, 1975) was an effective stain for seabuckthorn chromosomes. The squash preparations were obtained in 45 % glacial acetic acid. Microscopic investigation was carried out by a Nikon Eclipse 600 microscope. For morphometric analysis, the cells with well-spread metaphase chromosomes were photographed with digital camera Cool Pix Nikon, 1600x1200 dpi, 100x objective. The images were processed by Adobe Photoshop programmer.

Chromosome measurements included *length of individual chromosomes* (C), *long arm length* (L), *short arm length* (S), *arm ratio*, r (r = L/S), *centromeric index*, CI (CI=S/C x 100), and *the relative length of each chromosome, %* (C/length of haploid complement x 100).

Karyotypes were performed according to Levan *et al*., 1964 nomenclature. The chromosome homology and the establishment of the chromosome types were assigned on the basis of centromere position, respectively on CI and r values: the chromosomes are metacentric (r<1.70, CI=37.5-50.0), submetacentric (r=1.70–2.99, CI=37.5–25.0), subtelocentric (r= 3.00–6.99, CI=25.0–12.5), and telocentric (r=7.00–∞, CI<12.5).

Dry matter was determined by gravimetric method – biologic material is kept at 105°C to constant weight. The results are expressed in g dry matter/100 g fresh biological material.

Reducing carbohydrates were analyzed by method with 3,5-dinitrosalicylic acid based on the capacity of reducing carbohydrates to reduce 3,5-dinitrosalicylic acid to 3-amino, 5-nitrosalicylic acid, orange coloured, that is spectrophotometrically determined (Miller, 1959).

Total lipids are quantified by Soxhlet gravimetric method. The lipids are extracted at heat, by repeated washing (percolation), with specific organic solvents, under reflux in a special glassware. Results are expressed as g biological material/100 g dry matter (Artenie and Tănase, 1981).

Soluble protein content in enzyme extracts was established by Bradford method (Bradford, 1976). Method is based on binding of Coomassie Brilliant Blue G-250 solution to the amino acids radicals and recording of absorbance at λ = 595 nm using UV-VIS 1700 Spectrophotometer PharmaSpec - Shimadzu. Extraction of soluble protein from seabuckthorn fruits was performed using a Tris-HCl buffer containing dithiothreitol, ascorbic acid, EDTA, TRITON X 100 and cysteine.

Carotenoid pigments are determined by spectrophotometric method. Plant material is triturated with a mixture of reagents which retain the coloured compounds – other then carotenoids – and prevent the decomposition of these. Carotenoids are extracted with acetone and then in petrol ether. In final extract, the carotenoids are spectrophotometrically evaluated (Artenie and Tănase, 1981).

Assimilatory pigments content was measured by spectrophotometrical assay, after extraction with acetone in more steps. The chlorophylls were read at wavelengths specific to each pigment type.

Ascorbic acid determination was carried out by titrimetric method. Principle of this method consists in titration of ascorbic acid from plant extract with 2,6-dichlorophenolindophenol (2,6-DCPIP) solution having an accurately known titre. 2,6-dichlorophenolindophenol is reduced to its leucoderivate by ascorbic acid (Artenie and Tănase, 1981).

RESULTS AND DISCUSSIONS

Hippophae rhamnoides L. is an important plant in Romania, although its valences are not yet entirely exploited. In the last years, the research interest was focused on the realization of an inventory of Romanian seabuckthorn resources by their complex phenotypic and genotypic characterization, construction of a large theoretical and practical basis for the selection of valuable genotypes, and establishment of a germplasm national fund.

The high level of variability amplitude is the result of long term evolution and it constitutes the evolutive potential of the species, because it assures the basis for selection and amelioration activities. The marked polymorphism was revealed by a high number of genotypic and phenotypic studies; in last years the modern techniques of molecular biochemistry and

genetics were also used to determine the seabuckthorn genetic variability and to elucidate the genus taxonomy: *RAPD* (Random Amplified Polymorphic DNA) markers (Jeppsson *et al*., 1999; Bartish *et al*., 2000; Chowdhury *et al.*, 2000; Sheng *et al.*, 2006; Sun *et al.*, 2006), *cp DNA* (chloroplast DNA) (Bartish *et al.*, 2002), *ITS* (internal transcribed spacer) (Sun *et al.*, 2003), *AFLP* (Amplified Fragment Length Polymorphism) markers (Ruan and Li, 2005; Ruan, 2006), *ISSR* (inter-simple sequence repeats) markers (Tian *et al*., 2004), *DNA microsatellite loci* (Wang *et al.*, 2008), *intron sequences* (chalcone synthase intron – *Chsi*) (Bartish *et al.*, 2006). However, in spite of these numerous studies, there are still undeciphered zones concerning the seabuckthorn classification, genetic constitution and sex determination.

As previously was shown, the phenotypic and genotypic studies revealed a very large heterogeneity in all seabuckthorn provenances; the Romanian seabuckthorn resources are not the exception concerning the high morphological, biochemical and cytogenetic diversity in this species (Olteanu *et al*., 2009; Oprică *et al*., 2009; Truţă *et al.*, 2009; Zamfirache *et al*., 2009). This impressive phenotype heterogeneity depends on seabuckthorn origin, age, harvest moment, phenophase, methods used for extraction and determination, pedoclimatic factors and genotype characteristics (Li and McLoughlin, 1997; Li, 2002; Zeb, 2004).

Morphological and biochemical characterization is a conventional technique used for evaluation of the plant genetic diversity, although the morphological and biochemical traits are limited in number, are modified by the environment and may be controlled by epistatic and pleiotropic gene effects (van Beuningen and Busch, 1997). Despite these limitations, morphological and biochemical traits have been successfully used for genetic diversity analyses. Analysis of biochemical parameters in different seabuckthorn genotypes could therefore result in evaluation of genotype – phenotype relationship and in accumulation of useful information for selection of desired combinations in further breeding studies. A substantial part of the phenotypical variability is associated with the respective genotypes.

If literature regarding morphology and seabuckthorn chemical composition is very rich, there is a paucity of data on the chromosome constitution of this species. The cytogenetic studies establish the chromosome number of a species and help to decipher the morphological particularities of chromosomes and the metric characteristics of these, followed by karyotype construction. The pattern of chromosome formula, the presence of ploidy level, the existence of some chromosome anomalies can be discussed in relation with respective phenotypes and can direct the activities of selection and amelioration.

To conclude if it is possible that some of cytogenetic traits to be considered as markers in identification of one or more phenotype characters of bioproductive interest, we will comment on some aspects concerning the manner in which the chromosome variability reflects in phenotypisation of quantitative traits in analyzed ecotypes.

The detailed analysis of karyotypes evidenced a relatively high degree of intraspecific uniformity for all studied variables in the five studied ecotypes (Table 1). The metaphases of somatic cells displayed 24 chromosomes. 2n=24 is the diploid number repeatedly reported in the literature, the data being quasi-unanimous to sustain the existence of this chromosome number for all studied varieties, independently of their Asian or European provenance. Its widespread occurrence suggests that it is probably the true diploid number of *Hippophaë rhamnoides* species.

According to the values of arm ratios and centromeric indexes, the five karyotypes have exclusively metacentric (m) and submetacentric (sm) chromosomes, the metacentrics being more frequent; some differences are present in the proportion of these chromosome morphotypes (Truţă *et al.*, 2010, *in press*). Our data on the existence in seabuckthorn of only metacentric and

submetacentric chromosome types are in agreement with the reports of Cireaşă and Dascălu, 1983-1984 (2n = 8m + 16sm); Cao and Lu, 1989; Cimpeanu *et al*., 2004 (2n = 20m + 4sm).

The sea buckthorn chromosomes can be considered as small, they having sizes lower than 4 µm. Only HR-L8 ecotype has one chromosome pair exceeding 4 µm, all the other chromosomes having sizes smaller than 3.5 µm. The mean absolute length of individual chromosomes varies between 4.08 µm (HR-L8 ecotype) and 1.05 µm (HR-Bu2 and HR-L4 ecotypes). The length of haploid complement is comprised between 27.87 µm and 21.73 µm. No secondary constrictions and satellites were evidenced.

The results of analyses performed on seabuckthorn Romanian ecotypes confirm the great morphological, biochemical and cytogenetical variability of this species. Large phenotype heterogeneity was evidenced concerning colour (yellow, orange, or red), size and weight, shape (spherical, cylindrical, ovate, ellipsoidal, or irregular) and number of berries, their placement and degree of agglomeration on branch as well as the frequency and shape of leaves and thorns (Table 1). Thorns show large variation in terms of density, shape and sharpness.

Seeds also showed differences between ecotypes regarding their length, width and length/diameter ratio (Fig. 1), although these differences are not pronounced. Letea ecotypes (HR-L3, HR-L4, HR-L8) are close regarding the three parameters, comparatively with the other two analyzed ecotypes.

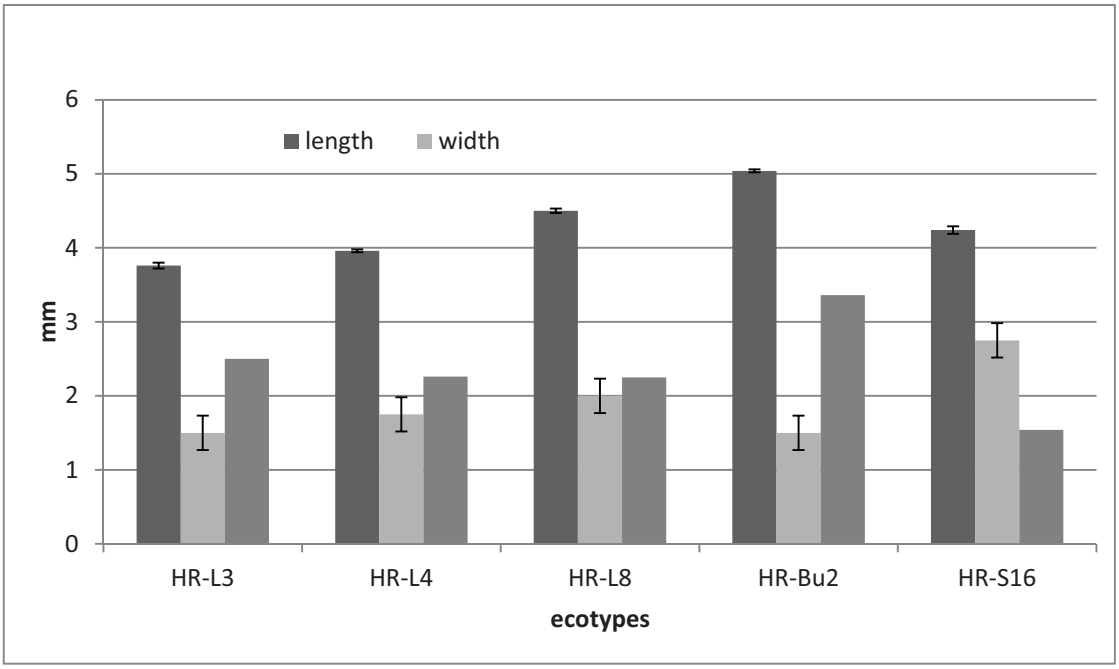

Fig. 1. Graphic representation of metric characters of seeds of *Hippophae rhamnoides* L. ecotypes (x±SE)

For example, they range in length from 3.76±0.04 mm (HR-L3) to 4.5±0.02 mm (HR-L8), with the length/width ratio ranging from 2.25 to 2.50, whereas this parameter is 3.36 for

HR-Bu2, respectively 1.54 for HR-S16. The mean length for HR-Bu2 ecotype is 5.04±0.02 mm and 4.24±0.05 mm for HR-S16 ecotype.

The biochemical results included in this study are an additional proof for the large scale of phenotypisation of studied characters, as reflection of genotype expression of each ecotype, in the specific environmental conditions. Next, a discussion on relation between chromosome constitution and some biochemical and morphological traits will be presented.

Karyotype characteristics of HR-L3 and HR-L8 ecotypes are very close. So, length of haploid complement is 27.87 μm, for HR-L3, respectively 26.98 μm, for HR-L8, whereas the formula of haploid complement is identical – n = 8m + 4sm – for both ecotypes. They are also similar concerning the average chromosome length (2.32±0.19 μm, respectively 2.24±0.23 μm). These features differentiate these ecotypes by HR-L4 which has a smaller length of haploid complement (HCL – 21.73 μm) and a different haploid chromosome formula – n = 11m + 1sm. The mean length of chromosomes is also different (x±Sx = 1.81±0.16 μm).

For these reasons it could be expected that phenotype expression of HR-L3 and HR-L8 ecotypes to be similar. On the contrary, morphological and biochemical indicators are different. Thus, HR-L3 has ovoid, yellow-orange fruits and small thorns (1-2 cm), while HR-L8 ecotype carried light orange, almost round and large sized fruits; rare leaves and 4-5 cm in length thorns. Among pulp chemical compounds, the first ecotype showed 14.84 mg% carotenoid, but the second had only half from this amount.

Also, the other constituents conferring value to seabuckthorn berries – ascorbic acid, carbohydrates, lipids, proteins – show different levels in the two ecotypes. The differences are significant for ascorbic acid (281.40 mg% for HR-L3, 405.20 mg% for HR-L8), pulp lipids (30.35 g% - HR-L3, 25.88 g% - for HR-L8), pulp protein (38.07 mg% – HR-L3, 22.39 mg% - HR-L8).

HR-Bu2 ecotype is characterized by phenotypisation at high levels of ascorbic acid content (698.96 mg%), exceeding 2 times that of HR-L8 (Letea karyotype with the biggest value of ascorbic acid), and of soluble protein (45.35 mg%), surpassing 2 times the protein amount of HR-L8 and HR-S16 ecotypes. However, it not shows – at least in the case of the present used analysis methods – any cytogenetic marker which allows us to establish a direct, definitive and undisputable relationship between genetic background and one trait of interest, in this case one biochemical character.

