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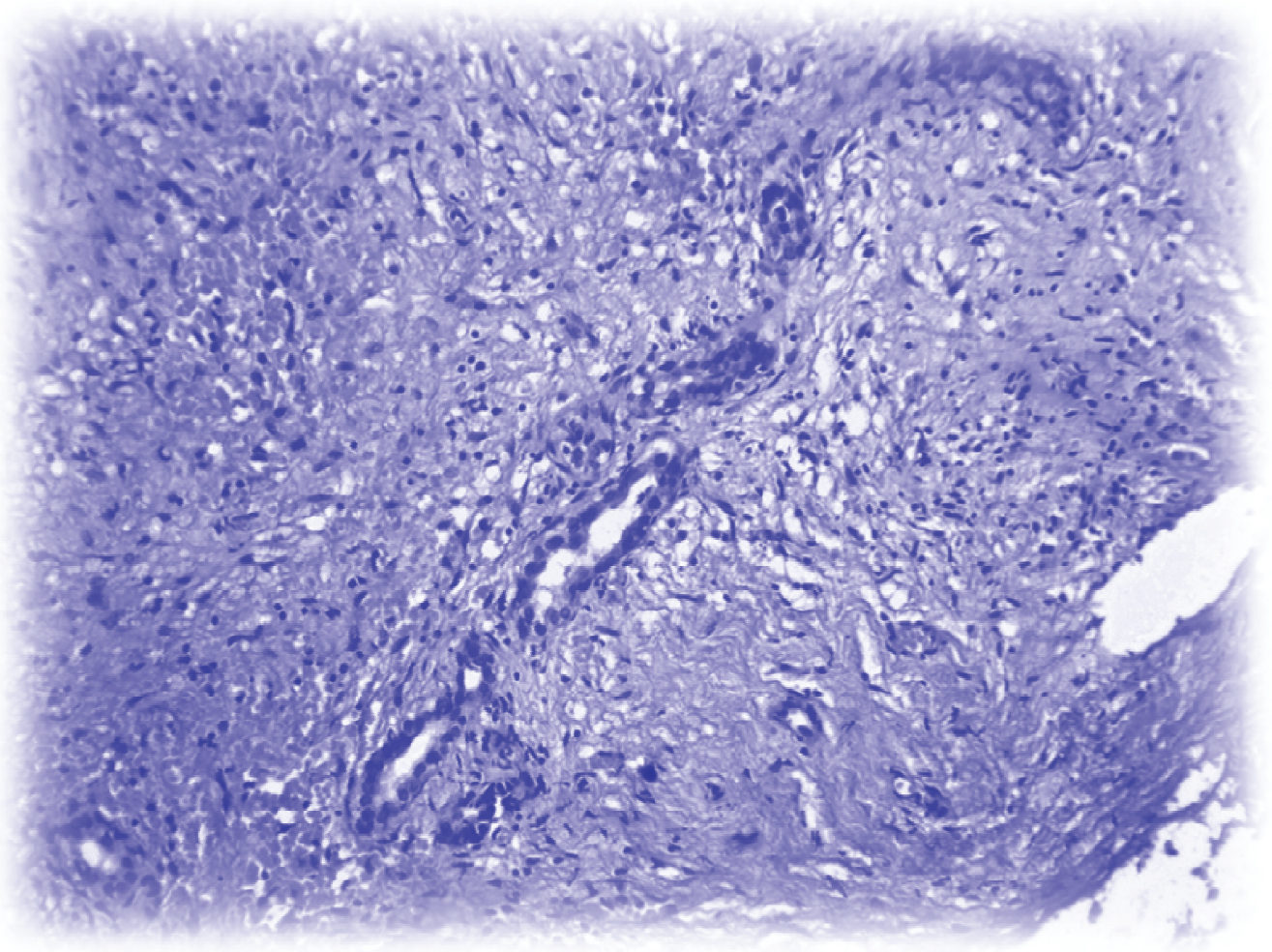

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REGULAR ARTICLES

REVIEW

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Original pictures on front cover: Luschka ducts, chronic inflammation and microhemorrhage in the gallbladder wall © Ştefan-Claudiu Mirescu

STUDIA UNIVERSITATIS BABEŞ-BOLYAI, BIOLOGIA, LIX, 2, 2014 (p. 5-16)

COMPARATIVE ANALYSIS OF D1 PROTEIN SEQUENCES IN CYANOBACTERIA

CIPRIAN CHIŞ[1, 2], IULIANA CHIŞ[1, 2], OANA SICORA[1], DALTON CARMEL[1, 3], AURICA GREC[3], AUREL ARDELEAN[3], NICOLAE DRAGOŞ[2] and COSMIN SICORA[1,✉]

SUMMARY. The D1 protein of Photosystem II (PSII), encoded by the *psbA* genes, is an indispensable component of oxygenic photosynthesis. Due to strongly oxidative chemistry of PSII water splitting, the D1 protein is prone to constant damage and requires its replacement by a new copy every 5 h under low light conditions and every 20 minutes under intense illumination, whereas most of the other PSII subunits remain ordinarily undamaged. In cyanobacteria the D1 protein is encoded by a *psbA* gene family, ranging from 1 to 6 members. The presence of multiple *psbA* genes encoding different D1 isoforms is an indication of their importance in regulatory mechanisms responsible for maintaining a functional PSII upon changing environmental conditions in natural habitats of cyanobacteria.

Herewith, we present a comparative analysis of the protein sequences encoded by *psbA* gene family in model cyanobacteria strains with sequenced genomes, highlighting their characteristic features that give indication of their putative function.

Keywords: cyanobacteria, D1 protein, Photosystem II, photosynthesis;

Introduction

Photosynthesis is one of the most important processes responsible for the maintenance of the oxygen level in the atmosphere and the reduction of CO_2 to carbohydrates (Loll et. al., 2005). It creates organic matter out of inorganic compounds. In plants, as well as in algae and cyanobacteria, photosynthesis is initiated in Photosystem II (PSII) using light energy to drive two distinct chemical reactions - the photo-oxidation of water and the reduction of plastoquinone (Singhal *et. al*., 1999).

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PSII is a large homo-dimeric protein-cofactor complex consisting of 20 protein subunits and more than 77 co-factors. Two proteins form the reaction center core of PSII: the D1 and D2 proteins. While higher plants have one *psbA* gene encoding the D1 protein, cyanobacteria generally display a small *psbA* gene family, consisting of 1 to 6 members, differentially expressed, according to environmental conditions (Mulo *et. al*., 2009). The D1 protein of PSII is an indispensable component of oxygenic photosynthesis. The aminoacid structure of the *PsbA* peptide gives clues about the hydrophobic nature for D1 protein with five membrane spanning helices (Dwivedi and Bhardwaj, 1995). The D1 protein is photodamaged because of the strongly oxidative chemistry of the Photosystem II, requiring its replacement, whereas most of the other subunits of PSII remain undamaged. The D1 protein is the primary component damaged and replaced in all organisms that perform oxygenic photosynthesis. In normal growth conditions a D1 protein is replaced in the PSII every 5 h, and under different stress conditions the replacement occurs every 20 minutes (Mulo *et al*., 2009). When the D1 protein is damaged, the Photosystem II is inactivated; the dimers separate, followed by the partial disassembly of the Photosystem II. The damaged D1 protein is removed by an FtsH type protease and degraded. Subsequently a new copy of D1 protein is translated, and the new D1 protein is co-translational inserted into the membrane. The C-terminus of the D1 protein is post-translational processed. The monomers are assembled, then the functional dimers are formed and the PSII is active again (Mulo *et al*., 2009).

Due to the advance of sequencing techniques, new genomes of cyanobacteria are being sequenced and annotated all the time (*http://genome.microbedb.jp/cyanobase/*), so periodically new *psbA* gene sequences and of the proteins they encode become available. These sequences allow for putative functional identification of the D1 isoforms and offer clues about their role. The studies of gene expression showed that under stress conditions, cyanobacterial cells adopt different strategies to cope with the changes. One strategy involves the production of larger amount of the same D1 protein, compensating for the higher rate of damage under stress, behavior best characterized in *Synechocystis* sp. PCC 6803, where one type of D1 protein is encoded by two *psbA* genes (Mate *et al*., 1998). Another strategy involves the replacement of a D1 protein isoform present in normal growth conditions with another one under stress conditions, this strategy was first described at *Synechococcus* sp. PCC 7942 (Sane *et al*., 2002). The D1 protein isoforms exchange occurs helping the cell to resist better in stress conditions and increases the adaptability to different environmental conditions (Clarke *et al.*, 1993). A parallel strategy involves the induction of another *psbA* gene, considered silent for a long time (Sicora *et al*., 2009, Summerfield *et al*., 2008). It was shown that during the microaerobic conditions, a distinct D1 protein isoform is induced, the D1' isoform, hence its postulated role in adaptation of the cells to low

oxygen conditions (Sicora *et al*., 2009; Summerfield *et al*., 2008). Recently, a new class of D1 protein termed 'D1 rogue' (D1r), present in diazotrophic cyanobacteria, was postulated by Murray (2012). Based on its structural characteristics, the protein D1 rogue impairs the PSII function during night allowing for nitrogen fixation.

In conclusion, five, functionally different, D1 protein isoforms are known to date (Mulo *et al*., 2009):

D1m or D1major is an isoform present in PSII in normal growth conditions, and induced in most stress conditions.

D1:1 is expressed in PSII under normal growth conditions, repressed under stress conditions.

D1:2 are repressed in PSII under normal growth conditions, induced under stress conditions. The D1:1 and D1:2 proteins differ at 25 of 360 amino-acid positions, with a conserved change of Gln with Glu at position 130 (Campbell *et al*., 1998).

D1' protein is induced in microaerobic conditions. The sequence of amino-acid shows three conserved mutations at positions 80, 158 and 286; these changes are characteristic to the D1' isoform: position 80 is a Gly to Ala mutation, at position 158 is a Phe to Leu mutation and at position 286 is a Thr to Ala mutation (Sicora *et al*., 2009).

D1r protein is a hypothetical protein, which blocks the Photosystem II during night and has 8 characteristic amino-acids differences; the most relevant from them are at positions: 61 Asp to Glu, 170 Asp to Glu/Ser, 189 Glu to Asp/Ala/Arg, 333 Glu to Ala/Ser, 342 Asp to Thr/Leu/Val, 344 Ala to Ala/Ser (Murray, 2012).

While functional characterization is needed for proper D1 isoform identification, in this article we identify possible D1 protein isoforms inferred from the existing sequencing data available fitting them in the proposed functional classes. We also propose models for the gene family development in the studied cyanobacteria strains.

Materials and methods

All the cyanobacterial *psbA* gene sequences were obtained from CyanoBase (*http://genome.microbedb.jp/cyanobase/*) and imported in the CLC Sequence Viewer 6.9.1 software (freeware http://www.clcbio.com). Using the aminoacid sequences, we made an accurate multiple alignment of all 91 sequences, bassed on progressive alignment algorithm. The alignment was manual edited, the non-informative regions were deleted resulting a number of approximative 360 positions. This made possible the comparison of the aminoacids conserved at all D1 protein isoforms. Based on

the alignment, we summarized the amino-acid changes in tables 2, 3 and 4. The next step was the construction of phylogenetic trees by Neighbour Joining method, 1000 replicates, made with CLC Sequence Viewer 6.9.1 program. A Venn diagram, showing the grouping and distribution of the studied D1 protein isoforms was constructed.

Results and discussion

In this study we analyzed a number of 30 cyanobacterial strains with sequenced genomes, from Cyanobase and identified a total of 91 *psbA* genes which encode different isoforms of the D1 protein (Table 1). There are cases when several genes encode one D1 protein isoform and other cases when each gene encodes a different D1 protein.

As the functional data accumulated it had become evident that all D1 protein isoforms have specific changes in the peptide structure. Based on this experimental observations it has been concluded that the presence at position in the position 130 of a glutamic acid (Glu), instead of glutamine (Gln) suggests the presence of the D1:2 isoform of the D1 protein strongly induced under stress conditions (Table 2).

Usually, one cyanobacterial strain posessing the D1:2 isoform also has the D1:1 isoform of the D1 protein expressed under optimal growth conditions, so we can see that from the total of 30 cyanobacterial strains studied, 17 strains have 45 *psbA* genes which encode the D1:2 isoform of the D1 protein.

Another specific isoform of the D1 protein (D1') is induced in microaerobic conditions (Sicora *et al.,* 2009). Based on the alignment, we searched the sequenced with the three changes in the aminoacid structure characteristic cu D1' isoform (Table 3). This table, shows that out of thirty cyanobacterial strains studied, ten have the D1' isoform of the D1 protein, the isoform that is induced in microaerobic conditions and probably helps the cells adapt to low oxygen conditions.

By bioinformatic analysis of D1 aminoacid compositionMurray (2012) concluded that a possible new isoform of the D1 protein that putatively blocks the Photosystem II during night and allows for nitrogen fixation. This isoform of the D1 protein ('D1 rogue') has 6 major changes in the aminoacids structure. The presence at the position 61 of glutamic acid (Glu) instead of aspartic acid (Asp), at position 130 of a glutamic acid (Glu) or serine (Ser) instead of an aspartic acid (Asp), at position 189 of an aspartic acid (Asp) or alanine (Ala) or arginine (Arg) instead of a glutamic acid aminoacid, are only 3 changes that suggest the presence of the D1 rogue isoform. From what we can see below (Table 4), five out of thirty cyanobacterial strains analyzed have three, four or five changes in the peptide structure characteristic to the D1 rogue protein.

Table 1.

Cyanobacterial strains analyzed in this study and the proposed number of *psbA* genes within.

No.	Cyanobacterial strain	Number of *psbA* genes
1	*Synechocystis* sp. PCC 6803	3
2	*Anabaena* sp. PCC 7120	5
3	*Thermosynechococcus elongatus* BP-1	3
4	*Gloeobacter violaceus* PCC 7421	5
5	*Mycrocystis aeruginosa* NIES-843	5
6	*Prochlorococcus marinus* SS120	1
7	*Prochlorococcus marinus* MED4	1
8	*Prochlorococcus marinus* MIT9313	2
9	*Synechococcus sp*. WH8102	4
10	*Synechococcus elongatus* PCC 6301	3
11	*Synechococcus sp*. CC9311	2
12	*Synechococcus sp*. PCC 7002	3
13	*Acaryochloris marina* MBIC11017	3
14	*Prochlorococcus marinus* str. NATL2A	3
15	*Anabaena variabilis* ATCC 29413	6
16	*Synechococcus sp*. CC 9902	4
17	*Synechococcus elongatus* PCC 7942	3
18	*Synechococcus sp*. JA-2-3B'a(2-13)	3
19	*Synechococcus sp*. JA-3-3Ab	4
20	*Prochlorococcus marinus* str. AS9601	1
21	*Prochlorococcus marinus* str. MIT9515	1
22	*Prochlorococcus marinus* str. MIT9303	1
23	*Prochlorococcus marinus* str. NATL21A	2
24	*Prochlorococcus marinus* str. MIT 9301	1
25	*Synechococcus sp*. RCC307	4
26	*Synechococcus sp*. WH7803	4
27	*Prochlorococcus marinus* str. MIT9215	1
28	*Cyanothece sp*. ATCC 51142	5
29	*Nostoc punctiforme* ATCC 29133	4
30	*Arthrospira platensis* NIES-39	4
TOTAL	30	91

Table 2.

List of proteins (emphasized by bold characters below strain's name) from studied cyanobacterial strains exhibiting aminoacid changes characteristic to D1:1 and D1:2 isoforms.

D1:2		
130 Gln to Glu		
Synechococcus sp. PCC 7002	*Synechococcus* sp. PCC 7002	*Anabaena* sp. PCC 7120
SynPCC7002_A1418	**SynPCC7002_A0157**	**Alr_4592**
Synechococcus elongatus PCC 7942	*Synechococcus elongatus* PCC 7942	Anabaena sp. PCC 7120
Synpcc7942_1389	**Synpcc7942_0893**	**Alr_3727**
Synechococcus elongatus PCC 6301	*Synechococcus elongatus* PCC 6301	*Anabaena* sp. PCC 7120
Syc0166_d	**Syc0647_d**	**All_3572**
Anabaena variabilis ATCC 29413	*Anabaena variabilis* ATCC 29413	*Anabaena variabilis* ATCC 29413
Ava_1597	**Ava_2460**	**Ava_3553**
Nostoc punctiforme ATCC 29133	*Nostoc punctiforme* ATCC 29133	*Nostoc punctiforme* ATCC 29133
Npun_F3544	**Npun_R5188**	**Npun_R2273**
Synechococcus sp. WH8102	*Synechococcus* sp. WH8102	*Synechococcus* sp. WH8102
SYNW0983	**SYNW1919**	**SYNW2151**
Synechococcus sp. CC9902	*Synechococcus* sp. CC9902	*Synechococcus* sp. CC9902
Syncc9902_1814	**Syncc9902_2036**	**Syncc9902_1817**
Synechococcus sp. CC9311	*Synechococcus* sp. WH 7803	*Synechococcus* sp. WH 7803
Sync_2384	**SynWH7803_0366**	**SynWH7803_0790**
Synechococcus sp. WH 7803	*Synechococcus* sp. RCC307	*Synechococcus* sp. RCC307
SynWH7803_2084	**SynRCC307_1440**	**SynRCC307_2009**
Synechococcus sp. RCC307	*Arthrospira platensis* NIES-39	*Arthrospira platensis* NIES-39
SynRCC307_2183	**NIES39_K03030**	**NIES39_03080**
Arthrospira platensis NIES-39	*Thermosynechococcus elongatus* BP-1	*Thermosynechococcus elongatus* BP-1
NIES39_R00140	**Tlr1477**	**Tlr1844**
Synechococcus sp. JA-2-3B'a(2-13)	*Synechococcus* sp. JA-2-3B'a(2-13)	*Synechococcus* sp. JA-3-3Ab
CYB_0371	**CYB_0433**	**CYA_1811**
Synechococcus sp. JA-3-3Ab	*Synechococcus* sp. PCC 7002	*Arthrospira platensis* NIES-39
CYA_1849	**SynPCC7002_A2164**	**NIES39_O04720**

Table 2. continued

Gloeobacter violaceus PCC 7421 **Glr0779**	*Gloeobacter violaceus* PCC 7421 **Glr2322**	*Gloeobacter violaceus* PCC 7421 **Glr2656**
Gloeobacter violaceus PCC 7421 **Glr1706**	*Gloeobacter violaceus* PCC 7421 **Gll3144**	*Cyanothece* sp. ATCC 51142 **Cce_3411**

Table 3.

List of proteins from studied cyanobacterial strains that exhibit aminoacid changes characteristic for the D1' isoform.

D1'		
80 Gly to Ala	**158 Phe to Leu**	**286 Thr to Ala**
Thermosynechococcus elongatus BP-1 **Tlr1844**	*Thermosynechococcus elongatus* BP-1 **Tlr1844**	*Thermosynechococcus elongatus* BP-1 **Tlr1844**
Synechococcus sp. PCC 7002 **SynPCC7002_A2164**	*Synechococcus* sp. PCC 7002 **SynPCC7002_A2164**	*Synechococcus* sp. PCC 7002 **SynPCC7002_A2164**
Arthrospira platensis NIES-39 **NIES39_O04720**	*Arthrospira platensis* NIES-39 **NIES39_O04720**	*Arthrospira platensis* NIES-39 **NIES39_O04720**
Synechocystis sp. PCC 6803 **Slr1181**	*Anabaena* sp. PCC 7120 **Alr3742**	*Synechocystis* sp. PCC 6803 **Slr1181**
Anabaena sp. PCC 7120 **Alr3742**	*Anabaena variabilis* ATCC 29413 **Ava_1583**	*Anabaena* sp. PCC 7120 **Alr3742**
Anabaena variabilis ATCC 29413 **Ava_1583**	*Cyanothece* sp. ATCC 51142 **Cce_3411**	*Anabaena variabilis* ATCC 29413 **Ava_1583**
Cyanothece sp. ATCC 51142 **Cce_3411**	*Synechococcus* sp. JA-3-3Ab **CYA_1748**	*Cyanothece* sp. ATCC 51142 **Cce_3411**
Synechococcus sp. JA-3-3Ab **CYA_1748**	*Synechococcus* sp. JA-2-3B'a(2-13) **CYB_0216**	*Synechococcus* sp. JA-3-3Ab **CYA_1748**
Synechococcus sp. JA-2-3B'a(2-13) **CYB_0216**	*Anabaena variabilis* ATCC 29413 **Ava_4121**	*Synechococcus* sp. JA-2-3B'a(2-13) **CYB_0216**
Cyanothece sp. ATCC 51142 **Cce_3477**	*Acaryochloris marina* MBIC11017 **AM1_0448**	*Cyanothece* sp. ATCC 51142 **Cce_3477**
Anabaena variabilis ATCC 29413 **Ava_4121**		*Anabaena variabilis* ATCC 29413 **Ava_4121**

Table 3. continued

Acaryochloris marina MBIC11017 **AM1_0448**	*Acaryochloris marina* MBIC11017 **AM1_0448**

Table 4.

List of proteins from studied cyanobacterial strains that exhibit aminoacid changes characteristic to the D1rogue isoform.

D1r		
61 Asp to Glu	**170 Asp to Asp/Glu/Ser**	**189 Glu-Asp/Ala/Arg**
Cyanothece sp. ATCC 51142 **Cce_3477** *Acaryochloris marina* MBIC11017 **AM1_0448**	*Synechococcus* sp. JA-3-3Ab **CYA_1748** *Synechococcus* sp. JA-2-3B'a(2-13) **CYB_0216** *Cyanothece* sp. ATCC 51142 **Cce_3477** *Acaryochloris marina* MBIC11017 **AM1_0448**	*Cyanothece* sp. ATCC 51142 **Cce_3477** *Anabaena variabilis* ATCC 29413 **Ava_4121**

D1r		
333 Glu to Ala/Ser	**342 Asp to Thr/Leu/Val**	**344 Ala to Ser**
Synechococcus sp. JA-3-3Ab **CYA_1748** *Synechococcus* sp. JA-2-3B'a(2-13) **CYB_0216** *Cyanothece* sp. ATCC 51142 **Cce_3477** *Anabaena variabilis* ATCC 29413 **Ava_4121** *Acaryochloris marina* MBIC11017 **AM1_0448**	*Synechococcus* sp. JA-3-3Ab **CYA_1748** *Synechococcus* sp. JA-2-3B'a(2-13) **CYB_0216** *Cyanothece* sp. ATCC 51142 **Cce_3477** *Anabaena variabilis* ATCC 29413 **Ava_4121** *Acaryochloris marina* MBIC11017 **AM1_0448**	*Anabaena variabilis* ATCC 29413 **Ava_4121**

Based on the analysis of the tables 2, 3 and 4, we observed the possible presence of several types of D1 protein at the same cyanobacteria strain, without a very obvious relation between them. To better highlight the number of cyanobacterial strains which contain several different isoforms of the D1 protein, we built a Venn diagram (Fig. 1). We observed that from the total of thirty cyanobacterial strains analyzed, four strains have all the three isoforms of D1 protein (D1:2, D1' and D1r).

Seventeen cyanobacterial strains contain the D1:2 isoform of D1 protein. From these seventeen genomes, nine have just the D1:2 isoform, four have both the D1:2 and D1' isoform and four of them have all the 3 isoforms discussed.

As concerns the D1' isoform, ten cyanobacteria contain this isoform of D1 protein; one strain has only the D1' isoform out of the three studied isoforms. Four strains contain the D1:2 and D1' isoform, four strains contain all the three isoforms and only one strain contains the D1' together with the D1r isoform.

The D1 rogue isoform is present in five cyanobacterial genomes, from which four genomes have all the three isoforms and one have the D1r and D1' isoforms.

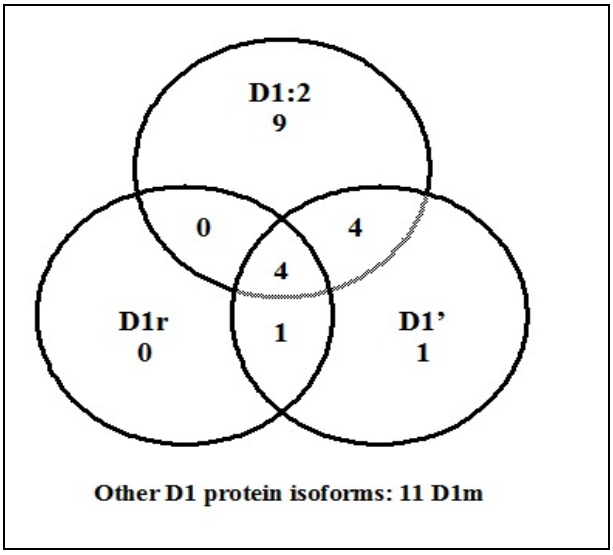

Figure 1. Venn diagram summarizing the distribution of D1 protein isoforms among the cyanobacterial strains analyzed.

Based on the alignment done with the sequences imported in the CLC sequence Viewer 6.9.1 software, a phylogenetic tree including all 91 sequences was constructed by using Neighbour Joining method, (Fig. 2).

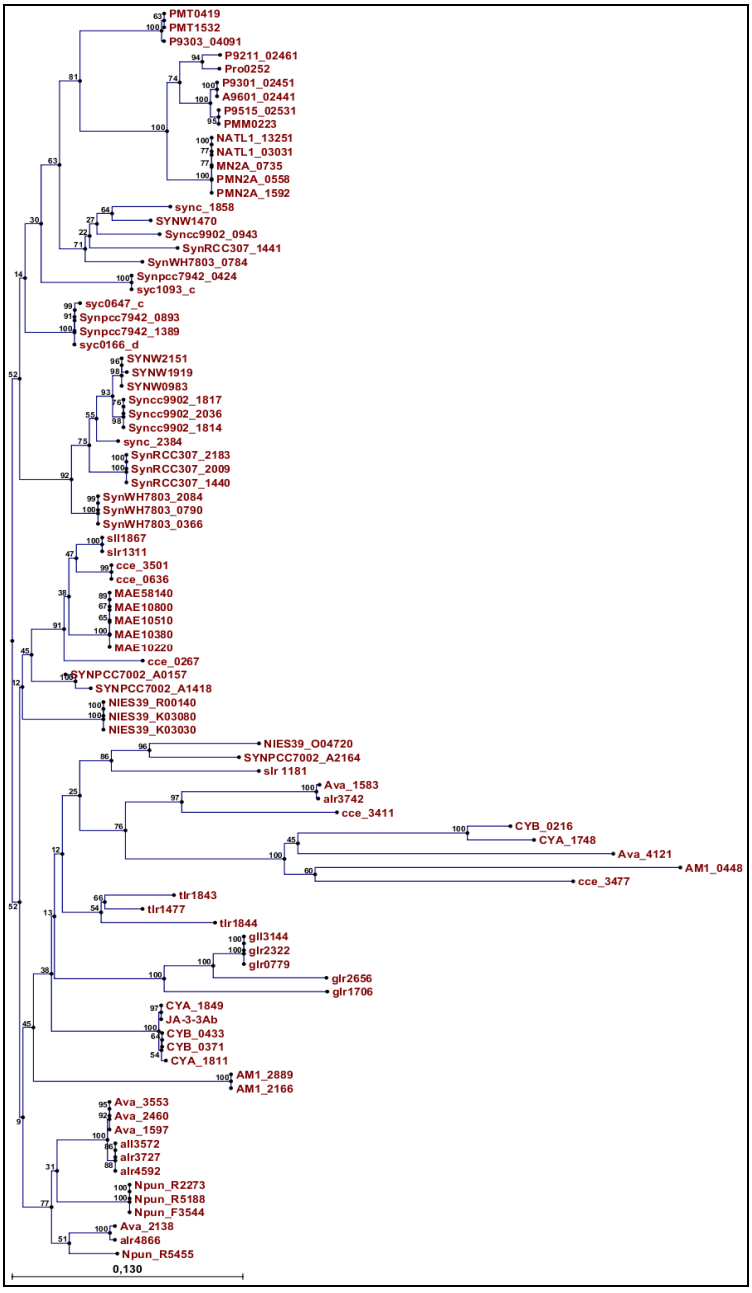

Figure 2. Phylogenetic tree of the 91 protein sequences from Cyanobase, made with Neighbour Joining method, 1000 bootstrap replicates. The scale bar represents number of insertions, deletions/time.

Analyzing the tree it becomes evident that specific isoforms are grouped together independent of the cyanobacterial strain they belong to. D1' protein from *Arthrospira platensis* NIES-39 (NIES39_O04720) is grouped with the one from *Synechococcus sp*. PCC 7002 (SYNPCC7002_A2164), *Synechocystis sp*. PCC 6803 (Slr1181), *Anabaena variabilis* ATCC 29413 (Ava_1583), *Anabaena sp*. PCC 7120 (Alr3742) and *Cyanothece sp*. ATCC 51142 (Cce_3411). There are, however, several D1 protein families grouped after the strain they belong. Examples for this case are the D1 protein family from *Gloeobacter violaceus* PCC 7421 (glr0779, gll3144, glr2322, glr2656, glr1706) or *Thermosynechococcus elongatus* BP-1 (tlr1843, tlr1477, tlr1844).

Based on our results, we can conclude that there are two possible ways in which the *psbA* genes and the D1 protein encoded by them developed.

One strategy is by horizontal transfer, when the proteins are more closely related to each other than to the other members of the gene family from the same strain, it can be observed that the D1' protein from different species that group together (Fig. 2). An explanation can be the fact that the strains sharing the same environment conditions managed to adapt by the importing a gene that will give them an evolutionary edge. The transfer can be intermediated by common phages infecting a specific community.

The second strategy is by intraspecific duplication of the genes. In this way the strains create new forms of D1 protein by duplication from the existing ones as a response to the need to adapt to changes in the environment (Sajjaphan *et al*., 2002).

Conclusions

Herewith, we reviewed the available new information regarding the D1 protein isoforms from newly sequenced cyanobacteria genomes and we presented a possible functional distribution of these forms based upon the available knowledge. This information can trigger further functional studies leading to characterization of new D1 protein families. We also processed these data into an evolutionary tree that gives indications regarding the evolutionary strategies of the *psbA* gene family from cyanobacteria. The presented data bring, original interpretation of updated information regarding the *psbA* gene families and the encoded D1 protein isoforms available from the newly sequenced cyanobacterial genomes.

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REFERENCES

Campbell, D., Eriksson, M-J., Oquist, G., Gustafsson, P., Clarke, A.K. (1998) The cyanobacterium Synechococcus resists UV-B by exchanging photosystem II reaction-center D1 proteins, *Proc. Natl. Acad. Sci. USA* **95**, 364-369

Clarke, A.K., Hurry, V.M., Gustafsson, P., Oquist, G. (1993) Two functionally distinct forms of the photosystem II reaction-center protein D1 in the cyanobacterium *Synechococcus* sp. PCC 7942, *Proc. Natl. Acad. Sci. USA* **90**, 11985-11989

Dwivedi, U., Bhardwaj, R. (1995) D1 protein of photosystem II: The light sensor in chloroplasts, *J. Biosci*. **20**, 35-47

Loll, B., Kern, J., Saenger, W., Zouni, A., Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II, *Nature*, **438**, 1040-1044

Mate, Z., Sass, L., Szekeres, M., Vass, I., Nagy, F. (1998) UV-B induced differential transcription of *psbA* genes encoding the D1 protein of photosystem II in the cyanobacterium *Synechocystis* 6803, *J. Biol. Chem.* **273** 17439-17444

Mulo, P., Sicora, C., Aro, E-M. (2009) Cyanobacterial *psbA* gene family: optimization of oxygenic photosynthesis, *Cell. Mol. Life. Sci* **66**: 3697-3710

Murray, J.W. (2012) Sequence variation at the oxygen-evolving centre of photosystem II: a new class of 'rogue' cyanobacterial D1 proteins, *Photosynth Res* **110**, 177–184

Sajjaphan, K., Shapir, N., Judd, A.K., Wackett, L.P., Sadowsky, M.J. (2002) Novel *psbA1* Gene From a Naturally Occuring Atrazine-Resistant Cyanobacterial Isolate, *Appl. Environ. Microbiol.,*, **68**, 1358-1366

Sane, P.V., Ivanov, A.G., Sveshnikov, D., Huner, N.P.A., Oquist, G. (2002) A transient exchange of the photosystem II reaction center protein D1:1 with D1:2 during low temperature stress of *Synechococcus* sp. PCC 7942 in the light lowers the redox potential of Q (B), *J. Biol. Chem.* **277**, 32739-32745

Sicora, C.I., Ho, F.M., Salminen, T., Styring S., Aro, E-M. (2009) Transcription of a "silent" cyanobacterial *psbA* gene is induced by microaerobic conditions, *Biochim.Biophys. Acta.* **1787**, 105-112

Singhal, G.S., Renger, G., Sopory, S. K., Irrgang, K. D., Govindjee. (1999) Introduction to Photobiology, Photosynthesis and Photomorphogenesis, In: Concepts in photobiology, Photosynthesis and Photomorphogenesis, *Narosa Publishing House*, 1-7

Summerfield, T. C., Toepel, J., Sherman, L. A. (2008) Low-oxygen induction of Normally Cryptic *psbA* genes in Cyanobacteria, *Biochemistry*, **47**, 12939-12941

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FORWARD ELECTRON TRANSPORT MEASURED *IN SITU* IN MICROBIAL MATS FROM A HOT SPRING IN N-W ROMANIA

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SUMMARY. Cyanobacteria, green algae and higher plants have highly conserved photosynthetic machinery. Cyanobacteria have a great plasticity and can be found in different environments, from Arctic zones to hot springs; they can be part of microbial mats. The hot spring near Ciocaia, in Bihor County has a range of different temperatures, from 65.5°C at the spring well, 60.5°C at 2 meters away from the spring well and between 32-38°C in the area where microbial mats are formed. In this study we showed changes in PSII function in mats grown at the location of the hot spring, comparative with microbial mats grown in laboratory conditions. We also showed a comparative study of some basic parameters of chlorophyll fluorescence. These results are very important to understand the functionality of photosystem II in the larger context of adaptation to environmental cues.

Keywords: chlorophyll, cyanobacteria, fluorescence, hot spring

Introduction

Photosynthesis is the fundamental process by which cyanobacteria, algae and higher plants use water as a source of electrons in reducing CO_2 to various organic compounds, this way being responsible for maintaining the oxygen level in atmosphere, and in a larger context, sustaining life on earth (Loll *et. al*., 2005). In organisms performing oxygenic photosynthesis, the linear electron transport (light reactions) takes place in the thylakoid membrane-embedded proteins, which are Photosystem II (PSII), Cytochrome b_6f ($Cytb_6f$) and Photosystem I (PSI). Photosynthesis is initiated in PSII, a dimeric protein-cofactor membrane embedded complex. By capturing the light energy, PSII produces the energy required to oxidize water to atmospheric oxygen (Singhal *et al*., 1999). PSII is a complex that consists of more than 20 protein subunits and 77 co-factors (Loll *et. al*., 2007). The D1 and D2 proteins are

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involved in binding the most redox active components of PSII. The factors which damage the linear electron transport equilibrium can lead to the accumulation of toxic intermediates that will cause irreversible damage to the PSII reaction center, especially to protein D1. The D1 protein of Photosystem II, encoded by the *psbA* genes, is indispensable for oxygenic photosynthesis. The aminoacid structure of the *psbA* gene predicts a hydrophobic nature for D1 protein with five membrane spanning helices (Dwivedi and Bhardwaj, 1995). Due to strongly oxidative chemistry of PSII water splitting, the D1 protein is prone to constant photodamage requiring its replacement, whereas most of the other PSII subunits remain ordinarily undamaged. When the photoinhibition occurs, the D1 protein is accessed by the FtsH proteases and degraded. Subsequently, a new copy of D1 protein is inserted in the membrane, the dimers are formed, and the functional PSII is activated again (Mulo *et al*., 2009). In normal growth conditions a D1 protein is replaced in the PSII at every 5h, and in different stress conditions the replacement occurs in 20 minutes.

During the light reactions of photosynthesis is described the pathway of electron transport from water to $NADP^+$, resulting in release of oxygen, the reduction of $NADP^+$ to NADPH and the accumulation of a high concentration of hydrogen ions in the thylakoid lumen, needed for ATP production. During the light reactions, fluorescence measurement reveal functional changes in the acceptor and donor side of PS II (Fig. 1). Mn is a Manganese center, has 4 manganese atoms and splits 2 water molecules into 4 protons ($4H^+$), 4 electrons ($4e^-$) and 2 oxygen atoms. Tyrosine (Tyr) shuttles electrons to the reaction center of PSII, P680. P680 absorbs a photon of light energy and reaches the excited state. Whereas P680 is the primary electron donor of PSII, Pheo is a pheophytin molecule, the primary electron acceptor of PSII. After absorbing the light, P680 sends one electron to Pheo; Pheo accepts the electron, it is reduced, and transfer than the electron to Q_A. The electron transfer until Q_A is very fast, and cannot be detected. The transfer between Q_A and Q_B takes a few milliseconds, and can be detected during chlorophyll fluorescence measurement. Q_A is a plastoquinone molecule fixed into the membrane which accepts and transfers one electron at a time. Q_B is a loosely bound plastoquinone, which at 2 cycles accepts 2 electrons from Q_A, than becomes mobile and goes to the plastoquinone pool from the thylakoid membrane. In cyanobacteria, the photosynthetic system is tightly connected to the other principal metabolic paths; therefore, chlorophyll fluorescence signals can provide rapid, real-time information on both photosynthesis and the overall acclimation status of cyanobacteria (Campbell *et al*., 1998).

At the hot spring drilling of Ciocaia, in Bihor County, Romania, a microbial community mat was formed, adapted to high water temperatures Visually, the microbial mats formed here have different colors: green, red and white, based on the temperatures they grow at. In this article we performed for the first time PSII functional studies *in situ* on a hot spring microbial mat, using chlorophyll fluorescence measurements with a submersible measuring head attached to the double modulation

fluorometer. We were able to get indications relative to the photosynthetic function of the microbial communities in their natural environment. Based upon the specific conditions of studied cyanobacterial mat we developed a fluorescence measurement protocol that can be further used for mat measurements. We also show a comparative analysis of the forward electron transport between the measurements *in situ* of microbial communities from Ciocaia, and laboratory grown *Phormidium* strains.

Materials and methods

The measurements were performed *in situ* at Ciocaia thermal drilling. The hot spring near Ciocaia (Bihor County, Nort-Western Romania) is geographically located at E 22°02.744' and N 47°20.471'. The chemical composition of water is 812 mg/l Cl^-, 7,283 mg/l $HCO3^-$, 27.9 mg/l $SO4^{2-}$, 7.9 mg/l $NH4^+$, 3,525 mg/l Na^+, 30 mg/l K^+, 790 mg/l Ca^{2+}, 2.6 mg/l Mg^{2+}, 0.2 mg/l Fe^{2+} and 3.7 wt.% total salinity (Ţenu *et al*., 1981). The fluorescence measurements were made with an FL3500 Fluorometer (Photon Systems Instruments, Brno, CZ), using a Q_{A-} reoxidation protocol . The field fluorescence measurements were performed with a submersible head, designed to measure fluorescence of underwater organisms, from Photon Systems Instruments. The submersible head was powered by 220 V car adapted convertor. We optimized the protocol for on field measurements considering the specific conditions of the mat-building communities: we had to modify the intensity of measuring flashes in order to decrease the actinic effect of the measuring flashes as well as the saturation of basic fluorescence caused by the huge cell density specific to the bacterial mat (Table 1).

The double-modulation fluorometer from PSI (CZ) can measure the chlorophyll fluorescence with a resolution of 4 micro-seconds, it is adapted for liquid sample measurement, can measure many photosynthetic parameters and in combination with DCMU (3-(3, 4-dichlorophenyl)-1, 1-dimethylurea), provides information about both the donor and the acceptor side of PSII (Fig. 1).

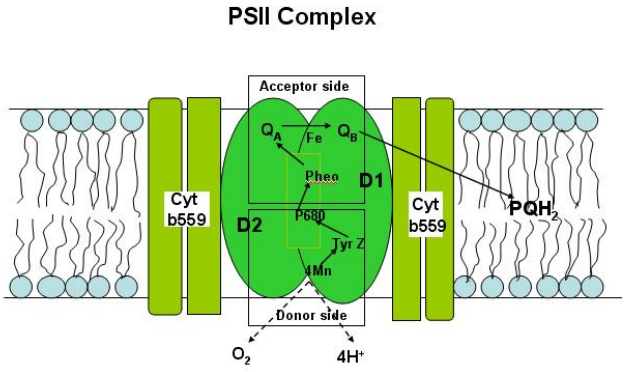

Figure 1. Functional structure of the Photosystem II.(original)

The transfer from Q_A to Q_B represents the acceptor side of PSII, so that the fluorescence measurement in absence of DCMU will give information about the functionality of the acceptor side of PSII (Fig. 1). DCMU is an herbicide that binds to the Q_B locus; this leads to the return of the electrons to the water oxidation complex (Metz *et al*., 1986). In presence of DCMU, any changes in the functionality of the water oxidation complex will be observed at fluorescence measurement in presence of DCMU (Fig. 1).

Below we are showing the measurement protocol used in our experiments. The Double-Modulation Fluorometer FL 3500 measures Chl-fluorescence signal with a time resolution of up to 4 μs in the Standard FL3500/S version. Measured fluorescence emission is excited by a set of light-emitting diodes that generate short measuring flashes. The photochemistry is driven by single-turnover flashes. Chlorophyll fluorescence is detected by a PIN photodiode and digitized by a 16-bit A/D converter. PSI fluorometers can measure various chlorophyll fluorescence parameters, ratios, and quenching coefficients that provide information on the functionality of the photosynthetic apparatus. In our experiments we followed the reoxidation of Q_A^- after a saturating actinic flash, probed with series of measuring flashes on a logarithmic time scale from microseconds up to 100 seconds. In addition we recorded three basic fluorescence parameters: F0, Fm and Fv. A short description of these parameters is given below:

F0 represents the minimum chlorophyll fluorescence yield measured on a dark-adapted sample.

Fm represents the maximum chlorophyll fluorescence yield measured on a dark-adapted sample.

Fv represents the variable fluorescence obtained: Fv=Fm-F0.

All samples used for fluorescence measurements were dark-adapted for 6 minutes before measurement was done in absence and presence of DCMU.

Table 1.

Fluorescence measurement protocol parameters used in field and laboratory measurements.

Sample Measured	Ciocaia - Field sample	Ciocaia- laboratory sample
Measuring Flash (μs)	8	8
Measuring delay (μs)	6	6
Actinic Flash (μs)	20	20
Pre-Flash (μs)	0	0
M_Voltage	10	30
F_Voltage	100	100
A_Voltage	100	100

M_voltage= relative power of Measuring flashes, relative voltage of the Measuring LED's. The signal is proportional to the voltage in 20%-100%.

F_Voltage= relative voltage of the actinic Flash or relative power of actinic flashes.

A_voltage = relative voltage of the actinic light LED's.

The laboratory *Phormidium* sp. culture from Ciocaia was grown in the BG11 medium, at 38ºC and 50 µmol photons $m^{-2}s^{-1}$ light. The culture was mixed by bubbling air all the time.

Finally, data were processed in OriginPro 8 (OriginLab Corporation, Northhampton, MA 01060, USA) software.

Results and discussion

Recording the function of PSII, the key component of the photosynthetic electron transport chain, gives useful insight into cell metabolism and allows for understanding of molecular effect of stress factors. Use of the Flash induced Chlorophyll Fluorescence methods using modern fluorometers allow for functionally dissecting of PSII photochemistry. These measurements are usually performed under laboratory controlled conditions. We devised a measurement protocol allowing the measurement of the reoxidation of Q_A- under field conditions using a submersible measuring head.

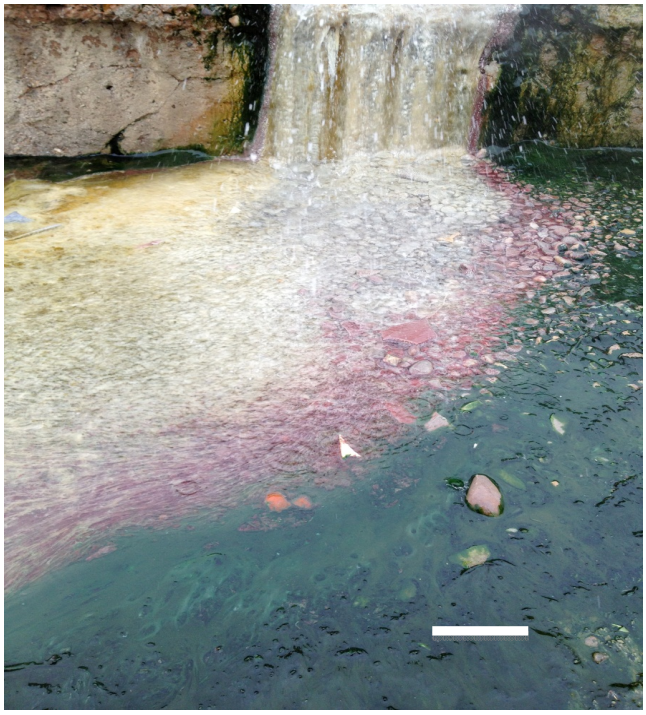

Figure 2. Image of microbial mats formed around the Ciocaia hot spring. Depending on the temperature gradient the composition of the mat and the color change from white at highest to green at lower temperatures (the bar is 20cm long).

The hot waters from Ciocaia thermal drilling flow directly on the ground forming a small hot spring. Close to the point of emergence a series of microbial mats are formed depending on water temperature, between 35°C and 60°C (Fig. 2). Closest to the drill and at highest temperature white color mat is present, followed by a red color mat at intermediate temperatures and a green mat at lower temperatures (Fig. 2).

A sample was collected from the green mat and photo-autothrophically grown in the laboratory at 38°C and BG-11 medium. Under light microscope the mat proved to be dominated by a *Phormidium* sp. strain (Fig. 3) (Coman, 2011). We used this strain as a control for our fluorescence measurements. Repetitions of fluorescence measurements were done in all of the above-mentioned mats.

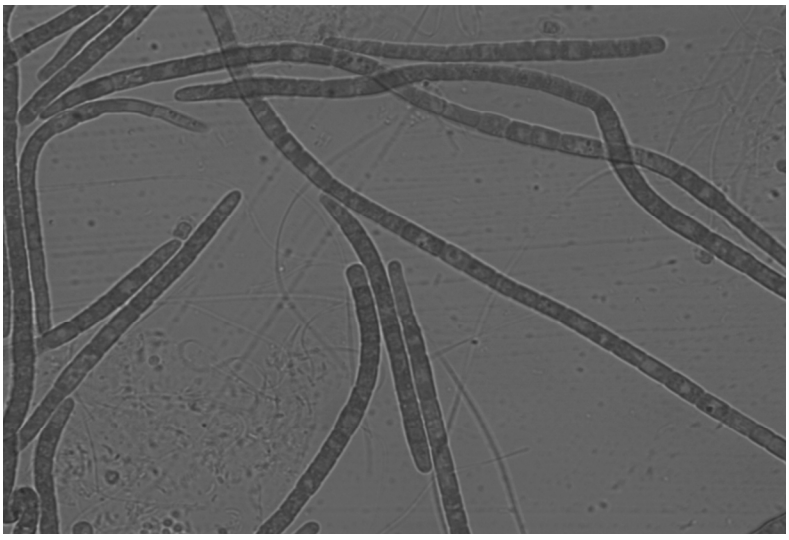

Figure 3. Typical shape of *Phormidium* sp. containing laboratory culture.
Laboratory culture isolated from the green mat from Ciocaia hot spring.
Optical microscope image at 100x magnification.

Initially F0, Fm and Fv parameters were measured and calculated in all three microbial mats as well as the laboratory sample (Fig. 4). The highest F0 value was recorded in the laboratory grown sample followed by the green mat from the field. The red and white mats showed small F0 values (Fig.4, panel A). The highest Fm value was recorded at the laboratory grown sample, followed by the value from the green mat, while the values from the red and white mat are close to 0 (Fig.4, panel B). Also, the variable fluorescence parameter, Fv follows the same decrease, from the highest value at the laboratory sample, followed by the green mat from the field. The red and white microbial mats have small Fv values (Fig.4, panel C).

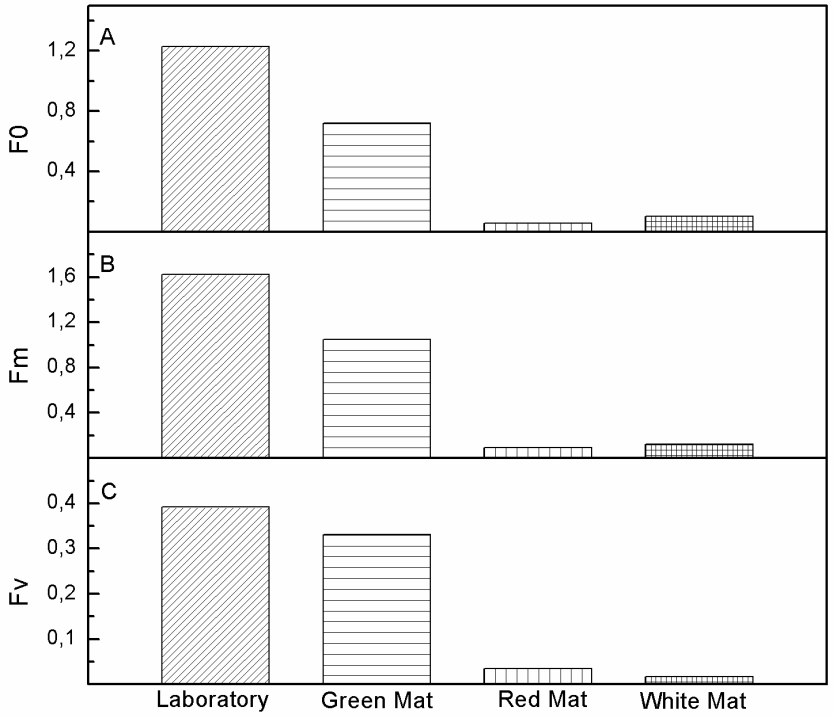

Figure 4. Basic fluorescence parameters F0 (panel A), Fm (panel B) and Fv (panel C) measured in all three microbial mats from Ciocaia as well as the laboratory grown sample.

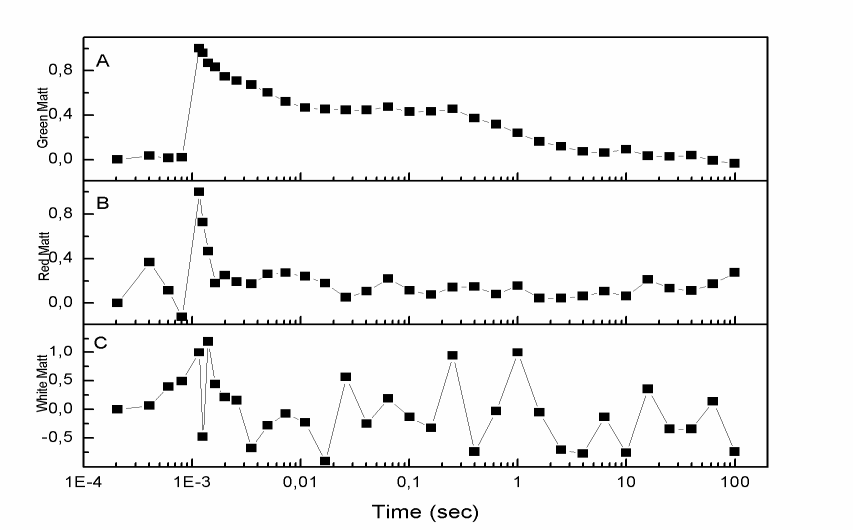

Figure 5. Changes of forward electron flow in the acceptor side of photosystem II at the green mat (A), red mat (B) and white mat (C) from Ciocaia hot spring.

Using the modified measuring protocol for the field measurements, we followed the forward electron transfer within PSII. In case of the green mat from the field (Fig. 5, panel A), the fluorescence decay curve looks close to the control one showing the typical features of a Q_A^- reoxidation decay curve. Following this experimental protocol we can evaluate the acceptor side of PSII. In our case there is a distinct slowdown of Q_A to Q_B transfer, and the Fm value is close to the 1.2 value.

The red mat (Fig. 5, panel B) displays a clear charge separation at the time of the saturating flash showing the formation of Q_A^-. However, the re-oxidation of Q_A^- is massively distorted, without the typical characteristics of a fluorescence decay curve. This will be consistent with an organism that does not exhibits typical photosynthesis, possibly bacteriochlorophyll-dependent photosynthesis still relying on energy from the light.

The white mat from Ciocaia did not show any signs of response to light excitation with the measurement being consistent with bacteria that has no light absorbance capacity but is probably adapted to high temperature (Fig. 5, panel C).

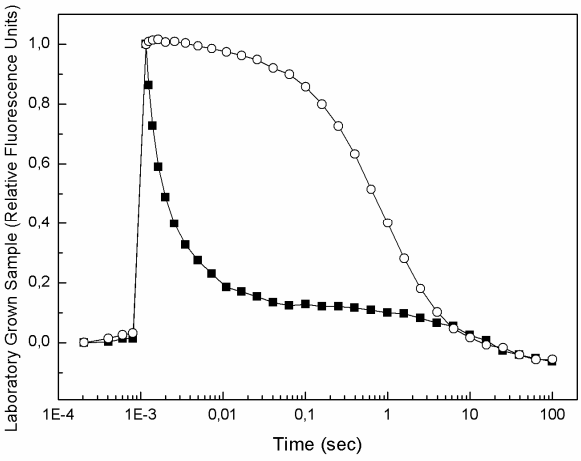

Figure 6. Characteristics of donor and acceptor side functionality of the photosystem II measured in the absence (solid squares) and presece of DCMU (open circle) in the laboratory sample isolated from the green mat from Ciocaia.

The laboratory-grown *Phormidium* sample started from the green mat from Ciocaia (Fig. 6), displayed a faster electron transfer between Q_A and Q_B corresponding to a "normally" functioning acceptor side of the photosystem II. In the presence of DCMU, that blocks the Q_A to Q_B transfer and prompts the back-recombination of Q_A^- with the donor-side components of PSII in particular the water-oxidation complex the fluorescence level is maintained at high values for

longer time before the recombination. The laboratory-grown sample showed a typical, unmodified DCMU curve giving indication of a standard PSII donor side in this cyanobacterium. This type of measurement could not be performed in the field samples, as we could not apply a DCMU treatment to the mat without fatally damaging the cells.

The fact that in the lab sample the fluorescence is decaying faster than the field measurement indicates the partial impairment of the electron transport that naturally occurs when the cells are exposed to sunlight. To our knowledge, this is the first measurement of forward electron transfer in a hot-spring cyanobacterial mat. This observation is very important, functionally, as most experiments are done under laboratory conditions and usually are claimed to mimic natural conditions.

Conclusions

Ciocaia hot spring is a good environmental model to study the physiology of microbial mat as it offers access to stable functional mats all year round. In our case, we performed field fluorescence measurements for the first time in three microbial mats of a hot-spring. From our experiments, we can conclude that:

- Due to the very high cell density, characteristic to a microbial mat, the optimization of the fluorescence measurement protocol was necessary in order to reduce noise and increase signal quality due to the field conditions;
- The fluorescence method we optimized, allows for the identification in the green microbial mat of members which have the capacity to use light as source of energy;
- A laboratory-grown sample isolated from the green mat was used to highlight the differences in forward electron transport between the laboratory condition and the natural conditions in the field. The first, showed a high amplitude of fluorescence characteristic to a high number of PSII active centers with a fast Q_A to Q_B electron transfer while in the field these traits were modified probably due to the effect of environmental conditions.

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REFERENCES

Campbell, D., Eriksson, M. - J., Oquist, G., Gustafsson, P., Clarke, A.K. (1998) The cyanobacterium *Synechococcus* resists UV-B by exchanging photosystem II reaction-center D1 proteins, *Proc. Natl. Acad. Sci. USA* **95**, 364-369

Coman, C. (2011) Studiul Biodiversitatii Comunitatilor Cianobacteriene Asociate Izvoarelor Termominerale din Campia de Vest a Romaniei prin Tehnici Moleculare, Cluj-Napoca, Teza de doctorat

Dwivedi, U., Bhardwaj, R. (1995) D1 protein of photosystem II: The light sensor in chloroplasts, *J. Biosci*. **20**, 35-47

Loll, B., Kern, J., Saenger, W., Zouni, A., Biesiadka, J., (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II, *Nature* **438**, 1040-1044

Loll, B., Kern, J., Saenger, W., Zouni, A., Biesiadka, J. (2007) Lipids in Photosystem II: Interactions with proteins and cofactors, *Biochim Biophys Acta* **1767**: 509-519

Metz, J.G., Pakrasi, H.B., Seibert, M.S., Arntzen, C. J. (2005) Evidence for a dual function of the herbicide-binding D1 protein in Photosystem II, *FEBS Lett*, **205**: 269-274

Mulo, P., Sicora, C., Aro, E. - M. (2009) Cyanobacterial *psbA* gene family: optimization of oxygenic photosynthesis, *Cell. Mol. Life. Sci* **66**: 3697-3710

Singhal, G.S., Renger, G., Sopory, S.K., Irrgang, K.D., Govindjee (1999) Introduction to Photobiology, Photosynthesis and Photomorphogenesis, InConcepts in photobiology, Photosynthesis and Photomorphogenesis, *Narosa Publishing House*, 1-7

Ţenu, A. (1975), Cercetări hidrogeologice complexe asupra apelor termale din zona băilor Felix şi 1 Mai, . *Studii de Hidrogeol.IMH* **12**:75-132

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CONTRIBUTIONS TO THE BRYOPHYTE FLORA OF THE "VALEA MORII" NATURE RESERVE

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SUMMARY. The paper contribute to the species inventory of the nature reserve "Valea Morii", with the first bryological approach. Despite its small area, a total of 63 bryophyte taxa (9 liverworts and 54 mosses) have been recorded on the available substrate, mainly on soil and tree bark. Phytogeographical analysis reveals the dominance of the temperate elements. Ecological requirements of the species show a great variability of microhabitats. Among all the approached ecological parameters, species requirements for temperature are the most stringent, lacking the moderately thermophilous and thermophilous species. The prevalence of microthermal species is related to the occurrence of glacial relicts, as a result of the particular cooler microclimatic conditions. The species sensitive to human impact: oligohemerobous and ahemerobous, reveal a low impact in the protected area and a favorable conservation status.

Keywords: bryophyte, indicator, inventory, nature reserve.

Introduction

Floristic studies contribute to the species inventories in nature reserves, to species distribution and biodiversity analysses, as instruments in nature conservation.

"Valea Morii" (Mill Valley) nature reserve has been founded in 1974, by Decision 9757 of Cluj Local Council, covering an area of 1 ha, extended in 1994 to 1.7 ha and included in the "Valea Căprioarelor" landscape reserve. It became a nature reserve of botanical interest (IUCN IV) according to the Law 5/2000. Nowadays, it is part of "Făgetul Clujului-Valea Morii" Nature 2000 site (ROSCI0074).

"Valea Morii" is located in the north – western part of Transylvania, 8 km south of Cluj-Napoca city, in the Transylvanian foothills. Administratively, it belongs to the Feleac commune. The elevation of the nature reserve is almost 630 m a.s.l. surrounded by 700-800 m hills that creates particular cooler microclimatic conditions. The climate is temperate continental, with an average annual temperature of 7.5°C and multiannual rainfalls of 782 mm.

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In "Valea Morii" nature reserve sarmatian sedimentary deposits are predominant, represented by sands or sandstones with "Feleacu concretions", on marly to sandy clays substrate.

The vegetation of "Valea Morii" has been studied by Pop, 1960, Pop *et al*., 1962, Csűrös-Káptalan, 1965, Ruprecht, 1998, Ruprecht and Botta- Dukát, 1999, 1999/2000, Goia *et al*., 2005. The forest vegetation is represented on the southern slopes by *Querco petraeae-Carpinetum* Soó *et* Pocs 1957, *Quercetum robori-petraeae* Borza 1959 phytocoenosis and on the northern ones by *Carpino-Fagetum* Paucă 1941 communities. Along the valley are patches of *Aegopodio - Alnetum glutinosae* Kárpáti *et* Jurkó 1963. In the hay meadows and pastures dominant species are *Trisetum flavescens, Festuca rubra, Agrostis capillaris* and on small stands *Nardus stricta*. According to Pop *et al*., 1962 the nature reserve is dominated by wetland communities: *Cladietum marisci* (All. 1922) Zobrist 1935 *phragmitetosum* Pop *et al.*, 1962, *Schoenetum nigricantis* (All. 1922) W. Koch 1926, *Carici flavae-Eriophoretum latifolii* Soó 1944, *Caricetum vulpinae* Soó 1927, *Schoenoplectetum tabernaemontani* Soó 1947.

Four habitats have been identified in the protected area: 7210* - calcareous fens with *Cladium mariscus* and species of the *Caricion davallianae*, 7230 - alkaline fens, 91E0* - alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion, Alnion incanae, Salicion albae*) and 6520 - mountain hay meadows.

A comprehensive vascular plant inventory of the nature reserve consist of 277 species, including some glacial relicts: *Cladium mariscus, Tofieldia calyculata* (the only certain population on the Romanian territory), *Schoenus nigricans*, *Swertia perennis* or threatened species: *Ophioglossum vulgatum, Arnica montana, Dactylorhiza incarnata, D. maculata, Epipactis palustris, Trollius europaeus, Salix rosmarinifolia* etc. (Oltean *et al*., 1994, Habitats Directive, Bern Convention).

Our main object was to complete the species inventory of the nature reserve "Valea Morii" with bryophyte species, since such studies are lacking. Establishing the habitats that host the identified bryophyte species are essential for developing certain recommendations that also take into account the bryophytes as components of the vegetation and as best indicators of environmental conditions from the studied area.

Materials and methods

The bryofloristic studies have been carried out in the frame of protected area, in 2005. The bryophytes were analyzed in terms of phytogeographic, ecological and anthropic impact. The ecological behavior of taxa and phytocoenoses was estimated based on the ecological indices (U, T, R, L) according to Düll, 1992. The phytogeographical elements follow Düll, 1983, 1984, 1985. The tolerance of mosses to the anthropic impact was used according to Dierβen, 2001.

The taxonomy and nomenclature was updated according to Hill *et al*., 2006.

Results and discussion

Despite its small area, a total of 63 bryophyte taxa (9 liverworts and 54 mosses) were on the available substrate, soil and tree bark. The alluvial forest host 41 bryophyte taxa. *Brachytheciaceae, Hypnaceae* and *Amblystegiaceae* were families with the largest number of species.

Species inventory

Liverworts

Leiocolea badensis (Gottsche) Jorg.
Lophocolea bidentata (L.) Dumort.
Lophocolea heterophylla (L.) Dumort.
Marchantia polymorpha L.
Pedinophyllum interruptum (Nees) Kaal.
Pellia endiviifolia (Dicks.) Dumort.
Plagiochila asplenioides (L. emed. Taylor) Dumort.
Plagiochila porelloides (Torr. ex Nees) Lindenb.
Radula complanata (L.) Dumort.

Mosses

Abietinella abietina (Hedw.) M. Fleisch.
Amblystegium serpens (Hedw.) Schimp.
Amblystegiella subtile (Hedw.) Schimp.
Atrichum undulatum (Hedw.) P. Beauv.
Brachytheciastrum velutinum (Hedw.) Ignativ & Huttunen
Brachythecium campestre (Müll.Hall) Schimp.
Brachythecium glareosum (Bruch ex Spruce) Schimp.
Brachythecium mildeanum (Schimp.) Schimp.
Brachythecium rutabulum (Hedw.) Schimp.
Brachythecium salebrosum (Hoffm ex F.Weber & D.Mohr) Schimp.
Bryum pseudotriquetrum (Hedw.) P. Gaertn. et al.
Bryum torquescens Bruch et Schimp
Calliergonella cuspidata (Hedw.) Loeske
Campylium stellatum (Hedw.) Lange & C.E.O. Jens.
Campyllophyllum calcareum (Crundw. & Nyholm) Hedenäs
Ceratodon purpureus (Hedw.) Brid.
Cirriphyllum piliferum (Hedw.) Grout
Climacium dendroides (Hedw.) F.Weber & D.Mohr.
Ctenidium molluscum (Hedw.) Mitt.

Didymodon ferrugineus (Schimp. ex. Besch) M.O.Hill
Encalypta streptocarpa Hedw.
Eurhynchium angustirete (Broth.) T.J. Kop.
Fissidens dubius P.Beauv.
Fissidens taxifolius Hedw.
Homalothecium lutescens (Hedw.) H.Rob.
Homalothecium sericeum (Hedw.) Schimp.
Hygramblystegium varium (Hedw.) Mönk.
Hylocomiadelphus triquetrus (Hedw.) Ochyra & Stebekl.
Hylocomium splendens (Hedw.) Schimp.
Hypnum cupressiforme Hedw.
Hypnum cupressiforme var. *filiforme* Hedw. (Brid.)
Kindbergia praelonga (Hedw.) Ochyra
Mnium stellare Hedw.
Orthotrichum striatum Hedw.
Oxyrrhynchium hians (Hedw.) Loeske
Palustriella commutata (Hedw.) Ochyra
Plagiomnium elatum (Bruch & Schimp.) T.J.Kop.
Plagiomnium rostratum (Schrad.) T.J.Kop
Plagiomnium undulatum (Hedw.) T. Kop
Plagiothecium denticulatum (Hedw.) Schimp.
Plagiothecium laetum Schimp.
Plagiothecium latebricola Schimp.
Plagiothecium succulentum (Wilson) Lindb.
Pleurozium schreberi (Willd. ex Brid.) Mitt.
Pohlia drummondii (Müll. Hal.) A.L.Andrews
Pohlia lescuriana (Sull.) Ochi
Pohlia wahlenbergii (F.Weber & D.Mohr.) A.L.Andrews
Pseudoscleropodium purum (Hedw.) M.Fleisch
Pylaisia polyantha (Hedw.) Schimp.
Rhytidiadelphus sqarrosus (Hedw.) Warnst.
Sanionia uncinata (Hedw.) Loeske
Scorpidium revolvens (Sw. ex anon.) Rubers
Thuidium assimile (Mitt.) A.Jaeger
Thuidium recognitum (Hedw.) Lindb.

Limited offer regarding the nature of the substrate is reflected by the predominance of terricolous species (39.68%). 50.79% are strictly adapted to one type of substrate, 28.57% can be recorded on two types of substrata, 14.25% occur on three types of substrate, 1.59% species can be found on 4 types of substrate and 4.76% haven't any substrate preference (Fig. 1).

Ecological requirements of the species show a great variability of microhabitats (Fig. 2), despite the small area of the nature reserve.

Regarding light preferences scio-heliophilous (L4, L5 – 33.53%) and helio-sciophilous species (L6, L7 – 33.33%) are prevalent, since most species are sheltering in the shade of the forest or in the herbaceous layer.

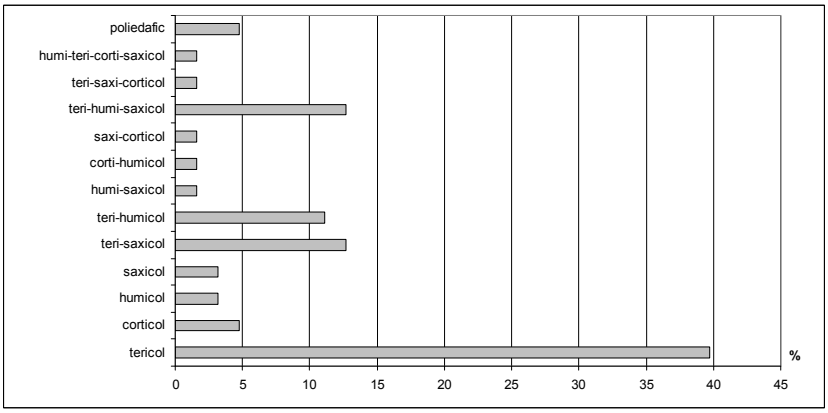

Figure 1. Species distribution regarding substrate preferences

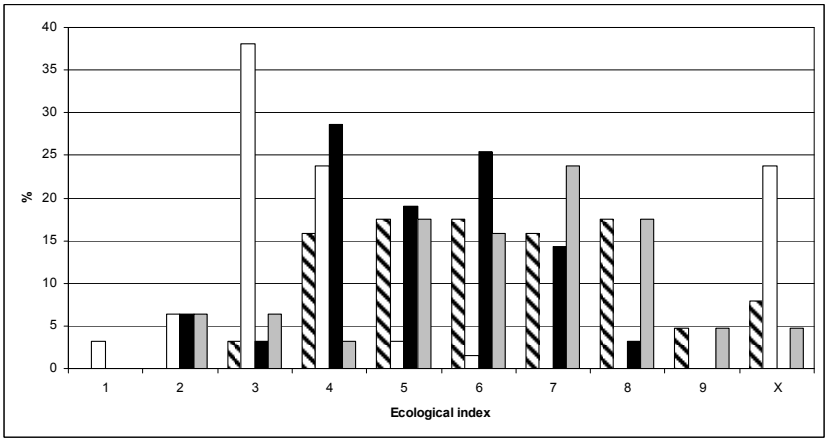

Figure 2. Ecological spectrum depending on the species preferences for light (L), temperature (T), humidity (U) and chemical reaction of the substrate (R)

The temperature requirements reveal the microthermal species prevalence (T3, T4 – 61.90%), another argument of climate peculiarities of "Valea Morii" nature reserve.

Referring to the ecological requirements for humidity most of the species are mesophilous (U5, U6, – 44.44%), but well represented are also xero-mesophytes (U4 – 28.57%) and meso-hygrophytes (U7 – 14.29%). Even if we expected a good percentage of meso-hygrophytes or hygrophytes, this fen is located at lower elevation and relatively far from similar ones. Relatively good prevalence of xero-mesophytes is a consequence of human pressure, manifested by drainage for meadows or human settlements expansion (construction of cottages, holiday or permanent houses).

Concerning the pH of the substrate, the base rich substrate is highlighted by the predominance of weak acid-neutrophilous (R5, R6 – 33.33%), followed by weak acid-neutrophilous (R7 – 23.81%) and neutro-basiphilous species (R8 – 17.46%).

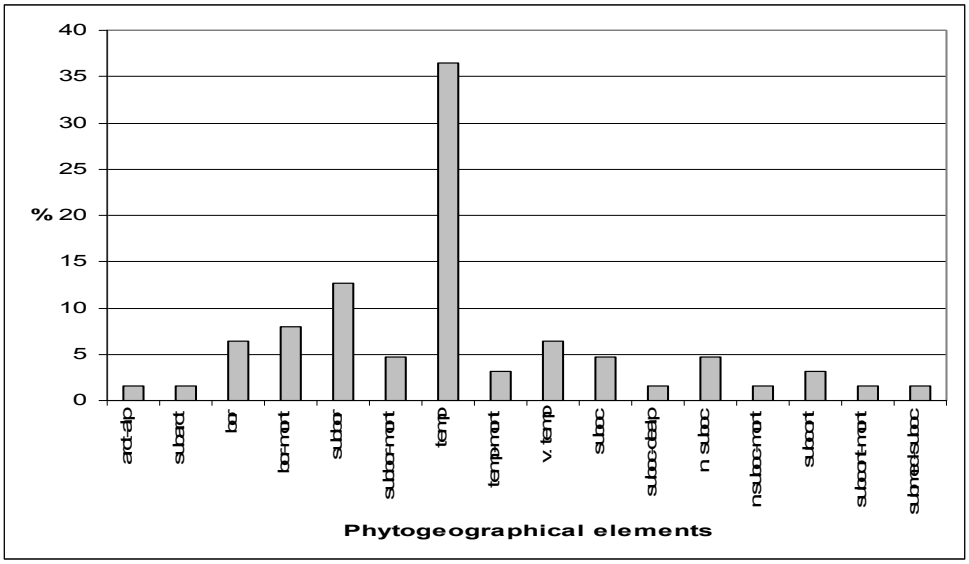

Figure 3. Phytogeographical spectrum for the bryophyte flora from "Valea Morii"

Phytogeographical analysis reveals the dominance of the temperate elements (46.03%) in accordance with the region's temperate climate (Fig. 3). Arcto-alpine (1.59%), subarctic (1.59%), boreal (6.35%), boreal-montane (7.94%), subboreal (12.70%), subboreal-montane elements (4.76%) are tipical for a cool microclimate, clues to the climate particularities of the nature reserve.

Regarding to the human impact the best represented are species with a mean tollerance - mesohemerobous (32.20%), but are well represented species sensitive to human impact: oligohemerobous (29.38%) and ahemerobous (20.90%), which reveal a low impact in the protected area (Fig. 4).

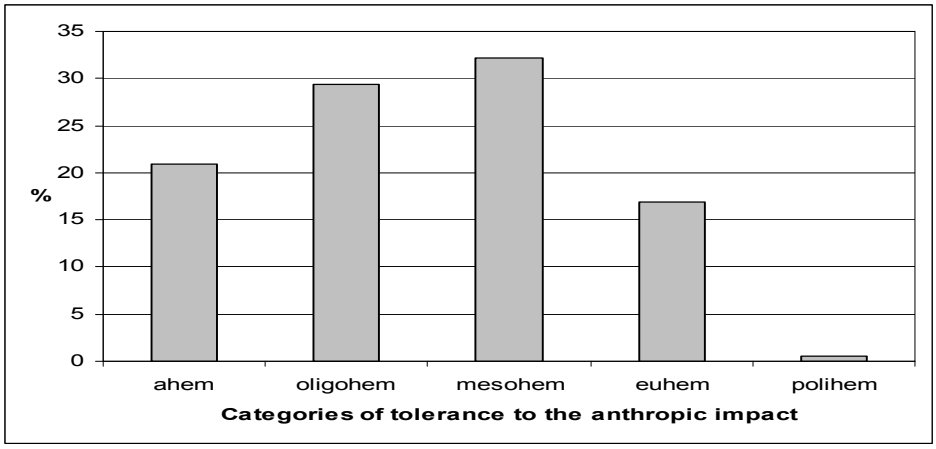

Figure 4. Distribution of bryophytes depending on their tolerance to the anthropic impact

Conclusions

The floristic inventory of "Valea Morii" consists of 63 bryophyte taxa, 9 hepatics and 54 mosses. It is a relatively small number of species compared to other small reserves (eg. Turda Gorges 136 species, Plămadă and Goia, 1993-1994; Someşul Cald Gorges 156 species, Goia and Mătase, 2001), due to low variability of the substrate types. Forest management is not considering leaving dead wood and the exposed rocks are lacking.

Even if we didn't recorded rare or relict species, bryophytes are important indicators of habitat changes. The prevalence of microthermal species is related to the occurrence of glacial relicts, as a result of the particular cooler microclimatic conditions. Among all the approached ecological parameters, species requirements for temperature are the most stringent, lacking the moderately thermophilous and thermophilous species.

The species indicator value reveals a favorable conservation status. The amount of xero-mesophilous, xerophilous, euhemerobous species, indicate an incipient degradation caused mainly by drainage and construction. This process of construction, due to the location in the vecinity of Cluj-Napoca, advances rapidly towards the midle of the valley and soon will damage the nature reserve. The upstream fens host some rare or relict species, but the nature reserve host the most important species and plant communities.

The inclusion of this protected area and the upstream marshes in the Natura 2000 site "Făgetul Clujului-Valea Morii" Nature 2000 site (ROSCI0074), is probably the most important step for "*in situ*" conservation of existing natural values.

Acknowledgements. The studies have been carried out in the frame of the Grants: CNCSIS A12/172/2004, A17/172/2005 provided by Romanian Ministry of Education and Research.