Although the cytogenetic parameters of HR-L4, HR-Bu2 and HR-S16 ecotypes are relatively similar (chromosomes are smaller than 3 μ, HCL is about 22 μ, two chromosome types are present) their morphological and biochemical parameters are very different. To argue this, the average values of some biochemical determinations are presented, in order for HR-L4, HR-Bu2 and HR-S16 ecotypes:

- ascorbic acid (mg%): 284.85 – 698.96 – 58.34
- lipids in fruit pulp (g%): 39.97 – 19.71 – 32.77
- protein in fruit pulp (mg%): 31.73 – 45.35 – 23.05
-protein in seeds (mg%): 33.28 – 97.00 -8.66.

Carotenoids range between 12.22 - 14.84 mg%, in HR-L4, HR-Bu2 and HR-L3, but show much smaller values in HR-S16 (3.84 mg%) and HR-L8 (7.25 mg%). Concerning carbohydrates, their limits of variability are small (1.47 – 1.66 g%).

The similarity of karyotypes pledges for the idea that the respective ecotypes are the expression of the same genotype constitution; they are in fact representatives of a single species.

Table 1. Karyotype and phenotype variability in five *Hyppophae rhamnoides* L. ecotypes (HCL=haploid complement length; CL=mean chromosome length; m=metacentric chromosomes; sm=submetacentric chromosomes; x±SE = mean±standard error)

ecotype	karyotype			morphological traits	chemical phenotype									
	2n	HCL (µm)	CL(µm) x±SE		ascorbic acid (mg%)	glucides in pulp (g%)	lipids (g%)		soluble protein (mg%)		carotenoids in pulp (mg%)	chlorophyll in leaf (mg/g)		dry matter –pulp- (g%)	water –pulp- (g%)
							seed	pulp	seed	pulp		a	b		
HR-L3	16m+8sm	27.87	2.32±0.19	ovoid, yellow-orange fruits; 1-2 cm thorns	281.40	1.55	30.35	21.23	38.07	33.35	14.84	4.18	1.46	19.13	80.87
HR-L4	22m+2sm	21.73	1.81±0.16	relatively small, orange fruits; long, thin thorns (8-9 cm)	284.85	1.47	39.97	17.33	31.73	33.28	12.22	4.78	2.29	19.24	80.76
HR-L8	16m+8sm	26.98	2.24±0.23	light orange; almost round and large sized fruits; rare leaves; thorns 4-5 cm	405.20	1.66	25.88	18.02	22.39	34.75	7.25	3.96	1.42	15.97	84.03
HR-Bu2	18m+6sm	22.57	1.88±0.17	numerous, intensely orange oblong fruits; discrete thorns; rare leaves	698.96	1.53	19.71	18.92	45.35	97.00	13.17	4.57	2.00	15.35	84.65
IIR-S16	18m+6sm	22.20	1.85±0.16	round, yellow fruits	58.34	-	32.77	17.83	23.05	8.66	3.84	-	-	20.04	79.96

*Except lipids, the other results are reported to fresh plant material

In this study, some differences in morphotype or chromosome size can be result from various factors such as the high chromosome stickiness and the reduced size of chromosomes which can generate errors in cytogenetic determinations. Because of the small and very small sizes of chromosomes, it is somewhat difficult to make a very exact determination of centromere position especially for the chromosomes smaller than 2 μm where the details are few distinguishable.

The phenotype heterogeneity both at morphological and biochemical level could be the consequence of a differentiated gene expression in some environmental conditions, specific for each ecotype; it is known that any phenotype character is a resultant of the circulation of hereditary information on genetic channel, in concrete environmental conditions.

This kind of approaches resuming to the analysis of morphological and biochemical characters not always provides clear responses in relation to genotype features.

For this reason, sooner or later, thoroughgoing molecular approach becomes necessary and obligatory to be made in order to identify some specific markers allowing the deciphering of still unsolved problems, although relatively recent studies have demonstrated that even relationships established on molecular markers do not always accurately agree with the phenotype reality (Fufa et al., 2005; Li et al., 2009).

For example, till now, utilization of molecular markers - especially RAPD - closely linked to sex determination is irrelevant. It is known that in seabuckthorn, it is very important to determine the plant sex before anthesis. For this reason, the identification of one specific genetic marker to allow early identification and removal of superfluous male plants may be helpful in seabuckthorn breeding programmes. But, in the research of Persson and Nybom, 1998, although in the F1 descendance of one cross, the RAPD marker was present both in male parental and in all male descendants and was absent in all female individuals, it can not be considered universal, because in F1 progenies of another cross it was present in only one of the males.

CONCLUSIONS

Analysis and interpretation of cytogenetic results in relation with morphological and biochemical traits of studied seabuckthorn ecotypes led to the following conclusions:

The five studied ecotypes have 2n=2x=24 chromosomes; chromosomes have small sizes (<4 μm), and karyotypes are symmetric.

There is intraspecific chromosome variability. Although two types of chromosomes are present (metacentric, with median placed centromere, and submetacentric, with submedian placed centromere), the formula of haploid complement is different concerning their preponderance.

Currently the results not allow us to establish a direct and reliable relationship between cytogenetical characteristics and certain morphological and biochemical parameters; approaches of molecular biology are necessary to be used to evidence specific markers.

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BEHAVIOUR OF ANTIOXIDATIVE ENZYMES AND OF SOLUBLE PROTEIN IN WHEAT SEEDLINGS AFTER LEAD-INDUCED STRESS

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Keywords. antioxidative enzymes, lead-induced stress, *Triticum aestivum* cv. *Maruca* L.

Abstract. The amplitude of antioxidative enzymatic response was investigated in *Triticum aestivum* cv. *Maruca*. Pb^{2+} was provided as solutions of lead acetate [$Pb(C_2H_3O_2)_2.3H_2O$] and lead nitrate [$Pb(NO_3)_2$], at four concentrations (10, 25, 50, 100 μM) containing 2.07, 5.18, 10.36, respectively 20.72 μg ml^{-1} Pb^{2+}. The results support idea that mainly superoxide dismutase and peroxidase are involved in the defence mechanism of wheat seedlings against Pb^{2+} toxicity, by scavenging reactive oxygen species. All Pb^{2+} concentrations enhanced SOD activity (the increase rates range between 24.59%-65.19%, for Pb^{2+} acetate, and between 20.88%-175.40%, for Pb^{2+} nitrate treated variants, comparatively to control). Pb^{2+} induced the decline of soluble protein level in all variants, indifferently of compound type and lead concentration.

INTRODUCTION

The problem of heavy metal toxicity acquired new dimensions in the industrial era. Besides the beneficial component, the progress of human society had destructive effects on environment, with disastrous repercussions on biological systems. Increment of civilization degree has meant the irrational exploitation of the nature, the increase of non-biodegradable waste, amplification of physical (radioactive, thermal, noise), chemical (heavy metals and other noxious agents) and biological (pathogen agents such as viruses, bacteria, fungi) pollution, increasing of greenhouse effect (global warming) by depletion of the earth’s stratospheric ozone layer etc. Heavy metals come from natural (volcanoes and continental dusts) and anthropogenic activities (mining operations, combustion of fossil fuels, metalworking industries, domestic garbage dumps, utilization of fertilizers etc.) resulting in their emission and accumulation in ecosystems. Such metals are released in the biosphere through air, water and soil and ultimately affect the plant, animal and human systems.

Lead is the most common heavy metal contaminant in aquatic and terrestrial ecosystems having various natural and anthropogenic sources (Sharma *et al*., 2005; Liu *et al*., 2009 a, b). It is naturally found in small amounts in the earth crust and is largely used in the production of containers of foods, stills, batteries, paints, and leathers. Human activities like burning of fossil fuels, mining, and manufacturing are lead sources. Its use as tetraethyl and tetra methyl additives in gasoline to increase octane rating has transformed lead into one of the metals of high toxic risk. In 1965 – 1990 lead consumption increased in the world to 5.6×10^6 tones (OECD, 1993), its concentration in biosphere being 1,000 – 100,000 times higher than the natural level (WHO, 1995). Since the half-life in biological systems is one of the longest among metals (150 – 5000 years), the consequences of lead pollution can be devastating.

Although lead has no known biological function, numerous investigations show that plants can accumulate lead via root and shoot. Important alterations have been reported in structure, biochemistry and physiology of plant cells in lead excess. In *Helianthus annuus* L., Pb^{2+} showed the highest phytotoxicity comparatively with Al, Cd, Cu, Ni, Pb and Zn (Chakravarty and Srivastava, 1992). This metal alters the transcriptional process, denatures the proteins (Rathore et al., 2007) and disturbs photosynthesis (Akinci *et al*., 2010). It causes changes in lipid composition of thylakoid membranes and modifies membrane permeability (Stefanov et al., 1995). Root elongation, plant growth, seed germination, transpiration, photosynthesis, mineral nutrition, plant water status and enzymatic activities can be also negatively influenced by lead treatment (Jiang and Liu, 2010; Kaznina *et al.*, 2005; Pinero *et al*., 2002).

An important feature of lead toxicity is the generation of reactive oxygen species (ROS) and free radicals, such as superoxide anion radical ($O_2^{\cdot-}$), singlet oxygen, hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) which cause oxidative stress to plants. Lead and other heavy metals promote oxidative damage not only by direct increasing of the cellular concentration of reactive oxygen species but also by the diminution of the cellular antioxidant capacity (Pinto et al., 2003). For a long time, ROS have been considered only as dangerous molecules, whose levels need to be kept as low as possible. Now it has been realized that they play important roles in the defence against pathogens, in plant development and in regulation of gene expression. Therefore, it is necessary for cells to control the level of ROS tightly, but not to eliminate them completely (Pitzschke *et al*., 2006). To minimize the damaging effects of ROS, aerobic organisms evolved non-enzymatic defence systems (ascorbic acid, reduced glutathione, carotenoids, tocopherols, flavonoids, alkaloids) and enzymatic protection mechanisms (superoxide dismutase, SOD, E.C. 1.15.1.1; peroxidases, POD, E.C. 1.11.1.7; catalase, CAT, E.C. 1.11.1.6).

Wheat is a plant of a worldwide economic importance, a main link in trophic chain and a pathway of pollutant ingestion for animals and humans. The main objectives of the present investigation are to evaluate the antioxidative response in *Triticum aestivum* L. cv. *Maruca* seedlings, by analyzing the activity patterns of antioxidative enzymes and the protein level after lead treatment, provided as lead acetate and lead nitrate. We tested two lead compounds, with important industrial uses, at different concentrations, to establish if the chemical structure in which lead is included induces significant differences in the studied parameters. *Lead acetate trihydrate*, the common form of lead acetate, is called *sugar of lead* and it is used as a mordant in dyeing and as a drier in certain paints. *Lead nitrate* is used in heat stabilization of nylon and polyesters, in coatings of photothermographic paper, in gold cyanidation, as oxidizer in the dye industry, as a metal stain for ultra-thin sections and as medical astringent.

MATERIAL AND METHODS

Plant material and treatment conditions. Biological material is represented by wheat seeds (*Triticum aestivum* L. cv. *Maruca*), Agricultural Research Station, Podu Iloaie, Romania). The seeds were 4 h treated with four concentrations (10 μM, 25 μM, 50 μM, 100 μM) for each lead compound. The lead concentrations (μg ml^{-1}) in each solution are presented in Table 1. In control, distilled water was used.

Table 1 Lead concentration in tested solutions.

variant	molar concentration of salt solution	lead concentration (μg ml^{-1})
Control – distilled water		
Lead acetate trihydrate, $Pb(C_2H_3O_2)_2.3H_2O$, mol. weight=379.33 g/mol	10 μM	2.07
	25 μM	5.18
	50 μM	10.36
	100 μM	20.72
Lead nitrate, $Pb(NO_3)_2$, mol. weight=331.20 g/mol	10 μM	2.07
	25 μM	5.18
	50 μM	10.36
	100 μM	20.72

Determination of antioxidative enzyme activities. The activity of antioxidative enzymes and soluble protein content were evaluated in 7 days old wheat seedlings.

Superoxide dismutase (SOD) activity was measured according to spectrophotometric assay (Winterbourn *et a*l., 1975) with slight modifications (Artenie et al., 2008), based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide radicals generated upon reoxidation of photochemically reduced riboflavin. Absorbance was recorded at λ=560 nm using UV-VIS 1700 Shimadzu PharmaSpec spectrophotometer (Kyoto, Japan). One unit of SOD is defined as the enzyme amount producing 50% inhibition of NBT reduction in the standard conditions.

Catalase (CAT) activity was assayed by Sinha's procedure with minor adaptations (Artenie *et al.,* 2008). The method principle is based on spectrophotometrical determination of chromium acid, obtained by reduction of $K_2Cr_2O_7$, in acid medium, in the presence of non decomposed H_2O_2, at λ=570 nm, using UV-VIS 1700 Shimadzu PharmaSpec Spectrophotometer.

Peroxidase (POD) activity was established by method of Gudkova and Degtiari (1968), based on the measurement of the colour intensity of product of o-dianisidine oxidation with H_2O_2, in the presence of peroxidase. Colour intensity is measured at UV-VIS 1700 Shimadzu PharmaSpec Spectrophotometer (λ=540 nm). The calculus of results uses the coefficient of micromolecular extinction (0.0128). One peroxidase unit corresponds to the enzyme amount catalyzing the decomposition of 1 μM H_2O_2 min^{-1}, in optimal conditions.

Determination of soluble protein content. Soluble protein content in enzyme extracts was established by Bradford method (Bradford, 1976). Method is based on binding of Coomassie Brilliant Blue G-250 solution to the amino acids radicals and recording of absorbance at λ = 595 nm. Protein content was established with a standard curve constructed with bovine serum albumin.

In order to compare the sensitivity of each parameter (enzyme activities, protein content), changes in these values were calculated as a percentage of their control value (set to 100%).