REFERENCES

Csűrös-Káptalan, M. (1965) Studii asupra mlaştinii de lângă comuna Vâlcele (raionul Turda), *Studia UBB Biologia*, **4** (1), 31-35

Council of Europe (1979) Convention on the Conservation of European Wildlife and Natural Heritage (Bern Convention), Bern, Switzerland. Available at: http://conventions.coe.int/Treaty/EN/Treaties/Html/104.htm

Dierssen, K. (2001) Distribution, ecological amplitude and phytosociologial caracterization of European bryophytes, *Bryophytorum Bibliotheca*; **56** (1), 26- 206

Düll, R. (1983) Distribution of the European and Macaronesian Liveworts (Hepaticophytina), *Bryologische Beiträge*, **2**, 1-114

Düll, R. (1984) Distribution of the European and Macaronesian Mosses (Bryophytina), Part I, *Bryologische Beiträge* **4**, 1-113

Düll, R. (1985) Distribution of the European and Macaronesian Mosses (Bryophytina), Part II*, Bryologische Beiträge*, **5**, 101-232

Düll, R. (1992) Distribution of the European and Macaronesian Mosses (Bryophytina), Adnotations and Progress, *Bryologische Beiträge*, **8/9**, 1-223

European Union Habitats Directive (1992) Council Directives 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora

Goia, I., Mătase, D. (2001) Bryofloristical research in the Someşul Cald Gorges, *Contribuţii Botanice*, **34**, 15-24

Goia, I., Mihăilă, G., Moraru, I., Hentea, S., Cristea, V. (2005) Populational studies on *Ophioglossum vulgatum* related to the human impact, *Revue de Cytologie et Biologie végétales-Le Botaniste,* **28** (s. i.), 298 – 306

Hill, M.O., Bell, N., Bruggeman-Nannenga, M.A., Brugués, M., Cano, M.J., Enroth, J., Flatberg, K.I., Frahm, J.-P., Gallego, M.T., Garilleti, R., Guerra, J., Hedenäs, L., Holyoak, D.T., Hyvönen, J., Ignatov, M.S., Lara, F., Mazimpaka, V., Muñoz, J., Söderström, L. (2006) An annotated checklist of the mosses of Europe and Macaronesia, *J. Bryol.,* **28**, 198-267

Olteanu, M. (ed.) (1994) Lista roşie a plantelor superioare din România, *Studii, sinteze, documentaţii de ecologie,* 1, 1–52

Plămadă, E., Goia, I. (1993-1994) Flora şi vegetaţia briofitică din rezervaţia naturală Cheile Turzii (I), *Contribuţii Botanice*, 87-85

Pop, E. (1960) *Mlaştinile de turbă din R. P. România*., Acad. R.P.R., Bucureşti

Pop, I., Csűrö-Káptalan, M., Rațiu, O., Hodișan, I. (1962) Vegetaţia din Valea Morii-Cluj, conservatoare de relicte glaciare, *Contribuţii Botanice*, 183-205

Ruprecht, E. (1998) Egyes jégkori reliktumok és egyéb növényritkaságok elterjedésének és populációméretének vizsgálata a Kolozsvár környéki Malom-völgyben, *Collegium Biologicum,* **2**, 37-43

Ruprecht, E., Botta-Dukát, Z. (1999) Talaj-növényzet kapcsolatok vizsgálata üde láprét-komplexekben. *Kitaibelia,* **4**(2), 331-340

Ruprecht, E., Botta-Dukát, Z. (1999/2000) Long-term vegetation textural changes of three fen communities near Cluj-Napoca (Romania), *Acta Botanica Hungarica*, **42,** 265-284

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IN VITRO STRESS SELECTION OF MARKER FREE TRANSGENIC POTATO LINES IN ORDER TO PRODUCE POTATO RESISTANT TO BOTH PVY AND DROUGHT STRESS

RALUCA-ALINA MUSTAŢĂ [1], IMOLA MOLNAR[1], TUNDE-EVA DENES[1] and ELENA RAKOSY-TICAN[1]✉

SUMMARY. Potato, an excellent staple food, is one of the main targets for genetic improvement by combinatorial biotechnology. The aim of the present study was to combine gene transfer with *in vitro* stress selection to obtain plants resistant to both PVY and drought stress. Our hypothesis is that one resistance to one type of stress might determine a state of better response to a second stress factor. Marker free, transgenic potato lines were tested for resistance to drought under *in vitro* polyethylene glycol (PEG) induced stress selection. Callus cultures have been obtained from internodes of potato cultivars: Baltica and Désirée, of control and transgenic lines, respectively. The drought stress was simulated *in vitro* for three weeks on MS-T medium with 5% PEG 6000. The regenerated plants were selected in a second round on RMB5 medium with 5%, 10% and 15% PEG. At the end of each stage, the plants were evaluated for viability, root development and the regeneration of axillary buds. Two clones of cv. Baltica putatively tolerant to drought and resistant to PVY were obtained.

Keywords: drought, PEG, potato stress selection.

Introduction

Potato (*Solanum tuberosum*) belongs to the Solanaceae family and is the third crop in the world after rice and wheat (Birch *et al*., 2012). It spreads to all continents and is cultivated in more than 150 countries, with varied uses in food, as animal feed and also in various industries (Muthoni *et al*., 2013). However the potato is very sensitive to diseases, pests and abiotic factors such as drought. Important losses in the annual potato production are also caused by pathogens or pests such as: Colorado potato beetle (*Leptinotarsa decemlineata*), late blight (*Phytophthora infestans*) and viruses (PVY, PVX, PRLV etc.).

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Potato virus Y (PVY) is an economically important virus, which is transmitted by aphids and infects crop species in the Solanaceae family. PVY has a high genetic variability, a significant number of strains being described so far: PVY^{O}, PVY^{C}, PVY^{Z}, PVY^{N} (Tribodet, *et al*., 2005). The recently identified strains, PVY^{NTN} (N-tuber-necrosis) and PVY^{N-W} (N-Wilga), have a rapid spread causing severe yield reductions, up to 80%. Both PVY^{NTN} and PVY^{N-W} induce Potato Tuber Necrotic Ringspot Disease (PTNRD) (Blanco-Urgoiti *et al*., 1998; Visser *et al*., 2012). Knowing that sources of natural virus resistance in plant are limited, novel resistance sources using transgenesis have been developed. Some genetic transformation methods including protein and RNA-mediated approaches (Prins, 2003) or virus-induced gene silencing (Lu *et al*., 2003; Godge *et al*., 2007) were used to induce virus resistance in plants (Baulcombe, 1999). In other studies the virus resistance in susceptible plants has been induced by transferring virus-derived genes which are including viral coat protein (CP) (Reddy *et al*., 2010) or CP genes in an intron- hairpin construct (Yan *et al.,* 2009). In previous experiments transgenic marker-free potato was produced using a two-step protocol. In the first step the transfer and expression of reporter gene *gfp* (green fluorescent protein) and marker gene *npt*II (neomycin phosphotransferase) were used to improve transformation of different cultivars of potato (Rakosy-Tican *et al.,* 2006). In the second step *A. tumefaciens* C58C1 pGV2260 with the hairpin construct pRGG YCPiPCY (35SCaMV enhancer and promoter, two repeated inverted PVY-CP sequences separated by an intron and pAnos terminator) was used for transforming the best responding potato cvs, Baltica and Désirée to resist PVY (Rakosy-Tican *et al.,* 2010). Resulting transgenic lines of two cultivars Baltica and Désirée were used in the present study for *in vitro* drought stress selection. Water deficit is a frequent stress in potato production, causing lower quality and reduced tuber yield (Steduto *et al*., 2012). Potato is very sensitive to water stress because of its shallow root system (Ekanayake and Midmore, 1992; Dalla Costa *et al*., 1997). One biotechnological method to obtain drought-resistant plants is *in vitro* selection under water stress conditions. To select *in vitro* drought resistant plants polyethylene glycol (PEG) is used as a component added to the culture media (Hassanpanah, 2010; Pino *et al.,* 2013). The treatment with PEG 6000 applied to seven potato cultivars allowed the selection of one drought resistant cultivar (Hassanpanah, 2010). In another study the drought conditions were simulated *in vitro* by the addition of PEG 4000 in concentration of 0%, 4% and 8% added to MS medium (Murashige and Skoog). These authors used both *Solanum tuberosum* and *S. commersonii* transgenic lines which overexpress the gene *ScCBF1* and compared them with the wild type after *in vitro* drought stress. Three different concentrations of PEG reduced the viability, but the transgenic plantlets were 100% viable in both species (Pino *et al*., 2013).

In this study *in vitro* stress selection of transgenic potatoes integrating a hairpin construct, hence putatively resistant to PVY and of their wild type counterparts was applied with the aim to combine these two resistance traits. Plant viability and

root regeneration rate indicate that two transgenic clones of cv. Baltica do resist *in vitro* drought stress. These lines are going to be further analyzed and provide an example of combining different resistance traits by biotechnological means.

Materials and methods

Selection of drought resistant plants

In this study we used two potato cultivars: Désirée and Baltica, which have been previously transformed using *Agrobacterium tumefaciens*, C58C1 pGV2260 with the hairpin construct pRGG YCPiPCY (35SCaMV enhancer and promoter, two repeated inverted PVY-CP sequences separated by an intron and pAnos terminator), to obtain transgenic potato lines resistant to PVY. Putatively transgenic lines were identified by PCR amplification of the construct, using two different designed specific primers (Rakosy-Tican *et al*., 2007).

The wild type (wt) Désirée (http://www.europotato.org) and Baltica (Solana GmbH & Co K6, Germany), the transgenic lines of cv. Désirée coded 4D1, 6D4, 6D6 and putatively transgenic (not analyzed yet at molecular level) 1D6, 1D9 and 6D1, as well as transgenic lines of cv. Baltica 5B2, 5B8, putatively transgenic 1B4, 3B5, 4B9, 6B4, 6B8 and nontransgenic 7B3 were used in the experiments.

The selection of plants resistant to drought stress was performed in two rounds: at callus level and then on plantlets exposed to increasing concentrations of PEG. Callus was induced on internodes of cvs. Baltica and Désirée of both control and transgenic potato, cultivated on MS-T medium (MS-based medium with 16 g L^{-1} glucose, 0.5 mg L^{-1} folic acid, 0.05 mg L^{-1} biotin, 40 mg L^{-1} adenine, 0.02 mg L^{-1} GA3, 0.02 mg L^{-1} NAA, 2.0 mg L^{-1} zeatin riboside and 0.7 % (w/v) agar, at pH 5.8). The callus was cultured three weeks on MS-T medium with 5% PEG 6000. Then the callus with first regenerated shoots was transferred on MS-T medium, without PEG. Potato plants were maintained in a growth room at 21 ºC with a photoperiod of 16 h (90 µmol m^{-2} s^{-1}, daylight fluorescent illumination). Plants regenerated, after stress, were evaluated for viability and capability to regenerate new plants. In the second round of selection, the lines which regenerated plants from callus under stress conditions were transferred on RMB5 medium (Menczel *et al*., 1981) with 5%, 10% and 15% PEG, each applied for three weeks. The lines with viable plants were selected on RMB5 with 10% PEG, simulating the drought conditions for other three weeks. Finally, the lines with viable plants, after stress induced by 10% PEG, were tested, in the next step of selection, on RMB5 with 15% PEG for another three weeks. At the end of each step the tested plants were evaluated for viability and regeneration rate. The data were statistically analyzed using Microsoft Excel and R statistics software (*http://www.lsw.uniheidelberg.de/users/christlieb/teaching/UKStaSS10/R_refman.pdf*).

Results and discussion

Although plant selection efficiency on drought stress conditions was dependent on genotype, all tested genotypes successfully regenerated callus and further on, all genotypes regenerated plants under stress conditions i.e. when 5% PEG was added to the culture media.

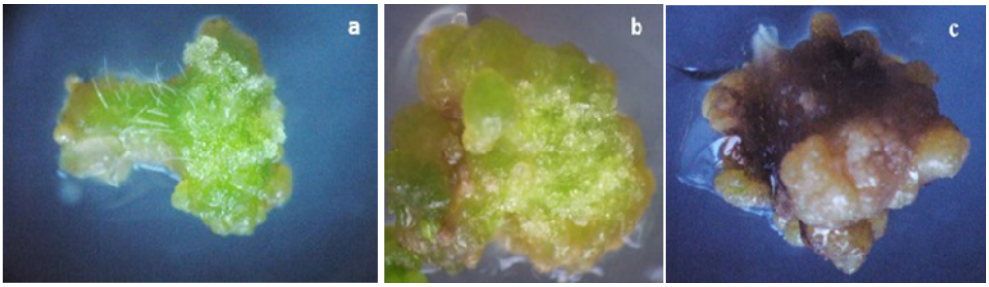

Figure 1. Internodal segments regenerating callus in different conditions: a) an example of polar regeneration of callus on internodal explant wihout drought stress; (b) callus extending on the entire inoculum; (c) callus necrosis under drought stress simulated by 5% PEG.

The callus regenerated on MS-T medium in both transgenic and control potato lines. Callus regeneration began at the apical pole of internodal fragment (Fig. 1 a) and expanded throughout the inoculum (Fig. 1 b). A part of the regenerated calli remained viable after treatment with PEG, but others lost their viability through necrosis (Fig. 1 c) during the first three weeks of testing on RMB5 media with 5% PEG.

Differences between the percentages of viability (%) of calluses derived from transgenic, non-transgenic and control (Désirée and Baltica) potato lines is depicted in Fig. 2. All transgenic genotypes have a higher viability when compared to the control cultivars Désirée and Baltica, but there are also differences between different transgenic genotypes, some performing better than others (Fig. 2).

It is possible that infection with *Agrobacterium* was stimulating organogenesis acting as a stress signal and did also influence plant response to *in vitro* regeneration under stress conditions also in next generations of plants, which means that an epigenetic mechanism should be involved. However, this hypothesis has to be further investigated. From these calluses, plants have been regenerated, under drought stress, in different percentages compared to controls. Based on the rate of regeneration, lines tested for resistance to drought stress were divided into two groups: resistant and susceptible to stress (Fig. 3).

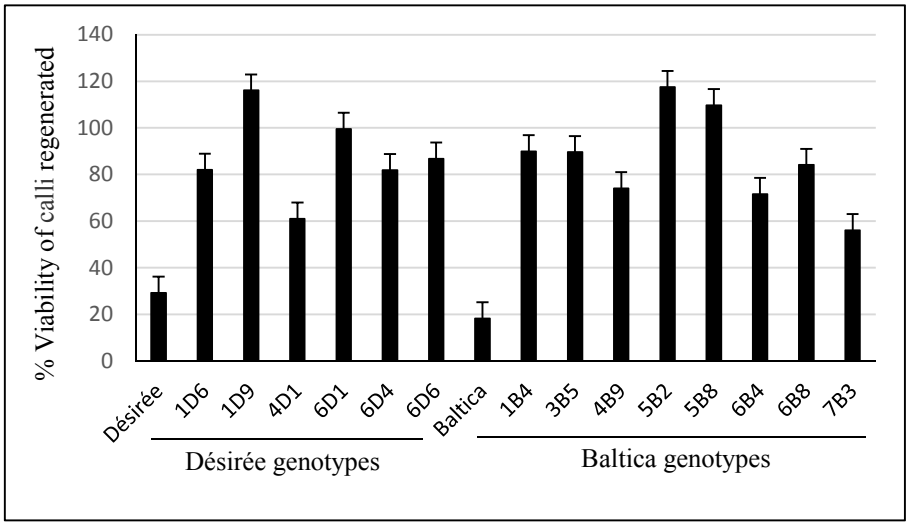

Figure 2. Percentage of viable calluses derived from potato lines transformed with *Agrobacterium* i.e. T1 generation maintained in *in vitro* culture, compared with the controls. Cultivars Désirée and Baltica were used as control. Values are means of percentage of viability of calli regenerated on MS-Tculture media with 5% PEG, n= 5; bars = SE.

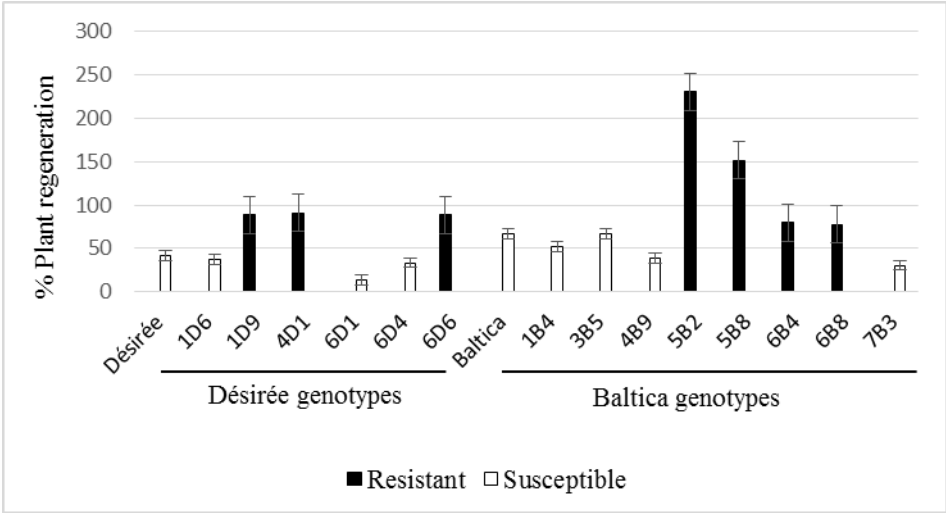

Figure 3. The percentage of potato plant regeneration from calli on MS-T media with 5% PEG, depending on genotype and compared with control cultivar. The values are means of five replicates; bars = SE.

Thus, lines with a regeneration rate higher than of control lines were considered resistant whereas lines with a regeneration percentage less than or equal to the controls were included in the susceptible group. Following the statistical analysis it was found, that there is a significant difference between these two groups (susceptible and resistant). After PEG induced drought stress, some regenerated shoots suffered necrosis (Fig. 4 a), formed kind of runners (Fig. 4 b) or even vitrified (Fig. 4 c). But some of the plants with integrated PVY resistance gene showed also resistance to drought stress (Fig. 5).

Figure 4. Potato plants regenerated under drought stress conditions from susceptible lines (a - 6D1, b - 1D6, c – 7B3 – exhibits vitrifying leaves).

In the second stage of stress selection, potato lines, which regenerated viable plants from callus under drought stress, were tested on three different concentration 5%, 10%, 15% of PEG 6000 added to the culture media.

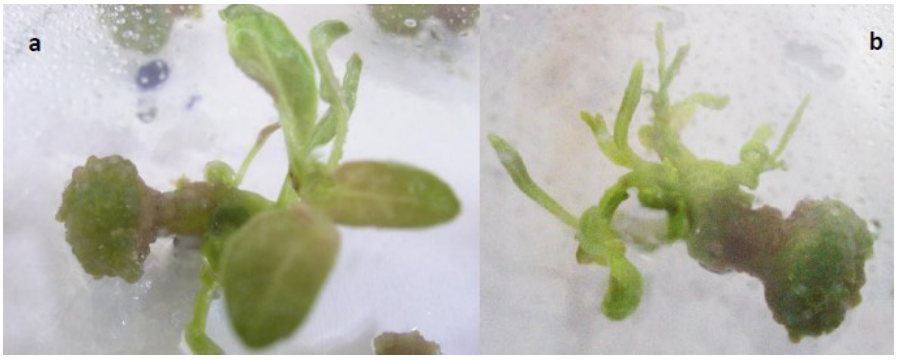

Figure 5. Regenerating plants from drought stress resistant lines (a – line 5B2, b – line 5B8).

Upon selection on 5% PEG, four putative drought resistant lines were selected all belonging to Baltica cultivar (4B9, 5B2, 5B8, 6B4). These lines had higher viability as compared with the control. Also, using T test, it was observed that it is a significant difference between plant viability of the lines 5B2, 5B8, 6B4 and the control wt Baltica cultivated on RMB5 +5% PEG ($p = 0.004 < 0.01$, $p = 0.002 < 0.01$, $p = 0.001 < 0.01$) (Fig. 6).

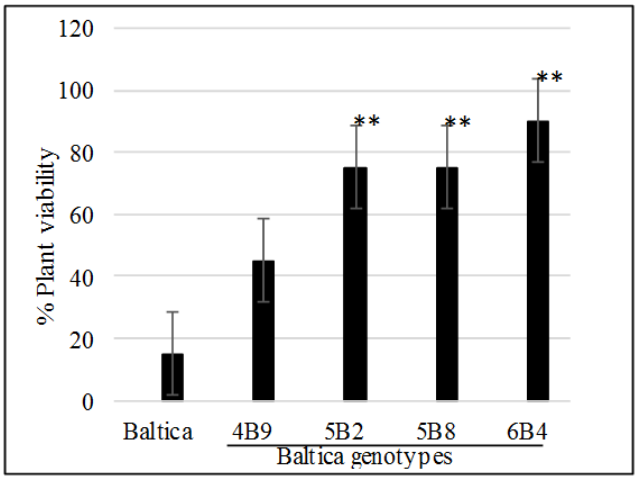

Figure 6. Percentage of plant viability of transgenic and putatively-transgenic genotypes and of control wild-type potato line (Baltica) under drought stress (RMB5 + 5% PEG). There are significant differences between the percentages of plant viability of transgenic and putatively-transgenic genotypes and the control Baltica (** $p < 0.01$).
Values are means of percentage of plant viability, n=5, bars=SE

After selection of the four potato lines on RMB5 media supplemented with 10% PEG, the control (Baltica) completely lost viability and line 4B9 had a viability much lower than the other three genotypes (5B2, 5B8, 6B4) that had a percentage of viability over 50% (Fig. 7).

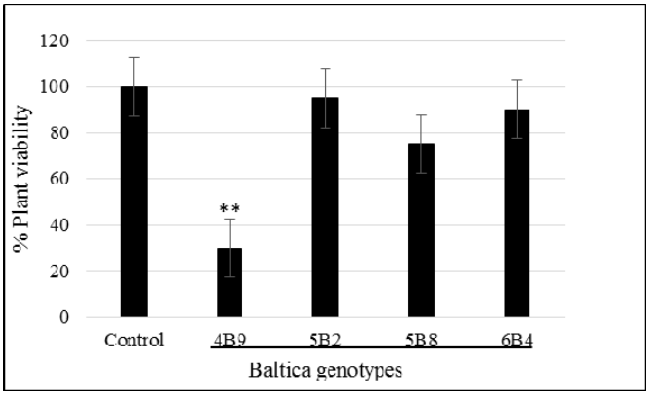

Figure 7. The percentage of plant viability of transgenic and putatively-transgenic potato genotypes under drought stress (RMB5 + 10% PEG) and of control (the same lines cultivated in control conditions: RMB5 without PEG). **Significant difference: $p < 0.01$.
Values are means of percentage of plant viability, n=5, bars=SE

Moreover, the statistical analysis showed that the difference between the plant viability of line 4B9 on RMB5 with 10% PEG and the same line on control conditions (RMB5 without PEG), is significant ($p=0.002< 0.01$). At the same time, the difference between the plant viability of lines 5B2, 5B8 and 6B4 under stress conditions (RMB5 + 10% PEG) and control conditions (RMB5 without PEG), is not significant. Thus it was observed that the line 4B9 became susceptible to drought stress (Fig. 8). This aspect was also confirmed during the stress selection on media with 15% PEG, when this line lost its viability. At the same time, during the stress selection on media with 15% PEG, the lines 5B2, 5B8 and 6B4 had percentage of plant viability close to 100% (Fig. 9) and formed roots (Fig. 10) with the average length between 0.5 and 1.5 cm.

The statistical analyses showed that there is no significant difference between the plant viability of these three lines cultivated in both drought stress conditions (RMB5 +15% PEG) and control conditions (RMB5 without PEG).

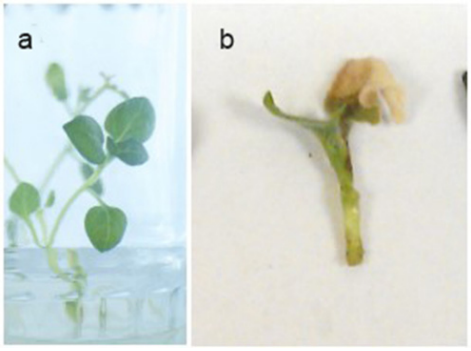

Figure 8. Differences between resistant plant (A - line 5B2) and susceptible plant (B - line 4B9) during the stress selection on RMB5 medium with 10% PEG.

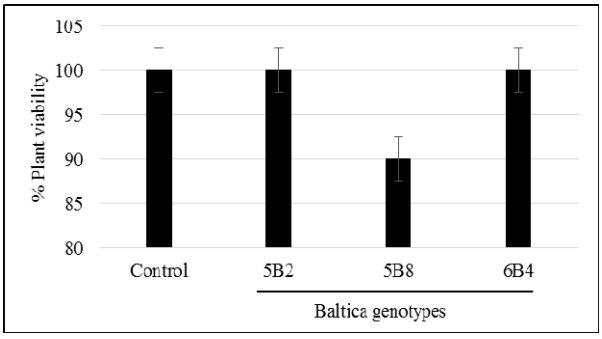

Figure 9. The percentage of plant viability of transgenic and putatively-transgenic potato genotypes under drought stress (RMB5 + 15% PEG) and of control (the same lines cultivated in control conditions: RMB5 without PEG). Values are means of percentage of plant viability, n=5, bars=SE.

After the test with 15% PEG plants have small size (Fig. 11 a, 11 d) but formed roots (Fig. 11 b). These results suggest that *in vitro* stress selection is sufficient to discriminate between susceptible and drought resistant plants in terms of viability and regeneration rate. Our findings are in accordance with previous studies which showed that *in vitro* stress selection is a useful technique to select drought resistant genotypes derived from different plant species such as sunflower (Punia and Jain, 2002), *Rubus* (Orlikowska *et al*., 2009) and potato (Gopal and Iwama, 2007). Previous PCR analysis showed that the five genotypes: 4D1, 6D4, 6D6, 5B2, and 5B8 were positive for the hairpin construct and hence putatively resistant to PVY.

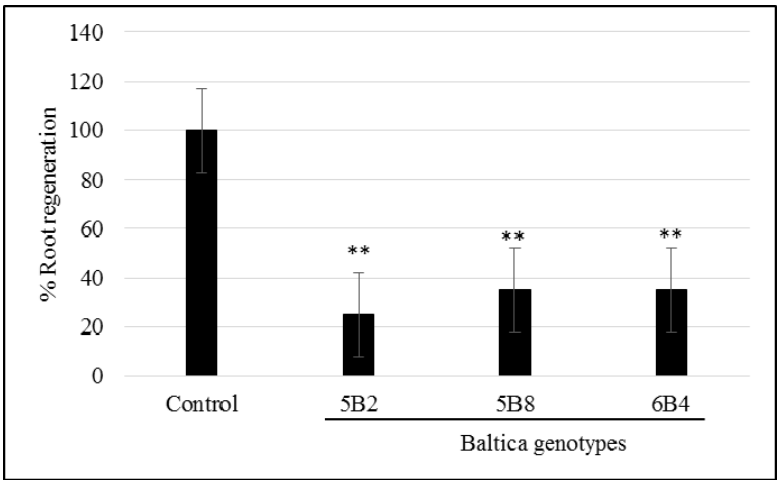

Figure 10. The percentage of root regeneration of transgenic and putatively-transgenic potato genotypes under drought stress (RMB5 + 15% PEG) and of control (the same lines cultivated in control conditions: RMB5 without PEG). There are significant differences between the lines tested on drought conditions and control ($p < 0.01$).
Values are means of percentage of root regeneration, n=5, bars=SE

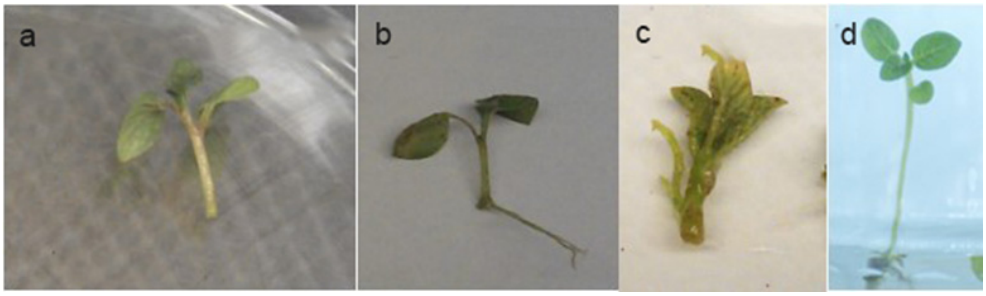

Figure 11. Viable potato plants selected on RMB5 medium with 15% PEG
(a, d – line 6B4; b – line 5B2; c – line 5B8).

In the current experiments, upon applying *in vitro* drought stress selection, two transgenic potato lines 5B2 and 5B8 proved to be also resistant to drought stress determined by PEG in all the three concentrations analyzed at plantlet level (5%, 10% and 15%). In other words, these two genotypes have been induced to carry a double resistance, to biotic (PVY) and abiotic (drought) stress (Table 1).

Table 1.

The response of genotypes undergoing transformation with *Agrobacterium* to drought stress, after all rounds of *in vitro* selections.

Genotype	Transgenic (T) / Not Transgenic (NT)	Resistant (R) / Susceptible (S) to drought stress
Désirée	**Control**	**-**
1D6	**ND***	**S**
1D9	**ND**	**S**
4D1	**T**	**S**
6D1	**ND**	**S**
6D4	**T**	**S**
6D6	**T**	**S**
Baltica	**Control**	**-**
1B4	**ND**	**S**
3B5	**ND**	**S**
4B9	**ND**	**S**
5B2	**T**	**R**
5B8	**T**	**R**
6B4	**ND**	**R**
6B8	**ND**	**S**
7B3	**NT**	**S**

*ND = Not Determined

Conclusions

The results obtained reveal that it is possible to combine transgenesis and *in vitro* stress selection in order to induce more resistant traits in potato, in our case PVY resistance mediated by RNAi and drought resistance.

In addition, it seems that one stress factor already existing in a plant can induce a general mechanism, which prepares the faster response to an additional stress factor.

This strategy might be applied to obtain other potato cultivars or other plant species with resistance to different stress factors.

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REFERENCES

Baulcombe, D. (1999) Viruses and gene silencing in plants, *Archives of Virology* Suppl., **15**, 189-201

Birch, R.J.P., Bryan, G., Fenton, B., Gilroy, E.G., Hein, I., Jones, J.T., Prashar, A., Taylor, M.A., Torrance, L., Toth, I.K. (2012) Crops that feed the world 8: Potato: are the trends of increased global production sustainable?, *Food Security*, **4**, 477-508

Blanco-Urgoiti, B., Tribodet, M., Leclere, S., Ponz, F., Perez de San Roman, C., Legorburu, F.J., Kerlan C. (1998) Characterization of potato potyvirus Y (PVY) isolates from seed potato batches. Situation of the NTN, Wilga and Z isolates, *European Journal of Plant Pathology*, **104**, 811–819

Dalla Costa, L., Delle Vedove, G., Gianquintoi, G., Giovanardi, R., Peressotti, A. (1997) Yield, water use efficiency and nitrogen uptake in potato: influence of drought stress, *Potato Research*., **40**, 19-34

Ekanayake, I.J., Midmore, D.J. (1992) Genotypic variation for root pulling resistance in potato and relationship with yield under water-deficit stress, *Euphytica*, **61**, 43-53

Godge, M.R., Purkayastha, A., Dasgupta, I., Kumar P.P. (2007) Virus-induced gene silencing for functional analysis of selected genes, *Plant Cell Reports*, **27**, 209-219

Gopal, J., Iwana, K. (2007) *In vitro* screening of potato against water-stress mediated through sorbitol and polyethylene glycol, *Plant Cell Reports*, **26**, 693-700

Hassanpanah, D. (2010) Evaluation of potato advanced cultivars against water deficit stress under *in vitro* and *in vivo* condition, *Biotechnology*, **9**, 164-169

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., Baulcombe, D.C. (2003) Virus-induced gene silencing in plants, *Methods*, **30**, 296-303

Menczel, L., Nagy, F., Kiss, Z.P., Maliga, P. (1981) Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: correlation of resistance to *N. tabacum* plastids, *Theoretical and Applied Genetics*, **59**, 191-195

Muthoni, J., Shimelis, H., Melis, R. (2013) Alleviating potato seed tuber shortage in developing countries: Potential of true potato seeds, *Australian Journal of Crop Science*, **7**, 1946-1954

Orlikowska, T., Kucharska, D., Horbowicz, M. (2009) The reaction of raspberry and blackberry cultivars to drought stress simulated *in vitro* by polyethylene glycol (PEG) 6000, *Acta Horticulturae*, **839**, 337-342

Pino, M.T., Avila, A., Molina, A., Jeknic, Z., Chen, T. H. H. (2013) Enhanced *in vitro* drought tolerance of *Solanum tuberosum* and *Solanum commersonii* plants overexpressing the ScCBF1 gene, *Ciencia e Investigacion Agraria*, **40**, 171-184

Prins, M. (2003) Broad virus resistance in transgenic plants, *Trends in Biotechnology*, **21**, 373-375

Punia, M., Jain, A. (2002) *In vitro* selection for drought tolerance in sunflower (*Helianthus annus L.*), *National Journal of Plant Improvement*, **4**, 27-30

Rakosy-Tican, E., Aurori, A., Dijkstra, C., Maior, M. C. (2010) Generating marker free transgenic potato cultivars with an hairpin construct of PVY coat protein, *Romanian Biotechnological Letters*, **15**, 63-71

Rakosy-Tican, E., Aurori, C, M., Dijkstra, C., Thieme, R., Aurori, A., Davey, M.R. (2006) The usefulness of the *gfp* reporter gene for monitoring *Agrobacterium*-mediated transformation of potato dihaploid and tetraploid genotypes, *Plant Cell Reports*, **26**, 661-671

Reddy, D.V., Sudarshana, M.R., Fuchs, M., Rao, N.C., Thottappilly, G. (2010) Genetically engineered virus-resistant plants in developing countries: current status and future prospects, *Advances in Virus Research*, **75**, 185-220

Steduto, P., Hsiao, T.C., Fereres, E., Raes, D. (2012) Crop yield response to water, *Food and Agriculture Organization of the United Nations*, **66**, 184-187

Tribodet, M., Galais, L., Kerlan, C., Jacquot, E. (2005) Characterization of Potato virus Y (PVY) molecular determinants involved in vein necrosis symptom induced by PVY^N isolates in infected *Nicotiana tabacum* cv. Xanthi, *Journal of General Virology*, **86**, 2101-2105

Visser, J.C., Bellstedt, D.U., Pirie, M.D. (2012) The Recent Recombinant Evolution of a Major Crop Pathogen, Potato virus Y, *PLoS ONE*, **7**, 1-12

Yan, P., Shen, W., Gao, X., Duan, J., Zhou, P. (2009) Rapin one-step construction of hairpin RNA, *Biochemical and Biophysical Research Communications*, **383**, 464-468

R Development Core Team. Version 2.11:
http://www.lsw.uniheidelberg.de/users/christlieb/teaching/UKStaSS10/R_refman.pdf.
http://www.europotato.org/display_description.php?variety_name= Désirée
http://www.potatopro.com/companies/solana-gmbh-and-co-kg

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CONSIDERATIONS REGARDING THE FOREST LANDSNAIL FAUNA OF THE CIUCAŞ MOUNTAINS

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SUMMARY. Part of the southern branch of Eastern Carpathians, the Ciucaş Mountains have a substratum of Cretaceous limestones, gravel and rounded blocks of crystalline schists, gneiss, limestone, sandstone, caught in a limestone matrix. This paper analyses the land snail fauna of deciduous and mixed forests, a habitat type where the geological substrate is completed with favourable humidity conditions that allow the development of specific land snail communities. In the research area a 55 species of terrestrial gastropods were identified. *Zonitoides nitidus, Mastus venerabilis, Faustina faustina, Balea fallax* and *Bulgarica cana* prevail in the communities, being abundant in most sampling sites. Other species such as *Balea stabilis* and *Monachoides incarnatus* develop large populations and reach high values of relative abundances in spite of their low frequency. The European interest species *Drobacia banatica* was identified in five of the ten sampling sites. This species is in the Ciucaş Mountains at the eastern limit of its distribution. One of the two species of the genus *Alopia* known as being present in the area was also found, represented by two subspecies*, Alopia nefasta nefasta* and *Alopia nefasta helenae.*

Keywords: biodiversity, the Ciucaş Mountains**,** communities, limestone, snails.

Introduction

Part of the decomposition biota in most terrestrial ecosystems, land snails are also preyed upon by many vertebrates and invertebrates, having therefore a central position in many food chains.

Several studies have documented the importance of edafic factors in land snail distribution. Traditionally the pH and calcium level are considered limiting factors (Agócsy, 1967; Cameron, 1973; Radea and Mylonas, 1992). Land-snails have high calcium requirements for the production of both their eggs and their shells, hence lime-rich substrates generally support abundant and diverse land-snail communities (Kerney and Cameron, 1979; Gärdenfors, 1992; Nekola, 1999).

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Several studies have demonstrated the strong positive correlation between individual abundance, species richness and the pH of the organic litter and soil (Wäreborn, 1969, 1970; Waldén, 1981; Nekola, 1999; Nekola and Smith, 1999; Pokryszko and Cameron, 2005).

Part of the southern branch of Eastern Carpathians, the Ciucaş Mountains have a development of the relief on three levels, with Cretaceous limestones, but also with gravel and rounded blocks of crystalline schists, gneiss, limestone, sandstone etc., cemented in a limestone matrix. These geological conditions allow the development of a rich land snail fauna. Our research aims to analyze the terrestrial snail communities inhabiting deciduous and mixed forests, habitats which combine the presence of soil calcium with favourable humidity conditions.

Materials and methods

Samples were taken from 10 sampling sites located in deciduous and mixed forests of Ciucaş Mountains as presented in Fig. 1 and described below.

S 1 10 km to Săcele, 45.32592 N, 25.49398 E, altitude: 843 m.
S 2 Cheia – Văleni, 45.2488 N, 25.56673 E, 791m altitude.
S 3 Valea Stânei: 45.2520 N, 25.57590 E, 885 m altitude.
S 4 one km upstream Valea Stânei Gorges: 45.2998 N, 26.0370 E, 1273 m altitude.
S 5 Muntele Roşu: 45.30110 N, 25.5690 E, 1336 m altitude.
S 6 Pârâul Berii: 45.3004 N, 25.5625 E, 1219 m altitude.
S 7 A tributary of Cheiţa River: 45.2853 N 25.5480 E, 1057 m altitude.
S 8 Babarunca: 45.3074 N, 25.5125 E, 952 m altitude
S 9 Dălghiu, near the forest range, 45.5366 N, 25.9049 E, 905 m altitude.
S10 Dălghiu, 45.5492 N, 25.9127 E, 932 m altitude.

The samping points were selected in forests with different microhabitat conditions considering the humidity level, the vegetation and the presence of limestones:

- sampling points with swampy areas near the river: S9,
- mixed forests: S1, S 5, S10,
- moderately humid areas: S2, S3, S7, S8,
- sampling points with limestone and limestone-cimented blocks: S 4, S 6,

Samples were collected from plots of 100 m^2, by four people for about an hour. All the living snails and freshly empty shells from vegetation, litter and decomposing wood were collected. The visual searching was quantified in effort. The same amount of time (one hour) was used to collect samples in all the sites. In order to collect the small species living in the litter, one 20 l sample of litter was sieved in every site (Cameron and Pokryszko, 2005). Standardisation of the collecting methods is necessary in order to perform an analysis of the land snail communities in terms of relative abundance.

The snails or empty shells found in the samples were identified using Grossu (1981, 1983, 1987, 1993) and Kerney and Cameron (1979). The classification follows Fauna Europaea (Bank, 2011).

The frequency, and the relative abundance were calculated and used in Table 1. The relative abundance was also used to build the affinity diagram of the sampling stations (single linkage method, Euclidean distance).

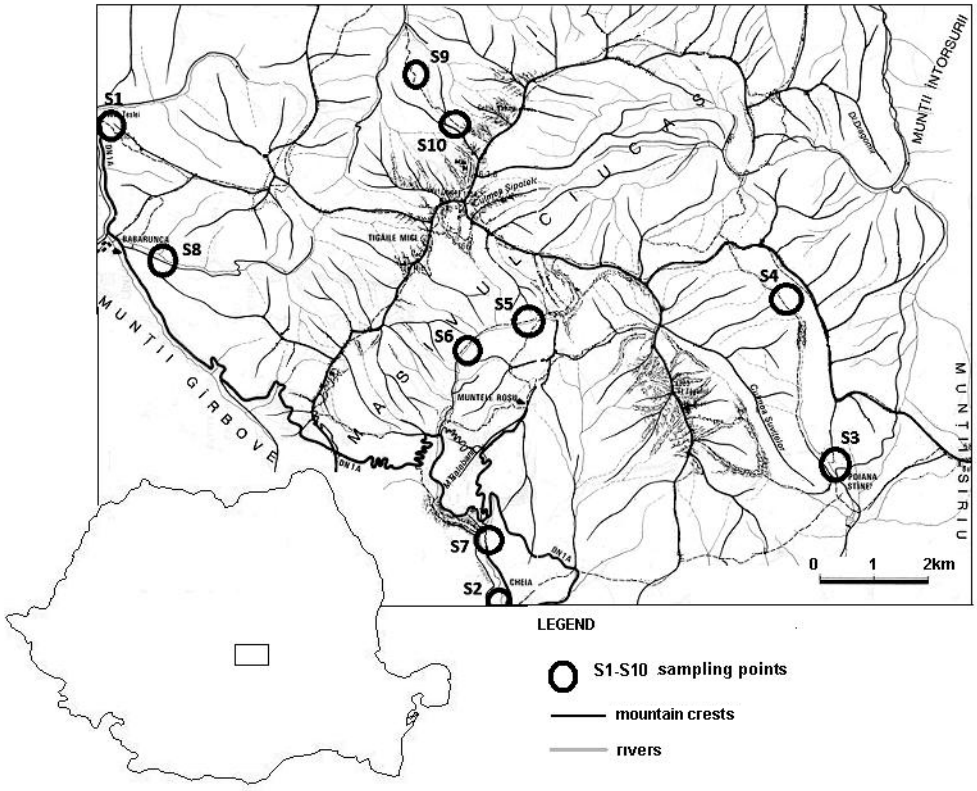

Figure 1. Location of the 10 sampling sites

Results and discussion

54 species of land snails were found in the sampling sites. The systematic list is presented below.

The systematic list of terrestrial gastropod species from deciduous and mixed forests of the Ciucaș Mountains

Carychiidae Jeffreys, 1830

Carychium tridentatum (Risso, 1826) - S1: 10 km to Săcele, S3: Valea Stânei, S4: Valea Stânei Gorges, S7: tributary of Cheiţa, S8: Babarunca, S9: Dălghiu.

Succineidae H. Beck, 1837

Succinea putris (Linnaeus, 1758) - S9: Dălghiu.

Cochlicopidae Pilsbry, 1900 (1879)

Cochlicopa lubrica (O.F. Müller, 1774) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei tributary of Cheiţa, S8: Babarunca, S9: Dălghiu.

Orculidae Pilsbry, 1918

Sphyradium doliolum (Bruguière, 1792) : S1 10 km to Săcele.

Valloniidae Morse, 1864

Vallonia costata (O.F. Müller, 1774) : S3: Pritvale - Valea Stânei.

Acanthinula aculeata (O.F. Müller 1774): S1: 10 km to Săcele.

Enidae B.B. Woodward, 1903 (1880)

Ena montana (Draparnaud, 1801) - S4: la one km upstream Valea Stânei Gorges , S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S9: Dălghiu.

Merdigera obscura (O.F. Müller, 1774) - S3: Pritvale – Valea Stânei.

Mastus venerabilis (L. Pfeiffer) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S4: one km ustream Valea Stânei Gorges, S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S8: Babarunca, S10: Dălghiu.

Clausiliidae J.E. Gray, 1855

Alopia nefasta helenae (R. Kimakowicz): S4: one km upsream Valea Stânei Gorges.

Alopia nefasta nefasta (M. Kimakowicz): S5: Muntele Roşu to Pârâul Berii.

Cochlodina (*Cochlodina*) *laminata* (Montagu, 1803) : S2: Cheia – Văleni tributary of Teleajen.

Cochlodina (*Paracochlodina*) *orthostoma* (Menke, 1828): S4: la one km upsream Valea Stânei Gorges, S6: Pârâul Berii.

Ruthenica filograna filograna (Rossmässler, 1836). Original data: S5: Muntele Roşu to Pârâul Berii.

Macrogastra (*Pyrostoma*) *plicatula* (Held, 1836): S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S10: Dălghiu.

Clausilia cruciata (Studer, 1820) - S2: Cheia – Văleni tributary of Teleajen, S4: la one km upstream Valea Stânei Gorges, S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii.

Clausilia dubia A. Schmidt, 1856 - S6: Pârâul Berii.

Laciniaria plicata (Draparnaud, 1801) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S8: Babarunca.

Balea fallax (Rossmässler, 1836): S2, Cheia; S3: Pritvale – Valea Stânei, S4: one km upstream Valea Stânei Gorges , S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S9, S10: Dălghiu.

Balea (*Pseudalinda*) *stabilis* (L. Pfeiffer, 1847) - S3: Pritvale – Valea Stânei.

Vestia (*Vestia*) *turgida* (Rossmässler, 1836) - S3: Pritvale – Valea Stânei.

Vestia (Vestia) elata (Rossmässler, 1836) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S13 : Babarunca, S15: Dălghiu.

Bulgarica (*Strigilecula*) *vetusta* (Rossmässler, 1836) - S5: Muntele Roşu to Pârâul Berii.

Bulgarica (*Strigilecula*) *cana* (Held, 1836) - S1: 10 km to Săcele, S2: Cheia – Văleni triutary of Teleajen, S3: Pritvale – Valea Stânei, S4: one km upstream Valea Stânei Gorges, S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca.

Punctidae Morse, 1864

Punctum (*Punctum*) *pygmaeum* (Draparnaud, 1801).

Original data: S4: one km upstream Valea Stânei Gorges, S7: tributary of Cheiţa, S8: Babarunca.

Pristilomatidae T. Cockerell, 1891

Vitrea diaphana (S. Studer, 1820) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale- Valea Stânei, S4: one km upstream Valea Stânei Gorges , S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S9, S10: Dălghiu.

Vitrea transsylvanica (Clessin, 1877) - S3: Pritvale – Valea Stânei, S6: Pârâul Berii, S8: Babarunca, S9, S10: Dălghiu.

Vitrea crystallina (O.F. Müller, 1774) - S7: tributary of Cheiţa.

Euconulidae H.B. Baker, 1928

Euconulus (*Euconulus*) *fulvus* (O.F. Müller, 1774) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S7: tributary of Cheiţa.

Gastrodontidae Tryon, 1866

Zonitoides (*Zonitoides*) *nitidus* (O.F. Müller, 1774) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S4: one km upstream Valea Stânei Gorges, S7: tributary of Cheiţa, S8: Babarunca, S9, S10: Dălghiu.

Oxychilidae P. Hesse, 1927 (1879)

Daudebardia transsylvanica (Draparnaud, 1805) - S1: 10 km to Săcele, S3: Pritvale – Valea Stânei, S8: Babarunca, S10: Dălghiu.

Oxychilus depressus (Sterki, 1880) - S8: Babarunca.

Oxychilus glaber (Rossmässler, 1835) - S1: 10 km to Săcele; S8-Babarunca.

Cellariopsis deubeli (Wagner, 1914) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S4: one km upstream Valea Stânei Gorges, S7: tributary of Cheiţa.

Aegopinella pura (Alder, 1830) - S1: 10 km to Săcele, S9: Dălghiu.

Aegopinella minor (Stabile, 1864) - S6: Pârâului Berii, S7: tributary of Cheiţa.

Aegopinella epipedostoma (Fagot, 1879) - S3: Pritvale – Valea Stânei, S4: one km upstream Valea Stânei Gorges, S6: Pârâul Berii, S7: tributary of Cheiţa, S10: Dălghiu.

Nesovitrea petronella (Pfeiffer, 1853) - S3: Pritvale – Valea Stânei, S6: Pârâul Berii, S7: tributary of Cheiţa.

Vitrinidae Fitzinger, 1833

Semilimax semilimax (J. Férussac, 1802) - S7: tributary of Cheiţa, S8: Babarunca.

Oligolimax annularis (S. Studer, 1820) - S7: tributary of Cheiţa.

Vitrina pellucida (Clessin, 1877) - S2: Cheia – Văleni tributary of Teleajen, S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S7: tributary of Cheiţa.

Limacidae Lamarck, 1801

Limax cinereoniger (Wolf, 1803) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S6: Pârâul Berii, S8: Babarunca.

Lehmannia marginata (O.F. Müller, 1774) - S2: Cheia – Văleni tributary of Teleajen.

Arionidae J.E. Gray, 1840

Arion subfuscus (Draparnaud, 1805) - S4: one km upstream Valea Stânei Gorges, S8: Babarunca, S9: Dălghiu.

Arion (*Carinarion*) *circumscriptus* (Johnston, 1828) – S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S10: Dălghiu.

Bradybaenidae Pilsbry, 1934 (1898)

Fruticicola fruticum (O.F. Müller, 1774) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S9: Dălghiu.

Hygromiidae Tryon, 1866

Euomphalia strigella (Draparnaud, 1801) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S8: Babarunca.

Monachoides incarnatus (O.F. Müller, 1774) - S4: one km upstream Valea Stânei Gorges, S9, S10: Dălghiu.

Monachoides vicinus (Rossmässler, 1842) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca.

Helicidae Rafinesque, 1815

Arianta arbustorum arbustorum (Linnaeus, 1758) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S5: Muntele Roşu traseu Pârâul Berii – izvor, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S9, S10: Dălghiu.

Drobacia banatica (Rossmässler, 1838) S1: 10 km to Săcele, S2: Pritvale – Văleni tributary of Teleajen, S8: Babarunca, S9, S10: Dălghiu.

Faustina (faustina) faustina (Rossmässler, 1835) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S4: one km upstream Valea

Stânei Gorges, S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S9, S10: Dălghiu.

Isognomostoma isognomostomos (Schröter, 1784) - S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S4:one km upstream Valea Stânei Gorges, S5: Muntele Roşu to Pârâul Berii, S6: Pârâul Berii, S7: tributary of Cheiţa, S8: Babarunca, S9, S10: Dălghiu.

Helix (*Helix*) *pomatia* (Linnaeus, 1758) - S1: 10 km to Săcele, S2: Cheia – Văleni tributary of Teleajen, S3: Pritvale – Valea Stânei, S4: one km upstream Valea Stânei Gorges, S6: Pârâul Berii, S7: tributary of Cheiţa.

The number of species and specimens for each sampling station is represented in Figure 2. The presence of limestones and lime-cimented blocks in a sampling site is associated with the decrease in humidity, hence there is a reduced diversity, as it is the case of S4, S5 and S6, while an increased diversity is found in sampling points with higher humidity level. The highest diversity and also the highest number of specimens were recorded in S3 - Pritvale-Valea Stânii, S8 - Babarunca and S9 - Dălghiu. The calcareous nature of the substratum is underlined by the presence of species characteristic for open calcareous habitats like *Mastus venerabilis,* which is one of the most abundant species in the analyzed area (A%=10.038, Table 1), and species of *Alopia* genus - *Alopia nefasta nefasta* and *Alopia nefasta helenae.* Analysing the land snail communities in term of relative abundance of individual species differences in habitat conditions are evident. The tree diagramm performed on relative abundance (Fig. 3) reveal a first group of stations located in mixed forests, were the lowest diversity was recorded (S1, S5, S10). A second group includes stations with limestone and limestone-cemented blocks (S4 and S6), while the most humid station (S9) and the one with the highest diversity (S3) are independent, joining the group at a great distance.

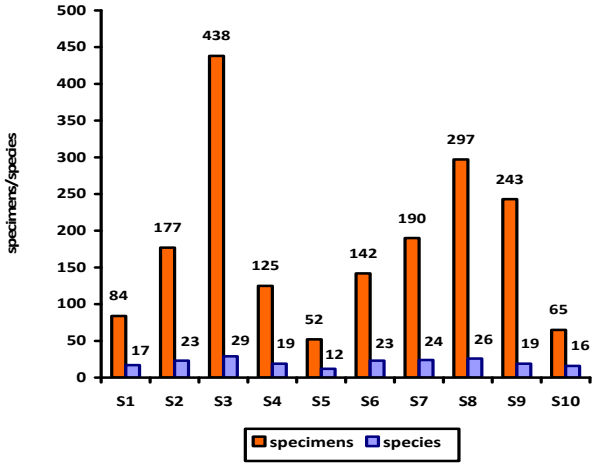

Figure 2. The number of specimens and species identified in each sampling site.

Table 1.

The identified species: zoogeographical elements (Hol. - Holarctic; Eur. - European; E-Sib.- Euro-Siberian; C-Eur. - Central-European; C-N Eur, – Central-North European; C-S Eur. – Central-South European; C-E Eur. – Central-East European; Balc. - Balcanic; Carp. – Carpathic; End. - endemic), their demands towards humidity (H – hygrophylous; MH mesohygrophylous; M – mesophylous; MX - mesoxerophylous), number of individuals, frequency (F%) and relative abundance (A%).

species	Zoogeo-graphy	Humidity	no. ind.	F%	A%
Carychium tridentatum	Eur.	H	32	70	1.765
Succinea putris	E-Sib.	H	1	10	0.005
Cochlicopa lubrica	Hol	H	12	60	0.661
Sphyradium doliolum	C-S Eur.	M	4	10	0.220
Vallonia costata	Hol.	MH	2	10	0.110
Acanthinula aculeata	Eur.	M	5	20	0.275
Mastus venerabilis	Balc.	MH	182	**80**	**10.038**
Ena montana	Eur.	MH	13	40	0.717
Merdigera obscura	Eur.	MH	3	10	0.165
Alopia nefasta helenae	End.	M	2	10	0.110
Alopia nefasta nefasta	End.	M	8	10	0.450
Cochlodina laminata	Eur.	MH	18	60	0.450
Cochlodina orthostoma	Eur.	MH	13	40	1.020
Ruthenica filograna	C. Eur.	M	3	10	0.165
Macrogastra plicatula	Carp.	M	41	40	2.261
Clausilia cruciata	C-N Eur.	M	23	10	1.875
Clausilia dubia	Eur.	MH	1	40	0.005
Laciniaria plicata	Eur.	MH	17	10	0.937
Balea fallax	Carp.	MH	74	**80**	**4.081**
Balea stabilis	Carp.	MH	215	**10**	**11.858**
Vestia elata	Carp.	M	11	40	0.606
Vestia turgida	Carp.	M	49	10	2.702
Bulgarica cana	C-N Eur.	M	66	80	3.640
Bulgarica vetusta	C-SE Eur	M	48	10	2.647
Punctum pygmaeum	Eur.	M	4	30	0.220
Vitrina pellucida	Hol.	M	8	40	0.441
Semilimax semilimax	Eur.	H	6	30	0.330
Phenacolimax annularis	Eur	MH	1	10	0.005
Vitrea diaphana	Eur.	MH	99	**80**	**5.460**
Vitrea transsylvanica	C-E Eur.	MH	21	50	1.158
Vitrea crystallina	Eur.	MH	1	10	0.005
Euconulus fulvus	Hol.	MH	3	30	0.165
Daudebardia transsylvanica	Carp.	MH	5	40	0.275
Cellariopsis deubeli	Carp.	MH	14	40	0.772
Oxychilus glaber	Eur.	MH	18	10	0.992
Oxychilus depressus	C-S Eur.	MH	5	10	0.275

Table 1 continued

Aegopinella pura	Eur.	M	5	20	0.275
Aegopinella minor	C-S Eur.	MX	10	20	0.551
Aegopinella epipedostoma	C Eur.	MH	40	50	2.206
Nesovitrea petronella	Eur.	M	7	30	0.386
Zonitoides nitidus	Hol.	H	206	**80**	**11.362**
Arion circumscriptus	Eur.	MH	100	**90**	**5.514**
Arion subfuscus	Eur.	MH	4	10	0.220
Limax cinereoniger	Eur.	MH	8	10	0.441
Lehmania marginata	Eur.	H	18	20	0.992
Bradybaena fruticum	Pal.	MH	48	30	2.647
Monachoides incarnatus	Eur.	M	127	**30**	**7.004**
Monachoides vicinus	C Eur.	M	23	60	1.268
Euomphalia strigella	Eur.	M	59	30	3.254
Arianta arbustorum	Eur.	M	14	80	0.772
Faustina faustina	C-E Eur.	M	130	**90**	**6.40**
Drobacia banatica	C-E Eur.	M	23	50	1.13
Isognomostoma isognomostomos	Eur.	M	59	90	2.90
Helix pomatia	Eur.	M	14	60	0.689

In five of the ten sampling points *Drobacia banatica* was present. *Drobacia banatica* is a species of European interest which reaches here the eastern limit of its distribution. The species is present both on the northern and southern part of Ciucaş Mountains.

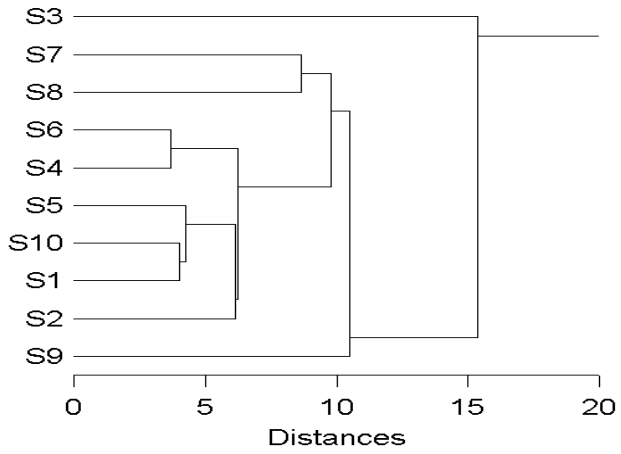

Figure 3. Cluster dendrogram of sampling sites based on species relative abundance (single linkage, Euclidean distance).

Of special interest are the species of the endemic genus *Alopia* H&A. (Adams, 1855). Except for one subspecies occurring in Slovakia, the genus *Alopia* is entirely endemic to Romania. It is a strongly calciphilous genus with the center of diversity in the eastern limestone-rich area of the Southern Carpathians, where endemic species have evolved within the major mountain ranges (Soós, 1943). Most of the taxa have a limited distribution and their conservation is important both at national and European level.

This genus is present mostly on limestone rocks in open habitats. This is the reason why in our samples only a few specimens belonging to a single species with two subspecies *Alopia nefasta nefasta* and *Alopia nefasta helene,* previously considered *Alopia helenae helenae* (Grossu, 1981), were present. A recent molecular study of the genus *Alopia* (Fehér et al., 2013) made some changes in its taxonomy. Consequently *Alopia helenae helenae* (Grosu, 1981; Nordsieck, 2008) was found to be a subspecies of *Alopia nefasta.*

Conclusions

The forests of Ciucaș Mountains show favourable conditions both of substratum and humidity and therefore shelter a rich land snail fauna. In that area both calciphilous and hygrophilous and mesohygrophilous snails are present. Species like *Zonitoides nitidus, Mastus venerabilis, Faustina faustina, Balea fallax, Bulgarica cana,* are present in most sampling points having high values of relative abundance. Other species such as *Balea stabilis* and *Monachoides incarnatus* develop large populations and reach high values of relative abundances in spite of their reduced frequency.

For both *Drobacia banatica* and the endemic subspecies of *Alopia nefasta* it is important to describe the current distribution, in order to establish the appropriate management measures for these species' conservation.

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REFERENCES

Agócsy, P. (1968) Data on quantitative conditions in the mollusk faunas of two different substrates in central Hungary, *Acta Zoologica Academiae Scientarium Hungaricae,* **14**, 1–6

Bank, R.A. (2011) Fauna Europaea: Mollusca, Gastropoda. Fauna Europaea, Version 2.4, http://faunaeur.org (accessed 10.V.2013)

Cameron, R.A.D. (1973) Some woodland mollusc faunas from southern England, *Malacologia,* **14**, 355–370

Fehér, Z., Németh, L., Nicoară, A., Szekeres, M. (2013) Molecular phylogeny of the land snail genus Alopia (Gastropoda: Clausiliidae) reveals multiple inversions of chirality, *Zoological Journal of the Linnean Society,* **167**, 259–272

Gärdenfors, U. (1992) Effects of artificial liming on terrestrial gastropod populations, *Journal of Applied Ecology,* **29,** 50–54

Grossu, A.V. (1981) *Gastropoda Romaniae*, *Ordo Stylommatophora Vol. 3: Suprafamiliile Clausiliacea şi Achatinacea,* Ed. Litera Bucureşti

Grossu, A.V. (1983) *Gastropoda Romaniae. Ordo Stylommatophora, Vol.4, Suprafam. Arionacea, Zonitacea, Ariophantacea şi Helicacea*, Ed. Litera Bucureşti

Grossu, A.V. (1987) *Gastropoda Romaniae Vol.* 2, Subclasa Pulmonata, Ord. Basommatophora şi Ord. Stylommatophora: Suprafam. Succineacea, Cochlicopacea, Pupillacea, Edit. Litera (1987), Bucureşti

Grossu, A.V. (1993) The catalogue of the Molluscs from Romania. *Travaux du Muséum d'Histoire Naturelle" Grigore Antipa",* **23**, 291- 366

Kerney, M.P., Cameron, R.A.D. (1979) *A field guide to the Land Snails of Britain and North-West Europe*, Collins, London, pp 288

Nekola, J.C. (1999) Terrestrial gastropod richness of carbonate cliff and associated habitats in the GreatLakes region of North America, *Malacologia,* **41**, 231–252

Nekola, J.C., Smith, T.M. (1999) Terrestrial gastropod richness patterns in Wisconsin carbonate cliff communities*, Malacologia,* **41***,* 253-269

Nordsieck, H. (2008) The system of the genus Alopia H. & A. Adams 1855 (Gastropoda: Stylommatophora: Clausiliidae), *Mitteilungen der Deutschen Malakozoologischen Gesellschaft,* **79/80**, 7–18

Pokryszko, B., Cameron, R.A.D. (2005) Geographical variation in the composition and richness of forest snail faunas in northern Europe, In: *Pattern and process in land mollusc diversity*, Cameron, R.A.D, Nekola, J.C., Pokryszko, B.M., Wells, F.E., (ed), Supplement No. 68 Perth, Western Australia: Records of the Western Australian Museum, pp 115-132

Radea, C., Mylonas, M. (1992) Landsnails in the organic horizon of a mediterranean coniferous forest, *Pedobiologia,* **36**, 187–192

Soós, L. (1943) *A Kárpát-medence Mollusca faunája*, Hungarian Academy of Sciences, Budapest

Waldén, H.W. (1981) Communities and diversity of land molluscs in Scandinavian woodlands. I. High diversity communities in taluses and boulder slopes in SW Sweden, *Journal of Conchology,* **30**, 351–372

Wäreborn, I. (1969) Land molluscs and their environments in an oligotrophic area in southern Sweden, *Oikos,* **20**, 461-479

Wäreborn, I. (1970) Environmental factors influencing the distribution of land molluscs of an oligotrophic area in southern Sweden, *Oikos,* **21**, 285-291

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BEHAVIORAL REACTIONS AND STRUCTURAL ALTERATIONS OF HIPPOCAMPAL TISSUE AFTER REPETITIVE MILD TRAUMATIC BRAIN INJURY IN MICE

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SUMMARY. Mild traumatic brain injury (TBI), often identified with concussion, is not considered to be life threatening in close perspective. However, the information about its delayed neurodegenerative effects is becoming more common. Especially this relates to repeated cases of concussion in military service members and athletes. The aim of our study was to show behavioral and histological changes in a mouse model of repetitive mild traumatic brain injury (rmTBI). No macroscopic brain abnormalities such as skull fractures or intracranial bleeding were found in mice after rmTBI. To detect behavioral alterations, we carried out the "Open field" test on day 5, 10 and 30 since the first impact of five. The behavioral data revealed decrease of motor activity and increase of anxiety level when compared to controls. Immunohistochemical data provided strong evidence of astro- and microgliosis that persisted for weeks after rmTBI. Our results indicate that new mouse model of human rmTBI can be useful in development of neuroprotective approaches for treatment of rmTBI consequences.

Keywords: hippocampus, immunohistochemistry, mouse model, "Open field" behavioral test, repetitive mild traumatic brain injury.

Introduction

Symptoms of mild traumatic brain injury (mTBI) are more difficult to detect than those observed in severe traumatic brain injury due to moderate range of noticeable abnormalities like skull fractures and intracranial bleeding. According to Centers for Disease Control and Prevention (CDC), common concept of TBI indicates brain damage due to bump, blow or jolt or a penetrating injury that disrupts normal function of the brain. However, TBI represents not a single event, but a whole cascade starting with initial damage (primary injury) and continuing with secondary processes (secondary injury) that can last from minutes to months and years (Xiong *et al*., 2013). Latter is mainly associated with cytotoxic and inflammatory processes accompanied by

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metabolic alterations of brain due to vascular failure (Werner and Engelhard, 2007; Beauchamp *et al*., 2008; Granacher, 2008). It should be noted that mild TBI is a type of closed trauma, and its outcome is primarily determined by secondary processes, while severe forms are much more dependent on initial mechanical injury (Betrus and Kreipke, 2012).

Acute signs of a concussion can include loss of consciousness, amnesia, irritability, slowed reaction times, sleep disturbances and emotional lability (McCrory *et al*., 2009). The critical fact is that these deficits are observed in the absence of structural brain damage when using such standard diagnostic methods as magnetic resonance imaging (MRI), computer tomography (CT) and electroencephalography (EEG) (Belanger *et al*., 2005; Davis *et al*., 2009). Nevertheless, there's still a danger of long-term consequences. Specifically, the results of the survey showed that retired professional football players who experienced three or more concussions were more likely to report about memory impairment and mild cognitive impairment (Guskiewicz *et al*., 2005). Moreover, the analysis of post-mortem data indicated that it was among retired football players who had played for five or more years there were elevated rates of death due to Alzheimer's disease (AD) or amyotrophic lateral sclerosis (ALS). However, the authors assumed that actual causes of death also might have included chronic traumatic encephalopathy − CTE (Lehman *et al*., 2012; McKee *et al*., 2014).

Taking into account all depicted above, modeling of mTBI and capturing its consequences at short and long time intervals after immediate impact appear to be extremely important. There are several kinds of models reproducing single and repeated mTBI. Mostly they represent variations of schemes that existed before and were used for mimicking more severe forms of trauma. On a similar principle, the model of repetitive mTBI (rmTBI) designed by Michael J. Kane et al. (Kane *et al*., 2012) and used in our research represents a modification of Marmarou's weight-drop model (Marmarou *et al*., 1994). The main point of its key features was to make experimental conditions of receiving a concussion closer to reality by lowering rates of mortality and cases of skull fractures and hemorrhages in animals. Since hippocampus is integral for multiple aspects of behavior and shows high sensitivity to any pathological changes of brain (Levita *et al*., 2014; Kim *et al*., 2014), we focused our research on this particular structure.

Materials and methods

All stages of the experiment were conducted in accordance to European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

Animals

Subjects were 56 8 to 12 weeks old males of wild-type mouse (24 – 40 g). Animals were housed in cages on a 12-hour light–dark cycle, with free access to food

and water. In the experiment, animals were divided into following groups: 1) not anesthetized, not injured; 2) anesthetized, not injured; 3) anesthetized, injured. Before injury mice were lightly anesthetized with diethyl ether. The positive effect of anesthesia was determined by absence of reaction to toe pinch.

Model of repetitive mild traumatic brain injury

We induced rmTBI by using a model proposed by Michael J. Kane et al. (Kane *et al*., 2012). Its design represents a modification of classical Marmarou's weight-drop model (Marmarou *et al*., 1994) which reproduced single diffuse TBI from mild to severe. Both in original scheme and in modified, the skull of a mouse is exposed to an impact with a weight guided by a vertical tube. The tube is pre-fixed to a stationary object like wall or rack. In both schemes, the severity of injury depends on the height from which the load falls and the weight of the latter. Respectively, in our study the 95 g weight fell from the 1 m height. Lightly anesthetized animal were not fixed in the apparatus, and after the weight fell, they fell too (through a piece of aluminium foil pre-attached on top of the H-shaped box), landing on a damper support. We avoided rebounding of the weight and, consequently, additional blows to the skull by using a nylon fishing line that stopped the load from falling after the impact due to its length. According to Kane's model, neither scalp incision nor attachment of helmet to the mouse skull were done. Mice were subjected to 5 head impacts (1/day for 5 successive days).

Recovery of righting reflex

After each of five impacts mice (n=29) were immediately placed on their backs on a flat surface, and time required for taking a normal position was fixed. Thus, we evaluated time required for neurological restoration. Measures of injured mice then were compared to controls (anesthetized, not injured, n=20).

Behavioral assessment

To investigate the behavioral effects of rmTBI, mice were tested in the Open field test before (control, n=16) and after the series of impacts (n=27) on day 5 (n=10), day 10 (n=9) and day 30 (n=8) since the first injury. In addition, we divided control animals into two groups, one of which was anesthetized without delivering the impact (n=8) in order to compare it with the group of animals tested shortly after the last injury (day 5). Presumably, animals from the group of day 5 could still be under the influence of anesthesia. Another control group (n=8) wasn't exposed to ether inhalations.

Animals were tested for changes in motor activity, rest duration, grooming activity and anxiety level. Each mouse was kept on a platform for 30 minutes. The area of the "field" was 60 cm x 60 cm and consisted of 16 equal squares. Motor activity was defined by counting lines (sides of the squares) crossed within respective period of time, rest duration was defined by fixing the time spent in corner zones of the platform, grooming activity was defined by number of acts of grooming and anxiety level was defined by number of acts of defecation.

Edema

Both controls (n=8) and injured mice (n=8) were sacrificed to detect possible signs of edema formation. The presence of edema was evaluated by comparing water content between controls and mouse brains after 4 hours since the last impact of five. We used the method which was previously described (Kawai et al., 2001), but slightly modified. Mice were decapitated, and brains were rapidly removed from the skull. Whole fresh brain was weighed on aluminum foil, dried for 90 hours at 73°C in an oven and reweighed. Percentage water content of each brain was calculated according to the formula: (wet weight – dry weight)/(wet weight) × 100.

Immunohistochemical staining and image processing

Immunohistochemical analysis was carried out as previously described (Pivneva *et al*., 2005). Controls (n=6) and mice of day 5 (n=5), 10 (n=5) and 30 (n=8) were anesthetized by injecting an overdose of calipsol and transcardially perfused with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde in 0,1 M PB. After perfusion, extracted brains were immersed in the same fixative and postfixed overnight at 4^{o}C. Frontal brain sections (50 μm) were prepared using a Leica VT1000 A vibratome (Wetzlar, Germany). The sections were left overnight at 4^{o}C for incubation with primary antibodies diluted in the solution (0.1 M PB, 1% bovine serum albumin, 0,3% Triton X-100). To visualize astrocytes and microglial cells, we used rabbit polyclonal antibodies anti-GFAP (1:1500, Dako, Denmark) and rabbit polyclonal antibodies anti-Iba-1 (1:1000, Wako, Japan). On the next day, brain sections were treated with secondary anti-rabbit Alexa Fluor 594-conjugated antibodies (1:1000, Invitrogen, USA). Stained sections then were mounted to glass slides using fluorescent mounting medium Immu-Mount (Thermo Scientific, Waltham, USA) and examined with a FluoView FV1000 confocal microscope (Olympus Inc., Japan).

Statistical Analysis

Statistical analysis was performed in StatSoft Statistica 6.0. The two-tailed Student's t–test was used to assess the significance of differences between samples ($p < 0.05$ was considered to indicate statistical significance). All data are shown as mean ± SEM.

Results and discussion

No cases of skull fractures, intracranial bleeding or seizures were registered. Preliminary data also showed no signs of general edema (Fig. 1).

To confirm the alterations of neurological state immediately after trauma, we compared time intervals necessary for righting reflex restoration in anesthetized controls and in injured mice after each impact (Fig. 2). Thus, we observed that receiving of an injury leaded to delay in full recovery of consciousness.

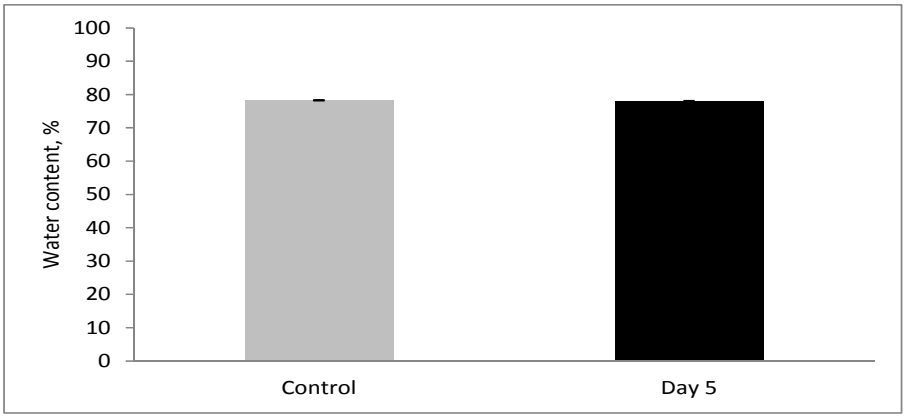

Figure 1. Edema measuring on day 5 since the first impact. Ocurrence of edema was defined by comparison of water content in control (n=8) and damaged (n=8) mouse brains.

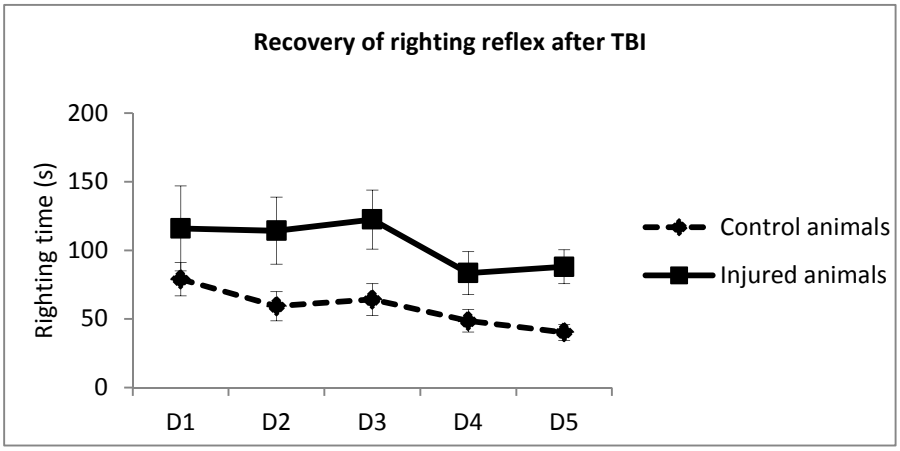

Figure 2. Recovery of righting reflex after each inhalational anesthezia (control animals, n=20) or after each impact (injured animals, n=28) on five successive days.
Results are the mean ± SEM.

Behavioral data indicated neurological deficits as well (Fig. 3). We observed decreasing of motor activity on day 5 and its gradual restoration by day 30 (Fig. 3 A). However, the level of control wasn't reached. Statistically significant difference was shown for groups of non-anesthetized control animals and injured animals tested at day 5 ($p < 0.01$). Since there was a decrease in motor activity of animals exposed to trauma, the time spent in corner zones increased (Fig. 3 B). Statistically significant difference was shown for groups of non-anesthetized control animals and injured animals tested at day 5 ($p<0.01$).

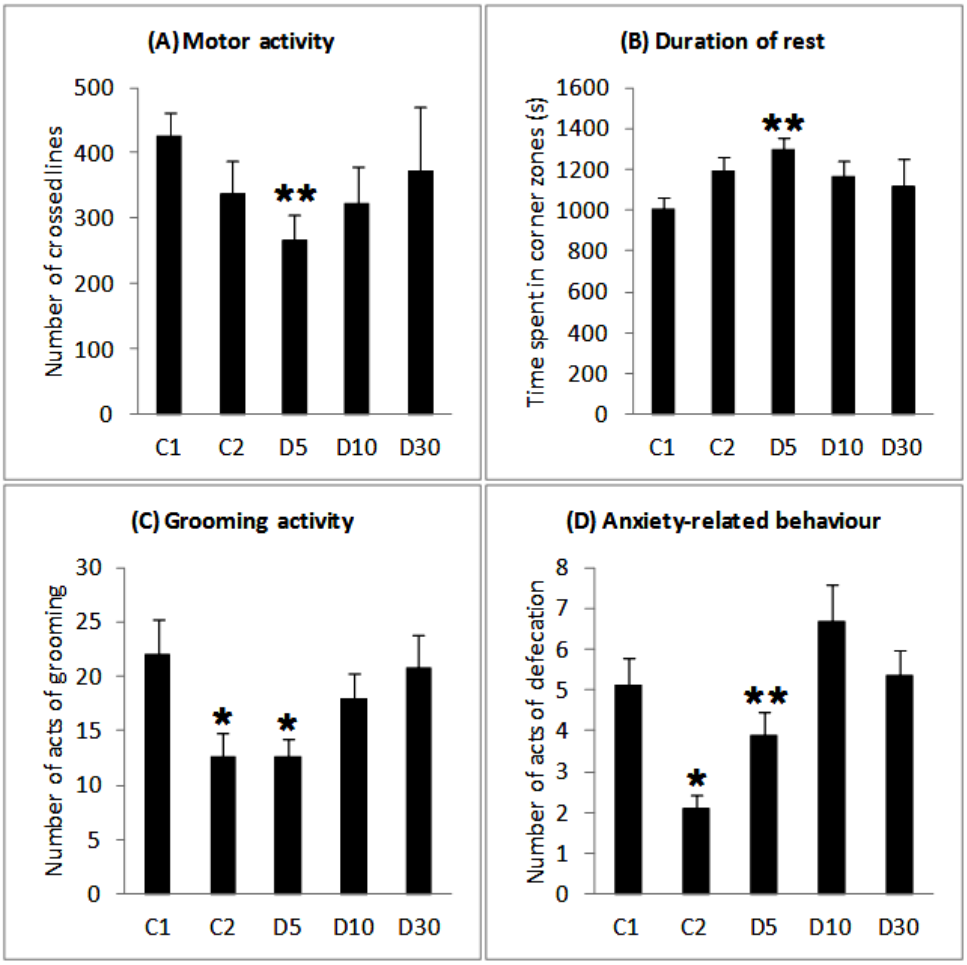

Figure 3. Results of behavioral test "Open field" in control (C1 – non-anesthetized, n=8, C2 – anesthetized, n=8), on day 5 (D5 – anesthetized, n=10), day 10 (D10, n=9) and day 30 (D30, n=8) since the first impact. * and ** - $p<0.05$ and $p<0.01$ in comparison with non-anesthetized control (t-test). Day 5 was compared to anesthetized control. Graph (D) shows statistically significant difference between anesthetized control and day 5. Rest of indicators represents the result of comparison with non-anesthetized control. Results are the mean ± SEM.