RESULTS AND DISCUSSIONS

Effects induced by Pb^{2+} on the activity of antioxidative enzymes in 7 days old wheat seedlings. Unlike iron, Pb^{2+} has no redox capacity. Therefore, lead-induced oxidative stress in treated plants seems to be an indirect effect of its toxicity leading to the production of ROS which enhance pro-oxidant status of cell by reducing the pool of reduced glutathione (GSH), activating Ca-dependent systems and influencing iron-mediated processes (Pinto *et al*., 2003). Increase of endogenous ROS levels and activation of antioxidant enzymes represent the most rapid indicators of oxidative stress resulted from the imbalance between production and elimination of ROS generated after lead treatment. Our investigations showed alterations in the activities of the three antioxidant enzymes in relation to lead exposure (Table 2).

Table 2 Increase/decrease rates of antioxidative enzyme activities and soluble protein levels in 7-days old wheat seedlings, after lead treatment.

Variant		SOD activity			CAT activity			POD activity		
		units mg^{-1} protein	%	–/+ rate (%)*	units mg^{-1} protein	%	–/+ rate (%)*	units mg^{-1} protein	%	–/+ rate (%)*
Lead acetate	control	4.31	100.00	0.00	393.82	100.00	0.00	6.24	100.00	0.00
	10µM	6.40	148.49	+48.49	284.30	72.19	-27.91	4.99	79.96	-20.04
	25 µM	6.75	156.61	+56.61	353.97	89.88	-10.12	7.78	124.67	+24.67
	50 µM	5.37	124.59	+24.59	446.32	113.33	+13.33	6.63	106.25	+6.25
	100µM	7.12	165.19	+65.19	350.84	89.08	-10.92	7.78	124.67	+24.67
Lead nitrate	control	4.31	100.00	0.00	393.82	100.00	0.00	6.24	100.00	0.00
	10µM	11.87	275.40	+175.40	559.73	142.12	+42.12	11.64	186.53	+86.53
	25 µM	5.58	129.46	+29.46	373.56	94.85	+5.15	6.94	111.21	+11.21
	50 µM	6.57	152.43	+52.43	317.67	80.66	-19.34	6.01	96.31	-3.69
	100µM	5.21	120.88	+20.88	355.06	90.31	-9.69	6.67	106.89	+6.89

*– decrease rate; + increase rate

Superoxide dismutase, the main scavenger of superoxide radicals, is a strong antioxidant which converts the toxic superoxide (O_2^-) to hydrogen peroxide and oxygen, by so called *dismutation reaction*: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. This enzyme represents the first line of cell defence against ROS generated by lead exposure, so preventing the tissue damage. Concerning SOD activity under lead stress (Table 2; Fig. 1), the treatment resulted in a considerable rise of enzyme activity in all variants, exposed either to lead acetate or lead nitrate, fact proving activation of wheat detoxification mechanisms. SOD increases are the result of the formation of superoxide radicals in lead exposed seedlings. This increment indicates the superoxide as being the central component of the signal transduction which triggers the genes responsible for antioxidant enzymes including SOD (Liu *et al.*, 2009b). SOD increased values were accompanied by significant lowering of protein level.

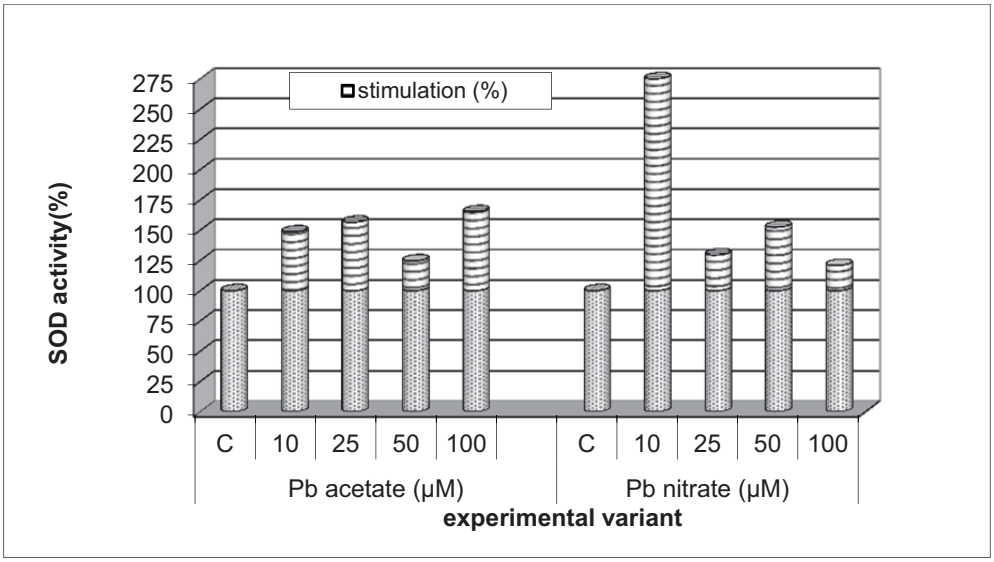

Fig. 1. Graphic representation of SOD activity in wheat seedlings, after lead treatment.

It was not observed a dose – SOD response relationship, but the differences from the controls are important. In 10 μM lead nitrate, SOD activity is 2.75 times higher than that of control (an increase of 175% in terms of percentage value) being an indicative of oxidative stress generated by superoxide radical production in the presence of Pb^{2+}. This concentration seems to be critical for wheat seedlings because this variant also showed the most increased activities of CAT and POD. CAT surpasses the control with more than 42%, while POD is almost 2 times higher than control. Soluble protein level has also one of the lowered values in 10 μM lead nitrate treated variant - 1.6 times smaller than control. The decrease both in peroxidase and catalase activity in 10μM lead acetate treated variant suggests a greater accumulation of H_2O_2 in the context of an amplified SOD activity. In literature, different trends of SOD activity (Dey *et al*., 2007) or significant increases of this enzyme (Pang *et al.*, 2001) have been noted in wheat seedlings exposed to lead stress.

SOD increase has been reported also in other plant species under lead stress, such as *Oryza sativa* (Verma and Dubey, 2003), *Medicago sativa* (Olteanu *et al*., 2008), *Sesbania drummondii* (Ruley *et al*., 2004), *Cassia angustifolia* (Qureshi *et al*., 2007), *Jatropha curcas* (Gao *et al*., 2009), and *Luffa cylindrica* (Jiang *et al*., 2010). SOD augmentation can be the result of two main factors: increase of amount of superoxide radicals and *de novo* enzyme synthesis which in turn can be associated with induction of SOD gene expression by superoxide mediated signalling transduction (Slooten *et al.,* 1995; Fatima *et al*., 2005).

Although lead was administrated as two different compounds, the effective level of metal was the same in the corresponding concentration variants (Table 1). The differences evidenced between correspondent variants of concentration can be due to the types of lead bindings to the other components in salt molecules.

Catalase is a major ROS-scavenging enzyme in all aerobic organisms, catalyzing the conversion of toxic H_2O_2 resulted in SOD dismutation reaction to H_2O and O_2, in peroxisomes.

In these conditions, SOD and CAT are complementary in their action to diminish the effects of oxidative stress. Depending on H_2O_2 concentration, catalase exerts a dual function (Scandalios, 2005). At low H_2O_2 concentrations (<1 μM) and in presence of increased levels of other substrata (ethanol, ascorbic acid etc.), catalase acts like a peroxidase: $RH_2 + H_2O_2 \rightarrow R + 2H_2O$. At high H_2O_2 concentrations, catalase degrades extremely rapid the hydrogen peroxide, by specific catalasic reaction: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$.

In the presence of Pb^{2+}, high levels of catalase activity have been reported in *Pteris vittata* L. (Fayiga *et al*., 2004) or *Sesbania drummondii* (Ruley *et al*., 2004), but in our experiments the mechanism of protection by catalase action is rather inefficient in wheat seedlings because, except the variant treated with 10 μM lead nitrate which shows a significant positive reaction and with 50 μM lead acetate which exceeds control with 13.33%, the others variants registered a more or less marked decline comparatively with control (Fig. 2). Lead-induced accumulation of H_2O_2 can result in the inactivation of catalase followed by decrease of its activity (Qureshi *et al*., 2007). CAT is more sensitive to Pb^{2+} than SOD and POD, as shows the decline in its activity. Decrease of CAT activity indicates a limited ability of this enzyme to eliminate the formed ROS and to decrease the oxidative state. Possibly, CAT is a less efficient H_2O_2 scavenger than POD because of its low substrate affinity.

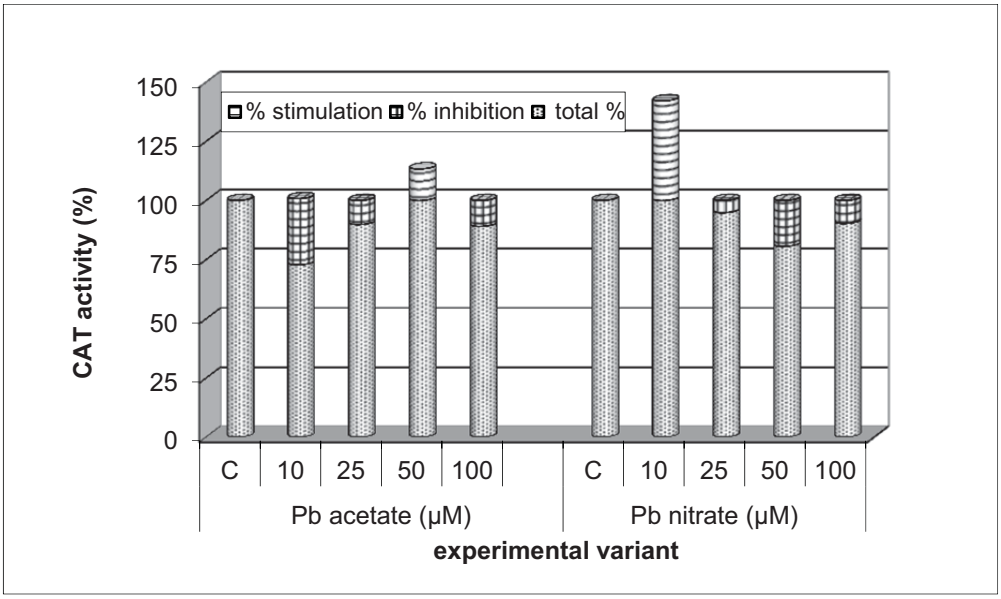

Fig. 2. Graphic representation of CAT activity in wheat seedlings, after lead treatment.

Data from literature differ on CAT behaviour in wheat seedlings exposed to lead treatment. Lead action determined either CAT decline (Dey *et al*., 2007) either moderate increases of this enzyme (Pang *et al*., 2001). The decline of CAT activity in Pb^{2+}-stressed plants can result not only from decrease of its synthesis but also from some changes in the assembly of enzyme subunits. Verma and Dubey (2003) observed lower intensity of two isozymic forms of catalase in

shoots of Pb^{2+}-stressed rice seedlings, consistent with decreased activity of the enzyme under Pb^{2+} treatment.

Peroxidase catalyzes the oxidation of many substrata (phenols, aromatic amines, ascorbic acid, glutathione, nitrites) in the presence of H_2O_2, with H_2O production: $AH_2 + H_2O_2 \rightarrow A + 2H_2O$. Peroxidase activity is considered as a potential biomarker of sublethal toxicity of heavy metals in plants, its role as a stress enzyme being widely accepted (Zhang *et al*., 2007). Peroxidase is stimulated by the accumulation of H_2O_2 in plant and it is able to scavenge this toxic compound. Compared to CAT, peroxidases possess a higher affinity towards H_2O_2, but have lower processing rate.

The pattern of peroxidase behaviour is different from that of catalase in our experiments (Fig. 3). Except 10 μM lead acetate treated variant, which shows an important POD decrease, and 50 μM lead nitrate, with small POD decrease, the other variants engaged this antioxidative defence system and POD levels registered more or less important increases. Since in contrast to CAT, POD activity increased, this enzyme seems to play a more significant role than CAT in detoxifying of the produced H_2O_2. POD augmentation can be also the result of release of those enzymes located in cell wall as response to the stress to which the plants are subjected (Gaspar *et al*., 1976).

Lead has been reported to induce peroxidase activity in soybean (Lee *et al*., 1976), rice (Verma and Dubey, 2003), *Sesbania drummondii* (Ruley *et al*., 2004), *Vicia faba* (Wang *et al.,* 2008) and *Luffa cylindrica* (Jiang *et al.*, 2010). In *Cicer arietinum* L. (cv. Radhey), successive increases and decreases of peroxidase activities were noted, depending on Pb^{2+} concentration (Reddy *et al*., 2005). In *Sonchus oleraceus* L., with increasing amounts of Pb^{2+}, the POD activity generally increased (Xiong, 1997), but in *Triticum aestivum* cv. *Maruca* it was not established a direct relation between increase of Pb^{2+} concentration and peroxidase activity.

In plants, multigene families encode the major antioxidant enzymes. This fact confers a great adaptive advantage by allowing a differential regulation of each gene family member in response to different endogenous and exogenous stimuli. Unlike most other organisms that have only one of each type of SOD in the various cell compartments, plants have multiple forms of each type encoded by more than one gene because they evolved more complex antioxidant defence strategies. As in the case of SOD, plant CATs are encoded by a small gene family constituted by three genes, as previously was described in maize, tobacco, cottonseed, *Arabidopsis*, and rice, whereas animals exhibit one CAT form. In *Eucalyptus grandis* L., 36 clusters as encoding antioxidant enzymes have been identified, 6 from these encoding POD isozymes, 3 encoding CAT proteins and 12 of them encoding SODs (Teixeira *et al*., 2005). Both *cat* and *sod* genes respond in a differential manner to various stresses known to generate ROS. This fact makes more difficult the explanation of some contradictory results relative to variable trends of the studied enzymes. Various behaviours of certain enzymes can be also due to the different degrees of tolerance or sensitivity of the plants to the heavy metal (Sharma and Dubey, 2005) or depend on tested organs (Jiang *et al*., 2010).