The observations of grooming activity showed that the number of grooming acts decreased when compared to non-anesthetized control, but remained the same when compared to the group of anesthetized control animals (Fig. 3 C). By day 10 it increased when compared to anesthetized control and day 5. By day 30 it became comparable to non-anesthetized control. Statistically significant difference was shown

between both control groups ($p<0.05$) and between non-anesthetized control and day 5 ($p<0.05$). The changes of the number of acts of defecation (Fig. 3 D) were analogous to those of grooming activity. Statistically significant difference was shown between both control groups ($p<0.01$) and between anesthetized control and day 5 ($p<0.05$).

Quantitive immunohistochemical analysis revealed that astro- and microglisosis in CA1-zone of hippocampus persisted for weeks (Fig. 4).

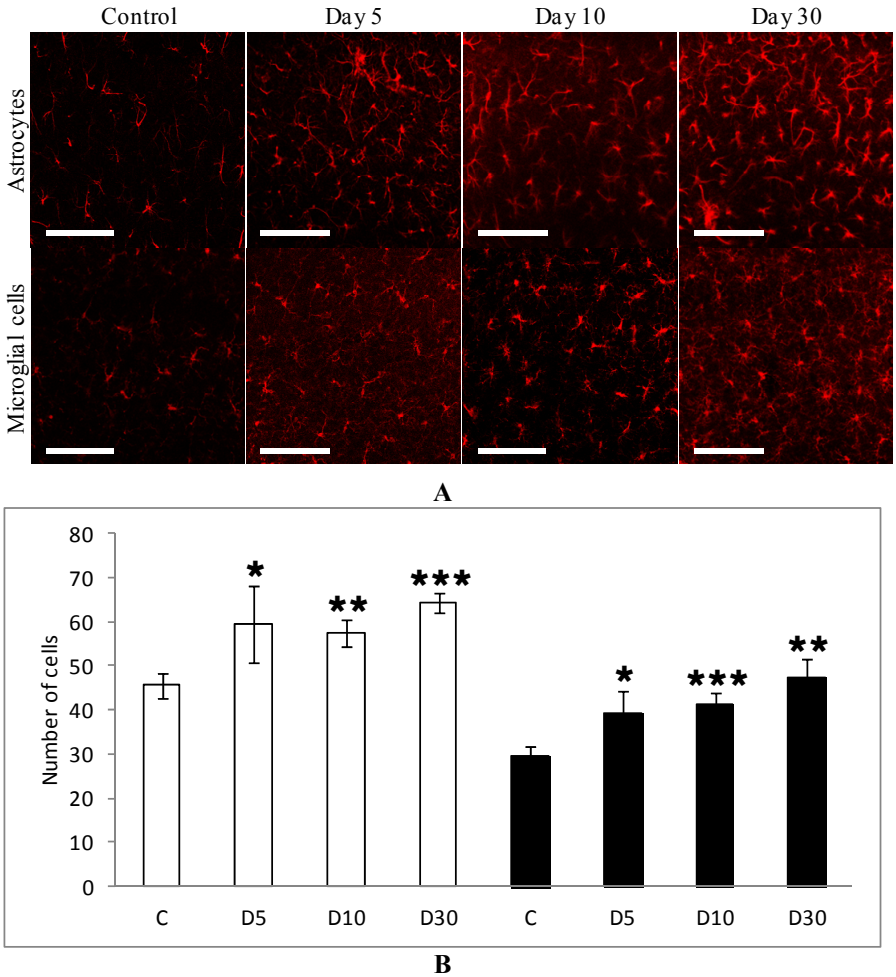

Figure 4. Changes in number of astrocytes and microglial cells of hippocampal CA1-zone after delivering brain injury in control, on day 5, day 10 and day 30 since the first impact. A – microphotographs of hippocampal CA1-zone. Bar=100 μm. The number of cells were counted in 0.1 mm^2. B – Comparative cell counts (astrocytes – white, microglial cells - black) in control and after delivering brain injury. *, ** and *** – $p<0.05$, $p<0.01$ and $p<0.001$ in comparison with control (t-test). Results are the mean ± SEM.

Specifically, the amount of astrocytes on day 5 since the first impact (Fig. 4 B) was significantly higher than in controls. Remaining roughly the same on day 10, the number of astrocytes increased by day 30. Statistically significant difference was shown between controls and each of injured groups: day 5 ($p<0.05$), day 10 ($p<0.01$) and day 30 ($p<0.001$). The tendency was similar for dynamics in microglia activation. In numbers of microglial cells, statistically significant difference was shown for all the groups related to rmTBI: day 5 ($p<0.05$), day 10 ($p<0.001$) and day 30 ($p<0.01$). Noteworthy, the activation of astro- and microglia was combined with restoration of behavioral performance.

Conclusions

In a novel model of rmTBI, no cases of skull fractures, intracranial bleeding or seizures were registered. "Open field" behavioral testing revealed two tendencies related to decrease of motor activity by day 5 and its gradual recovery by day 30, as well as increase of anxiety level by day 5 and day 10 and its return to control measures. Astro- and microgliosis took place on day 5, day 10 and day 30, which indicates the presence of secondary injury even after a long time interval. This allows us to use the model in further research of rmTBI features and selection of potential treatments.

REFERENCES

Beauchamp, K., Mutlak, H., Smith, W.R., Shohami, E., Stahel, P.F. (2008) Pharmacology of traumatic brain injury: where is the "golden bullet"?, *Mol. Med.*, **14**, 731–740

Belanger, H.G., Curtiss, G., Demery, J.A., Lebowitz, B.K., Vanderploeg, R.D. (2005) Factors moderating neuropsychological outcomes following mild traumatic brain injury: A meta-analysis, *Journal of the International Neuropsychological Society*, **11**, 215–227

Betrus, C., Kreipke, C.W. (2013) Historical Perspectives in Understanding Traumatic Brain Injury and in Situating Disruption in CBF in the Pathotrajectory of Head Trauma, In: *Cerebral Blood Flow, Metabolism, and Head Trauma*, Kreipke, C.W., Rafols, J.A. (ed), Springer Science+Business Media, New York, pp 1-29

Davis, G.A., Iverson, G.L., Guskiewicz K.M. et al. (2009) Contributions of neuroimaging, balance testing, electrophysiology and blood markers to the assessment of sport-related concussion, *Br. J. Sports Med.,* **43**, 36–45

Granacher, R.Jr. (2008) Traumatic Brain Injury: Methods for Clinical and Forensic Neuropsychiatric Assessment, 2nd Edition, CRC Press, Boca Raton, pp 586

Kane, M.J., Angoa-Pérez, M., Briggs, D.I., Viano, D.C., Kreipke, C.W., Kuhn, D.M. (2012) A mouse model of human repetitive mild traumatic brain injury, *J. Neurosci Methods*, **203(1)**, 41-49

Kawai, N., Kawanishi, M., Okauchi, M., Nagao, S. (2001) Effects of hypothermia on thrombin-induced brain edema formation, *Brain Res*., **895**, 50–58

Kim, J., Parker, D., Whyte, J., Hart, T., Pluta, J., Ingalhalikar, M., Coslett, H.B., Verma, R. (2014) Disrupted structural connectome is associated with both psychometric and real-world neuropsychological impairment in diffuse traumatic brain injury, *J. Int. Neuropsychol. Soc*., **20**, 887-896

Kunz, A., Dirnagl, U., Mergenthaler, P. (2010) Acute pathophysiological processes after ischaemic and traumatic brain injury, *Best Pract. Res. Clin. Anaesthesiol.*, **24(4)**, 495–509

Levita, L., Bois, C., Healey, A., Smyllie, E., Papakonstantinou, E., Hartley, T., Lever, C. (2014) The Behavioural Inhibition System, anxiety and hippocampal volume in a non-clinical population, *Biol. Mood Anxiety Disord.*, **4(4)**

Marmarou, A., Foda, M.A., van den Brink, W., Campbell, J., Kita, H., Demetriadou, K. (1994) A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics, *J. Neurosurg.,* **80**, 291–300

McCrory, P., Meeuwisse, W., Johnston K. et al. (2009) Consensus statement on concussion in sport - the 3rd International Conference on concussion in sport, held in Zurich, November, *J. Clin. Neurosci.*, **16(6)**, 755–763

McKee, A.C., Daneshvar, D.H., Alvarez, V.E., Stein, T.D. (2014) The neuropathology of sport, *Acta Neuropathol.*, **127(1)**, 29–51

Pivneva, T.A., Tsupikov, O.M, Pilipenko, M.N., Vasilenko, D.A., Skibo, G.G. (2005) Structural modifications of astrocytes in the hippocampus after experimental cerebral ischemia in gerbils, *Neurophysiology*, **37**, 359-364

Werner, C., Engelhard, K. (2007) Pathophysiology of traumatic brain injury, *Br. J. Anaesth.*, **99(1)**, 4–9

Xiong ,Y., Mahmood, A., Chopp, M. (2013) Animal models of traumatic brain injury, *Nat. Rev. Neurosci. Nature,* **14(2)**, 128–142

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIX, 2, 2014 (p. 69-76)

EFFECTS OF *ARTHROSPIRA (SPIRULINA)* ON HEMATOPOIESIS IN RATS

RODICA BERLEAN[1], CORINA L. ROŞIORU[1] and CORNELIU TARBA[1,✉]

SUMMARY.The objective of our study was to test the hematopoietic effects of an autohtonous strain of *Arthrospira* (*Spirulina)* added to the food of white rats. To investigate these effects we compared the reponse of anemiated and healthy rats to *Spirulina* feeding.The animals were divided into 3 groups which were fed bread and milk for the first week. The control group (C) received *Spirulina* powder (3% of the food) for two additional weeks. In the other two groups, during the first week, 25% of the blood was extracted 3 times, in days 1, 2 and 4, in order to induce anemia, while in days 3 and 5, small blood samplings were taken to establish the degree of the induced anemia. One of these two groups, denoted An (anemiated) received only bread and milk for the next two weeks, while the other one, denoted An-Sp, was also anemiated but received *Spirulina* powder. A blood sampling was also performed at the end of the 3rd week. The state of the animals was evaluated by measuring the blood content of 3 parameters: percent reticulocytes, red blood cell (RBC) count and hematocrit value. The percent reticulocytes and RBC count kept relatively constant for the C group, while hematocrit slightly increased. In the An group, the number of reticulocytes was highest at the second sampling (after anemia induction) and slightly decreased afterwards, while in the An-Sp group the number of reticulocytes at the second sampling was moderately lower than in the An group (but significantly higher than in the control) and decreased even more at the final sampling. As expected, the RBC count had a different evolution, increasing in a highly significant way in the An-Sp group, while in the An group it only approached the level of the control, a fact which can be explained by the hematostimulating effect of Spirulina in the An-Sp group. Due to the compensating changes between reticulocytes and RBCs, the hematocrit had only slight variations during the experiment. It is concluded that *Spirulina* had a hematostimulating effect not only in group An-Sp but also in group C.

Keywords: antianemic effect, hematocrit, red blood cells, reticulocytes, *Spirulina*.

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Introduction

Arthrospira (Spirulina) is a blue-green cyanobacterium which contains a complex of vitamins (A, B, D, E, K), minerals (calcium, potassium, sodium, posphorus, magnesium, iron, iodine), amino acids, fatty acids, pigments (phycocyanin, allophycocyanin, chlorophyll and carotenoids), all necessary to an equilibrated diet (see, for example, Belay *et al*., 1996; Sánchez *et al*., 2003; Becker, 2004; Spolaore *et al*., 2006). It represents the richest natural source of easily digestible proteins that contain all the essential amino acids (Guil-Guerrero *et al*., 2004)

Previous studies have also reported that *Arthrospira* (*Spirulina)* can reduce the severity of anemia, inducing an increase of hematologic parameters (Kostic *et al.*, 1993; Zikic *et al.*, 1997; Simsek *et al.*, 2009). Apparently, its content of phycocyanin stimulates hematopoiesis and mimics the effect of endogenous erythropoietin (Epo). The role of Epo consists in stimulating the proliferation, growth and differentiation of erythroid precursors, with the consequent increase of the erythrocyte count, the endogenous Epo being normally inversely proportional with the erythrocyte volume.

The objective of the present study was to test the hematopoietic effects of the locally isolated wild strain of *Arthrospira* (*Spirulina)* added as biomass to the food of white rats. More exactly, we investigated the stimulating effects of this cyanobacterium on both anemiated and normal/healthy rats.

Material and Methods

In our experimental variant, white rats of about 200 g each were monitored for 3 weeks. They were divided into 3 groups: group **C** (control), which was fed for the first week only bread and milk, to which *Spirulina* powder (3% of the food) was added for the last two weeks; group **An** (anemiated), which consumed all the time only bread and milk; group **An-Sp**, also anemiated, but treated with *Spirulina* for the last two weeks, similar to the C group. The anemia in groups **An** and **An-Sp** was induced through 3 blood takings in days 1, 2 and 4. In days 3 and 5, small blood samplings were performed to check for the level of the induced anemia. A final sampling (at the end of the 3rd week) was also performed for all groups. For each intervention, the animals were slightly anesthsized with ethyl ether. The blood was taken from the orbital plexus. For inducing anemia, 25% of the blood volume was extracted each time (the blood volume was estimated to 6 ml for each 100 g body weight, according to Chiricuţă *et al*., 1992). For blood samplings, only 0.5 ml were taken each time.

The state of the animals was evaluated by measuring the blood content of 3 parameters: percent of reticulocytes, red blood cell (RBC) count and hematocrit value, using standard clinical procedures.

Results and discussion

At the first/initial blood sampling, the 3 groups displayed very similar/close results, while in the subsequent samplings the results differed statistically significant in a manner suggestive for an antianemic effect of *Spirulina*. The evolution of the 3 parameters monitored, along with the appropriate statistical data for the 3 groups are presented in Table 1 (group C), Table 2 (group An) and Table 3 (group An-Sp). Suggestive bar illustrations of the measured parameters are also presented comparatively in Fig. 1 (reticulocytes), Fig. 2 (RBCs), and Fig.3 (hematocrit).

As can be seen from Table 1, at the final sampling (performed after *Spirulina* administration), the count of both reticulocytes and RBCs increased in a highly significant way, in comparison with either of the previous samplings. The hematocrit also increased significantly.

Table 1.

Evolution of the blood and statistical parameters in group C

Blood parameter (unit of measure)	**Statistical parameter**	**Sampling before *Spirulina* administration (S_2)**	**Final sampling (S_3)**
Reticulocytes (/100 erythrocytes)	$\bar{x}$±ES	0.59±0.02	1.08±0.06
	n	6	5
	±D%(S_2)	0	+83.05
	$p_{3/2}$		< 0.001 ***
RBCs ($x10^6/mm^3$)	$\bar{x}$±ES	9.34±1.69	12.69±2.29
	n	6	5
	±D%(S_2)	0	+35.86
	$p_{3/2}$		< 0.001 ***
Hematocrit (%)	$\bar{x}$±ES	43.27±0.98	48.26±1.55
	n	6	5
	±D%(S_2)	0	+11.53
	$p_{3/2}$		< 0.05 *

Explanations: **$\bar{x}$±ES** – mean ± standard error of mean; n – number of animals tested; **±D%(S_2)** – percent difference between final sampling and the sampling before *Spirulina* administration; **$p_{3/2}$** – value of **p** for the above defined difference.

The evolution of the blood parameters in the An group, along with the appropriate statistical parameters are presented in Table 2. As can be seen from this table, at the second sampling (after anemia was produced), a highly significant decrease in RBC count was recorded in the An group, as compared to the initial sampling, while at the final sampling this decrease was smaller, but still statistically

significant. However, as compared to the second sampling, there was a very significant increase in RBC count at the final sampling. This increase is most likely due to the induction of Epo synthesis and its effect on erythropoiesis, as a reaction of the organism to anemia. As regards the reticulocytes, there is a highly significant increase of their number at both final and second sampling, although the increase is somewhat smaller at the final sampling. This can be attributed to the conversion in time of some reticulocytes into adult erythrocytes, as indicated by the increase of RBC count at the last sampling, as mentioned above. Significant increases can also be observed for hematocrit. All these results demonstrate the natural capacity of hematologic recovery for the animals tested.

Table 2.

Evolution of blood and statistical parameters in group An

Blood parameter	**Statistical parameter**	**Initial sampling (S_1)**	**Sampling before *Spirulina* administration (S_2)**	**Final sampling (S_3)**
Reticulocytes (/100 erythrocytes)	$\bar{x}$±ES	0.64±0.03	3.67±0.46	3.03±0.36
	n	7	7	7
	±D%(S_1)	0	+473.43	+373.43
	$p_{2/1}$		< 0.001 ***	
	$p_{3/1}$			< 0.001 ***
	±D%(S_2)	-82.56	0	- 17.43
	$p_{3/2}$			NS
RBCs ($x10^6/mm^3$)	$\bar{x}$±ES	11.4±0.54	8.30±0.15	10.07±0.24
	n	7	7	7
	±D%(S_1)	0	-27.19	-11.66
	$p_{2/1}$		< 0.001 ***	
	$p_{3/1}$			< 0.05 *
	±D%(S_2)	+37.35	0	+21.32
	$p_{3/2}$			< 0.001 ***
Hematocrit (%)	$\bar{x}$±ES	38.18±3.05	40.26±1.32	44.54±2.11
	n	7	7	7
	±D%(S_1)	0	+5.44	+16.78
	$p_{2/1}$		NS	
	$p_{3/1}$			< 0.05 *
	±D%(S_2)	-5.16	0	0
	$p_{3/2}$			

Explanations: ±D%(S_1) – corresponding difference between S_2 or S_3 and S_1 $\mathbf{p_{2/1}}$ – value of p for the difference between the second sampling (performed before the administration of *Spirulina*) and the initial one; $\mathbf{p_{3/1}}$ – value of p for the difference between the final and the initial sampling; $\mathbf{p_{3/2}}$ – value of p for the difference between the final and the second sampling. The rest of the notations are identical to those in Table 1.

For the An-Sp group, the results are presented in Table 3, below. As can be seen, all three blood parameters tested show increased values at the final sampling, as compared to the initial one. The same statement is valid if comparison is made with the second sampling, with one notable exception: the reticulocytes. Their count is higher after the induction of anemia (before *Spirulina* addition) than at the final sampling. This can be explained by the fact that phycocyanin has an Epo-like effect, stimulating the hematopoiesis, i.e. the conversion of reticulocytes into RBCs. Indeed, the RBC count is much larger in the An-Sp group than in the An group and even higher than in the control (C) group. Since the control group also received *Spirulina*, the difference should be ascribed to the combined effect of the endogenous Epo (induced by anemia) and the Epo-like effect exerted by *Spirulina*. The value of the other blood parameters is also in line with this conclusion. For example, the hematocrit is significantly increased at the final sampling.

Table 3.

Evolution of blood and statistical parameters in group An-Sp

Blood parameter	**Statistical parameter**	**Initial sampling (S_1)**	**Sampling before *Spirulina* administration (S_2)**	**Final sampling (S_3)**
Reticulocytes (/100 erythrocytes)	$\bar{x}$±ES	0.58±0.04	3.03±0.20	1.53±0.08
	n	7	7	6
	±D%(S_1)	0	+422.41	+163.79
	$p_{2/1}$		< 0.001 ***	
	$p_{3/1}$			< 0.001 ***
	±D%(S_2)	-80.85	0	-49.50
	$p_{3/2}$			< 0.001 ***
RBCs ($x10^6/mm^3$)	$\bar{x}$±ES	10.85±0.60	6.97±1.29	13.19±2.39
	n	7	6	6
	±D%(S_1)	0	-35.76	+21.56
	$p_{2/1}$		< 0.01 **	
	$p_{3/1}$			< 0.001 ***
	±D%(S_2)	+55.66	0	+89.23
	$p_{3/2}$			< 0.001 ***
Hematocrit (%)	$\bar{x}$±ES	46.39±1.16	41.93±1.14	46.60±1.57
	n	7	6	6
	±D%(S_1)		-9.61	+0.45
	$p_{2/1}$		< 0.01 **	
	$p_{3/1}$			NS
	±D%(S_2)	+10.63		+11.13
	$p_{3/2}$			< 0.05 *

The same expalanations are valid as for Tables 1 and 2, but it should be remembered that this group was both anemiated and given *Spirulina*.

In order to have a general image of our results, a synthetic comparison across the 3 groups at all 3 samplings is also illustrated by bar diagrams in Figs.1-3, separately for each of the 3 blood parameters tested.

Fig. 1 illustrates and compares the reticulocyte count of the 3 groups at the moments of the 3 samplings. As can be seen, there are highly significant increases (***) in both An and An-Sp group as compared to the control, at the second and third (final) sampling, whereas a very significant decrease (+++) can be observed at the final sampling for the An-Sp group as compared to the An group. The significance of these observations has been already discussed.

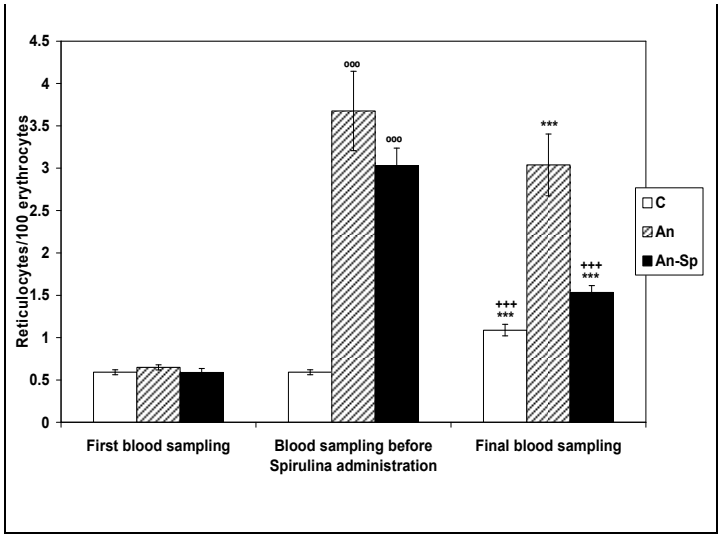

Figure 1. A comparative illustration of the evolution of the reticulocyte count in the 3 rat groups. º - denotes the significance of the differences between the sampling before *Spirulina* administration and the initial sampling; * - denotes the significance of the differences between the final sampling and the initial sampling while the symbol + is used to show significant differences between the final sampling and the one before *Spirulina* administration.

In Fig. 2 we illustrate the evolution of RBC count in the 3 rat groups. As can be seen, the differences that exist between the groups at each sampling do not appear statistically significant, although there are differences among the 3 moments of sampling as already discussed when we presented Tables 1-3.

Hematocrit values of the 3 groups are presented comparatively in Fig. 3. As already discussed, due to different compensating effects, the hematocrit has a rather slight variation which is either not significant or hardly significant. Occasionally, the hemoglobin concentration was also measured and its values seem to be more relevant than the hematocrit. As expected, the hemoglobin concentration parallels the increase of the RBC count (not shown here).

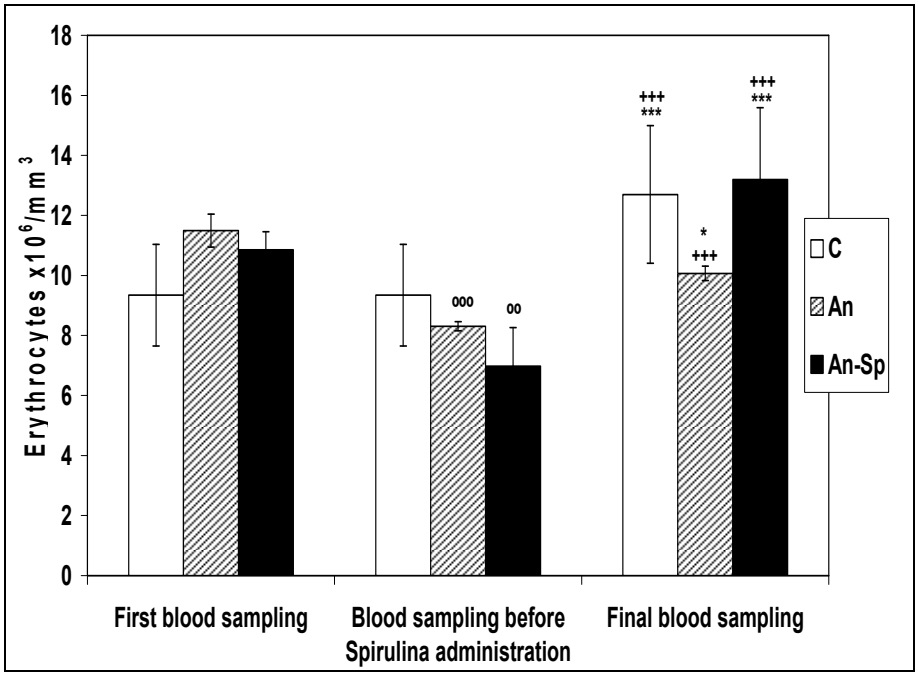

Figure 2. The evolution of the RBC count in the 3 rat groups

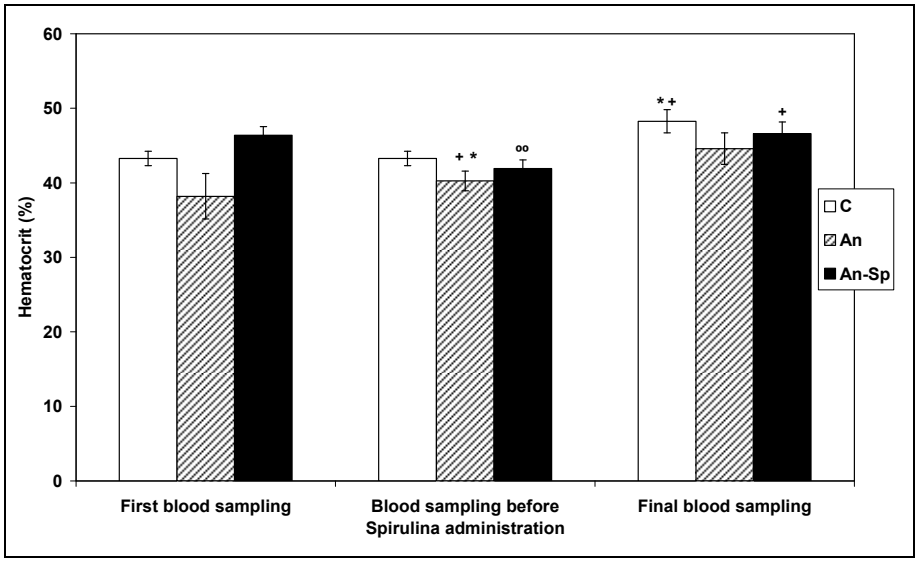

Figure 3. Hematocrit values in the 3 groups of rats at the 3 moments of sampling

Conclusions

The hematostimulating effect of our strain of *Arthrospira (Spirulina)* was observed even in group C (control), in the absence of anemia.

In the rats of the An (anemiated) group, after 14 days since the induction of anemia, the measured parameters (% reticulocytes, RBC count, and hematocrit) partially recovered, as a result of erythropoiesis intensification under the action of the induced endogenous Epo.

The animals in the An-Sp group were more efficient in restoring the affected parameters, as compared to the An group, because the normal reaction of the organism to anemia was supplemented by the effect of phycocyanin contained by the cyanobacterium *Spirulina.*

REFERENCES

Becker, W. (2004) Microalgae in human and animal nutrition, In: *Handbook of microalgal culture*, Richmond, A. (ed), Blackwell, Oxford, pp 312-351

Belay, A., Kato, T., Ota, Y. (1996) *Spirulina (Arthrospira)*: potential application as an animal feed supplement, *J. Appl. Phycol.* **8**, 303-311

Chiricuţa, I., Risca, R., Bologa, S., Ghergariu, S. (1992) Biologia animalului de laborator şi oncologie comparată: Material de informare şi îndrumare metodologică, *Enciclopedia Oncologică*, **19,** Cluj-Napoca, pp 116

Guil-Guerrero, J.L., Navarro-Juárez, R., López-Martínez, J.C., Campra-Madrid. P., Rebolloso-Fuentes, M.M. (2004) Functional properties of the biomass of three microalgal species, *J. Food Eng.* **65**, 511-517

Kostic, M.M., Ognjanovic, B., Dimitrijevic, S., Zikic, R.V., Stajn, A., Rosic, G.L., Zivkovic, R.V. (1993) Cadmium-induced changes of antioxidant and metabolic status in red blood cells of rats: in vivo effects, *Eur. J. Haematol.,* **51**(2), 86-92

Sánchez, M., Castillo, B.J., Rozo, C., Rodríguez, I. (2003) *Spirulina (Arthrospira)*: An edible microorganism. A review, *Universitas Scientiarum*, **8**(1), 1-16

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. (2006) Commercial application of microalgae, *J. Biosci. Bioeng.* **101**(2), 87-96

Simsek, N., Karadeniz, A., Keles, O.N., Unal, B. (2009) *Spirulina platensis* feeding inhibited the anemia- and leukopenia-induced lead and cadmium in rats, *J. Hazard. Mater.*, **164**(2-3), 1304-1309

Zikic, V., Stajn, A. S., Ognjanovic, B.I., Pavlovic, S.Z., Saicic, Z.S. (1997) Activities of superoxide dismutase and catalase in erythrocytes and transaminases in the plasma of carps (*Cyprinus carpio L.*) exposed to cadmium, *Physiol. Res.*, **46**(5), 391-396

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIX, 2, 2014 (p. 77-87)

EFFECTS OF ADRENALINE INDUCED STRESS AND FLUOCINOLONE-TREATMENT ON SOME ENDOCRINE GLANDS IN MALE WISTAR RATS

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SUMMARY. Synthetic glucocorticoids are widely used as antiinflammatory and antiallergic drugs. Since drugs contaning glucocorticoid are effectively absorbed, a prolonged glucocorticoid treatment leads to a surplus of endogen glucocorticoids. Long-term glucocorticoid treatments disrupt the endocrine system of the body causing hormonal and metabolic side effects. For this reason, it is necessary to specify the endocrino-metabolic effects of these drugs. Starting from the above findings and from the important physiological roles of glucocorticoids, we investigated the reactions of some endocrine glands after adrenaline-stress and fluocinolone treatment. For this purpose, it was important to compare the effects of high glucocorticoid level produced by stressors with the effects of high glucocorticoid level which occur during dermocorticoid treatment. In the present study adrenaline treatment was the source of glucocorticoid excess on the one hand, and fluocinolone on the other. In both cases, significant physiological changes were observed, the most sensitive gland in changes of glucocorticoid levels being the adrenal gland. Adrenaline stress caused atrophy of the cortical zone of adrenal gland. The fluocinolone-treatment determined hypertrophy of the cortical zone of the adrenal gland. In both cases we observed significant weight changes for the whole body and for each gland.

Keywords: adrenalin, adrenals, glucocorticoids, pancreas, thymus.

Introduction

Organisms survive by maintaining equilibrium with their environment. The stress system is critical to this homeostasis. Hormone secretion by the hypothalamic-pituitary-adrenocortical (HPA) axis is modulated by multiple factors which include the circadian rhytm, various types of stressors and glucocorticoids. It is also well demonstrated that multiple stimulatory agents can independently affect the function of the HPA axis (Kis *et al*., 2001b).

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These stimulatory agents include catecholamines, vasopressin and the corticotropin-releasing hormone (CRH). Stress induced activation of HPA axis is attended by release of CRH from paraventricular nucleus (NPV) of hypothalamus, followed by adrenocorticotropine (ACTH) and glucocorticoid release (Cole and Sawchenko, 2002). Among many possible neurotransmiters released in the PVN during exposure to various stressors, noradrenaline and adrenalin are thought to be the potent stimulators of CRH neurons in PVN (Kis and Crăciun, 2003a, 2003b).

Treatment with synthetic glucocorticoids e.g. dexamethasone or dermocorticosteroids, or repeated immobilization stress, decreases the total body weight gain of animals by disturbing the HPA axis function and accelerating the catabolism of the organism (Kis *et al*., 2001a, Gruver-Yates and Cidlowski, 2013, Noguchi, 2014).

Synthetic glucocorticoids are widely used as anti-inflammatory and anti-allergic drugs. Nevertheless, their administration may cause side effects related to the normal functioning of several organs. Glucocorticoids modulate the stress response at a molecular level by altering gene expression, transcription and translation, among other pathways. Glucocorticoids also modulate growth, reproductive and thyroid axes (O'Connon *et al.,* 2000, Kis *et al.,* 2001a, Kis and Crăciun, 2006).

Excessive glucocorticoid secretion or treatment, on the other hand, has been reported to have deleterious effects on the organism: it can induce tissue injury and even cell death (Craciun *et al.,* 1997, 1998, Kis and Crăciun, 2006). Madar and co-workers (1993) have studied the effect of excessive glucocorticoid levels generated through repeated formaldehyde stress or Fluocinolon acetonid-N (FC) treatment in rats. Formaldehyde was used as an endogenous glucocorticoid inducer, while FC was an exogenous source of the hormone. Both formaldehyde- and FC-treated groups showed significant metabolic disorders.

The exact mechanism of glucocorticoid-induced cell death is unknown, but several reports indicate that glucocorticoid-mediated generation of reactive oxygen species (ROS) occurs with the concomitant increase of calcium influx and morphological degeneration of the cell (Landfield and Eldridge, 1994, Viegas *et al*., 2008).

In recent studies elsewere we have reported that short-term and long-term epicutaneous applications of halogenated glucocorticosteroids in pregnant rats induced changes in thymus oxidative status of dams and newborn animals (Kis, 2010, 2012a, 2012b).

In our experiments we followed changes in bodyweight as well as the weight of some glands, histological aspects of rat thymus and adrenals during adrenaline (ADR) and FC treatment. We used adrenalin and Fluocinolon-N ointment, as endogenous and exogenous sources of elevated glucocorticoid levels.

Materials and methods

Experiments were carried out in male Wistar rats. Animals were kept under standardized bioclimatic conditions and fed on common rat chow, with water ad libitum.

Commercial Fluocinolone-acetonid-N ointment containing 25 mg Fluocinolon-acetonid-N/100 g excipient was applied topically to the skin at 2 cm2 for five consecutive days, by smearing 50 mg ointment/100 g b.w on the inguinal region, the daily dose of ointment being equal to 12.5 µg/100 g b.w. Epinephrine-treated animals were injected subcutaneously with 42 µl/100 g b.w. of Adrenaline solution.

Animals were divided into the following groups: K-control group-untreated animals, FC-Fluocinolone-N-treated animals and ADR- adrenaline-treated group.

After 16 hours of fasting and 24 hours following the cessation of treatments, the treated animals together with controls were sacrificed by exsanguination.

Body, pancreas, adrenals and thymus weights of male rats were measured with an accuracy of 0.00001 g immediately after excision. Data are presented as mean ± standard error of the mean. The comparison between the three groups was performed using Student t test for unpaired data.

The thymus and adrenal gland were fixed in Bouin liquid and afterwards processed in view of being embedded in paraffin. The fragments were sectioned at the Reichart microtome with a tickness of 7 µ. The staining of glands was carried out with the method of Hurduc and co-workers (Muresan *et al*., 1974). Histological preparations obtained were examined under an IORC4 optical microscope. Photos were taken using 10x and 20x objectives with an Olympus digital camera.

Results and discussion

The results presented in Figure 1 show that in our experiment FC treatment and ADR stress caused a progressive inhibition of total body weight gain in animals compared to the controls. Body weight loss in the ADR-treated group was lower than in the FC-treated one, indicating that ADR induces a milder stress than the direct cortisol treatment.

The fluocinolone treatment caused a significant weight loss in contrast with the control group (Fig. 2). However, significant weight loss in the FC group was observed as compared to the ADR treated animals. The relative weight of the pancreas in FC treated animals showed a slight increase. This result is in agreement with the literature, according to which short-term dexamethasone administration induces increased pancreatic rat beta-cell proliferation (Rafacho *et al*., 2009, 2010, 2011)

In the adrenaline treated group the weight of the gland showed a significant reduction compared with the control as well as in the FC treated groups (Fig. 3). GC excess induced by different stressors causes decreased b-cell insulin production and

insulin resistance; furthermore, it reduced effectiveness of insulin in suppressing hepatic glucose production and in increasing glucose uptake in muscle and fatty tissue (Andrews and Walker, 1999). Persons who have a diminished b-cell function or are low-insulin responders are predisposed to develop diabetes during glucocorticoid excess (Henriksen *et al*., 1997; Wajngot *et al*., 1992).

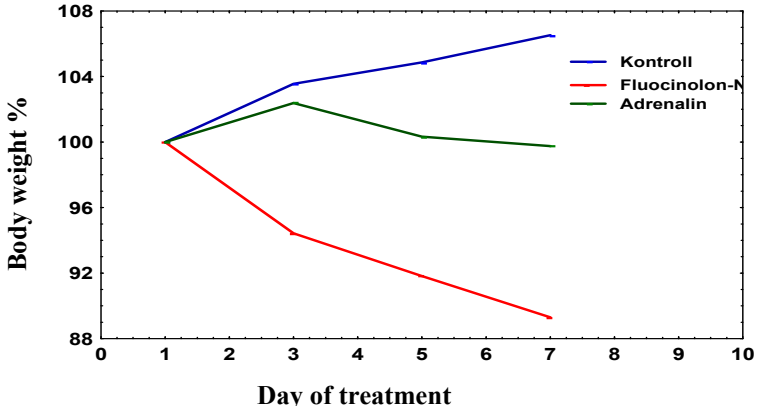
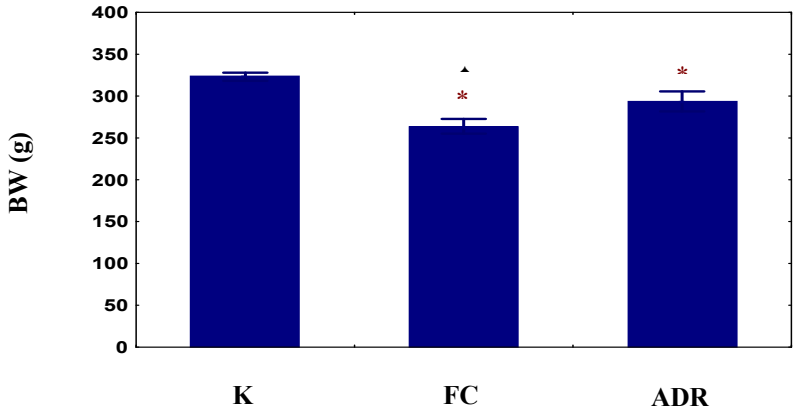
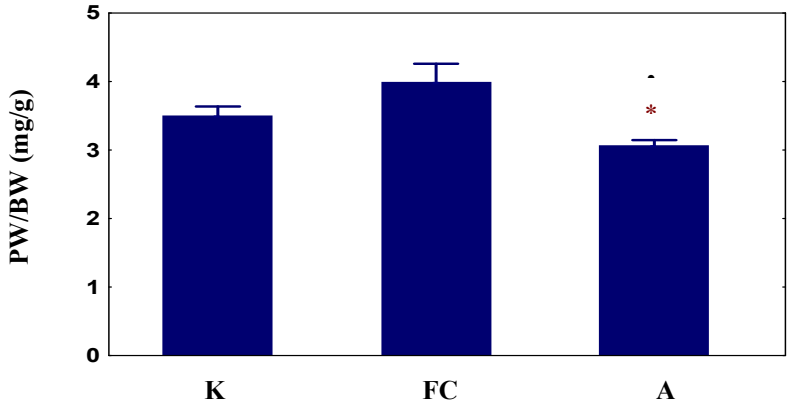


Figure 1. Evolution of the body weight.