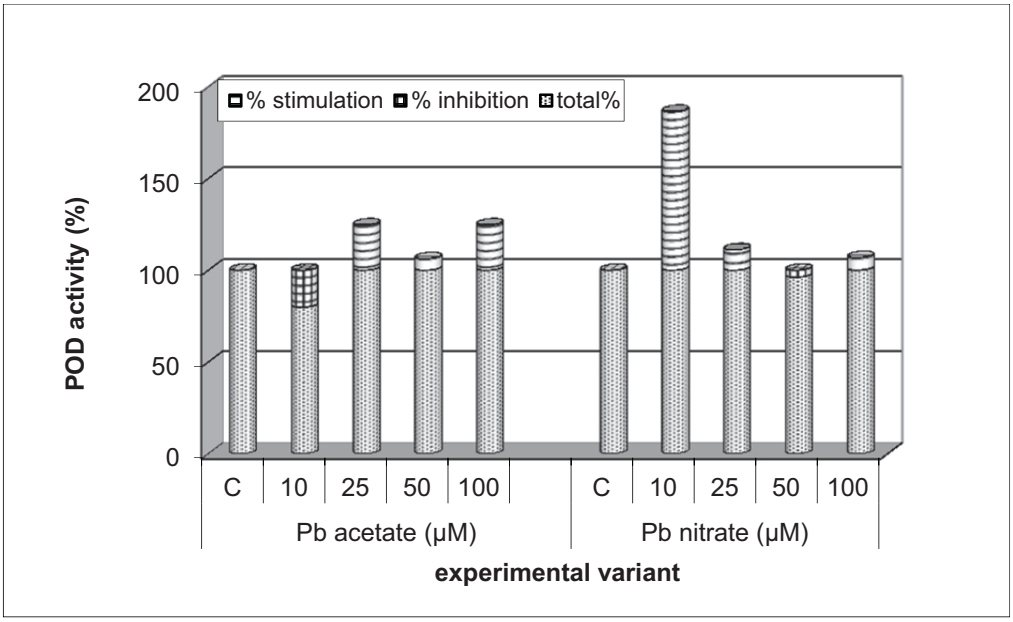

Fig. 3. Graphic representation of POD activity in wheat seedlings, after lead treatment.

Our results generally are in accordance with those published for wheat or other plant species, with some differences in enzyme behaviour. Lowering values of antioxidative enzyme activities are equivalent with a reduced protection against ROS, fact promoting accumulation of these and allowing the materialization of cytotoxic and eventually of genotoxic effect. The rise of enzyme activity as response to lead exposure is a proof of detoxifying ability of wheat seedlings by removal of ROS generated in stressed organisms. So, plants are able to overcome metal stress using an effective antioxidant defence mechanism in order to maintain the balance between ROS generation and their elimination. Therefore, in our experiments, for all tested concentrations of lead acetate and lead nitrate a stimulation of SOD activity was produced, but a distinct trend of CAT and POD activities in relation to Pb^{2+} concentration or compound type was not found. Various lead concentrations resulted either in inhibition or in increase activity of these enzymes. This situation is also signalled in literature for other heavy metals and plant species (Parmar *et al.*, 2002). The results published until now reveal a large diversity of antioxidative responses to heavy metal stress not only plant species but also a large intraspecific variability. Probably, the oxidative stress induced by heavy metals is a general phenomenon in plant species, but the antioxidative response is specific and depends on genetic potential of each cultivar or species.

Effects induced by Pb^{2+} on soluble protein level in 7 days old wheat seedlings. Oxidative damage of ROS on proteins refers to site-specific amino acid modifications such as formation of carbonyl derivatives on lateral chains of some amino acids (histidine, arginine, lysine, proline), fragmentation of the peptide chain, aggregation of cross-linked reaction products, alteration of electrical charge (Davies, 2003). In some cases, oxidation of susceptible residues such as cysteine and histidine lead to the production of *oxo* groups that can be assayed to provide an index of

oxidative damage to proteins (Babior, 1997). Oxidation of specific amino acids "marks" the proteins for degradation by specific proteases and can lead to cross-linkings and to an increased susceptibility to proteolysis. Regarding the mode of action with biological ligands, lead is included in the class of metals preferentially binding with sulphur- and nitrogen-rich ligands (e.g. amino acids) (Patra *et al*., 2004). Pb^{2+} acetate and Pb^{2+} nitrate inhibited the protein synthesis in wheat seedlings at all tested concentrations (Table 3, Fig. 4).

Table 3. Effects of Pb^{2+} treatment on soluble protein content in 7 days old wheat seedlings.

Variant		Soluble protein level		
		mg g^{-1} fresh weight	%	decrease rate (–), in %
Lead acetate	control	11.42	100.00	0.00
	10µM	8.17	71.54	-28.46
	25 µM	8.06	70.57	-29.43
	50 µM	9.18	80.38	-19.62
	100µM	6.69	58.58	-41.42
Lead nitrate	control	11.42	100.00	0.00
	10µM	7.12	62.34	-37.66
	25 µM	9.54	83.53	-16.47
	50 µM	7.93	69.43	-30.57
	100µM	9.76	85.46	-14.54

The greatest declines were present in the variants treated with 100 µM lead acetate (approximately 40 % inhibition, comparative to control), 10 µM and 25 µM lead acetate (approximately 30% inhibition), and also in 10 µM and 50 µM lead nitrate treated variants - both with more than 30% diminution of soluble protein level.

Decreasing effect of lead on protein level was also evidenced in *Vicia faba* L. (Mansour and Kamel, 2005), *Phaseolus vulgaris* L. (Hamid *et al*., 2010) or in *Oryza sativa* L. (Maitra and Mukherji, 1977). One explanation for the decrease of soluble proteins might be their increased susceptibility to proteolysis by specific proteases as result of the changes provoked by Pb^{2+}-generated ROS in side chains of specific amino acids, situation proved in *Hydrilla verticillata* under lead acetate stress (Jana and Choudhary, 1982). Also, gene expression is susceptible to be altered by lead treatment; so, it is possible that the repression mechanism of genes coding for protein synthesis became functional, fact evidenced by low level of proteins, as was present in *Brassica juncea* L. (Singh *et al*., 2002). Excessive damage to proteins under Pb^{2+} treatment could result from the attack of lipid peroxidation intermediates (Pinto *et al*., 2003). Loss of nuclear genetic material by chromosome fragmentation, micronuclei or laggards (see section concerning chromosome aberrations) can also have repercussions on protein synthesis afferent to those genes lost in this way.

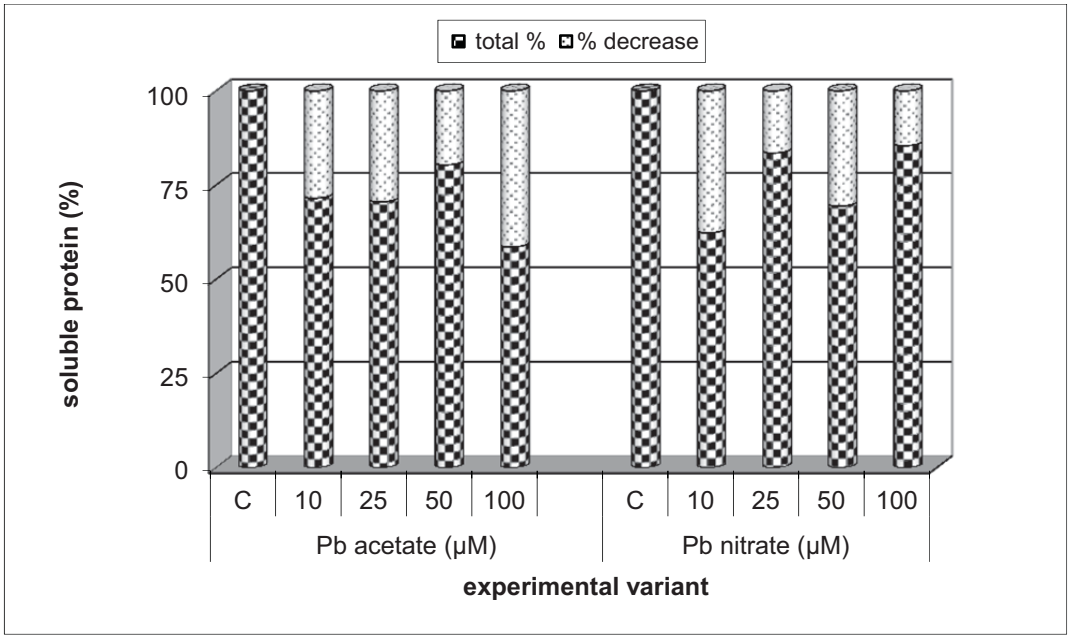

Fig. 4. Graphic representation of Pb^{2+} induced effects on soluble protein amounts in 7 days old wheat seedlings.

In literature, increases of some quantitative parameters including total protein amount were cited for common bean, alfalfa, oat and ryegrass, indifferently of lead concentration (Pinero *et al.*, 2002). In wheat and lens seedlings, the total protein content increased with the increase in lead concentration (Mesmar and Jaber, 1981). In other studies, different values of protein level have been noted in *Phaseolus mungo* and *Lens culinaris* seedlings, after lead treatment, depending on tested organs (Azmat and Haider, 2007). Increment of soluble protein amount can be a consequence of *de novo* synthesis of some stress proteins as result of exposure to exogenous factor (Gonçalves *et al*., 2007).

CONCLUSIONS

The results allow us to conclude that in *Triticum aestivum* cv. *Maruca* seedlings the protective mechanisms against Pb^{2+}-induced oxidative stress act by enhancing the antioxidant enzymes.
SOD and POD are mainly involved in the defence mechanism of wheat seedlings against Pb^{2+} toxicity.
For all tested concentrations of Pb^{2+} acetate and Pb^{2+} nitrate, a rise of SOD activity was registered.
Soluble protein level decreased in all variants, indifferently of compound type and Pb^{2+} concentration.
It was not established a direct relationship between Pb^{2+} concentration and enzyme activities.

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HEAVY METAL IONS INFLUENCE ON CONIFER SEEDS GERMINATION AND MITOTIC DIVISION

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Keywords: germination, manganese, spruce, larch, mitotic index

Abstract: The seed biostructure presents a differential response of both at the biochemical action of manganese ions - the inhibition or stimulation of specific enzymatic reactions - as at the osmotic characteristics of seeds tegument, depending on the studied plant species. The analysis of the cytological slides showed increased values of the mitotic index (MI) at dilution 10^{-3} $MnCl_2$ solution for larch (exposure time 48 hours) and 10^{-2} $MnCl_2$ solution for spruce (exposure time 21 days).

INTRODUCTION

It is well known the role of manganese in the cellular oxidative processes and also in the running of some enzymatic systems (Davidescu D. and all., 1988, Khan A. A., 1980, Roat-Malone, Rosette M., 2002), in connection with the iron one (Crichton, R., 2001). Thus, the bivalent manganese is part of the prokaryotic superoxide dismutase (SOD), enzyme which neutralize in the mitochondria the superoxide anions that induces numerous negative effects in the cells due to the formation of the hydrogen peroxide (Roat-Malone, Rosette M., 2002).

In the green plants, the photo system II uses another manganese enzyme that induces the water splitting and the production of molecular oxygen.

In a previous paper (Rîşca, I.M. şi colab., 2008) we studied the effect of manganese ions on the germination of wheat seeds, observing a number of specific changes during germination of wheat biostructure under the influence of Mn^{2+} ions. The present paper aims to study the effects that manganese has on seed germination of some forest species, especially of the spruce (*Picea abies*) and larch (*Larix decidua*) and the impact on the dynamics of mitotic division of the root apical meristems.

MATERIAL AND METHODS

Equipments. Germination was fulfilled in a growth chamber CONVIRON G30 whose parameters were set the following values: temperature 20^0C, humidity 90%, without lighting.

Biological material. Samples of spruce (*Picea abies*) and larch (*Larix decidua*) used were from UP 75A, respectively UP 45 P, 2009 harvest, 5.5 g/1000 seeds, respectively 5.0 g/1000 seeds. The following parameters of the germinated plants were determined: germination (FG), according to current standards (SR1634, 1999), length of hypocotyls (L_H) and rootlets (L_R).

Reagents. Reagents. $MnCl_2$ p.a. (Chimopar) and bi-distilled water were used.

Treatments. Seeds were treated with $MnCl_2$ solutions of seven concentrations: 1 m, 0.5 m, 0.1 m, 5 x 10^{-2} m, 10^{-2} m, 5 x 10^{-3} m and 10^{-3} m, against a distilled water witness. 50 seed, in three repetitions, were used, including for the witness, in Petri dishes on filter paper.

Four treatment regimes used, namely: the seeds were immersed in solutions of varying treatment periods (24 hours, 48 hours, 7 days and, respectively, for the entire duration of germination) and then, for the first three schemes treatment, the seeds were put to germinate in distilled water.

At 21 days the above mentioned parameters were determined, namely: the number of germinated seeds (FG) and the length and rootlets(L_R) and hypocotyls (L_H) at the germinated plants.

In order to perform microscopic squash-type preparations the rootlet meristem tips of spruce and larch were stained with Carr reagent (amended carbolic fuchsine). The samples were examined under the optical microscope at 10x and 40x objectives, and the cells in mitotic division were recorded. They were reported the total number of cells examined and the mitotic index, for both witness and treated samples.

RESULTS AND DISCUSSIONS

Experiments were conducted in order to determine the biological answer of the seeds of spruce and larch, respectively, under the influence of Mn^{2+} ions. The results are summarized in Tables 1 and 2 and Figures 1-5.

Table 1: values of the germination and hypocotyls and rootlets lengths of *Picea Abies* under the influence of the treatments with $MnCl_2$ solutions

Measured parameter (average values)	Concentration of Mn^{2+} / Immersion period	Witness	1 m	0,5 m	0,1 m	$5*10^{-2}$ m	10^{-2} m	$5*10^{-3}$ m	10^{-3} m
L_R (mm)	24 h	26,796	10,735	11,049	16,86	16,036	11,29	9,708	10,644
	48 h		2,471	5,81	7,839	10,12	19,487	17,025	12,98
	7 d		0,83	2,902	4,476	5,45	9,985	10,386	3,284
	21 d		0	0	1,505	5,955	27,31	27,804	29,884
L_H (mm)	24 h	28,706	16,238	21,154	25,032	25,252	21,237	19,466	18,213
	48 h		1,383	7,69	10,161	14,04	16,735	18,69	17,79
	7 z		0,933	3,025	8,154	9,569	21,877	17,818	6,337
	21 z		0	0	0,461	1,895	28,51	27,276	34,715
FG (%)	24 h	71,33	47,33	44	58,66	60	62,66	68	61
	48 h		18	48	39,33	61,33	52,66	65,33	56,66
	7 z		6,66	30	48	62,66	46,66	58	45,33
	21 z		0	0	13,33	39,33	80,66	78,66	64,66

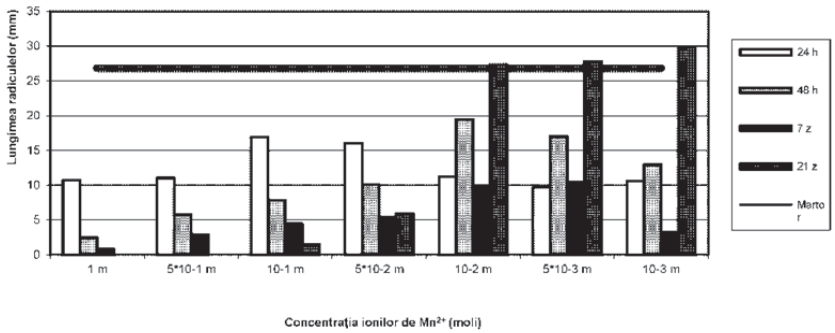

Fig. 1: influence of manganese ions against spruces of *Picea Abies*

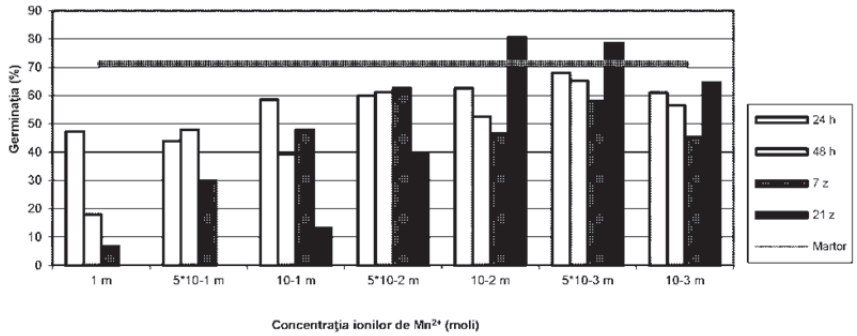

Fig. 2: influence of manganese ions against the seeds germination of *Picea Abies*

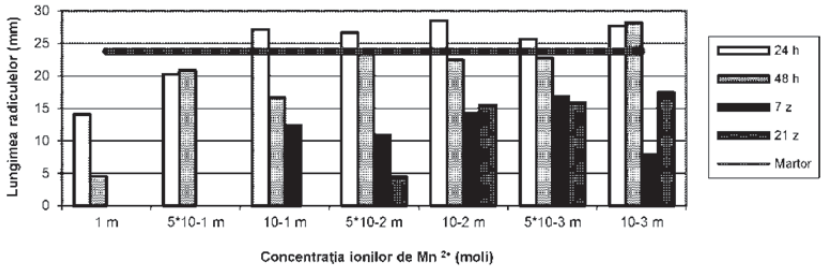

Fig. 3: influence of manganese ions against the rootlets of *Larix decidua*

Table 2: values of the germination and hypocotyls and rootlets lengths of *Larix decidua* under the influence of the treatments with $MnCl_2$ solutions

Measured parameter (average values)	Concentration of Mn^{2+} / Immersion period	witness	1 m	0,5 m	0,1 m	$5*10^{-2}$ m	10^{-2} m	$5*10^{-3}$ m	10^{-3} m
L_R (mm)	24 h	23,777	14,079	20,256	27,107	26,665	28,477	25,649	27,687
	48 h		4,566	20,839	16,662	24,171	22,442	22,76	28,155
	7 z		0	0	12,394	10,919	14,323	16,846	7,944
	21 z		0	0	0	4,53	15,518	15,89	17,433
L_H (mm)	24 h	24,972	5,095	20,003	27,319	22,567	24,463	24,72	29,937
	48 h		5,733	20,679	25,639	28,875	27,812	33,573	27,005
	7 z		0	0	16,344	14,567	24,872	21,063	22,348
	21 z		0	0	0	6,666	25,259	24,031	25,996
FG (%)	24 h	16,66	6	14	22,66	21,33	13,33	14,33	16
	48 h		9	16,66	19,33	15,33	24,67	15,33	18,66
	7 z		0	0	10	13,33	18,33	15,33	13,33
	21 z		0	0	0	6	13,33	12,67	16

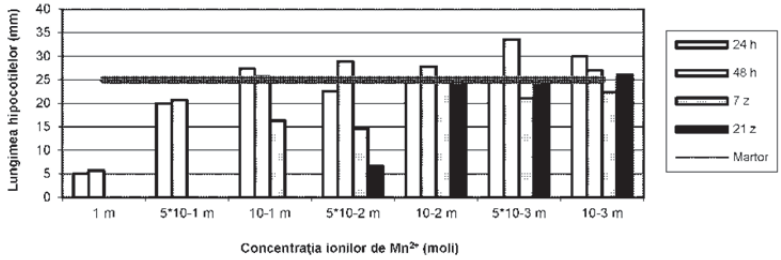

Fig. 4: influence of manganese ions against spruces of *Larix decidua*

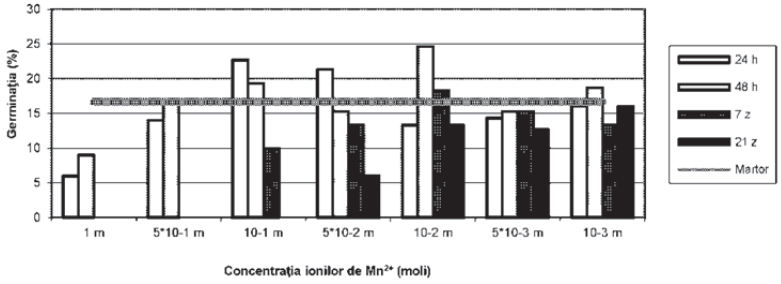

Fig. 5: influence of manganese ions against the seeds germination of *Larix decidua*

Table 3. Mitotic index in larch

Nr. of cells	Division cells					Mitotic index
Blank		P	M	A	T	
1350	170	130	17	10	13	0,13
Sample						
1280	210	166	21	16	7	0,18

Table 4. Mitotic index in spruce

Nr. of cells	Division cells					Mitotic index
Blank		P	M	A	T	
1350	155	127	12	6	10	0,11
Sample						
1280	189	156	16	11	6	0,14

A first finding is that at high concentrations manganese has - without exception - inhibitory effects on seed germination, the more pronounced effects as the seed to manganese exposure is longer. Individual tolerances vary, larch showing - for the short time of treatment - a

greater tolerance to high concentrations of manganese ions (10^{-1} m), unlike spruce shows stimulant symptoms at higher dilutions 10^{-2} m, and only long-stroke treatment. On the other hand, the effects of manganese occurs – depending on treatment periods and dilution - differentiated for spruce and larch. Thus, if the spruce is stimulated by high dilution and long immersion times, the larch has the reverse effect.

Spruce has an increased sensitivity to phytotoxic effects of manganese, but - paradoxically - maintained the entire period in dilute solutions of Mn^{2+} - it has significantly growth, especially in terms of length rootlets and, to a lesser extent, the hypocotyls (Fig. 1 and 2).

Larch is stimulated by dilution from 10^{-1} m, but only for short treatment times (Figures 3-5); the stimulation is manifested both in terms of germination and the size and hypocotyls and rootlets. For long exposure periods the larch resists at concentrations smaller than 10^{-2} m, with the exception of roots that have a pronounced inhibition (Fig. 4).

CONCLUSIONS

The above data lead us to the idea of a differential response of seed biostructure both at the biochemical action of manganese ions - the inhibition or stimulation of specific enzymatic reactions - as at the osmotic characteristics of seeds tegument, depending on the studied plant species.

Thus, the larch tegument is more permissive to the diffusion of manganese ions, inducing the rapidly activating of the enzyme systems (exposure times of 24 and 48 hours) but prolonging exposure to manganese causes an evident toxic accumulation. High concentrations of manganese have brutal effects, unbalancing irreversible the seed biostructure. At dilutions greater than $5x10^{-3}$ m changes are practically insignificant compared with witness.

For spruce seeds, their tegument presents an increased resistance against the diffusion of manganese ions, so that at short diffusion times significant results are not achieved. On the other hand, as already stated, spruce seeds are more sensitive so that, once "pierced" the osmotic defense line of the tegument, the seeds biostructure can maintain homeostasis parameters much closer limits, reflected by a more pronounced inhibition for all the measured parameters (Fig. 1-2) the most affected seemed to be root systems of spruce (Fig. 1).

The analysis of the cytological slides showed increased values of the mitotic index (MI) at dilution 10^{-3} for larch (exposure time 48 hours) and 10^{-2} for spruce (exposure time 21 days). In these conditions were registered 0,18 (0,13 blank) MI in larch mitotic division and 0,14 (0,13 blank) MI in spruce mitotic division (tab. 3 and 4).

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A SEMIQUANTITATIVE ANALYSIS TECHNIQUE REGARDING IMMUNOHISTOCHEMICAL DETECTION FOR MATRIX METALLOPROTEINASES

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Keywords: matrix metalloproteinses, immunohistochemistry, color deconvolution
Abstract: Modern image processing techniques are used today in order to evaluate immunohistochemical detection for various markers, especially those important for malignant tumor diagnosis and remodeling processes. Evaluating the immunohistochemical markers detection may be completed by a quantitative analysis. We have used samples of normal and fibrous tissue from surgical scars harvested after 2 months from surgery. We have investigated the immunohistochemically marked areas and we have performed a semiquantitative image analysis, using an academic, open sourcesoftware, ImageJ v. 1.38. After image adjustments (binarization) and correction, we have applied a deconvolution filter after which we have performed and analyzed a histogram of the selected area. The binarized areas were measured and compared for three samples of each tissue. We have followed the semiquantitative analysis of MMP-2 and MMP-9 presence on the investigated samples. This technique, even if controversial, allows us a fast analysis of common markers detected by immunohistochemistry

INTRODUCTION

Modern image processing techniques are used today in order to evaluate immunohistochemical detection for various markers, especially those important for malignant tumor diagnosis and remodeling processes. Evaluating the immunohistochemical markers detection may be completed by a quantitative analysis. However, due to difficult access to these markers and the reduced amount of tissue that can he harvested, we can use complementary techniques for image processing that may allow us to better quantify tumor markers detection by immunohistochemistry [1].

Matrix metalloproteinases (MMPs) are a family of zinc-containing endoproteinases that have been traditionally characterized by their collective ability to degrade all components of the extracellular matrix [2]. These enzymes are postulated to regulate the homeostasis of a variety of tissues under the control of tissue inhibitor of metalloproteinases (TIMPs), which bind to and inhibit the activity of MMPs. Accordingly, an imbalance between MMPs and TIMPs can lead to a variety of pathological states, such as metastasis of cancer [3], or diseases including, rheumatoid arthritis and multiple sclerosis.

In the 60s, Cross and Lapiere [4], were the first to describe the collagenolytic activity responsible for tadpole resorption. In 2010 there are 28 well defined classes of MMP, most of them are involved in human health and diseases. Cell-matrix interactions are critical for cells and tissue behaviors during normal development and also for pathological processes including benign and malignant tumors. MMPs are essential regulators for signals represented by matrix molecules involved in cell growth and development. Uncontrolled remodeling induced by MMP overexpression or malfunction result in abnormal development and induction of many pathological conditions in which excessive degradation or a lack of degradation of ECM components occurs [5,6].

The MMPs can be divided into collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), and membrane-type MMPs (MMP-14, -15, -16, and -17). For more than 30 years MMPs have been seen as promising targets for the treatment of the above-mentioned diseases because collagenase (MMP-1), gelatinases (MMP-2 and -9), and stromelysin-I (MMP-3) have been shown to play a key role in cancer invasion and metastasis [7].

MATERIAL AND METHODS

We have used samples of normal and fibrous tissue from surgical scars harvested after 2 months from surgery. MMP2 and 9 were detected by immunohistochemical means with DAB-labbeled antibodies. We have investigated the immunohistochemically marked areas and we have performed a semiquantitative image analysis, using an academic, open sourcesoftware, ImageJ v. 1.38.

This technique was imagined and applied by Ruifrok and Johnston; it evaluates the intensity of the imunohistochemical labeling for any color less than grey. The images were captured at the same size in pixels, 3264x2448 pixels, in RGB color coding system. We have selected 3 areas following pathologist advice in order to avoid large vessels. After image adjustments (binarization) and correction, we have applied a deconvolution filter after which we have performed and analyzed a histogram of the selected area.

RESULTS

The binarized areas were measured and compared for three samples of each tissue. We have followed the semiquantitative analysis of MMP-2 and MMP-9 presence on the investigated samples. For each sample we have followed the same protocol for three regions of interest on the same slide.