* significant differences from the C-group ($p < 0.004$), ♠ significant differences from the ADR-group ($p<0.007$)

Figure 2. Body weight (BW) on the last day of treatment.



* significant differences from the C-group (p< 0.04), • significant differences from the FC group (p< 0.02)

Figure 3. Relative weight of the pancreas (PW).

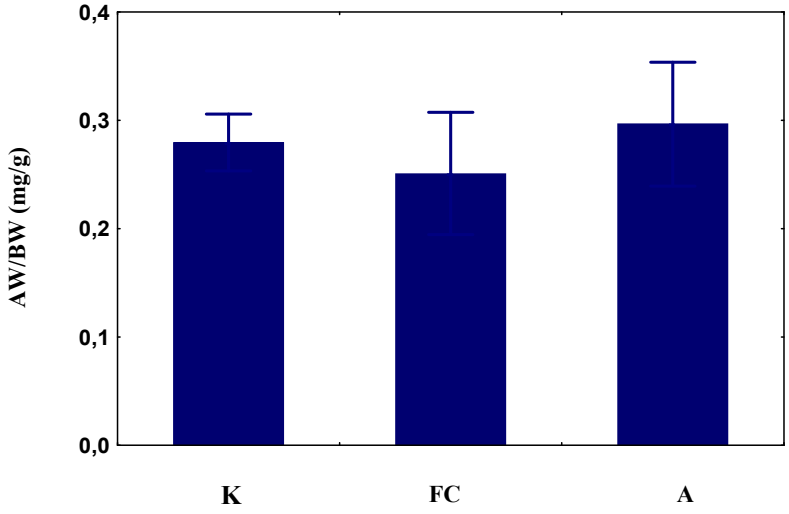
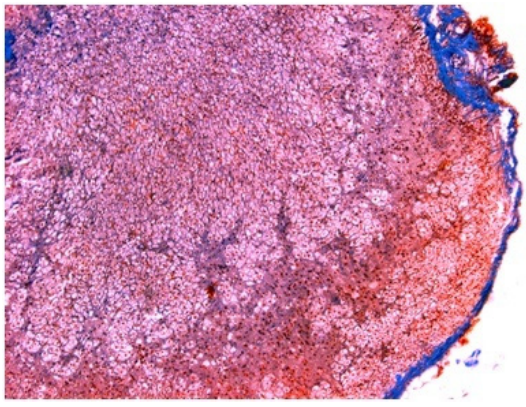


Figure 4. Relative weight of the adrenal gland (AW)

The relative weight of the adrenal gland in the FC group showed a slight decrease compared with the control group (Fig. 4). In the ADR treated group we observed an increase of the gland weight compared with the control one.

These are confirmed with the results of microscopical examination (Fig. 5).

a.



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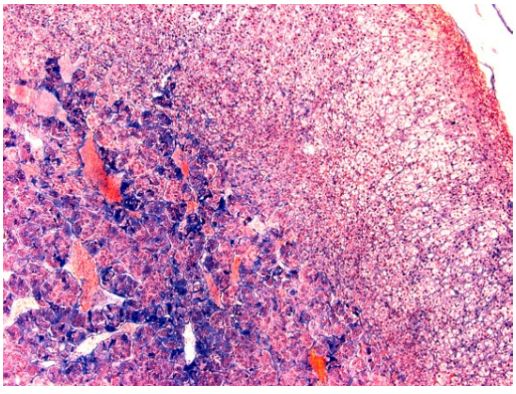


c.

Figure 5. Histological aspects of adrenal gland (20x).: a) normal aspect, b) hypertrophy of cortical zone in ADR stressed animals, c) atrophy of cortical zone in fluocinolone-treated rats

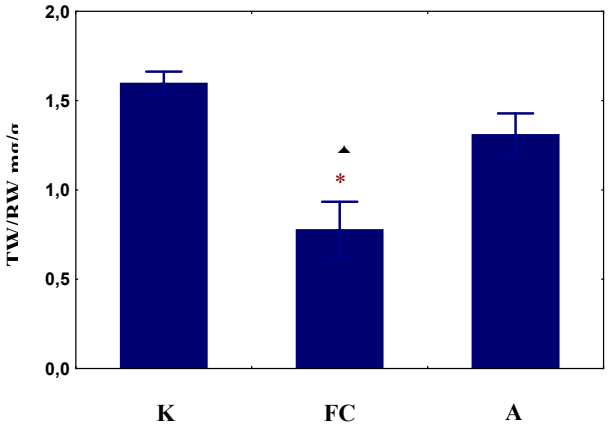
Results of our microscopical examination confirm these changes. The adrenals of animals treated with FC are atrofiated (Fig. 5b), whereas we observed hipertrophy of the gland cortex in animals treated with ADR drenaline (Fig. 5c).

Glucocorticoid administration results in a negative feedback effect via glucocorticoid receptors in the anterior hypothalamus, which, in turn, suppresses the production of corticotrophin-releasing hormone and the release of POMC/ACTH from adenohypophysis. The prolonged suppression of adrenocorticotropine levels leads to atrophy of the adrenal cortex and secondary adrenal insufficiency (Kis and Crăciun, 2003a, 2003b).

Glucocorticoids mediate these effects via both DNA binding-dependent and DNA binding-independent mechanisms (Schaöcke *et al.,* 2002, Anacker *et al*., 2013).

The hypertrophy of the cortex under ADR treatment suggests increases of glucocorticoids synthesis. ADR treatment as a stressor leads to glucocorticoid secretion through the induction of sympathoadrenal system, which is followed by the activation of hypothalamo - pituitary - adrenal axis (Kis and Crăciun, 2003a, 2003b). Noradrenalin microinjection in NPV causes elevation in plasma corticosteron levels (Cole, 2002, Harris *et al.,* 2001, Leibowitz, 1998).

The relative thymus weight was significantly reduced in the FC group (Fig. 6). During ADR treatment we observed a slight decrease of the thymus weight.

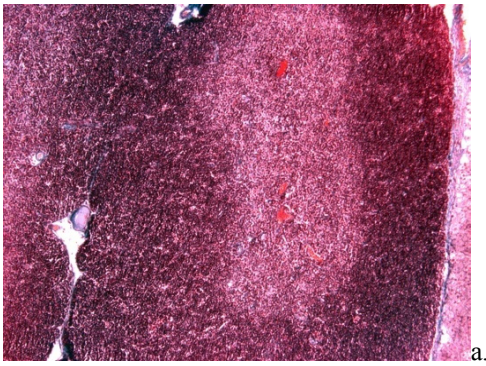


* significant differences from the K-group ($p < 0.02$), ♠ significant differences from the ADR-group ($p < 0.03$)

Figure 6. Relative weight of the thymus

This result is in agreement with the literature according to which stress-induced thymic involution is characterised by reduction in thymus size caused by acute loss of cortical thymocites and reduced output of native T cells to the periphery (Crăciun *et al.,* 1997, 1998, Wang *et al.*, 1994).

Microscopic studies confirm these results. FC treatment caused the atrophy of the thymus, while thymus structure in the animals treated with adrenaline did not differ from the control group (Fig. 7).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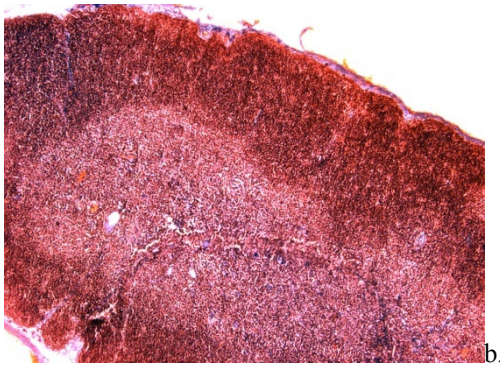
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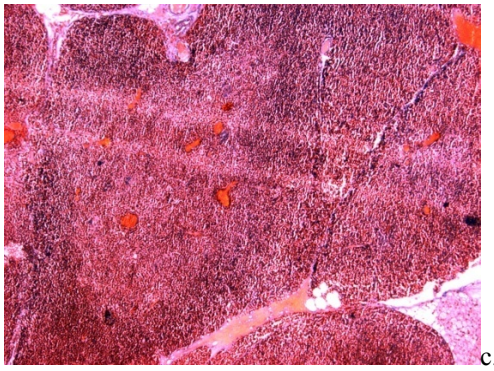
c.

Figure 7. Histological aspects of thymus (10x): a) normal structure, b) relatively normal aspect of thymus in ADR group, c) moderately alterated in the FC group, slightly rarefied cortical zone of the thymus lobules.

Acute stress-induced thymic atrophy is a complication of many environmental stressors as well in which transient reduction in thymus function persists until the physiological stressor is removed (Gruver and Sempowsky, 2008).

Conclusions

1. In summary, we can conclude that glucocorticoids can lead to tissue injury even at relatively low doses.
2. Milder organ atrophies were observed in the thymus and pancreas of the ADR treated group.
3. Severe adrenal hypertrophies were observed in the ADR treated group.
4. More severe atrophies were observed in the thymus and adrenals of the FC-treated group.
5. The response to elevated glucocorticoid levels was tissue dependent, thymus and adrenals being the most susceptible to injury.

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REFERENCES

Anacker, C., Cattaneo, A., Musaelyn, K., Zunszain, A.P., Horowitz, M., Motteni, R., Luoni, A., Calabrese, F., Tausey, K., Gemarelli, M., Thuret, S., Price, J., Uher, R., Riva, M. A., Parinate, M.C. (2013) Role for the kinase SGK1 in stress, depression, and glucocorticoid effects on hippocampal neurogenesis, *Proc. Natl. Acad. Sci*., **110 (21),** 8708–8713.

Andrews, R.C., Walker, B.R. (1999) Glucocorticoids and insulin resistance: old hormones, new targets. *Clin. Sci*., **96**, 513– 523.

Cole R.L, Sawchenko P.E. (2002) Neurotransmitter regulation of cellular activation and neuropeptide gene expession in paraventricular nucleus of the hypothalamus, *J. Neurosci*., **22(3):**959-969.

Crăciun, C., Madar, I., Tarba, C., Frăţilă, S., Ardelean, A., Crăciun, V., Ilyes, I. (1998) Correlation betwen ultrastructural thymus modification, thymolisis, thymus and blood-serum lipids in response to epicutaneously applied dermacorticosteroids in pubertal rats, in *Cur. Probl. Tech. Cel. Mol. Biol.,* Crăciun, C., Ardelean, A., (eds.), Risoprint, Cluj-Napoca, 218-235.

Crăciun, C., Ardelean, A., Madar, I., Tarba, C., Şildan, N., Crăciun, V., Fărcaş,T. (1997) Ultrastructural studies of the secondary effets induced at the level of thymus by topic application of Fluocinolon-acetonid-N in prepubertal rats, in *Cur. Probl. Tech. Cel. Mol. Biol.,* Crăciun, C., Ardelean, A., (eds.), Mirton, Timişoara, 176-186.

Gruver, L.A., Sempowsky G.D. (2008) Cytokines, leptin, and stress-induced thymic atrophy, *J. Leukoc. Biol*., **84,** 915–923.

Gruver-Yates, L.A., Cidlowski, J.A. (2013) Tissue-specific actions of glucocorticoids on apoptosis: A double-edged sword, *Cells*, **2**, 202-223.

Harris, R.B.S., Tiffany D.M., Simpson, J., Redmann, S.M., Youngblood, B.D., Ryan, D.H. (2002) Weight loss in rats exposed to repeated acute restraint stress is independent of energy or leptin status. *Am. J. Physiol. Regulatory Integrative Comp. Physiol.*, **282**, R77–R88.

Henriksen, J.E., Alford, F., Ward, G.M., Beck-Nielsen, H. (1997) Risk and mechanism of dexamethasone-induced deterioration of glucose tolerance in non-diabetic first-degree relatives of NIDDM patients, *Diabetologia,* **40,** 1439–1448.

Kis, E. (2010) Fluocinolon therapy induced oxidative stress effects on pregnant and neonatal rat thymus, In *Annals of RSCB*, Ardelean, A., Crăciun, C. (eds.) Ed. Risoprint **15 (2),** 130-134.

Kis, E. (2012a) Fluocinolon therapy induced oxidative stress on pregnant rat adrenal, pancreas and liver, In *Annals of RSCB*, Ardelean, A., Crăciun, C. (eds.) Ed. Risoprint, **17 (1),** 149-153.

Kis, E. (2012b) Glucocorticoid excess and fetal development in white Wistar rats. In *Annals of RSCB*, Ardelean, A., Crăciun, C. (eds.) Ed. Risoprint, **17 (2),** 82-85.

Kis, E., Crăciun, C. (2003a) Atenuarea modificărilor structurale şi metabolice induse de excesul glucocorticoidic prin administrarea de propranolol, *Studia UBB, Biologia*, **48 (1),** 67-79.

Kis, E., Crăciun, C. (2003b) Atenuarea modificărilor gravimetrice induse de tratamentul cu Fluocinolon- acetonid N prin administrarea de propranolol, *Studia UBB, Biologia,,* **48 (2),** 83-88.

Kis, E., Crăciun C. (2006) *Efecte secundare ale unor glucocoticoizi topici*, Ed. Risoprint, Cluj-Napoca.

Kis, E., Crăciun, C., Paşca, C., Sandu, V.D., Crăciun, V., Madar, I. (2001a) Comparative studies of the ulrastructure of somatotrope, gonadotrope and corticotrope cells in mature rats treated with topical dermocorticoids, *Studia UBB, Biologia*, **56(1),** 99-110.

Kis, E., Sandu, V.D., Paşca, C., Crăciun, C. (2001b) Efectul antiglucocorticoid al propranololului, *Studia UBB, Biologia*, **56 (2),** 83-93.

Landfield, P.W., Eldridge I.C. (1994) The glucocorticoid hyphotesis of age related hippocampal neurodegeneration: role of dysregulated neuronal calcium, *Ann. NY Acad. Sci.*, **746,** 308-321.

Leibowitz, S.F., Diaz, S., Tempel, D. (1998) Norepinephrine in the paraventricular nucleus stimulates corticosterone release, *Brain Res.,* **496,** 219-227.

Madar, I., Şildan, N., Frecuş, G. (1993) Studiul comparativ al efectului stresului şi tratamentului cu fluocinolon-acetonid asupra unor parametri endocrino-metabolici la şobolani Wistar tineri, *St. şi cerc. biol., Seria Biol. Anim*., **t. 45, 1,** 53-59.

Mureşan, E., Gaboreanu, M., Bogdan, A.T., Baba, A.I. (1974) *Tehnici de histologie normală şi patologică*, Ed. Ceres, Bucureşti.

Noguchi K.K. (2014) Glucocorticoid induced cerebellar toxicity in the developing neonate: implications for glucocorticoid therapy during bronchopulmonary dysplasia, *Cells,* **3,** 36-52.

O'Connor, T.M., O'Halloran, D.J., Shanan, F. (2000) The stress response and the hypothalamic-pituitary adrenal axis: from molecule to melancholia, *Q.J.Med.,* **93,** 323-333.

Rafacho A., Marroquí L., Taboga S.R., Abrantes J.L., Silveira L.R., Boschero A.C., Carneiro E.M., Bosqueiro J.R., Nadal A., Quesada I. (2010) Glucocorticoids in vivo induce both insulin hypersecretion and enhanced glucose sensitivity of stimulus-secretion coupling in isolated rat islets, *Endocrinol.*, **151,** 85 – 95.

Rafacho, A., Cestari T.M., Taboga, S.R., Boschero A.C., Bosqueiro J.R. (2009) High doses of dexamethasone induce increased beta-cell proliferation in pancreatic rat islets, *Am. J .Physiol. Endocrinol. Metab*, **296 ,** E681 – E689.

Rafacho, A., Abrantes J.L.F., Ribeiro, D.L., Paula, F.M., Pinto, M.E., Boschero, A.C., Bosqueiro, J.R. (2011) Morphofunctional alterations in endocrine pancreas of short- and long-term dexamethasone-treated Rats, *Horm. Metab. Res.,* **43,** 275 – 281.

Sapolsky R.M., Romero L.M., Munck A.U. (2000) How do glucocorticoids influence stress responses? Integraing, permissive, supressive, stimulatory and preparative actions, *Endocrine Reviews,* **21(1),** 55-89.

Viegas, L.R., Hoijmana, E., Beatod, M., Pecci, A. (2008) Mechanisms involved in tissue-specific apopotosis regulated by glucocorticoids, *J. Steroid Biochem. & Mol. Biol*., **109,** 273–278.

Wajngot, A., Giacca, A., Grill, V., Vranic, M., Efendic, S. (1992) The diabetogenic effects of glucocorticoids are more pronounced in low- than in high-insulin responders, *Proc. Natl. Acad. Sci. USA.,* **89,** 6035–6039.

Wang, S.D., Huang, K.J., Lin, Y.S., Lei, H.Y. (1994) Sepsis-induced apoptosis of the thymocytes in mice, *J. Immunol.,* **152,** 5014 –5021.

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CHRONIC ADMINISTRATION OF RED BULL AFFECTS BLOOD PARAMETERS IN RATS

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SUMMARY. Energy drinks are commercial drinks conceived in order to improve physical and intellectual performance. Short term effects of energy drinks are known and do not seem to affect the state of health, but long term effects are less known and raise serious health questions. In this work we investigated if Red Bull affects morphologic and some of the biochemical parameters of the blood.

Twenty adult Wistar rats, weighing 186.6±3.15 g were organized into two groups. The Control (C) group received a standard diet and tap water. The animals in the energy drink (ED) group were orally administrated 1.5 ml/100 g b.wt. of Red Bull daily, for 4 weeks. After 4 weeks of treatment, the rats were killed by exsanguination and blood samples were collected for morphologic and biochemical parameter analysis. A significant reduction in red blood cell count was noticed in the ED group, while the hemoglobin concentration and the hematocrit slightly increased in the experimental group, as compared to the control. The concentrations of glucose and total cholesterol increased significantly after Red Bull administration, as did the activities of serum LDH, AST and ALT. These results indicate that a long-term consumption of energy drinks can affect certain morphological and biochemical blood parameters. Furthermore, these drinks may be risk factors for the development of a metabolic disease.

Keywords: blood, caffeine, energy drinks, Red Bull

Introduction

Red Bull is a commercial energy drink conceived in order to improve physical and intellectual performance. This beverage was launched in 1987, but in 1992 it was banned in France and other countries, in Europe and South America, due to its negative effects. In 2008, the commercialization of Red Bull restarted in France, as a result of growing pressure from the European Union (Reissing *et al.,* 2009). The stimulating properties of Red Bull stem from the combined action of its

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ingredients: taurine, caffeine, carbohydrates (glucose and sucrose), B12 vitamin, glucuronolactone etc. Each ingredient, taken separately, may have protective or therapeutic effects, if taken in appropriate dose.

Caffeine, the main ingredient of the energy drink, is an ergogenic compound (Zang *et al*., 2014). It improves physical activity (Davis and Green, 2009; Lara *et al.,* 2014) and stimulates diuresis and lipolysis, reduces intrahepatic lipid content and stimulates fatty acid β-oxidation in liver parenchimal cells (Sinha *et al*., 2014). It also stimulates the release of calcium from the sarcoplasmic reticulum, which is indispensable for muscle contraction. Furthermore, caffeine is a nonselective competitive inhibitor of phosphodiesterase enzymes (Echeverri *et al.,* 2010) and it could be an effective therapeutic agent against Alzheimers disease (Arendash and Cao, 2010).

Taurine is involved in the regulatory processes of a broad spectrum of biological functions. Electrophysiological studies conducted on mouse brain have shown that taurine induces long-term synaptic potentiation, which is believed to underline learning and memorizing (Ito *et al*., 2010). It is known that taurine has a cholesterol-lowering effect and decreases fat deposits. These beneficial effects are due to acceleration of cholesterol conversion into bile acids (Yamori *et al.,* 2010). It has also been demonstrated that taurine acts as an antioxidant and anti-inflammatory agent, in addition to its vasodilatory properties. In endothelial cells, taurine inhibits apoptosis, inflammation and oxidative stress (Maia *et al*., 2014). However, the effects of chronic administration of taurine in food supplements are controversial. It has been shown that taurine either improves the capacity of learning and memory (Lu *et al*., 2014), or it has no effect on these processes (Bichler *et al*., 2006).

B vitamins have benefic effects on the nervous system. They are involved in the regulation of the cognitive processes and affective state (Duthie *et al*., 2002; Mattson and Shea, 2003). Cobalamin (vitamin B12) is indispensable for DNA synthesis (Gueant *et al.,* 2013; Zhao *et al*., 2014) and erythrocytes formation (Koury *et al*., 2004; Zhao *et al*., 2014). Vitamin B12 is a cofactor of methionine synthase in the synthesis of methionine, which is the precursor of the universal methyl donor S-adenosylmethionine (Gueant *et al*., 2013). It plays a crucial role in maintaining neurological function (Zhao *et al.,* 2014). It has been proven that niacin (vitamin B3) decreases total serum cholesterol and oxidative stress (Hamound *et al*., 2013). Niacin has been used in the treatment of dyslipidemia and cardiovascular disease for more than 50 years (Digby *et al.,* 2012). It decreases the levels of all atherogenic lipoproteins and increases the level of protective HDL.

Short term effects of the energy drinks are relatively well known and do not seem to affect the state of health, but the effects on the long term are less known and raise serious questions. There are several studies that reported moderate improvements in physical endurance and mental performance, including choice reaction time, concentration and memory after acute ingestion of energy drinks (Alford *et al*., 2001; Seidl *et al.,* 2000; Scholey and Kennedy, 2004).

The long term effects of energy drinks consumption are controversial. Only a few studies have shown that chronic ingestion of energy drinks are beneficial to one's health (Schrader *et al*., 2013). Other studies mentioned that chronic administration of energy drinks did not influence physical activity (Forbes *et al.,* 2008; Candow *et al*., 2009). Most investigations have reported adverse health effects of energy drinks due, most probably, to caffeine (Malinauskas *et al*., 2007; Kurtz *et al*., 2014). Central nervous system, cardiovascular, gastrointestinal and renal dysfunctions have been associated with chronic caffeine ingestion (Bichler *et al*., 2006; Maulinaukas *et al*., 2007; Clauson *et al*., 2008). Excessive caffeine consumption elevates blood levels of cholesterol and homocysteine (Dworzanski *et al*., 2011).

Animal studies have shown that chronic oral administration of energy drinks is associated with changes in blood chemistry and liver enzymes activities. For example, the concentrations of total cholesterol, triglycerides, high density lipoproteins (HDL), low density lipoproteins (LDL) and glucose increased after energy drinks consumption. The activities of transaminases increased in both rats and rabbits (Akande *et al*., 2011; Ebuehi *et al.,* 2011; Khayyat *et al*., 2014).

Long term studies should aim to better define maximum safe doses, the effects of chronic use and effects in at-risk population, and better document and track of adverse health effects (Seifert *et al*., 2011). Energy drinks may exacerbate risk factors for heart disease, since studies suggest that energy drinks may serve as a gateway to other forms of drug dependence (Higgins *et al*., 2010).

Knowledge of energy drink consumption effects on health is very important, especially given its prevalence among young people. Therefore, the aim of our study was to investigate if chronic administration of energy drinks affect morphologic and certain biochemical parameters of the blood.

Materials and methods

Chemicals. All reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich Chemie GmbH, Germany, Nordic Invest S.R.L., Romania and S.C. BioZyme S.R.L, Romania. The Red Bull energy drink was bought from the local market.

Animals. The animals were twenty albino male Wistar rats, weighing 186.6±3.15 g, housed in the zoobase of the Department of Molecular Biology and Biotechnology, School of Biology and Geology, Babes-Bolyai University. They were kept in hygienic conditions, under 12/12 h light/dark cycle, received a standard diet (S.C. Siamond Prod. S.R.L., Cluj Napoca, Romania) and had *ad libitum* access to tap water. Rats were organized into two experimental groups. The control (C) group received a standard diet and tap water. The animals from the energy drink (ED) group were orally administered 1.5 ml/100 g b.wt. of Red Bull daily, for 4 weeks. The animals were gently handled, without causing them stress or pain, and after 4 weeks, they were killed under anesthesia by exsanguination.

Assays. Blood was collected from the jugular vein and processed for hematological and biochemical examinations. Counts of red and white blood cells were carried out using a Bürker-Türk haemocytometer. The hematocrit was determined by the microhematocrit technique and the results were expressed as volume percentage (%) of red blood cells in blood. Haemoglobin concentration was measured with Drabkin reagent (Drabkin *et al*., 1935). Glycaemia was determined using Somogy-Nelson method (Somogy, 1945; Nelson, 1944). Serum cholesterol was assayed using ferric chloride (Zlatkins *et al*., 1953). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were assessed according to the method described by Reitman and Frankel (1957), and lactate dehydrogenase (LDH) activity according to Bergmeyer and Bernt (1974).

Data analysis. The results are presented as mean ± standard error (SE). The data were analyzed for statistical significance using unpaired Students *t* test. A value of $p < 0.05$ was considered significant.

Results and discussion

The aim of our study was to investigate if energy drinks affects morphologic and certain biochemical parameters of blood in rats. Blood serves as a transport medium, carrying essential nutrients to cells and removing metabolic waste products. The biochemistry of the blood plays a key role in maintaining health.

As seen in Fig. 1a, the red blood cell count decreased significantly after energy drink administration (ED 7.45±0.39 *vs* control 9.09±0.27; $p<0.01$). Perhaps energy drinks, due to a high content of carbohydrates increased osmotic pressure (Gottlieb *et al*., 2006). On the other hand, Khayyat *et al.* (2014) mentioned that depletion of red blood cells count could be attributed to disturbed haematopoiesis, destruction of erythrocytes, reduction in the rate of their formation and/or their enhanced removal from circulation. Destruction of red blood cells reflects a failure of hepatocellular functions that could be caused by caffeinated energy drink consumption (Akande *et al*., 2011; Khayyat *et al*., 2014).

Interestingly, the hematocrit (ED 45.98±0.46 *vs* control 44.96±0.49) (Fig. 1b) and haemoglobin concentration (ED 20.76±0.63 *vs* control 18.83±0.65) (Fig. 1c) increased after Red Bull intake. These changes may be a result of dehydration caused by caffeine (Pennay *et al.,* 2011). Treatment with Red Bull led to a significant decrease in the total white blood cell count (ED 8.85±0.22 *vs* control 9.90±0.27; $p<0.05$) (Fig. 1d). This modification may also suggest the existence of a moderate dehydration (Nakyinsige *et al*., 2013).

Red Bull increased significantly the plasma glucose concentration (ED 133.81±6.53 *vs* control 115.86±3.22, $p<0.05$) (Fig. 2a). The same effect was seen by Ebuehi *et al*. in rabbits (2011). The increment might result from the fact that the

glucose content of Red Bull is high. Regarding that, Red Bull chronic consumption could have adverse effects, because it is showed that consumption of sweetened beverages is associated with health risk as metabolic syndrome and type 2 diabetes (Malik *et al*., 2010).

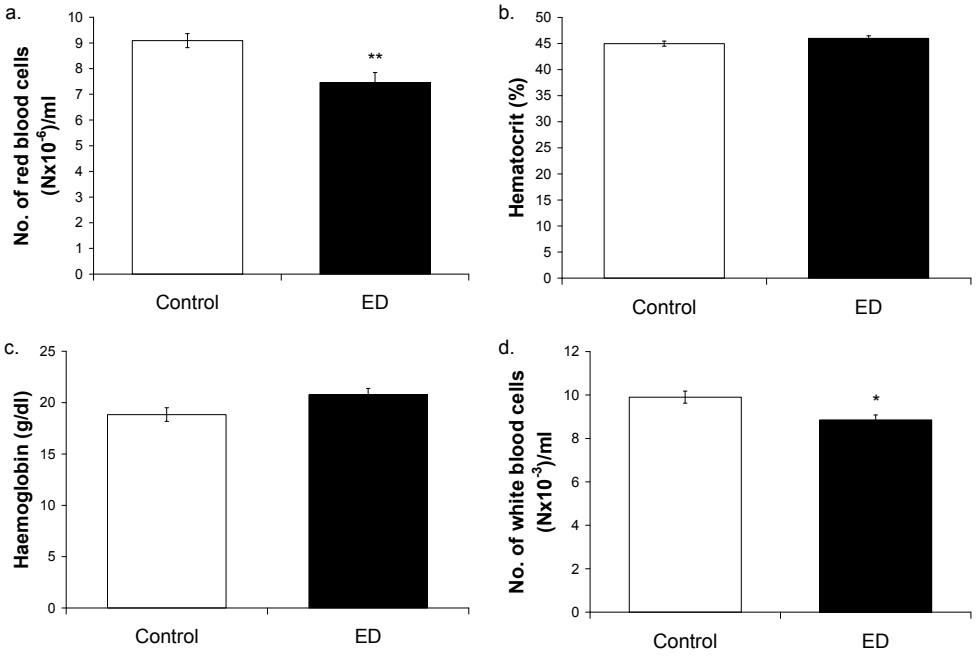

Figure. 1. Erythrocyte number ($p<0.01$) (a), hematocrit (b), haemoglobin concentration (c) and white blood cell count ($p<0.05$) (d) in the two experimental groups. n=10 in each group. The results are expressed as mean ± SE.

Energy drinks may, in fact, be a risk factor for metabolic syndrome development due to their high sugar content. In this study, we have observed that the experimental groups had a similar evolution concerning body weight: all animals gained continuous weight (Fig. 2b), but the difference between the beginning and the end of experiment was higher in ED group (ED from 143.82 to 176.35 *vs* control from 155.8 to 177.43). This finding is confirmed by Ugwuja (2014), who also attributed it to the high sugar content.

As depicted in Fig. 2c, serum total cholesterol increased slightly after the energy drink administration (ED 155.95±5.33 *vs* control 150.06±6.13). This result seems surprising since Red Bull contains taurine and niacin, two ingredients that are

commonly used for lowering lipids concentration (Yamori *et al*., 2010; Hamound *et al*., 2013). However, Red Bull contains caffeine which elevates the blood level of cholesterol and homocysteine (Dworzanski *et al*., 2011).

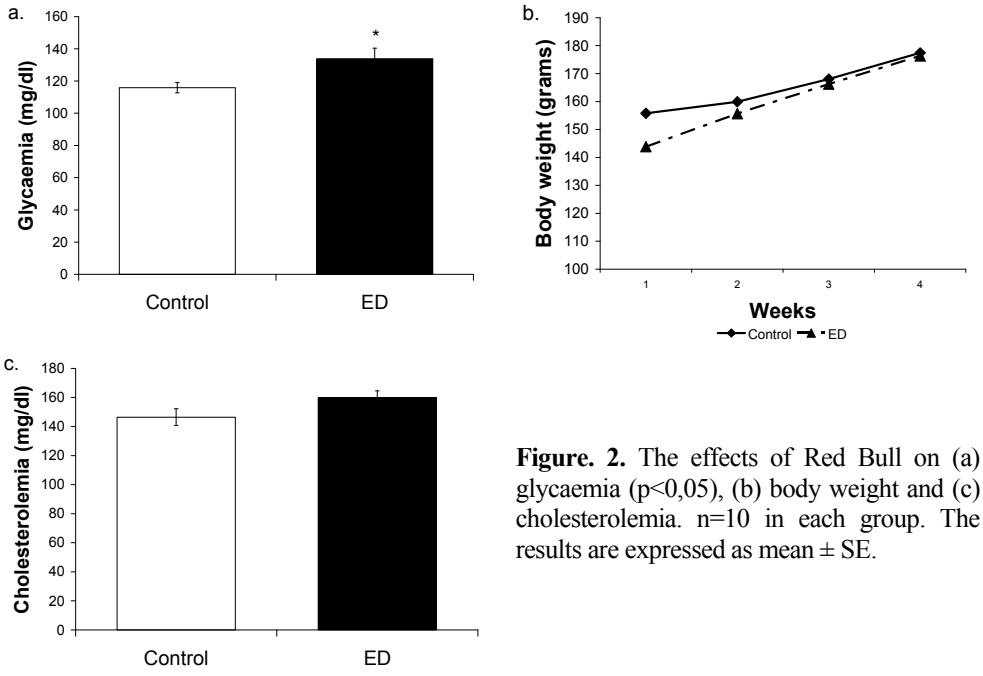

Figure. 2. The effects of Red Bull on (a) glycaemia ($p<0,05$), (b) body weight and (c) cholesterolemia. n=10 in each group. The results are expressed as mean ± SE.

The liver enzyme markers (LDH, ALT and AST) activities are shown in Fig. 3a, b and c. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST), together with lactate dehydrogenase (LDH) are common markers of plasma membrane integrity. Their concentration in the plasma is normally low, as they are considered "liver enzymes", although they can be also found in lower concentrations in other tissues. A rise of the serum transaminases is usually considered a sign of altered plasma membrane structure, causing the "leaking "of enzymes outside the cells.

LDH (ED 0.3153±0.0164 *vs* control 0.2433±0.0229; $p<0,05$), ALT (ED 130.01±12.44 *vs* control 87.71±5.57) and AST (ED 281.48±16.46 *vs* control 183.16±10.19; $p<0.001$) activities were elevated in the sera of the rats after Red Bull consumption. This is in agreement with the results of Ankade and Banjoko (2011), Ebuehi *et al*. (2011) and Khayyat *et al*. (2014). Moreover, Bukhar *et al*. (2012) mentioned that there is a relation between energy drink administration and the concentration of liver enzymes in normal and hyperglycemic rats.

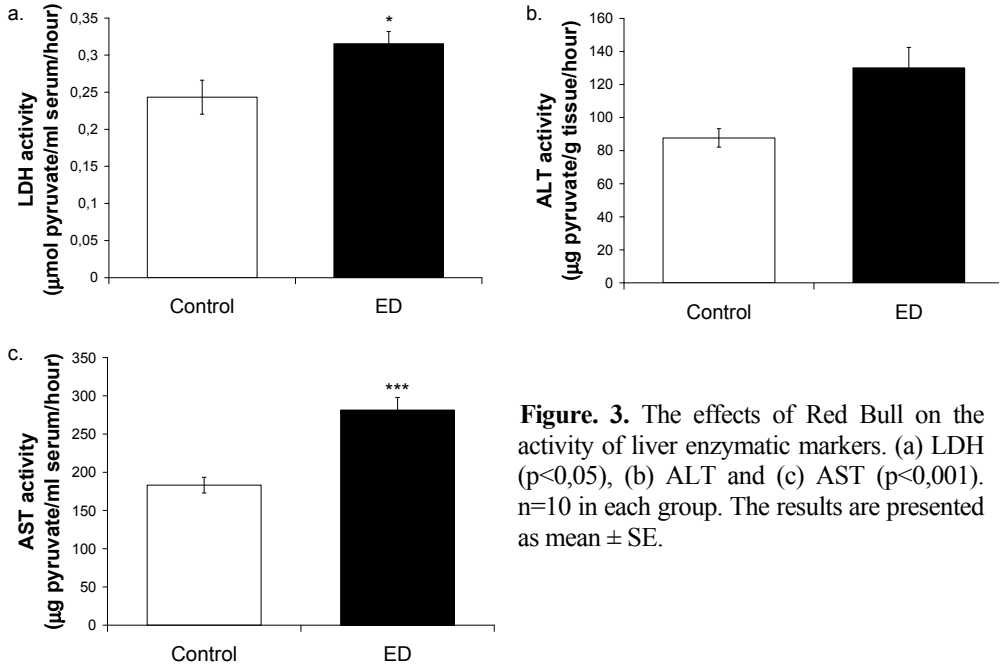

Figure. 3. The effects of Red Bull on the activity of liver enzymatic markers. (a) LDH ($p<0,05$), (b) ALT and (c) AST ($p<0,001$). n=10 in each group. The results are presented as mean ± SE.

Conclusions

The results of the present study indicate that energy drinks such as Red Bull can affect certain morphological and biochemical blood parameters. Furthermore, these drinks may be a risk factor for the development of metabolic diseases. Further research is needed and a raise of awareness in young people and athletes about the potential long term effects of energy drinks consumption.