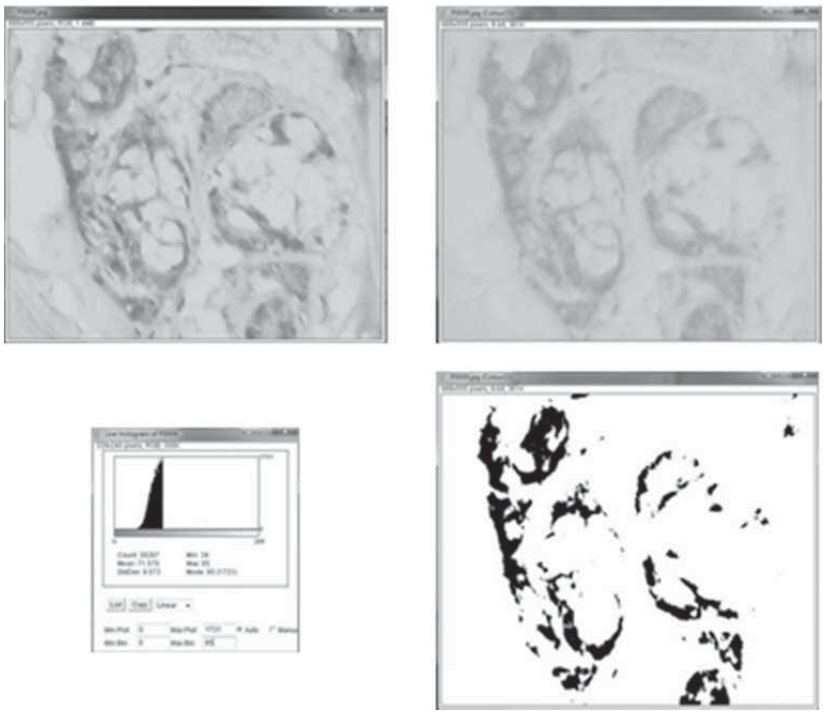

Figure 1. MMP9 detection, histogram and colour deconvolution for DAB

The final results expressed as ratios of marked areas for the two investigated antiMMP DAB-labbeled antibodies are shown in table 1 and figure 2.

Table 1. Percent areas for MMP2 and MMP9, detected by immunohisochemistry

	MMP2	**MMP9**
C1	0,245	38,092
	0,056	10,623
	0,249	16,722

	MMP2	MMP9
Mean case C1	**0,18**	**21,81**
C2	0	22,02
	0	27,457
	0	7,428
Mean case C2	**0,00**	**18,97**
C3	0	29,649
	0	23,265
	0	4,085
Mean case C3	**0,00**	**19,00**
C4	0	94,759
	0	73,904
	0	29,632
Mean case C4	**0,00**	**66,10**

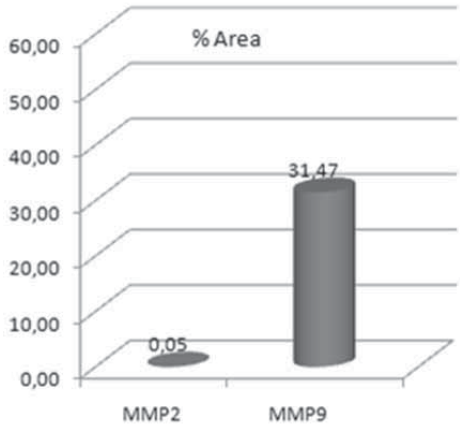

Figure 2. Graphic representation for the final results, MM2 and MMP9 detection expressed in percentage area

MMP2 appears to be expressed in insignificant amounts in the investigated scar tissues (percent marked areas - 0,05%). Even if it is actively involved in remodeling processes and similar as molecular structure with MMP9, MMP2 levels are really low in the investigated samples. This fact can be due to an important expression of TIMP2 that have inhibited pericellular MMP2. By comparison, MMP9 presence occupies 31,47% surface area.

CONCLUSIONS

As a conclusion, remodeling processes in scar tissues depend on presence and activity of MMP9 and not on MMP2 presence.

This technique itself, even if controversial, allows us a fast analysis of common markers detected by immunohistochemistry.

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XANTHAN/CHONDROITIN SULFATE HYDROGELS AS CARRIER FOR DRUG DELIVERY APPLICATIONS

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Keywords: hydrogels, codeine, drug delivery, biocompatibility

Abstract: Preparation, characterization and *in vitro* release studies of codeine from xanthan/chondroitin sulfate (X/CS) hydrogels prepared through a crosslinking technique are reported.

Swelling and drug delivery studies were conducted in phosphate buffer solution (pH=7.4) which simulates the pH of the intestinal fluid, at 37 °C.

The *in vitro* release test revealed that the percentage of codeine released in phosphate buffer solution increases with increasing the amount of chondroitin sulfate in the composition of hydrogels. The drug release behaviour of the hydrogels loaded with codeine fitted well with case II transport mechanism for all formulations.

The biocompatibility testing was made by hemolisys (plasma hemoglobin) technique.

INTRODUCTION

Hydrogels are insoluble, crosslinked polymer networks composed of hydrophylic homo- or hetero-co-polymers, which have the ability to absorb significant amounts of water. Due to their water content, hydrogels also possess a degree of flexibility very similar to natural tissue, which minimizes potential irritation to surrounding membranes and tissues.

Hydrogels have been used as prime carriers for pharmaceutical applications, predominantly as carriers for delivery of drugs, peptides or proteins. They have been used to regulate drug release in reservoir-based, controlled release systems or as carriers in swellable and swelling-controlled release devices (Peppas, 1987, 1997; Narasimhan, 1997).

Xanthan gum (Figure 1) is a high molecular weight extracellular polysaccharide, produced on commercial scale by the fermentation of gram negative bacterium *Xanthomonas campesteris.*

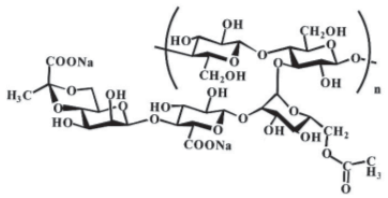

Figure 1. Xanthan structure

The molecule consists of a backbone identical to that of cellulose, with side chains attached to alternate glucose residues. It is a hydrophilic polymer, which until recently had been limited for use in thickening, suspending and emulsifying water based systems (Gwen, 1996). It appears to be gaining appreciation for fabrication of matrices, as it not only retards drug release, but also provides time-independent release kinetics with added advantages of biocompatibility and inertness, in ophthalmology (Ceulemans, 2002; Ludwig, 2005), implantology (Kumar, 2007) and tissue engineering (Silava, 2007), can also work effectively *in vivo* establishing constant drug plasma levels (Lu, 1991). It is also recommended for use in both acidic and alkaline systems. Xanthan gum has been evaluated as a hydrophilic matrix for controlled release preparation, using different model drugs including theophylline (Lu, 1991), cephalexin (Dhopeshwarkar, 1994), prednisolone (Watanabe, 1992), and indomethacin (Watanabe, 1993).

Chondroitin sulfate is an important structural component in connective tissues and cartilages. It provides compressive strength to connective tissues by regulating their water content, and possesses characteristic features, such as a high water absorption, multifunctionality and biodegradability suitable for bioapplications (Wang, 2007; Comper, 1990). In addition, the presence of active functional groups in chondroithin sulphate, such as –COO−, and –SO_3−, provides access to biological functionalities, which have been recently exploited in *in vivo* cartilage repair applications (Wang, 2007).

It is a copolymer of D-glucuronic acid and sulfated N-acetyl-D-galactosamine in C_4 or C_6 and belongs to the glycosaminoglycans (GAGs), which are primarily located on the surface of cells or in the extracellular matrix (Figure 2).

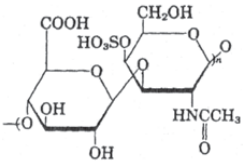

Figure 2. Chondroitin sulfate structure

Studies on chondroitin sulfate-based hydrogels were reported by Kuijpers et al. (Kuijpers, 2000) who evaluated chemically cross-linked gelatine-chondroitin sulfate hydrogels, impregnated in Dacron, as drug delivery systems for antibacterial proteins.

Varghesea et al. (Varghesea, 2008) synthesised a fast thermoresponsive hydrogel composed of poly(N-isopropylacrylamide) (PNIPAm) and chondroitin sulphate (CS) using precipitation polymerization suitable for controlled delivery applications of cationic drugs

The aim of this study is to combine the properties of X and CS in mixed hydrogels in order to obtain new materials for medical and pharmaceutical applications. With this aim have been also evaluated the biocompatibility of X/CS hydrogels and their applicability in codeine delivery systems, for achieving a controlled release profile suitable for oral and subcutaneous administration so the investigation were made in phosphate buffer solution (pH=7.4).

Codeine or metylmorphine (Figure 3) is an opiate used for its analgesic, antitussive and antidiarrheal properties. It is one of the most effective orally-administrated opioid analgesics and has a wide safety margin.

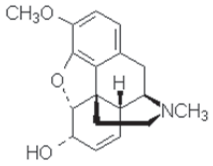

Figure 3. Chemical structure of codeine

It may be mentioned that these types of formulations based on xanthan and chondroitin sulfate have not been studied or proposed as hydrogels for biomedical applications.

MATERIALS AND METHODS

Xanthan gum (X) was purchased from Sigma Aldrich and chondroitin sulfate (CS) from Roth.

Xanthan/chondroitin sulphate (X/CS) hydrogels were produced by a crosslinking technique in various mixing ratios: 90/10, 80/20, 70/30, 60/40, 50/50 X/CS in presence of epichlorhydrin, as crosslinking agent, purified by washing with warm water and dried for 10 hours by using a LABCONCO FreeZone device.

In this study were used 80/20, 60/40 and 50/50 X/CS formulations.

Codeine was purchased from Centre for the Study and Therapy of Pain (CSTD), "Gr.T.Popa" Medicine and Pharmacy University, Iasi.

The *kinetics of the swelling* were carried out for all formulations by direct immersion in a phosphate buffer solution which simulate intestinal fluid (pH=7.4). The hydrogels samples were maintained for 24 hours at 37 ^{0}C, periodically removed from the solution, gently wiped with a soft tissue to remove surface water, weighed and than placed back into the vessel as quickly as possible. The swelling degree at equilibrium was calculated according to the equation (1).

$$Q_{\max} = (W_t - W_d)/W_d \times 100(\%) \qquad (1)$$

where W_t is the weight of the samples after swelling in phosphate buffer solution at time t and W_d is the dry weight of the sample.

To determine the kinetics of solvent diffusion into the matrices (swelling) the following equation was used: (Berens, 1978)

$$F_t = \frac{W_t}{W_{eq}} = k_{sw} t^{n_{sw}} \quad (2)$$

where W_t and W_{eq} represent the amount of phosphate buffer solution, absorbed by the matrices at time t and at equilibrium, respectively, k_{sw} is the swelling constant characteristic of the system and n_{sw} is the power law diffusion exponent which takes into account the type of solvent transport. Eq. (2) applies to initial states of swelling (swelling degree less than 60%) and linearity is observed when log Ft as a function of log t is represented.

The *drug loading* of the hydrogel matrices was carried out by mixing codeine with dried matrices in powdered form and then a certain quantity of the appropriate solvent (maximum amount of liquid uptaken during swelling) was added and left to swell at room temperature at least 25 ^{0}C, while the drug penetrates and/or attached into matrices. The drug-loaded samples were freeze-dried using a Labconco FreeZone device.

During the *in vitro drug release* studies, at predetermined time intervals, samples of 1 ml were withdrawn from the release medium and concentrations of codeine was determined at λ_{max} value 284 nm using a HP 8450A UV–visible spectrophotometer. In order to maintain the solution concentration the sample is reintroduced in the circuit after analyzing.

A simple, semi-empirical equation using Korsmeyer and Peppas model was used to kinetically analyze the data regarding the drug release from studied matrices system which is applied at the initial stages (approximately 60 % fractional release) (Higuchi, 1961; Ritger, 1987; Chen, 2007; Peppas, 1985, 1986, 1989) (3):

$$M_t / M_\infty = k_r t^{n_r} \quad (3)$$

where M_t/M_α represents the fraction of the drug released at time t, M_t and M_∞ are the absolute cumulative amount of drug released at time t and at infinite time (in this case maximum release amount in the experimental conditions used, at the plateau of the release curves), respectively; k_r is a constant incorporating characteristics of the macromolecular matrix and the drug n_r is the diffusion exponent, which is indicative of the release mechanism. In the equation above a value of $n_{sw}/n_r = 0.5$ indicates a Fickian diffusion mechanism of the drug from matrix, while a value $0.5 < n_{sw}/n_r < 1$ indicates an anomalous or non-Fickian behaviour. When $n_r = 1$ a case II transport mechanism is involved while $n_r > 1$ indicates a special case II transport mechanism (Katime, 2001; Korsmeyer, 1984; Serra, 2006; Berg, 2006).

The corresponding drug-release profiles were represented through plots of the cumulative percentage of drug release versus time.

Percent hemolysis test

Blood was obtained from healthy patients drawn by routine venipuncture from the antecubital vein in tubes containing EDTA. The blood was stored refrigerated for no more than 2 days until its use. Each hydrogel preparation was tested with blood from a single patient. Prior to hemolysis test all the hydrogel samples were sterilized by ultraviolet light trans-illumination for 2 min. Distilled water was used as positive control and plasma separated from the same blood as negative control. From each tube, 1.5 mL of blood were drawn and put into contact with hydrogels, in Eppendorf centrifuge tubes (2 mL). The blood samples in contact with the biomaterials were incubated at 37 °C for 2 h. After incubation, the samples were centrifuged at 5000 rpm for 6 min. The separated plasmas were diluted 11-fold with hydroxymethyl amynomethane – Sigma-Aldrich, (Tris) (62.5 mmol/L, pH 8.0 adjusted with HCl) prior to spectrophotometrical measurements. The remaining 0.5 mL of blood in each tube were centrifuged, again, at 5000 rpm and separated plasmas were diluted 11-fold with hydroxymethyl amynomethane (Tris) the resulting solutions being used as negative controls. The positive control was prepared by hemolysing blood with distilled water (1:11 dilution). The hemolysed solution was also incubated at 37 °C for 2 h. Finally, the positive control solution was diluted 100-fold for spectrophotometric analysis (ISO, 2002).