REFERENCES

Alford, C., Cox, H., Wescott, R. (2001) The effects of Red Bull energy drink on human performance and mood, *Amino Acid* **2**, 139-150

Akande, I.S., Banjoko, O.A. (2011) Assessment of Biochemical effect of "Power Horse" energy drink on hepatic, renal and histological functions in Sprague Dawley rats, *Annu. Rev. & Res. Biol*. **1**, 45-56

Arendash, G.W., Cao, C. (2010) Caffeine and coffee as therapeutics against Alzheimer disease, *J. Alzheimer Dis*. **1**, 117-26

Bergmeyer, H.U., Brent, E. (1974) UV-assay with pyruvate and NADH. In: *Bergmeyer, H.U., ed. Methods of enzymatic analysis.* New York: Academic Press, pp 574 –579

Bichler, A., Swenson, A., Harris, M.A. (2006) A combination of caffeine and taurine has no effect on short term memory but induces changes in heart rate and mean arterial blood pressure, *Amino Acids* **4**, 471-6

Bukhar, M.M., El-Sawy, N.A., Header, A.E. (2012) Biological effect of high energy drink on normal and hyperglycemic rats, *Pak J. Nutr*. **11**, 301-309

Candow, D.G., Kleisinger, A.K., Grenier, S., Dorsch, K.D. (2009) Effect of sugar-free Red Bull energy drink on high-intensity run time-to-exhaution in young adults, *Cond Res*. **4**, 1271-5

Clauson, K.A., Shields, K.M., McQueen, C.E., Persad, N. (2008) Safety issues associated with commercially available energy drink, *J Am Pharm Assoc*. **3,** 55-63

Davis, J.K., Green, J.M. (2009) Caffeine and anaerobic performance: ergogenic value and mechanisms of action, *Sports Med.* **10**, 813-32

Digby, J.E., Ruparelia, N., Choughury, R.P. (2012) Niacin in cardiovascular disease: recent preclinical and clinical developments, *Arterioscler Thromb Vasc Biol*. **3**, 582-8

Drabkin, D.L., Austin, J.H. (1935) Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide, hemoglobin and sulfhemoglobin. *J. Biol. Chem*. **112**, 51

Duthie, S.J., Whalley, L.G., Collins, A.R., Leaper, S., Berger, K., Deary, I.J. (2002) Homocysteine, B vitamin status and cognitive function in the eldery, *Am J. Clin Nutr*. **2**, 523

Dworzanski, W., Burdan, F., Szumilo, M., Jaskolska, A., Anielska, E. (2011) Coffee and caffeine-enemies or alliantes of a cardiologist, *Kardio Pol*. **2**, 173-6

Ebuehi, O.A., Ajayl, O.E., Onyeulor, A.L., Awelimolor, D. (2011) Effects of oral administration of energy drinks on blood chemistry, tissue histology and brain acetylcholine in rabbits, *Nig Q J Hosp Med*. **1**, 29-34

Escheverri, D., Montes, F.R., Carbrera, M., Galan, A., Prieto, A. (2010) Caffeines vascular mechanisms of action, *Int J Vasc Med*, 834060

Forbes, S.C., Candow, D.G., Little, J.P., Magnus, C., Chilibeck, P.D. (2008) Effect of Red Bull energy drink on repeated Wingate cycle performance and bench-press muscle ndurance, *Int J Sport Nutr Exerc Metab.* **5**, 542

Gottlieb, H.B., Ji, L.L., Jones, H., Penny, M.L., Fleming, T., Cunningham, J.T. (2006) Differential effects of water and saline intake on water deprivation-induced c-Fos staining in the rat, *Comp Biochem Physiol A Mol Integr Physiol.* **5**, 1251-61

Gueant, J.L., Caillerez-Fofou, M., Battaglia-Hsu, S., Alberto, J.M., Freud, J.N., Dulluc, I., Adjalla, C., Maury, F., Merle, C., Nicolas, J.P., Namour, F., Daval, J.L. (2013) Molecular and cellular effects of vitamin B12 in brain, myocardium and liver through its role as co-factor of methionine synthase, *Biochimie* **5**, 1033-40

Hamoud, S., Kaplan, M., Meilin, E., Hassan, A., Torqovicky, R., Cohen, R., Hayek, T. (2013) Niacin administration significantly reduces oxidative stress in patient with hypercholesterolemia and low levels of high-density lipoproteine cholesterol, *Am J Med Sci.* **3,** 159-9

Higgins, J.P., Tuttle, T.D., Higgins, C.L. (2010) Energy beverages content and safety, *Mayo Clin Proc*. **8,** 1033-1041

Ito, K., Matevz, A., Kawasuki, T., Kuwhara, M., Tsubone, H. (2009) The effect of subacute supplementation of taurine on spatial learning and memory *Exp animal* **2**, 175-80

Khayyat, I.L., Essawy, E.A., Al Rawy, M.M., Sorour, J.M. (2014) Comparative study on the effect of energy drinks on haematopoetic system in Wistar albino rats, *J. Environ. Biol.* **5**, 883-91

Koury, M.J., Ponka, P. (2004) New insights into erythropoesis: the roles of folate, vitamin B12, and iron, *Annu Rev Nutr.* **24**, 105

Kurtz, A.M., Leong, J., Anand, M., Darqush, A.E., Shas, S.A. (2013) Effects of caffeinated versus decaffeinated energy shots on blood pressure and heart rate in healthy young volunteers, *Pharmacoltherapy* **8**, 779-86

Lara, B., Gonzalez-Millan, C., Salinero, J.J., Abian-Vicen, I., Areces, F., Barbero-Alvarez, J.C., Munoz, V., Portillo, L.J., Gonzalez-Rave, J.M., Del Coso, J. (2014) Caffeine-containing energy drink improves physical performance in female soccer players, *Amino Acids* **5**, 1385-92

Lu, C.L., Tang, S., Meng, Z.Y., He, Y.Y., Song, L.X., Liu, Y.P., Ma, N., Li, X.Y., Guo, S. C. (2014) Taurine improves the spatial learning and memory ability impaired by sub-chronic manganese exposure, *J Biomed Sci.* **1**, 51

Maia, A.R., Batista, T.M., Victorio, J.A., Clerici, S.P., Carneiro, E.M., Davel, A.P. (2014) Taurine supplementation reduce pressure and prevents endothelial dysfunction and oxidative stress in post-weaning protein-restricted rats, *PLoS. One* **8**, 9

Malik, V.S., Popkin, B.M., Bray, G.A., Depres, J.P., Willet, W.C., Hu, F.B. (2010) Sugar-sweetened beverages and risk of metabolic syndrome and type 2 diabetes: a meta-analysis, *Diabetes Care* **11**, 2477-83

Maulinauskas, B.M., Aeby, V.G., Overton, R.F., Carpenter-Aeby, T., Barber-Heidol, K., (2007) A survey of energy drink consumption patterns among college students, *Nutr J.* **6**, 35

Mattson, M.P., Shea, T.B. (2003) Folate and homocysteine metabolism in neural plasticity and neurodegenerative disorders, *Trends Neurosci*. **26**, 137-146

Nakyinsige, K., Sazli, A.Q., Aghwan, Z.A., Zulkifli, I., Goh, Y.M., Fatimah, A.B. (2013) Changes in blood constituents of rabbits subjected to transportation under hot, humid and tropical conditions, *Asian-Australas J Anim.* **6**, 874-8

Nelson, N. (1944): A photometric adaptation of the Somogy method for the determination of glucose, *J. Biol. Chem.* **153**, 375-380

Pennay, A., Lubman, D., Miller, P. (2011) Combining energy drinks and alcohol – a recipe for trouble, *Aust Fam Physician* **3**, 104-7

Reissing, C.J., Strain, E.C., Griffiths, R.R. (2009) Caffeinated energy drinks – a growing problem, *Drug Alcohol Depend.* **1**, 99

Reitman, S., Frankel, S. (1957) A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases, *Am. J. Clin. Pathol*. **1**, 56-63

Schrader, P., Panek, L.M., Templ, J.L. (2013) Acute and chronic caffeine administration incresses activity in sedentary adults, *Nutr Res.* **6**, 457-63

Scholey, A.B., Kennedy, D.O., (2004) Cognitive and physiological effects of an energy drink: an evaluation of the whole drink and of glucose, caffeine and herbal flavouring fractions, *Psychopharmacology* **3-4**, 320-30

Seidl, R., Peyrl, A., Nicham, R., Hauser, E. (2000) A taurine and caffeine-containing stimulated cognitive performance and well-being, *Amino Acids* **3-4**, 635-642

Seifert, S.M., Schaechter, J.L., Hershorin, E.R., Lipshultz, S.E. (2011) Health effects of energy drinks on children, adolescents and young adults, *Pediatrics* **3**, 511-28

Sinha, R.A., Farah, B.L., Singh, B.K., Siddique, M.M., Li, Y., Wu, Y., Ilkayeva, O.R., Gooding, J., Chinq, J., Zhou, J., Martinez, L., Xie, S., Bay, B.M., Summers, S.A., Newqard, C.B., Yen, P.M. (2014) Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice, *Hepatology* **4**, 1366-80

Somogy, M. (1945) A new reagent for determination of sugars, *J. Biol. Chem.* **160**, 61-68

Ugwuja, E. (2014) Biochemical Effects of energy drinks alone or in combination with alcohol in normal Albino rats, *Adv Pharm Bull* **1**, 69-74

Yamori,Y., Taquchi, T., Hamada, A., Mori, H., Mori, M. (2010) Taurine in health and disease consistent evidence from experimental and epidemiological studies, *J Biomed Sci.* **10,** 1186-1423

Zang, Y., Coca, A., Casa, D.J., Antonio, J., Green, J.M., Bishop, P.A. (2014) Caffeine and diuresis during rest and exercises: A meta-analysis, *J. Sci. Med. Sport* **14**, 143-1

Zhao, H., Li, M., Ruberu, K., Garner, B. (2014) Impaired lysosomal cobalamin transport in Alzheimer disease, *Journal of Alzheimer Disease* **217**, 67-74

Zlatkis, A., Zak., B., Boyle, A.J. (1953) A new method for the direct determination of serum cholesterol. *J. Lab. Clin. Med.* **41,** 486-92

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIX, 2, 2014 (p. 99-104)

A HISTOPATHOLOGICAL STUDY OF CHOLECYSTECTOMY SPECIMENS SAMPLED FROM APRIL 2012 TO APRIL 2013 IN THE PATHOLOGY DEPARTMENT OF THE MUNICIPAL HOSPITAL, CLUJ-NAPOCA

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SUMMARY. Cholecystitis is the most common disease of the gallbladder, usually caused by calculi or, less frequent, by acute inflammations of the organ. In the vast majority of the cases, the treatment is surgical removal of the gallbladder. Cholecystectomy specimens are referred to the pathology department for microscopic confirmation of the disease and exclusion of a neoplastic disease. We analysed 167 specimens, seeking inflammation, the state of the epithelial lining and other markers used for cholecistectomy pathological reporting. We concluded that the majority of the analysed specimens were affected by chronic inflammation, with very few cases of acute involvement.

Keywords: cholecystitis, inflammation, pathological sampling.

Introduction

The gallbladder is a piriform bladder that is attached to the extrahepatic biliary system *via* the cystic duct and rests in a shallow depression located on the inferior surface of the posterior right lobe of the liver (Mills, 2007). The gallbladder functions are to store bile, concentrate bile and, when stimulated, to eject bile into the lumen of the duodenum. Its microscopic anatomy reflects all these functions (Vaughan, 2002). Its wall consists largely of a highly folded mucosa, with a simple columnar epithelium overlying a typical lamina propria; a muscularis with bundles of muscle fibers oriented in all directions to facilitate emptying the organ; an external adventitia where it is against the liver and a serosa where it is exposed (Mescher, 2010).

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Chronic cholecystitis is the most commonly encountered disease of the gallbladder; the overwhelming majority of cholecystectomies are performed for chronic cholecystitis. It is associated with cholelithiasis in more than 90% of the cases (Mills, 2010). Microscopically, the mucosa of a chronically inflammed gallbladder shows various degrees of mononuclear infiltration and fibrosis. The epithelium may be relatively normal or atrophic or show hyperplastic and metaplastic changes. The gallbladder may show fibrosis, muscle hypertrophy, encrusted stones, and nodular collections of foamy macrophages (Rosai, 2011). Sometimes, the mucosa epithelium forms diverticula which can reach the serosal surface, called Rokitansky-Aschoff sinuses (Levinson, 2008).

Acute cholecystitis is a clinically defined entity characterized by an abrupt injury of the organ. It does not necessarily imply acute inflammation of the gallbladder, in the sense that the hallmark of acute inflammation, polymorphonuclear leucocytes, is often lacking. Nevertheless, it is an acute destructive process typically associated with ischemia, congestion, edema, epithelial denudation, vascular leakage, and fibrinous changes (Mills, 2010).

Resection specimen sampling. Most cholecystectomy specimens are now done laparoscopically rather than by open surgery and submitted opened, or unopened and containing 5-10 ml of bile fluid. When received, the specimen is measured, opened (if not previously performed by the surgeon) and described. In a non-neoplastic specimen, three histology blocks are usually sufficient: the cystic margin, section from the corpus and section from the fundus of the gallbladder. Any other lesion is noted and sampled also (Westra, 2003).

Materials and methods

A total of 167 cholecystectomy specimens received between April 2012 and April 2013 were analyzed. The specimens were received in fresh state to the pathology department, opened and fixed in formalin 4% overnight. The organs were described according to the guidelines, and routine sampling was performed (sections from the cystic margin, the corpus and the fundus of the gallbladder). If the wall of the gallbladder was thickened, additional sections were performed in order to exclude a neoplastic condition. If present, cystic duct ganglia were also sampled.

The histology samples were embedded in paraffin blocks, sectioned at 3 micrometers thickness and stained with hematoxylin and eosin. The slides were analysed using a Leica DM500 microscope with white light and images were taken using a digital camera connected to the microscope. When appropriate, adjacent microscopic fields were photographed and stitched using *Hugin* free software, resulting in high resolution mosaic images of the slides.

The following issues were reported and included in the study:

- erosions of the lining epithelium;
- fibrosis;

- epithelial dysplasia;
- inflammatory infiltrate;
- hypertrophy/atrophy;
- presence of the foamy macrophages;
- vascular congestion;
- adipose infiltrate;
- hemorrhage;
- necrosis;
- presence of Rokitanski-Aschoff sinuses.

Statistical analysis and graphical output was performed using Microsoft Office Excel.

Results and discussion

Of all 167 cases of gallbladder specimens analysed, the vast majority was histologically diagnosed as chronic cholecystitis (91%), and only 9 % were diagnosed as acute or subacute cholecystitis. One case was regarded as scleroatrophic cholecystitis (the gallbladder wall was thickened over 5 mm and extended fibrosis was found microscopically) and one case presented epithelial dysplasia.

Considering the inflammatory infiltrate, 8% of the cases we examined presented lymphoid follicles with consistent germinal centers in the lamina propria of the mucosa and/or the serosa. Transmural inflammatory infiltrate was also a common finding.

A landmark of chronic cholecystitis was the presence of discontinuities of the cover epithelium (Fig. 1A, Fig. 3B).

One case presented transmural eosinophilic infiltrate (Fig. 2D), but not so abundant to be regarded as eosinophilic cholecystitis. In this particular case, the patient presented high eosinophilia, probably associated with the clinical diagnosis of chronic obstructive pulmonary disease (COPD). There was no evidence of intestinal parasites regarding this patient.

A common finding was the presence of nests of foamy macrophages in the lamina propria of the mucosa (53%) and pseudopyloric metaplasia (23%), the latter predominantly in the neck of the gallbladder (Fig. 1C).

As literature shows, acute cholecystitis is not compulsory associated with acute inflammatory infiltrate (neutrophils) but, more often, it is associated with extended erosions and ulcerations of the mucosa, thickened wall, transmural hemorrhage and a loss of the histological architecture of the gallbladder wall (Fig. 1B).

Rokitansky Aschoff sinuses were present in 13% of the cases, all of which were diagnosed as chronic cholecystitis.

One case was diagnosed with multiple adenomatous nodules (Fig 2C), which do not change the diagnosis and do not have any significance for the patient's outcome.

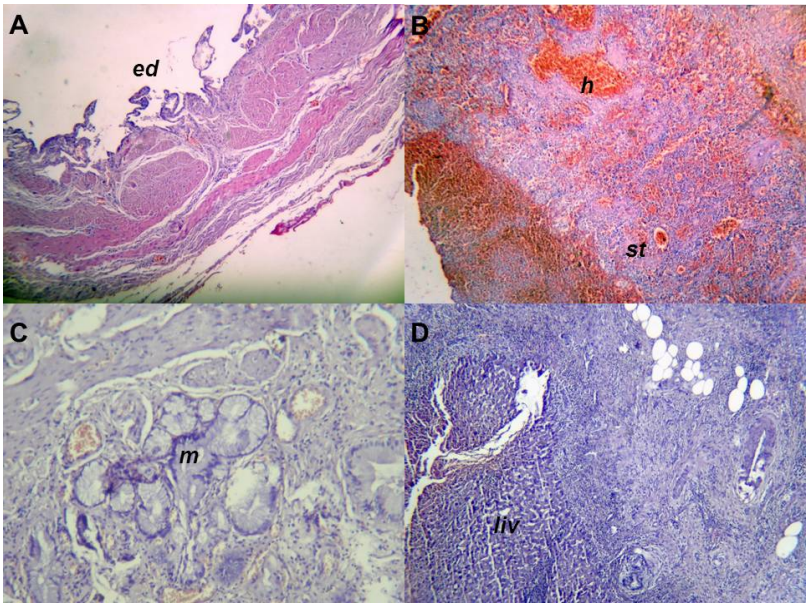

Figure 1. A – Focal epithelial discontinuities (*ed*); B – Hemorrhage (*h*) and stasis (*st*); C – Pseudopyloric metaplasia (*m*); D – Liver tissue (*liv*) attached to the gallbladder.

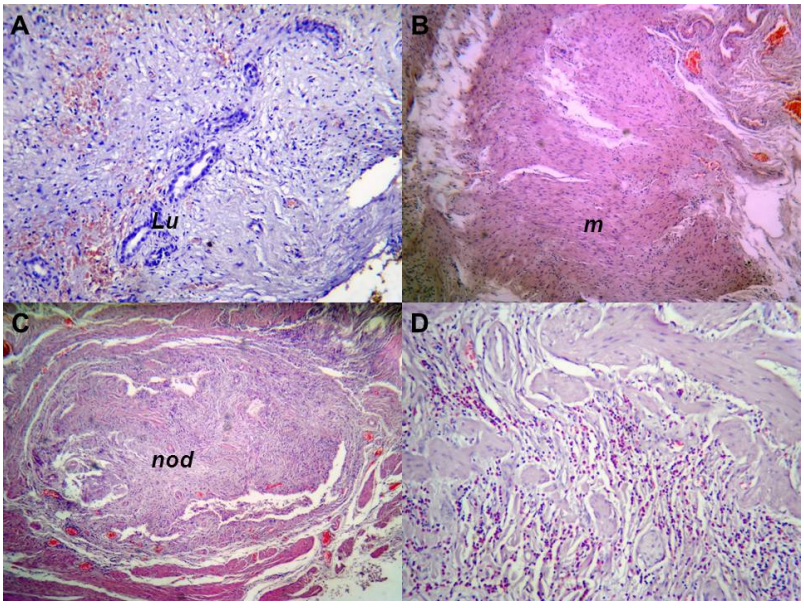

Figure 2. A – Luschka ducts (*Lu*); B – Hypertrophy of the muscular layer (*m*); C – Adenomyomatosis nodule (*nod*); D – Eosinophilic infiltrate.

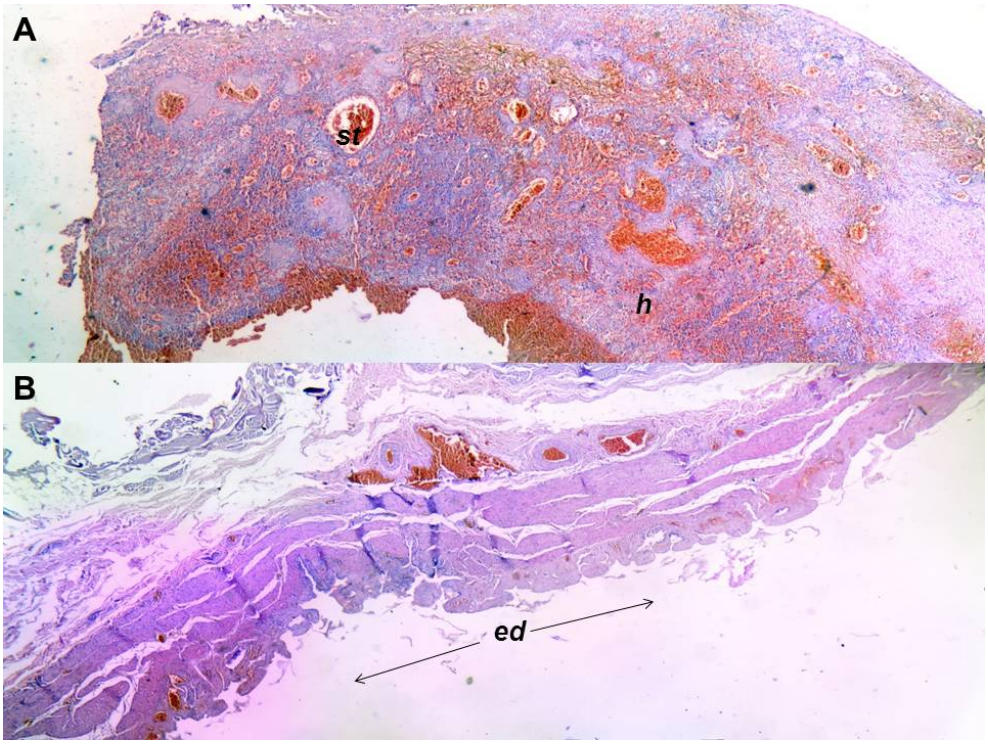

Figure 3. A – Hemorrhage (*h*) and stasis (*st*); B – Extended epithelial erosions (*ed*).

Conclusions

Cholecystitis remains a major cause of addressability in most surgical services, especially due to cholelithiasis. However, histopathlogical examination remains of undoubtful importance, mainly because of the risk of carcinoma.

REFERENCES

Levinson, D., Reid, R. (2008) *Muir's Textbook of Pathology*, Edward Arnold, London, p. 274

Mescher, A. (2010) *Junqueira's Basic Histology, Twelfth Edition*, McGraw-Hill, Printed in China, pp. 339-340

Mills, S. (2007) *Histology for Pathologists*, Lippincott Williams & Wilkins, Philadelphia, pp. 708-709

Mills, S. (2010) *Sternberg's Diagnostic Surgical Pathology, 5th Edition*, Lippincott Williams & Wilkins, Philadelphia, pp. 1603-1608

Rosai, J. (2011) *Rosai and Ackerman's Surgical Pathology*, Elsevier, Printed in China, pp. 985-986

Vaughan, D. (2002) *A Learning System in Histology*, Oxford University Press, New York, pp. 167

Westra, W. (2003) *Surgical Pathology Dissection: An Illustrated Guide, Second Edition*, Springer, New York, pp. 82-83

STUDIA UNIVERSITATIS BABEŞ – BOLYAI, BIOLOGIA, LIX, 2, 2014 (p. 105-113)

CHALLENGES IN IMPLEMENTING HEART RATE VARIABILITY TESTING IN A FAMILY MEDICINE PRACTICE: STRENGTHS, PITFALLS AND CAVEATS

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SUMMARY. Heart rate variability (HRV) measures beat-to-beat changes in the duration of the RR intervals in the electrocardiogram (ECG). According to many studies, HRV-derived methods can contribute a certain amount of data to the screening for cardiovascular diseases, and also predict the outcome for cardiovascular patients. The study focuses on a trial of HRV measuring in a family general practitioner's office, in two different groups: healthy subjects and patients with a varied collection of organic diseases. Differences were found between the two groups, especially concerning nonlinear parameters of HRV and Poincaré diagram analysis. Although promising, screening by HRV in the general practitioner's office has many pitfalls and caveats, especially regarding technical problems and the approach of patients with multiple comorbidities.

Keywords: family medicine, heart rate variability, screening.

Introduction

The term "heart rate variability" (HRV) refers to a measure of the beat-to-beat changes in duration of the RR intervals (RRIs) in the electrocardiogram (ECG). The RRIs, or interbeat interval, is the distance between one R-spike and the next in the ECG recording (Lagos, 2008). From a pathophysiological point of view, HRV analysis is a collection of various mathematical and computational techniques that characterizes biologic time-series with respect to overall fluctuation, spectral composition, scale-free variation, and degree of irregularity or complexity (Ahmad, 2009).

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The methods for analyzing and quantifying HRV parameters are categorized as: time domain, spectral or frequency domain, and nonlinear dynamics methods (Poincaré plot derived parameters), respectively:

1. In time domain analysis, the intervals between adjacent R waves are measured over a period of recordings and a variety of statistical parameters can be calculated: average RR interval, standard deviation of the RR intervals, average heart rate, standard deviation of the heart rate (Thalange, 2010);
2. The heart rate frequency domain analysis is used to evaluate the contribution of the autonomic nervous system to HRV; it is a sensitive and non-invasive method for evaluating the cardiovascular control system (Buccelletti, 2009);
3. The Poincaré plot (return map) is a scattergram, which is constructed by plotting each RR interval against the previous one. The Poincaré plot may be analyzed quantitatively by fitting an ellipse to the plotted shape (Kitlas, 2005).

Although ECG is considered the most accurate method for data acquisition used in HRV analysis (Berntson, 1997), there are studies proclaiming that periphery photoplethismography (PPG) can be used as a surrogate method for recording signals used in variability analysis (Gil, 2010; Greve, 2012; Mirescu, 2012). PPG is based on calculating the amount of infrared light that travels through peripheral capillaries (from a finger or the earlobe), thus tracing peripheral blood flow curve, which is synchronous with the heartbeats. Although more prone to moving and light artifacts, PPG is more comfortable from the patient's point of view, because it does not need the application of cutaneous electrodes, necessary in ECG recordings.

Heart rate variability has been used in different clinical settings, including diabetes (Poanta, 2010), coronary artery disease (Kleiger, 1987; Mirescu, 2012), sudden cardiac death (Dougherty, 1992) and chronic renal failure (Lerma, 2003).

Family medicine contributes to the care of patients at all levels, throughout all stages of life. Family physicians focus on each individual in his or her given situation, integrating mental and physical health, within each individual's own social context. Family medicine is not only dedicated to patients, but also to healthy individuals that need medical advice or specific documents that attest their health state. Thus, a large number of individuals visiting the family medicine office are not under observation for any disease.

A large amount of the family medicine practitioner's time should be allocated to prevention and screening. According to the World Health Organization (WHO), screening is defined as the presumptive identification of unrecognized disease or defect by the application of tests, examinations, or other procedures which can be applied rapidly (Wilson, 1968). Modern methods include screening for cervical cancer or dysplasia (cervical smear test), prostate cancer (prostate specific antigen from serum) or breast cancer (palpation and mammography).

The purpose of this study is to assess the practical value of HRV in screening for cardiovascular disease in the family medicine office using a PPG device.

Materials and methods

The study investigated 50 volunteer visitors at the family doctor's office, both healthy and with various conditions (78% females, aged 19 – 70 years old). After the patient has rested for 10 minutes, the PPG measurement was started for another 10 minutes, in a seated position. The sensor was placed on the index finger of the right hand and it consisted of an infrared LED and an infrared receiver, both placed on opposite sides of the finger. The infrared receiver was connected to a computer via an Arduino board, using an analogical input pin. The Arduino board was programmed to output the interval between beats directly though its serial port. The intervals were recorded in a Microsoft Excel sheet.

The volunteers were asked not to talk during recordings, in order to avoid the influence of forced respiration on HRV. Medical data (diagnostic and medication) were collected from the patients medical records.

Calculation of the HRV parameters was performed using Kubios HRV software. Table 1 summarizes the analyzed parameters.

After the recording, ectopic beats (due to arrhythmias, atrial and/or ventricular premature contractions) were removed.

Table 1.

HRV parameters that were analyzed in the study

Parameter	Unit	Description
Time domain parameters		
Average RR interval	ms	Average of the interval between two adjacent beats
RR standard deviation	ms	Standard deviation of the intervals between adjacent beats
Average heart rate	beats/minute	Average of heart beats
Heart rate standard deviation	beats/minute	Standard deviation of heart beats
Frequency domain parameters		
HF/LF	-	Ratio between high frequency spectral power and low frequency spectral power; it represents a measure of autonomic balance
Nonlinear parameters		
SD1	ms	Standard deviation 1 of the Poincaré plot (short axis)
SD2	ms	Standard deviation 2 of the Poincaré plot (long axis)

Results and discussion

One of the major problems we initially encountered was severe artifactuation in some of the recordings. Artifacts were mainly due to patient moving or shaking. In one instance, the recording was influenced by ambiental (sun) light, which was too intense and fell directly on the recording device. In this situation, the finger with the sensor mounted on it had to be covered. Fig. 1 depicts such an artifactuated recording, which could not be used for further interpretation.

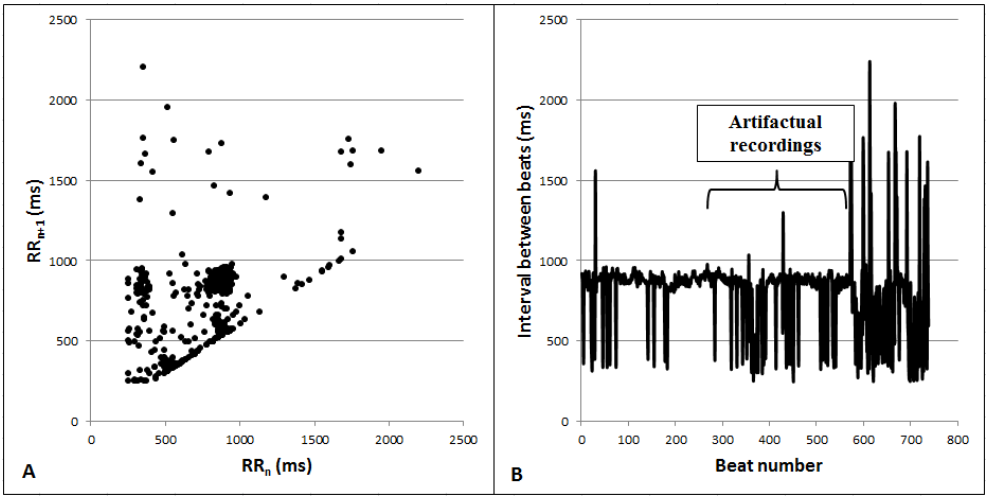

Figure 1. A strongly artifactual recording (A – the Poincaré scattergram shows very dispersed points, typical for an artifactual recording; B – a tachogram of the same subject with artifactual alterations).

One of the major pitfalls was confusing artifactual recording with a form of arrhythmia. Sometimes, the differentiation between the two requires interpretation by a cardiologist, who is familiar with the electrocardiographic aspect of arrhythmias. The method was not feasible in the patients whose hand trembled during measurement.

Mainly, tachograms containing many values that drop down to the baseline can be considered artifactual. If there are few basal values present in the trace, they can be manually removed. This operation has to be done carefully, in order not to delete any value that is correctly recorded, thus useful in variability analysis. Following this manual cleanup, the trace can be uploaded to Kubios HRV for parameter calculation.

A typical recording of a healthy patient, as reported by Kubios HRV software, is showed in Figs. 2 and 3.

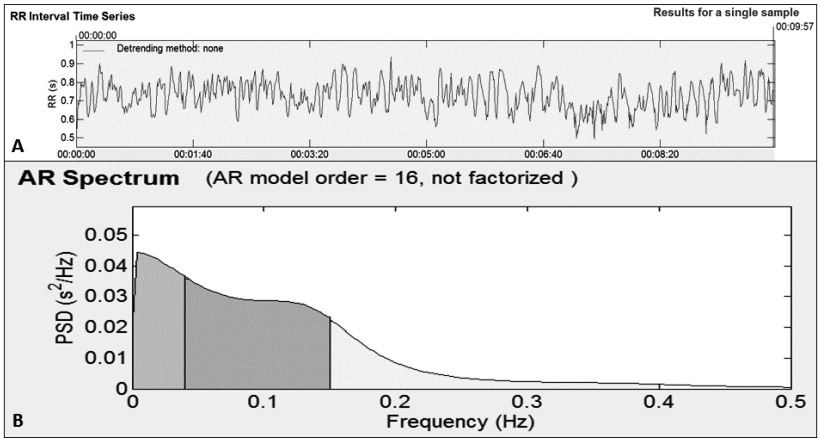

Figure 2. A. Typical tachogram of beat-to-beat intervals of a healthy subject; B. Power spectral density plot of the same subject.

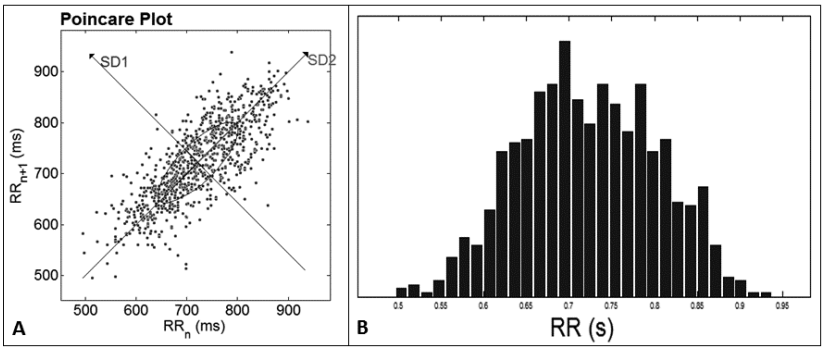

Figure 3. A. Typical Poincaré scattergram of a healthy subject; B. Histogram of RR intervals of the same individual.

Healthy vs. cardiac subject. According to the majority of the authors (Yukishita, 2010), increased HRV parameters are associated with a state of health of the individual, as compared to patients with cardiac diseases. This was also a finding of our study. Fig. 4A expresses a typical beat-to-beat interval plot (tachogram) of a healthy individual, while Fig. 4B represents the plot for a patient with a history of treated myocardial infarction (the plots use the same scale on both axes). The Poincaré scattergram also confirms this finding (Fig. 5).

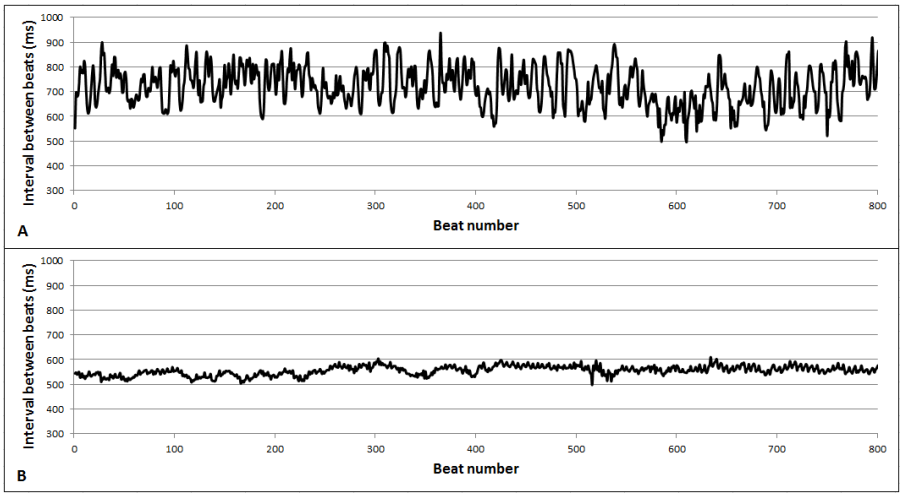

Figure 4. A. Typical Poincaré tachogram of a healthy subject; B. Tachogram of RR intervals of the same individual.

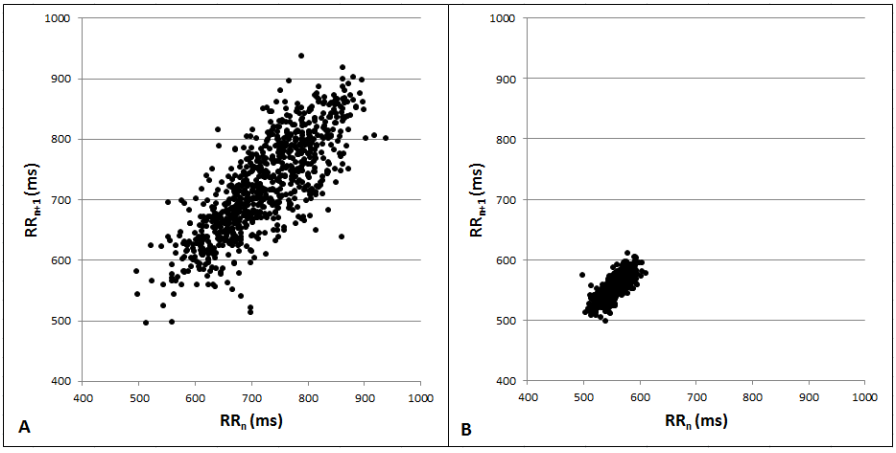

Figure 5. A Poincaré scattergram of a healthy individual (A), compared with the Poincaré plot of a patient with a history of treated acute myocardial infarction (B). Agglomeration of points can be observed in the second graph, due to decreased HRV parameters.

The mean values and standard deviation of HRV parameters of healthy subjects are summarized in Table 2.

Table 2.

Mean and stadard values of HRV parameters in healthy subjects

Parameter	**Unit**	**Mean and standard deviation**
Time domain parameters		
Average RR interval	ms	740 ± 91
RR standard deviation	ms	54 ± 27
Average heart rate	beats/minute	82 ± 5
Heart rate standard deviation	beats/minute	5 ± 2
Frequency domain parameters		
HF/LF	-	3 ± 1,6
Nonlinear parameters		
SD1	ms	25 ± 13
SD2	ms	72 ± 36

A case of a patient with advanced diabetic neuropathy worth special mentioning, because of the resulted Poincaré diagram (Fig. 6A). The classical (two-dimensional) Poincaré diagram exhibits a four-arm distribution of points, prior unseen by the authors in other cases. Because of this particular aspect, we tried a three dimensional (3D) modelling of the points, using a Microsoft Excel plugin for 3D scattergrams. The result is showed in Fig. 6B, where one can observe that the four arms of the Poincaré plot are not located in the same plane, but in three different plans, converging to a central point. Further studies will investigate whether this 3D aspect is characteristic or pathognomonic for advanced diabetic neuropathy.

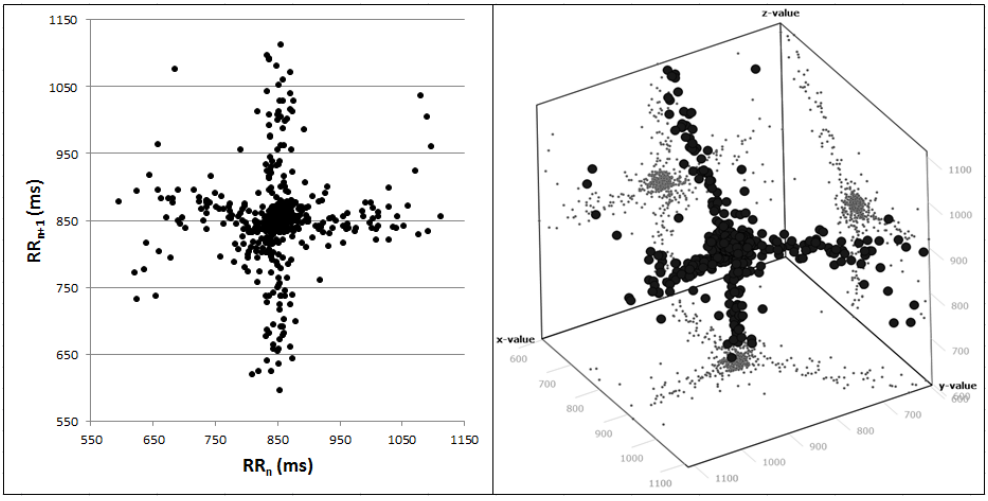

Figure 6. Two-dimensional Poincaré scattergram of a subject with advanced diabetic neuropathy (A). Three-dimensional Poincaré scattergram of the same patient (B).

Conclusions

The major outline of this pilot study is that HRV parameters are unique for each individual, and further studies will be performed to highlight patterns of connection with pathology. However, great caution must be taken in interpreting the results, because HRV parameters can be influenced by various conditions, even in healthy subjects: smoking or running prior to the measurements, anxiety or other emotions.

Further studies are needed in order to determine how HRV parameters vary in healthy subjects and how they can predict outcome or contribute to diagnostic in patients with certain pathologies. Also, in order to test the screening capacity of HRV parameter determination, double-blinded studies are needed.