The method used for measuring plasma hemoglobin concentration in all the specimens was the polychromatic method of Noe et al. (Noe, 1984). Absorbance was measured at 380 nm, 415 nm and 470 nm and the formula used for evaluation was:

$$C(mg/L) = 1.65\ mA_{415} - 0.93\ mA_{380} - 0.73\ mA_{470} \quad (4)$$

where C is the hemoglobin concentration in mg/L, mA_{380}, mA_{415} and mA_{470} are the absorbances at 380nm, 415nm and 470nm expressed in miliabsorbance units. The results were expressed as:

$$\text{hemolysis percent (\%)} = (C - C_n)/(C_p - C_n) \times 100 \quad (5)$$

where C is the concentration of hemoglobin in the sample, C_n the concentration of hemoglobin in the negative control and C_p the concentration of hemoglobin in the positive control.

RESULTS AND DISCUSSION

Swelling kinetic studies

Swelling studies were performed in phosphate buffer solution of pH = 7.4 which simulate the pH of intestinal fluids at 37 ^{0}C, the results being presented in table 1.

The values presented in table 1 show that the 50/50 X/CS hydrogel, with 50% xanthan and 50% CS, has the highest degree of swelling – 1642 wt% comparing with 80/20 X/CS composition with a swelling degree of 1134.3 wt%. It can be observed that all tested formulations are superabsorbants and the increase of CS content significantly increases the swelling degree of X/CS hydrogels. The high water quantity uptake and faster swelling can be attributed to the existence of groups with negative charges in CS structure, such as $–COO^-$ and $–SO_3^-$, which helps the gels to swell highly, conferring a high concentration of negative charge in the regions that contain them (Comper, 1990).

Table 1. The influence of composition, swelling degree and the values n_{sw} and k_{sw} of the X/CS hydrogel

Hydrogels	Xanthan (wt%)	CS (wt%)	Q_{max} (%)	n_{sw}	k_{sw} (min^{-nsw})
80/20 X/CS	80	20	1134.354	0.14	0.70
60/40 X/CS	60	40	1495.874	0.12	0.73
50/50 X/CS	50	50	1642.025	0.06	0.83

Table 1 presents the values obtained for the kinetic parameters of swelling, k_{sw} and n_{sw}, of the X/CS hydrogels swollen in a pH 7.4 phosphate buffer solution, at 37 °C.

The values obtained for swelling parameter, n_{sw}, in case of X/CS hydrogels with different mixing ratios, varies between 0.06 – 0.14 indicating an anomalous transport mechanism and the values of swelling rate constant, k_{sw}, are increasing with increasing of CS content.

In vitro codeine release studies

The release profiles of codeine from X/CS hydrogels are shown in figure 4.

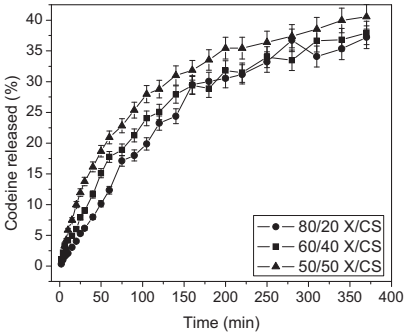

Figure 4. Release profiles of codeine from X/CS-based hydrogels with different compositions, in phosphate buffer solution (pH=7.4) at 37 ^{0}C

The release profiles showed that the percentage of codeine released increases with increasing the amount of chondroitin sulfate in the composition of hydrogels. Thus, the 50/50 X/CS hydrogel released approximately 43 % codeine comparative with 80/20 X/CS composition witch release about 38 % within 400 minutes.

The kinetic parameters for codeine released in from X/CS-based hydrogels with various compositions are presented in table 2.

Table 2. The kinetic parameters of codeine released from X/CS hydrogels

Hydrogels	First order kinetic model	
	$k\ 10^{-3}$ (min^{-1})	R
80/20 X/CS	3	0.99
60/40 X/CS	1.9	0.99
50/50 X/CS	4.83	0.99

From the obtained release profiles the diffusion exponent n_r was calculated according to Eq. 3 (Table 2). The release of codeine in phosphate buffer solution is described as a case II transport mechanism (zero order kinetics) for all formulations.

Hemolysis test

The hemolysis test showed that the hemolysis percentages of all the blood samples in contact with the hydrogels based on X/CS were negative. All hemolysis percentages were less than 1% (table 3) as compared to the positive control, value which is below 5% limit admitted for this test (ISO, 2002).

Table 3. Hemolysis percentage of the X/CS hydrogel formulations tested

Hydrogels	Hemolysis percentage (%)
80/20 X/CS	-0.036
60/40 X/CS	0.0381
50/50 X/CS	-0.0611

The results obtained for hemolysis test, for both types of formulations, showed a good biocompatibility between hydrogels and blood.

CONCLUSIONS

Xanthan/chondroitin sulfate hydrogels were produced by a crosslinking technique in presence of epichlorhydrin, as crosslinking agent.

The swelling of xanthan/chondroitin sulfate hydrogels shows a relationship with CS concentration, so an increase of CS content in hydrogels composition leads to a higher swelling ratio.

The results of controlled release tests showed that an increase of CS content leads to an increase of codeine percent released.

The release of codeine from X/CS hydrogels was described as case II transport mechanism for all formulations.

The biocompatibility testing was made by hemolysis (plasma hemoglobin) the results obtained showed a good biocompatibility with tested formulations.

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THE VARIABILITY OF SOME MORPHOLOGICAL CHARACTERS IN *RANA RIDIBUNDA* (PALL.)

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Keywords: *Rana ridibunda*, morphometry

Abstract: For the biometric analysis at *Rana ridibunda* (Pall.) we studied the 12 morphological features and we calculated the report between some analyzed biometric indexes. The interpretation of the obtained results was done using the comparative statistic tests. The analysis of the variability shows that most morphological features have a low variability. Only the interpalpebral distance and the length of the internal metatarsal tubercle presented an average variability. Another observation would be that, generally, male individuals had a slightly higher variability of the investigated parameters.

INTRODUCTION

Rana ridibunda species Pallas 1771 is part of the green frog complex together with the species *Rana esculenta* Linnaeus 1758 and *Rana lessonae* Camerano 1882. The general opinion regarding the three species emphasizes the hybrid nature of the *Rana esculenta* (L.) by expressing some morphological features intermediary to the parental species of the complex. It is the reason for which the hybrid identification methods for such features are used in the identification of the 3 species.

Besides, the global decline of biodiversity, in this case the amphibian – about 200 species are let down and 32 species are about to disappear (Blaustein and Wake, 1990, Alford and Richards, 1999, Houlahan et all., 2000) – impose the launching of some monitoring programs involving the use of some investigation methods and techniques as varied as possible to form a clear image upon the adaptation potential of these species. Thus, the variability may be considered a result of the interactions between the genotype and the environment.

This paper was meant to bring some contributions to the knowledge of biodiversity, by assessing the variability of some morphological features at *Rana ridibunda* (Pall.), using adult individuals collected from Balţii pool in Dorohoi (Botoşani county).

MATERIAL AND METHODS

To comply with the above mentioned study, 49 individuals from the *Rana ridibunda* (Pall.) species were collected (32 males and 17 females) from Balţii pool located in a swampy area (with groundwater located in little depths) from the east of Dorohoi.

Capturing individuals to be used in our study was done by means of a fish landing. We observed that, by submersion of the fish landing ring and attracting individuals by means of a vivid colored cork, fixed at the end of a fishing rod, the efficiency of the capturing action obviously increased. After the completion of biometric measurements, the individuals were released in their natural environment. To avoid a possible investigation of the same individuals in case of a re-capture, before their release, the individuals were marked. As marking technique, we used the band method (Elmberg, 1989; Rice and Taylor, 1993, quoted by Cogălniceanu, 1997). For the procurement of biometric data, we used a digital caliper, Mannesmann brand (Brüder Mannesmann Werkzenge GmbH, Germany), with 0.01 mm precision.

To complete the samples, all the data should be characteristic to the adult population. Therefore, we used as a criterion in the sample formation the length of the body of the collected individuals, according to the mentions from Fauna R.P.R. Amphibia, vol. XIV, 1(1960). Thus, the sample from *Rana ridibunda* (Pall.) contains individuals with the length between 62 – (76, 4) – 94 mm.

For the biometric analysis we studied the morphological features below: interpalpebral distance (Sp.p.), the eye length (L.o.), the head width (Lt.c.), the eardrum length (L.tymp.), the length of the head (L.c.), the length of the body (L.), the length of the anterior limb (L.m.a.), the length of the femur (F.), the length of the tibia (T.), the length of the shin articulation (L.tars.), the length of the metatarsal tubercle (C.int.), the length of the first finger (D.p.).

Based on the obtained results, we calculated the report between some analyzed biometric indexes. Among these, the most used in the specialty literature (Wijnands şi Van Gelder, 1976; Gubányi şi Kórsós, 1992; Csata, 1998; Zamfirescu, 2002; Krizmanić, 2008) are:

- The report between the length of the tibia and the length of the internal metatarsal length (T/C.int.);
- The report between the first finger and the length of the internal metatarsal tubercle (D.p./C.int.);
- The report between the length of the body and the length of the first finger (L/D.p.).

For each morphological feature analyzed, the minimum and maximum value were determined, as well as the arithmetic average, standard error and the variation coefficient. The interpretation of the obtained results was done using the comparative statistic tests. To determine the dispersion, we used the Fisher-Snedecor test and according to it, the Student homoscedastisc or heteroscedastisc.

The obtained results are presented in tables 1 and 2 and figure 1.

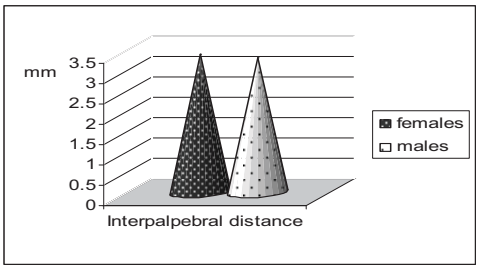
RESULTS AND DISCUSSIONS

After the completion of the biometric measurements, the registered data were centralized on each researched parameter and 2 sexes, setting the values of the statistic indicators for each of the morphometric features (tab. 1). In figure 1 the arithmetic averages of the researched morphological parameters are graphically represented at the 2 sexes.

Tab. 1 – Values of some morphological parameters (in mm) at *Rana ridibunda* (Pall.)

Biometric parameter	Statistic parameter	*R. ridibunda*	*R. ridibunda* ♀	*R. ridibunda* ♂
Interpalpebral distance (Sp.p.)	Minimum	2.8	3.0	2.8
	Maximum	4.5	4.5	4.2
	Average	3.414	3.511	3.362
	Standard error	0.052	0.100	0.059
	Variation coefficient	10.72	11.81	9.90
Length of the eye (L.o.)	Minimum	7.4	7.8	7.4
	Maximum	11.0	10.2	11.0
	Average	8.955	8.935	8.965
	Standard error	0.110	0.174	0.142
	Variation coefficient	8.59	8.03	9.00
Width of the head (Lt.c.)	Minimum	23.8	24.0	23.8
	Maximum	35.4	31.7	35.4
	Average	28.518	27.482	29.086
	Standard error	0.387	0.492	0.510
	Variation coefficient	9.49	7.38	9.92
Length of the eardrum (L.tymp.)	Minimum	4.5	5.0	4.5
	Maximum	6.6	6.4	6.6
	Average	5.628	5.594	5.646
	Standard error	0.049	0.079	0.085
	Variation coefficient	7.62	5.86	8.55
Length of the head (L.c.)	Minimum	23.6	24.1	23.6
	Maximum	37.0	32.3	37.0
	Average	28.891	28.464	29.118
	Standard error	0.399	0.571	0.533
	Variation coefficient	9.68	8.27	10.37
Length of the body (L.)	Minimum	62.6	62.6	67.6
	Maximum	91.7	83.7	91.7
	Average	75.932	74.317	76.79
	Standard error	0.872	1.529	1.046

Biometric parameter	Statistic parameter	*R. ridibunda*	*R. ridibunda* ♀	*R. ridibunda* ♂
	Variation coefficient	8.04	8.48	7.70
Length of the anterior member (L.ma.)	Minimum	16.5	16.5	18.0
	Maximum	24.2	23.4	24.2
	Average	21.030	20.470	21.328
	Standard error	0.259	0.496	0.289
	Variation coefficient	8.63	10.00	7.66
Length of the femur (F.)	Minimum	31.0	31.0	31.3
	Maximum	47.3	40.2	47.3
	Average	37.102	36.288	37.534
	Standard error	0.488	0.702	0.643
	Variation coefficient	9.22	7.97	9.69
Length of the tibia (T.)	Minimum	30.8	31.0	30.8
	Maximum	43.7	40.1	43.7
	Average	36.320	35.517	36.746
	Standard error	0.434	0.610	0.573
	Variation coefficient	8.37	7.08	8.82
Tars length (L.tars.)	Minimum	14.9	15.5	14.9
	Maximum	21.7	21.1	21.7
	Average	18.426	18.24	18.525
	Standard error	0.251	0.406	0.321
	Variation coefficient	9.54	9.17	9.81
Length of the metatarsal tubercle (C.int.)	Minimum	3.0	3.1	3.0
	Maximum	4.7	4.7	4.7
	Average	3.971	3.952	3.981
	Standard error	0.070	0.131	0.084
	Variation coefficient	12.46	13.66	12.00
Length of the first finger (D.p.)	Minimum	12.0	12.0	12.7
	Maximum	16.9	16.5	16.9
	Average	14.620	14.364	14.756
	Standard error	0.167	0.334	0.183
	Variation coefficient	7.99	9.59	7.04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2
1.5
1
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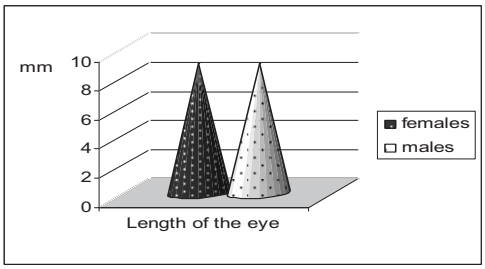
Interpalpebral distance
females
males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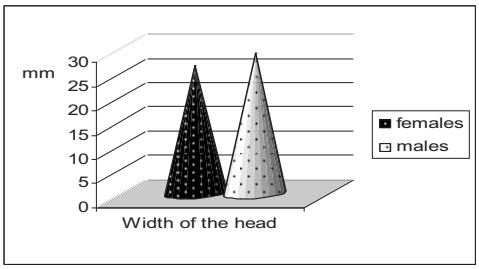
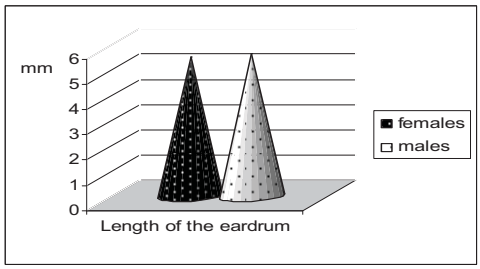
8
6
4
2
0
Length of the eye
females
males


mm
30
25
20
15
10
5
0
Width of the head
females
males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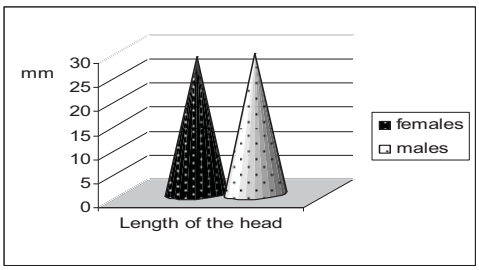
5
4
3
2
1
0
Length of the eardrum
females
males


mm
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25
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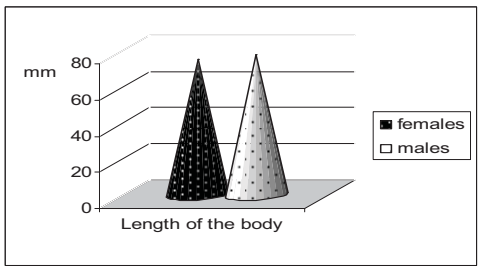
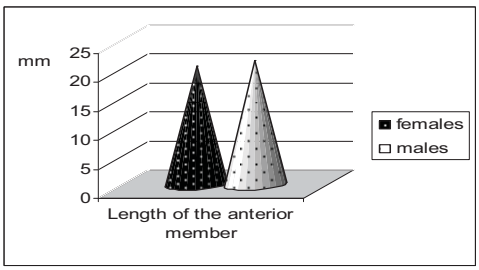
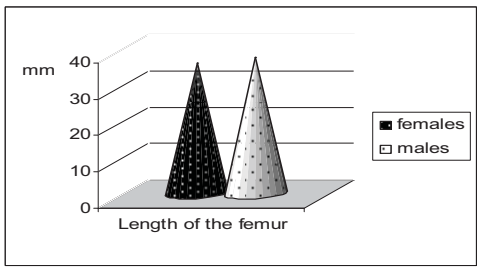
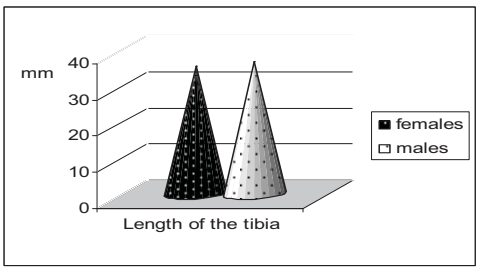
15
10
5
0
Length of the head
females
males


mm
80
60
40
20
0
Length of the body
females
males


mm
25
20
15
10
5
0
Length of the anterior member
females
males


mm
40
30
20
10
0
Length of the femur
females
males


mm
40
30
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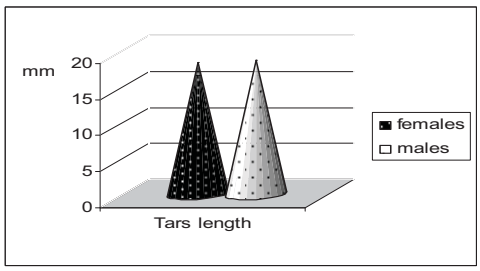
10
0
Length of the tibia
females
males


mm
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Tars length
females
males

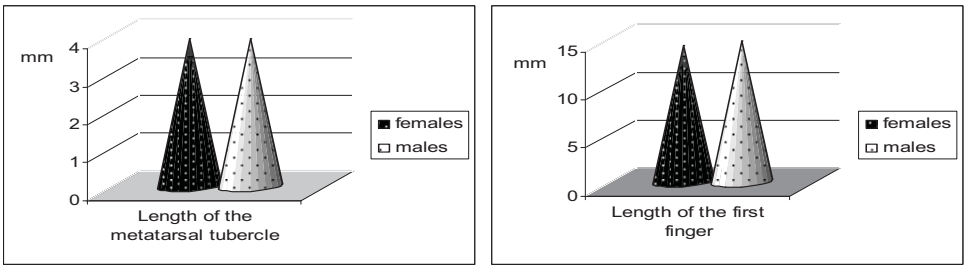

Fig. 1 Graphical representation of the average values of some biometric parameters at the *Rana ridibunda* Pall species, according to the sexes of the individuals

Interpalpebral distance (Sp.p.), measured between the ocular globes, at the middle of the eyelids, at the investigated population of *Rana ridibunda* (Pall.), is between 2.8 – 4.5 mm with an average of 3.41±0.05 mm, the variability of the individuals from that population being average to reduced (CV = 10.72), being a little bit increased to females as compared to males. Even with the calculated mean of the interpalpebral distance at the 2 sexes (3.51±0.10 mm to female and 3.36±0.06 mm to males) we cannot talk about the possibility of differentiation between the 2 sexes using as a criterion this biometric parameter, since the T test applied with the level of signification ($\alpha = 0.05$) has confirmed the null hypothesis (H_0) of the equality between the 2 averages.

The length of the eye (L.o.), measured at the ends of the ocular longitudinal diameter, varies at the sample of the *Rana ridibunda* (Pall.) population between 7.4 mm and 11 mm, with an average of 8.96±0.11 mm, the parameter variability being a reduced one both to males and females, according to the values of the variation coefficient (CV = 8.59). Comparative tests (F test and T test) confirm the existence of a non-significant difference between the averages, with an error of 0.05.

The width of the head (Lt.c.), measured between the mouth corners, varies between 23.8 mm and 35.4 mm. The average value of this feature is of 28.51±0.39 mm, and the variation coefficient (CV = 9.49) indicates a reduced variability of the parameter for both sexes. The head width can be used as a discriminator parameter of the sexes in the *Rana ridibunda* (Pall.) population, males having bigger average as compared to females with an error of 0.05.

The length of the eardrum (L.tymp.), represented by the horizontal diameter of the eardrum, varies between 4.5 şi 6.6 mm. The average length of the drum ear, calculated for the sample, was of 5.63±0.05 mm, and the variability of the feature is reduced (CV = 7.62). The averages of the eardrum length do not significantly differ according to the sexes of the individuals.

The length of the head (L.c.), measured from the top of the muzzles to one of the corners of the mouth, is a biometric parameter whose registered valued in the studied population of *Rana ridibunda* (Pall.) were between 23.6 mm and 37 mm. The dispersion interval of this parameter has an average of 28.89±0.4 mm, and the variability of the feature is reduced (CV = 9.68). If we consider the sex of the individuals, there are no important changes regarding the dispersion of the biometric data and the comparative analysis of the averages.

The length of the body (L.), measured from the top of the muzzle to the anus, at the population of *Rana ridibunda* (Pall.), had as variability limits between 62.6 and 91.7 mm, the average length of the body being of 75.93±0.87 mm. The variability of this feature was reduced

(CV = 8.04). In case of females, the length of the body varied between 62.6 and 83.7 mm, with an average of 74.32±1.53 mm. As for the males, the limits of the analyzed feature were between 67.6 and 91.7 mm, with a mean of 76.79±1.05 mm. Comparative analysis of the samples differentiated by sexes indicate an equal dispersion of the values between the 2 data rows (F test-non important – H_0) and the comparative analysis of the means with the help of the homoscedastic T-test present a greater possibility than the level of significance (0.05), which leads to the acceptance of the null hypothesis (H_0).

The length of the anterior member (L.ma.), measured from the wrist to the top of the longest finger (3rd finger), within the studied *Rana ridibunda* (Pall.) population, varied between 16.5 and 24.2 mm, with an average mean of 21.03±0.26 mm, the feature variability being reduced (CV = 8.63). Although the arithmetic means differ according to sex, the statistic applied tests do not indicate significant differences.

The length of the femur (L.), measured from the anus to the middle of the knee articulation, represents an often used parameter in the specialty literature from the biometry works at *Ranidae*. In the analyzed *Rana ridibunda* (Pall.) population, this parameter oscillated between 31 and 47.3 mm, having a sample average of 37.10±0.49 mm. The feature variability is reduced (CV = 8.37), including in the samples differentiated by sexes. Moreover, we support the equality of the averages of the 2 samples, with an error of 0.05.

The length of the tibia (T.), measured from the middle of the knee articulation to the middle of the tibia tarsal articulation, varies between 30.8 and 43.7 mm, having a sample average of 36.32±0.43 mm. The variability of the feature is reduced (CV = 8.37). Although the arithmetic averages of the tibia length are a bit different at the 2 sexes, the difference is not significant statistically.

The legth of the tars (L.tars.), measured from the middle of the tibia tarsal articulation to the middle of the tars metatarsal articulation, varies between 14.9 mm and 21.7 mm at the *Rana ridibunda* (Pall.) population. The average length of the tars is of 18.43±0.25 mm, and the variability of the feature is reduced (CV = 9.54). Statistic tests applied for the 2 differentiated samples according to sexes indicate equal dispersions of the data, and the average values of the tars length are statistically equal to the 2 sexes, with an error of 0.05.

The length of the internal metatarsal tubercle (C.int.) represents an important biometric parameter for the determination and characterization of the species of green frogs. Limit values for this parameter were of 3.0 and 4.7 mm, with an average calculated of 3.97±0.07 mm. It is a feature showing intermediate variability (CV = 12.46). As for the possibility of differentiating the 2 sexes using as discriminatory criterion the length of the internal metatarsal tubercle, the applied calculation are not important.

The length of the first finger (digittus primus – D.p.), measured from the top to the distal edge of the internal metatarsal tubercle, represent another morphological parameter extremely important in the biometric research of green frogs. It is a parameter oscillating between 12.0 and 16.9 mm, arithmetic average being of 14.62±0.17 mm. The feature variability is low (CV = 7.99), being similar to females and males. There are no statistic significant differences between the averages registered by this parameter to the 2 sexes.

The analysis of the variability of the 12 morphological features studied at *Rana ridibunda* (Pall.) show that most of them have a low variability, the variation coefficient displaying values under 10. Only 2 of the investigated parameters presented an average variability: the interpalpebral distance and the length of the internal metatarsal tubercle. Another observation would be that, generally, male individuals (although they outnumber the females in

the analyzed sample) had a slightly higher variability of the investigated parameters, but for the interpalpebral distance, length of the femur, length of the anterior limb, length of the internal metatarsal tubercle, and length of the first finger, with a much increased variability in females.

As for the report between one of the analyzed indicators (tab. 2), the calculated values are included in the intervals stated by the specialty literature (Wijnands and Van Gelder, 1976, Gubányi and Korsós, 1992, Zamfirescu, 2002, Krizmanić, 2008). The most extended of their variability interval was in the case of the T/C.int report. The minimal value of this report is of 6.57, maximum value of 12.09, and the average of 9.26±0.16. The situation does not change in case of the analysis of this report on sexes. The other studied reports vary in lower limits. Thus, the D.p./C.int. is somewhere between 3.02 and 4.91, with an average of 3.72±0.06 in the whole collected sample, (between 3.11 and 4.10 – with an average of 3.67±0.08 to females; between 3.02 and 4.91 – with an average of 3.75±0.08 to males. The L./D.p. report has values between 4.64 and 5.73 – with an average of 5.20±0.04 in the analyzed *Rana ridibunda* (Pall.) population (limit values of 4.64 and 5.62 – with a mean of 5.19±0.07 to females; limit values of 4.83 and 5.73 – with an average of 5.21±0.04 to males). Comparative analysis of these reports, according to the sex of the individuals, shows non important differences between the averages of the reports, with an error of 0.05.

Tab. 2 – Report value between some of the analyzed biometric indicators

Number of individuals (*Rana ridibunda*)	Statistic parameter	Report		
		T/C.int.	D.p./C.int.	L./D.p.
Total individuals (49)	Minimum	6.574	3.021	4.636
	Maximum	12.093	4.906	5.731
	Average	9.255	3.720	5.200
	Standard error	0.163	0.058	0.037
	Variation coefficient	12.32	10.99	5.03
Females (17 individuals)	Minimum	7.782	3.108	4.636
	Maximum	10.636	4.102	5.619
	Average	9.092	3.666	5.187
	Standard error	0.229	0.082	0.072
	Variation coefficient	10.40	9.24	5.78
Males (32 individuals)	Minimum	6.574	3.021	4.828
	Maximum	12.093	4.906	5.731
	Average	9.342	3.749	5.207
	Standard error	0.218	0.078	0.043
	Variation coefficient	13.25	11.87	4.69

CONCLUSIONS

The study of variability of 12 morphological features of individuals belonging to a *Rana ridibunda* (Pall.) population from Balţii pool (Botoşani county), showed that, except the width of the head, all the other investigated parameters register average values approximately similar, according to sex.

The variability of the researched features is reduced both at the level of the analyzed sample, and according to sex. Yet male individuals generally present slightly increased values of the variation coefficient than females for most of the studied parameters.

Analysis of the report of some of the parameters investigated at *Rana ridibunda* (Pall.) shows a low variability of the report between the length of the body and the length of the first finger (L./D.p.) to both sexes, which makes this report reliable in the characterization of *Rana ridibunda* (Pall.) species.

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