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R E F E R E N C E S

Ahmad, S., Tejuja, A., Newman, K., Zarychanski, R., Seely, A. (2009) Clinical review: a review and analysis of heart rate variability and the diagnosis and prognosis of infection, *Critical Care* **13**, available online at http://ccforum.com/content/13/6/232

Berntson, G., Tommas Bigger JR, J., Eckberg, D., Grossman, P., Kaufmann, P., Malik, M., Nagaraja, H., Porges, S., Philip Saul, J., Stone, P., Van Der Molen., M. (1997) Heart rate variability: origins, methods and interpretive caveats, *Psychophysiology* **34**, 623-648

Buccelletti, F., Gilardi, E., Scaini, E., Galiuto, L., Persiani, R., Biondi, A., Basile, F., Gentiloni Silveri, N. (2009) Heart rate variability and myocardial infarction: systematic literature review and metanalysis, *Eur. Rev. Med. Pharmacol. Sci.* **13**, 299-307

Dougherty, C., Burr, R. (1992) Comparison of heart rate variability in survivors and nonsurvivors of sudden cardiac arrest, *Am. J. Cardiol.* **70**, 610-615

Gil, E., Vergara, J., Laguna, P. (2010) Pulse time transit variability versus heart rate variability during decreases in the amplitude fluctuations of photoplethysmography signal, *Int. J. of Bioelectromagn.* **12**, 95-101

Greve, M., Kviesis-Kipge, E., Rubenis, O., Rubins, U., Mecnika, V., Grabovskis, A., Marcinkevics, Z. (2012) Comparison of pulse rate variability derived from digital photopletismography over the temporal artery with the heart rate variability derived from a polar heart rate monitor, *Proceedings of the 7th ESGCO 2012*, 22-25

Kitlas, A., Oczeretko, E., Kowalewski, M., Borowska, M., Urban, M (2005) Nonlinear dynamics methods in the analysis of heart rate variability, *Rokz. Akad. Med. Bialymst* **50**, 46-47

Kleiger, E., Miller, P., Bigger, T., Moss, A. (1987) Decreased heart rate variability and its association with increased mortality after acute myocardial infarction, *Am. J. Cardiol.* **59**, 256–262

Lagos, L., Vaschillo, E., Vaschillo, B., Lehrer, P., Bates, M., Pandina, R. (2008) Heart rate variability biofeedback as a strategy for dealing with competitive anxiety: a case study, *Biofeedback* **36**, 109-115

Lerma, C., Infante, O., Perez-Grovas, H., Jose, M. (2003) Poincaré plot indexes of heart rate variability capture dynamic adaptations after haemodialysis in chronic renal failure patients, *Clin. Physiol. Funct. Imaging* **23**, 72-80

Mirescu, C., Harden S. (2012) Photopletismography as a potential alternative to electrocardiography for recording heart rate intervals used in variability analysis, *Journal of Medicine and Life* **5 (special issue)**, 123-128

Mirescu, C., Harden, S. (2012) Nonlinear dynamics methods for assessing heart rate variability in patients with recent myocardial infarction, *Rom. J. of Biophys.* **22**, 117-124

Poantă, L., Damian, I., Albu, A. (2010) Variabilitatea frecvenței cardiace și toleranța la efort la pacienții cu diabet zaharat tip II, *Palestrica Mileniului III – Civilizație și Sport* **3**, 198-201

Thalange, A., Mergu, R. (2010) HRV analysis of arrhythmias using linear – nonlinear parameters, *Int. J. Comput. Appl.* **12**, 71-77

Wilson, J., Jungner, G. (1968) Principles and practice of screening for disease, *Public Health Papers* **34**, 11-12

Yukishita, T., Lee, K., Kim, S., Yumoto, Y., Kobayashi, A., Shirasawa, T., Kobayashi, H. (2010) Age and sex-dependant alterations in heart rate variability: profiling the charasteristics of men and women in their 30s, *J. Anti Aging Med.* 7, 94-99

*** (2013) The Family Medicine Milestone Project, a joint initiative of The Accreditation Council for Graduate Medical Education and The American Board of Family Medicine

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=== REVIEW ===

RNAi AS A TOOL TO OBTAIN POTATO RESISTANT TO VIRUSES

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SUMMARY. Potato is the third most important crop in the world but is very sensitive to pathogens. Viruses are pathogens which affect potato productivity causing important losses in the annual production. Consequently, potato is one of the main targets for genetic improvement by gene transfer and other biotechnological tools. RNA interference (RNAi) has recently been identified as a natural mechanism for regulation of gene expression and also as a natural antiviral defense mechanism. RNAi is a promising approach adopted to develop virus-resistant plants. The present paper gives an update of different approaches of using RNAi to obtain virus resistant potato plants.

Keywords: hairpin RNA, RNAi, potato resistant to viruses, PVY.

Introduction

Potato (*Solanum tuberosum*) the third most important crop in the world after rice and wheat (Birch *et al.,* 2012), is very sensitive to disease and pests. Viruses are one of the main pathogens of potato and represent a serious problem, not only because of effects determinate by primary infection, but also because potato crop is vegetatively propagated and this is a good way to transmit viruses through the tubers to other generations (Bushra *et al.*, 2012). The most important economically potato viruses are represented by PVY (*Potato Virus Y*), PVX (*Potato Virus X*), PVM (*Potato Virus M*) and PLRV (*Potato Leafroll Virus*).

Potato Virus Y is transmitted by aphides and has a high genetic variability, a significant number of strains being described so far: PVY^{O}, PVY^{C}, PVY^{Z}, PVY^{N} (Tribodet *et al*., 2005). The recently identified strains, PVY^{NTN} (N-tuber-necrosis) and PVY^{N-W} (N-Wilga), induce Potato Tuber Necrotic Ringspot Disease (PTNRD)

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(Blanco-Urgoiti *et al*., 1998; Visser *et al*., 2012). *Potato Virus X* is a common limiter of potato productivity around the globe, producing a severe mosaic and dwarf plants (Shafique *et al*., 2013).

It is known that plants have both passive (cell wall) and active mechanisms of defense against viruses. The active defense mechanisms are represented by hypersensitive response (HR) and extreme resistance (ER) during which the cells located near the virus infection die (Goldbach *et al*., 2003) due to the presence of naturally produced secondary metabolites (terpenes, phenolic compounds, ethylene, salicylic acid, jasmonic acid) which are toxic (Van Etten *et al*., 2001; Mazid *et al.,* 2011). More than that, plants have resistance genes (R genes), which are recognizing the specific avirulence genes (avr) from infecting virus (Bushra *et al*., 2013). R proteins (guards) are related with host cellular proteins (guardees), which are targets for avirulence proteins of the pathogen. During the infections, the avirulece proteins produce modifications to the guardee, and alter the interaction between the guard and guardee. In this situation, the guard initiates a signaling cascade that leads to defense (Soosaar *et al.*, 2005).

In the last decades a new defense system was detected in plants, which is known as post-transcriptional gene silencing (PTGS) or RNA interference (RNAi). The term RNAi was created by Fire and Mello to describe a mechanism of gene-silencing based on dsRNA (Fire *et al.,* 1998).

RNAi is a natural defense system evolved against parasites (viruses) and mobile genetic elements or transposons. This system recognize and degrade the double-strand RNA (dsRNA) (Kryldakov *et al.*, 2011) and in plants act as a RNA virus resistance mechanism (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Joseph *et al*., 2012; Kasschau *et al*., 1998).

Mechanism of RNAi

RNAi is a defense pathway in which the target RNA molecules are degraded at mRNA level based on homology. In this way the translation of target RNA is prevented. In plants two different functionally RNAs: micro RNA (miRNA) and small interfering RNA (siRNA) were characterized. miRNAs are small dsRNA genome coded, which have a double stranded region and a loop region which is single stranded. miRNA is involved in regulation of gene expression (Bartel, 2004) and can induce RNA silencing if it is introduced in the cell as artificial miRNA (Duan *et al*., 2012). siRNA i.e. small interfering RNA molecules are obtained from long dsRNA through RNAi, being involved in defense mechanisms (Lacellier *et al*., 2004).

RNAi act in plants as a defense system which is directed against viruses (Wang *et al*., 2012). During the viral attack, long dsRNA are obtained from viral RNAs through replication. These long dsRNA are substrates for a cytosolic endonuclease termed Dicer. Dicer recognizes dsRNA and cleaves them into duplex siRNA. The

siRNA duplex contain guide strand (complementary with target mRNA) and passenger strand. The guide strand goes into RNA-induced silencing complex (RISC) and then the viral mRNA is degraded (Bushra *et al*., 2013) (Fig. 1). RISC is made up of Dicer (endonuclease enzyme), accessory protein (ARGONAUTE), RNA binding protein (PDR) and transacting RNA-binding protein (TRBP) (Schwarz *et al*., 2003).

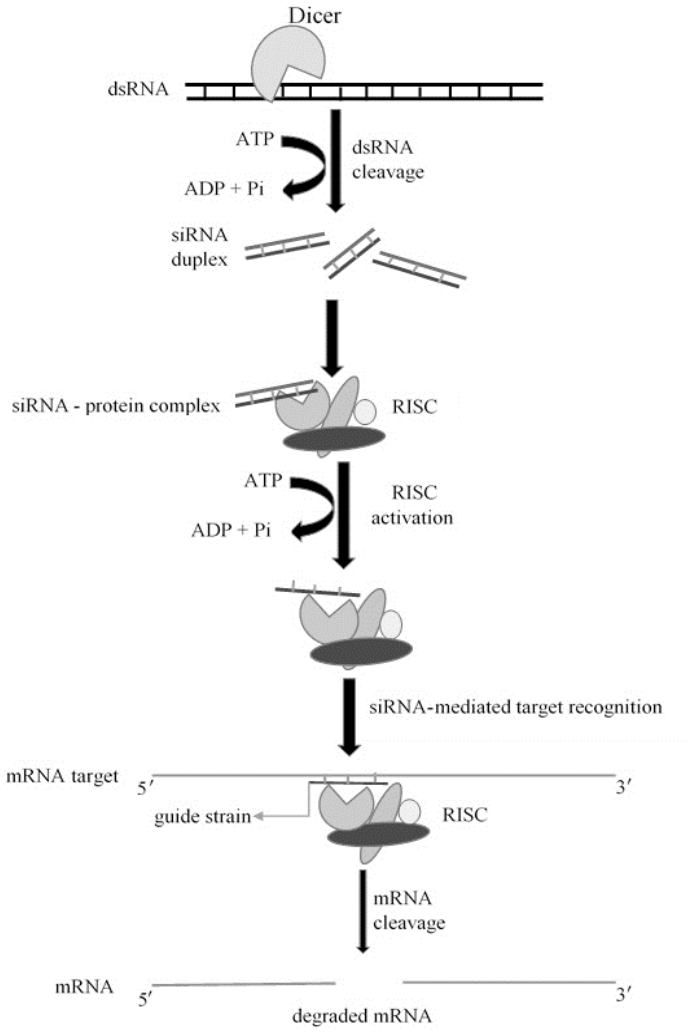

Figure 1. The mechanism of RNAi: Long double-stranded RNA is cleaved by Dicer endonuclease into siRNA duplex in an ATP-dependent way. The siRNAs are uptaken by RNA-induced silencing complex (RISC), which is activated by removing the passenger strand of the siRNA duplex. The guide strand (complementary with target mRNA) help RISC to recognize the mRNA target which is degraded.

RNAi mediated resistance in plants

Multiple approaches have been attempted to initiate the process of viral gene silencing. Initially, it was realized by separately expressing sense and antisense genes of viral origin in plants. The first attempt to obtain virus resistant potato plants through RNAi technology was made with Potato virus Y (PVY), where the complete immunity to the virus was reported after both sense and antisense transcripts of the viral helper-component proteinase (HC-Pro) gene, which was simultaneously expressed (Waterhouse *et al*., 1998).

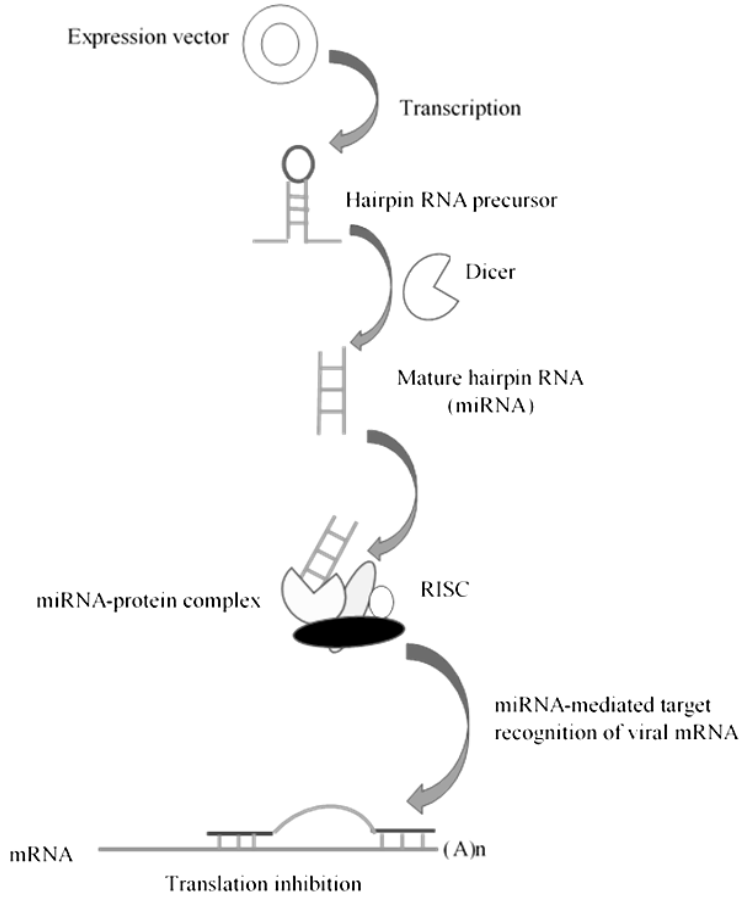

Figure 2. Inhibition of translation through the hairpin RNA mechanism: The hairpin RNA precursor, obtained through transcription of expression vector, is digested by Dicer resulting miRNA. The miRNA are up taken by RNA-induced silencing complex (RISC), would bind to the 3'untraslated region (UTR) of the target mRNA and then will block translation.

Hairpin RNA (hp RNA)

RNA silencing has been adopted to generate virus-resistant plants through expression of virus-derived hairpin RNAs (Fig. 2). The hairpin RNA construct contains inverted repeated sequences which are separated by another sequence termed spacer. The spacer region is used to stabilize the transgene construct (Surekha *et al*., 2013).

Because of the high sequence specificity of RNA silencing, this technology has been limited to the targeting of single viruses. To achieve simultaneous targeting of multiple viruses or plant genes a chimeric cassette was used. So it was developed a method to construct chimeric hairpin RNA rapidly and efficiently. This method splices two DNA fragments from viruses using Overlap Extension PCR (OE-PCR); then this chimeric sequence was assembled with an intron sequence to generate an intron-containing hairpin RNA construct (Yan *et al*., 2010).

Similar approach was developed in potato plants using a hairpin RNA construct corresponding to a conserved region of the *cp* (coat protein) gene of PVY (Missiou *et al*., 2004). Some genetic transformation methods including protein and RNA-mediated approaches (Prins, 2003) or virus-induced gene silencing (Lu *et al.,* 2003; Godge *et al.,* 2007) were also used to induce virus resistance in plants (Baulcombe 1999).

Intron hairpin RNA (ihp RNA)

The ihp RNA transgene is similar to the hairpin transgene except that the spacer region is an intron sequence because it increases the efficiency of silencing. Ihp RNAs are considered to be produced more easily and remain more stable by using a selection marker and thus provides an alternative to the use of noncoding spacers (Surekha, *et al*., 2013). The selection marker is a gene conferring suitable traits, especially antibiotic resistance, for artificial selection of transgenic plants. In different studies the virus resistance in susceptible plants have been induced by transferring primarily virus-derived genes which are including viral coat protein (Reddy *et al.,* 2010) or using a construct intron-containing hairpin RNA (Yan *et al.,* 2009). Besides these, in some experiments potato resistant to PVY were generated by using an environmentally safe construct which did not use a marker gene for antibiotic resistance to select the transgenic plants. Therefore the isopentenyl transferase gene (*ipt*) present on the Ti plasmid of *Agrobacterium* mutant strain was used as a phenotypic selection marker. A marker-free binary vector with a hairpin construct derived from the coat protein gene of PVY^{NTN} strain was then used for transformation (Bukovinszki *et al*., 2007). In other reports, the transgenic, marker-free potato was produced using a two-step protocol. In the first step was aimed to improve the transformation methods applied to different cultivars of potato, through the transfer and expression of reporter gene *gfp* (green fluorescent protein) and marker gene *npt*II (neomycin phosphotransferase) (Rakosy-Tican *et al*., 2006).

In the second step, the potato cultivars (Baltica and Désirée) which have the best response to transformation process from the first step, were transformed for PVY resistance using *A. tumefaciens* C58C1 pGV2260 with the hairpin construct pRGG YCPiPCY (35SCaMV enhancer and promoter, two repeated inverted PVY-CP sequences separated by an intron and pA nos terminator) (Fig. 3). The transgenic lines proved to integrate the hairpin construct with high efficiency. In this case maximum 50% of the regenerated plants, analyzed at molecular level by using gene specific primers, were proved to integrate the construct (Rakosy-Tican *et al*., 2010).

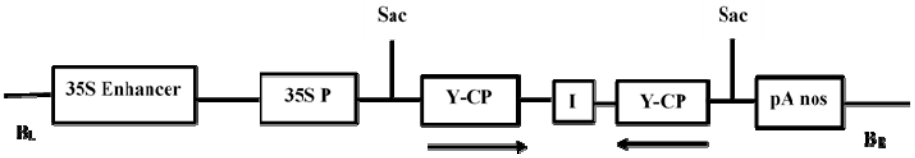

Figure 3. Schematic representation of the marker free PVY CP hairpin construct used to obtain transgenic potato resistant to PVY: B_L = left border and B_R= right border of T-DNA; 35S Enhancer = enhancer of 35S CaMV; P = promoter 35S CaMV; Y-CP = coat protein gene of PVY; I = intron; pA nos = poliadenilated nopaline synthase terminator; the arrows indicate antisense sequences that form hairpin structure; Sac = restriction site for the enzyme SacI (with authors permission Rakosy-Tican *et al*., 2010).

Conclusions

RNA interference (RNAi) is becoming an important tool for silencing the gene expression for potato crop improvement. Nevertheless, even if RNAi stability in plants is critical, the new constructs obtain by intron hairpin RNA construct provide an opportunity to develop new strategy to obtain plants resistant to viruses.

The RNAi is an interesting method which could be further exploited to analyze the function of target genes and regulation of gene expression for crop improvement.

The intron hairpin RNA can be considered an eco-friendly technology, because it eliminates even some risks related with the use of marker gene for antibiotic resistance, to select the transgenic plants. This risk refers to the possibility of the transfer of antibiotic resistance genes from plants to bacteria. But using a marker-free construct, this risk is eliminated. This strategy was also developed for a better acceptance of transgenic plants by consumers, particularly in Europe.

REFERENCES

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H., Vance, V. B. (1998) A viral suppressor of gene silencing in plants, *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 13079-13084

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell*, **116**, 281–297

Baulcombe, D. (1999) Viruses and gene silencing in plants, *Archives of Virology Supplement*, **15**, 189-201

Birch, R.J.P., Bryan, G., Fenton, B., Gilroy, E. G., Hein, I., Jones, J. T., Prashar, A., Taylor, M.A., Torrance, L., Toth, I.K.. (2012) Crops that feed the world 8 - Potato: are the trends of increased global production sustainable? *Food Security*, **4**, 477-508

Blanco-Urgoiti, B., Tribodet, M., Leclere, S., Ponz, F., Perez de San Roman, Legorburu1, C., F.J., Kerlan C. (1998) Characterization of potato potyvirus Y (PVY) isolates from seed potato batches. Situation of the NTN, Wilga and Z isolates, *European Journal of Plant Pathology*, **104**, 811–819

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., Baulcombe, D.C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*, *EMBO Journal*, **17**, 6739–6746

Bukovinszki, A., Diveki, Z., Csanyi, M., Palkovics, L., Balazs, E. (2007) Engineering resistance to PVY in different potato cultivars in a marker-free transformation system using a 'shooter mutant' *A. tumefaciens*, *Plant Cell Reports*, **26**, 459-465

Bushra, T., Idrees, A.N., Usman, A., Tayyab H. (2012) How RNA Interference Combat Viruses in Plants, In: *Functional Genomics*, Dr. Meroni, G., (ed), InTech, pp 113-130

Bushra, T., Zunaira, S., Muhammad, T., Anwar, K., Naila, S., Muhammad, B., Memoona, R., Muhammad, S.I., Idrees, A.N., Tayyab, H. (2013) Overview of acquired virus resistance in transgenic plants, *Experimental Agriculture and Horticulture*, **2**, 12-28

Duan, C.G., Wang, C.H., Guo, H.S. (2012) Application of RNA silencing to plant disease resistance, *Silence*, **3**, 1-8

Fire, A., Xu, S., Montgomery, M., Kostas, S., Driver, S., Mello, C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature*, **391**, 806–811

Godge, M.R., Purkayastha, A., Dasgupta, I., Kumar P.P. (2007) Virus-induced gene silencing for functional analysis of selected genes, *Plant Cell Reports*, **27**, 209-219

Goldbach, R., Bucher, E., Prins, M. (2003) Resistance mechanisms to plant viruses: an overview, *Virus Research*, **92**, 207-212

Joseph, B., Ajisha, S.U., Jeevitha, M.V. (2012) siRNA Mediated Gene Silencing: A Mini Review, *Aceh International Journal of Science and Technology*, **1**, 98-103

Kasschau, K.D., Carrington, J.C. (1998) A counter defensive strategy of plant viruses: suppression of posttranscriptional gene silencing, *Cell*, **95**, 461-470

Kryldakov, R., Akbergenov, R., Hohn, T., Iskakov, B. (2011) Identification of silencing suppressors of *Potato Virus M*, *Journal of Cell and Molecular Biology*, **9**, 15-20

Lecellier, C.H., Voinnet, O. (2004) RNA silencing: no mercy for viruses? *Immunology Reviews*, **198**, 285–303

Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I., Baulcombe, D.C. (2003) Virus-induced gene silencing in plants, *Methods*, **30**, 296-303

Mazid, M., Khan, T.A., Mohammad, F. (2011) Role of secondary metabolites in defense mechanisms of plants, *Biology and Medicine*, **3**, 232-249

Missiou, A., Kalantidis, K., Boutla, A., Tzortzakaki, S., Tabler, M., Tsagris, M. (2004) Generation of transgenic potato plants highly resistant to *Potato Virus Y* (PVY) through RNA silencing, *Molecular Breeding*, **14**, 185–197

Prins, M. (2003) Broad virus resistance in transgenic plants, *Trends in Biotechnology*, **21**, 373-375

Rakosy-Tican, E., Aurori, A., Dijkstra, C., Maior, M.C. (2010) Generating marker free transgenic potato cultivars with an hairpin construct of PVY coat protein, *Romanian Biotechnological Letters*, **15**, 63-71

Rakosy-Tican, E., Aurori, C, M., Dijkstra, C., Thieme, R., Aurori, A., Davey, M.R. (2006) The usefulness of the *gfp* reporter gene for monitoring *Agrobacterium*-mediated transformation of potato dihaploid and tetraploid genotypes, *Plant Cell Reports*, **26**, 661-671

Reddy, D.V., Sudarshana, M.R., Fuchs, M., Rao, N.C., Thottappilly, G. (2010) Genetically engineered virus-resistant plants in developing countries: current status and future prospects, *Advances in Virus Research*, **75**, 185-220

Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., Zamore, P.D. (2003) Asymmetry in the assembly of the RNAi enzyme complex, *Cell*, **115**, 199–208

Shafique, A., Nasir, I.A., Yaqub, T., Wassem, M., Bushra, T., Faiza, M., Anwar, K., Shahid, J.B., Tayyab, H. (2013) Molecular detection, phylogenetic analysis and designing of siRNA against *Potato Virus X, International Journal "Advancesments in Life Sciences"*, **1**, 37-44

Soosaar, J.L.M., Burch-Smith, T.M., Dinesh-Kumar, S.P. (2005) Mechanisms of plant resistance to viruses, *NATURE REVIEWS | MICROBIOLOGY*, **3**, 789- 798

Surekha, A., Satendra, K.M. (2013) Advances, challenges and prospects in small RNA mediated approaches of virus resistance in plants, *Journal of Genomes and Exomes*, **2**, 43-62

Tribodet, Mi., Galais, L., Kerlan, C., Jacquot, E. (2005) Characterization of *Potato virus Y* (PVY) molecular determinants involved in vein necrosis symptom induced by PVY^{N} isolates in infected *Nicotiana tabacum* cv. Xanthi, *Journal of General Virology*, **86**, 2101-2105

Van Etten, H., Temporini, E., Wasmann, C. (2001) Phytoalexin (and phytoanticipin) tolerance as a virulence trait: why is it not required by all pathogens? *Physiological and Molecular Plant Pathology*, **59**, 83-93

Visser, J.C., Bellstedt D.U., Pirie, M.D. (2012) The Recent Recombinant Evolution of a Major Crop Pathogen, *Potato virus Y*, *PLoS ONE*, **7**, 1-12

Wang, M.B., Masuta, C., Smith, N.A., Shimura, H. (2012) RNA silencing and plant viral diseases, *Molecular Plant Microbe Interaction*, **25**, 1275-1285

Waterhouse, P.M., Graham, M.W., Wang, M.B. (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA, *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 13959–13964

Yan, P., Shen, W., Gao, X., Duan, J., Zhou, P. (2009) Rapid one-step construction of hairpin RNA, *Biochemical and Biophysical Research Communications*, **383**, 464-468

Yan, P., Wang, S., Shen, W., Gao, X., Wu, J., Zhou, P. (2010) Simple construction of chimeric hairpin RNA for virus resistance in plants, *Journal of Virology Methods*, **166**, 101-105

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=== REVIEW ===

THE MOST COMMON HUMAN AUTOSOMAL TRISOMIES

GYÖNGYI SZÉKELY[1,✉]

SUMMARY. Trisomy is a chromosomal abnormality, characterized by the appearance of an extra chromosome in the genetic material. Usually, human autosomal trisomies are not compatible with live birth, but there are some exceptions of live born trisomies. The most common live born trisomies are: Down syndrome, Edwards syndrome and Patau syndrome. All of these trisomies cause growth retardation, mental retardation and several congenital abnormalities. All of them show distinctive features. Autosomal trisomies are caused especially by chromosomal nondisjunction during meiosis. Advanced maternal age is considered to be a main reason for non-disjunction disorder, but understanding the mechanisms of this effect still remains unclear. Influence of paternal age on trisomies is controversial, but it was demonstrated that advanced paternal age together with maternal age has a significant role in occurrence of Down syndrome.

Keywords: Down syndrome, Edwards syndrome, maternal age, Patau syndrome, trisomy.

Introduction

Trisomy is a form of aneuploidy, which means that a kariotype shows the occurrence of one or more extra or missing chromosomes. This abnormality leads to genetic disorders with different effects on embryos and live birth persons. Trisomy, a numerical chromosomal aberration, means that there is one extra chromosome in the affected kariotype instead of normal two.

The most common human autosomal trisomies are those which affect the numerical alterations of chromosomes 21, 18 and 13. Among these, the chromosome trisomy 21, known as Down syndrome (DS) named for John Langdon Down, the physician who first described the condition in 1866**,** is the most frequent chromosome

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aberration leading to mental retardation. 3-5% of mentally retarded people have DS. Nowadays the birth prevalence is 1/900 for DS. At the time of conception, the zygote frequency with chromosome 21 trisomy is high, but 85% of these abnormal embryos die in uterus leading to spontaneous abortion or late fetal loss (Papp, 1995). But, lately there are real possibilities to decrease the frequency of trisomy born infants by prenatal screening.

The connection between maternal age and human trisomies

During the last few decades different approaches have been used to investigate oocyte chromosomes and to recognize the presence of chromosomal aberrations in human female gametes, which include cytogenetic and different staining methods. The results of these screening methods indicated distinct variability in the occurrence of chromosomal aberrations, which reflect the difficulties of this kind of studies. However, the only unambiguous factor associated with human chromosome numerical variations is the maternal age (Pellestor *et al.,* 2005). At the 16-th week of pregnancy, which is the optimal time for amniocentesis, the frequency of chromosome 21 trisomy at 36 years old women is 1/200, at 39 age is 1/100 and at 42 age is 1/50. These data significantly change according to father's age (Table 1).

Table 1.

Chromosomal non-disjunction trisomy 21 and other chromosome aberrations depending on maternal and paternal age (Papp, 1995)

Paternal age	**Maternal age**			
	35 – 40		41 - 46	
	trisomy 21	total chromosome aberrations	trisomy 21	total chromosome aberrations
≤ 34	0.4	1.3	1.0	1.7
35 - 40	0.6	1.6	1.2	2.8
41 - 46	1.3	2.1	2.8	5.6
≥ 47	2.0	2.3	4.1	6.5

It has long been known that trisomies appear more frequent with increasing maternal age, but it depends not only of mother, but the father's age also (Table 1). As shown in table 1, at maternal age above 40, the cytogenetic risk is double compared with maternal age under 40, but there are the same data if father's age is above 40 (Papp, 1995; Fisch *et al.*, 2003).

Define reasons of Down syndrome formation

All people with DS have an extra chromosome 21 material. An extra whole chromosome 21 in all cells of an individual with DS is found in 92% of cases.

Beside advanced maternal age (Hassold and Chiu, 1985), altered chromosome recombination (Warren *et al*., 1987; Sherman *et al.*, 1991) constitutes the major risk to evolve DS when the appearance of an extra chromosome 21 in the oocytes during meiosis is responsible for this genetic condition (Sherman *et al.*, 2007). Studying a population from eastern part of India, Ghosh *et al.* (2009) reported a correlation between meiosis I nondisjunction and recombination in the telomeric 5.1 Mb of chromosome 21. Otherwise, in meiosis II the same authors observed preferential pericentromeric exchanges covering the proximal 5.7 Mb region, with interaction between maternal age and the location of the crossover. Generally, defective recombination independent of maternal age is also obvious in meiosis I cases (Ghosh *et al.*, 2009).

In 3-4% of DS individuals, a Robetsonian translocation happens. It is formed when one chromosome 21 (long arm) attaches to another chromosome, which is usually chromosome 14 (long arm) and forms a new chromosome (Benke *et al.*, 1995). If we analyze the DS carrying children of young mothers, we will found that in some occurrences the cause of appearance of syndrome is not simply the trisomy 21, but other numerical chromosome aberration: number of G group chromosomes (includes chromosomes 21 and 22) are normal four, number of D group chromosomes (includes chromosomes 13, 14 and 15) are only five (one chromosome is missing), and there is one extra C group chromosome (includes chromosomes 6 – 12). It is possible only if the extra chromosome 21 has translocated to D group chromosomes. In such cases one of the parents carries a translocation, but apparently one chromosome 21 is missing, meaning that this parent has only 45 chromosomes without any kind of affection (Szemere, 2001). Another kind of translocation happens within the G group chromosomes. In such cases there are only three G chromosomes, but there are five F group chromosomes (includes chromosomes 19 and 20), so the total number of chromosomes are 46, nevertheless the child has DS. The former translocation is called D/G, the latter is called G/C translocation (Szemere, 2001). Translocations involving chromosome 22 have a lower risk because trisomy 22 has very limited potential to be viable (Scriven *et al.*, 2001).

20% of the children from translocation carrier mother and 5-10% from translocation carrier father have the risk to evolve DS (Szemere, 2001).

Mosaic trisomy 21 is a form of DS when the individual has two populations of cells, the cells with trisomy 21, and a set of normal cell line. This form constitutes 2-4% of the persons with DS (Benke *et al.*, 1995).

Genes involved in Down syndrome development

There are several theories about which genes are required in DS development. One accepted theory affirms that only a small portion of chromosome 21, called DS Critical Region, is needed to be triplicated to get the effect of DS. The 21-st chromosome is the smallest chromosome in the human karyotype (together with chromosome 22) and contains only 200 -250 genes, but only a few of them are involved in producing the phenotype of DS. It is not clearly yet exactly which genes do

what to produce the syndrome (Leshin, 1997). It has been reported (Wiseman *et al.,* 2009) that *DYRK1A* (tyrosine-(Y)-phosphorylation-regulated kinase 1A) and *RCAN1* (regulator of calcineurin 1) genes may have a role on multiple tissues development. Overexpression of *DYRK1A* together with *SIM2* (synaptojanin 1 and single-minded homologue 2) genes and trisomy of neuronal channel proteins, (GIRK2), may contribute to learning difficulties in individuals with DS. Trisomy of *APP* (amyloid precursor protein) genes has a great contribution to the high frequency of dementia in persons with DS (Granholm *et al.*, 2000; Seo and Isacson, 2005; Salehi *et al.*, 2006). Trisomy of Hsa21 micro-RNA has-miR-155 may have a role in reduced incidence of hypertension in individuals with DS (Sethupathy *et al.*, 2007). Hsa-miR-155 is supposed to specifically target one allele of type-1 *AGTR1* (angiotensin II receptor) gene, downregulate the expression of gene, which may result in reduced risk of hypertension (Wiseman *et al.*, 2009). Leshin (1997) reported that *SOD1* (superoxide dismutase) gene may cause premature aging and decreased function of the immune system, *COL6A1* (collagen type VI) gene cause heart defects, *ETS2* causes skeletal abnormalities, *CBS* (cystathione beta synthase) disrupt metabolism and DNA repair process, *CRYA* may cause cataract, *IFNAR* affects normal function of the immune system.

Common symptoms of Down syndrome

People with DS show a wide range of symptoms, not everyone with the same symptoms, and they may have encounter different problems at different period of their life cycle.

Physical features of DS comprise: muscular hypotonia, short neck with loose skin on nape of neck, flat nasal bridge and facial profile, small head, ears and mouth, protruding tongue, upward slanting eyes, ring of iris speckles - Brushfield's spots, short broad hands, short little finger, single, deep crease across the palm, a deep grove between the first and second toes (Hassold *et al.*, 1996; O'Nuallain *et al.*, 2007). Beside these physical characteristics, the corporal development of children with DS can take longer compared to children without DS. Because of hypotonia, a child with DS may learn slower to turn over, sit, stand and walk, but these children are able to learn to participate in different physical exercise activities (Carothers *et al.*, 1999).

Intellectual development and learning capacities: frequent cognitive and behavioral problems of people with DS are poor judgment, impulsive behavior and slow learning capacity (Bull *et al.*, 2011; Ulrich *et al.*, 2011). Most of the affected children are able to develop communication skills they need, but usually it takes longer compared with other children (Martin *et al.*, 2009). Määttä *et al.* (2006) described that in a population of 129 persons with DS from Finland, females showed milder forms of intellectual disability, better cognitive abilities and more developed speech compared with males. Attention deficit was observed at children with DS. Young adults showed often depression (Määttä *et al.,* 2006). Depression and Alzheimer disease are common in people with DS (Burt *et al.*, 1992; Määttä *et al.*, 2006).

People with DS has increased risk to develop different affections, including autism, hormones and gland problems, poor immune function (Hummer, 2010), congenital heart defects, hearing and vision difficulties (Baekgaard Laursen *et al.*, 1976; Bull *et al.*, 2011; Sumar, 2011).

Fertility: males with DS usually are sterile; women with DS have lower rates of fertility compared to unaffected women (Pradhan *et al.*, 2006).

Individuals with DS can reach adulthood and even old age regularly and are commonly living till 50-60 or 70 years.

Edwards syndrome

Edwards syndrome (ES) is a chromosome disorder caused by the presence of an extra full, mosaic, or partial chromosome 18 (Goldstein *et al.*, 1988; Embleton *et al.*, 1996; Carey *et al.*, 2010). Full copy of trisomy 18 is the most common. In 95% of cases, Edwards syndrome is represented by trisomy; mosaicism and translocations are very rare. ES is named after John Hilton Edwards, being the first who described the syndrome in 1960 (Edwards *et al.*, 1960). This is the second most common autosomal trisomy after DS, the affected babies usually dye in the first half year of life. ES prevalence is around 1/6000 live births, and 80% of affected individuals are female. Many of affected fetuses die before birth (Won *et al.*, 2005; Morris *et al.*, 2008). The occurrence of trisomy 18 increases with advanced maternal age. After a comprehensive study, Savva *et al.*, (2010) suggested that the frequency is constant until age 30, after this age increases exponentially, and becomes constant at age 45.

Symptoms of trisomy 18: prenatal growth retardation is one of the most common sign in trisomy 18 (Yamanaka *et al.*, 2006; Cho *et al.*, 2009; Sepulveda *et al.*, 2010). The low weight and height continues in postnatal period also, and most of the newborn babies have feeding difficulties and may need tube feeding (Cereda and Carey, 2012). Other major physical abnormalities are: clenched hand and overlapping fingers. Gastrointestinal malformations are frequent in patients with Edwards syndrome (Rosa *et al.*, 2013). Congenital structural heart defects are found on a large scale (Carey, 2010), but it is controversial if heart defects affects survey of the patients. Conversely, respiratory problems are one of the most frequent causes of death (Baty *et al.,* 1994; Embleton *et al.,* 1996; Kosho *et al.*, 2006; Carey, 2010). The ears are small with small lobules, the helix is unfolded, simple and sometimes attached to the scalp; intra-abdominal tumors (Wilms tumor) are frequent (Carey, 2010). Beside mentioned physical malformation, individuals with trisomy 18 also suffer from severe mental retardation (Giaccardi *et al.*, 1991; Matthews, 1999).

Reaching adulthood with trisomy 18 is very rare. A few individuals reached their young ages, but they were not able to live independently and needed constant care (Ricki, 2013).

Genes involved in Edwards syndrome development

Several markers are used to detect Edwards syndrome from maternal serum: AFP (alpha-fetoprotein) and hCG (human chorionic gonadotrophin) (Ilnicka *et al.*, 1996; Barkai *et al.*, 1993), PAPPA (pregnancy associated plasma protein-A) (de Graaf *et al.*, 1999; Shiefa *et al.*, 2013), APCDD1 (adenomatosis polyposis coli down-regulated 1) and VAPA (vesicle-associated membrane protein) (Tsui *et al.*, 2010). Gene dose effect of *CNDP2* (CNDP dipeptidase 2 metallopeptidase M20 family) gene was demonstrated in a patient with trisomy 18 (Spano *et al.*, 1990). The detection of the presence of unmethylated (U-maspin) and methylated (M-maspin) formes of *MASPIN* (Serpin peptidase inhibitor, clade B (ovalbumin), member 5; SERPINB5) gene may be useful as potential biomarkers for non-invasive detection of fetal trisomy 18 (Lee *et al.,* 2013). Edwards syndrome is also associated with *BCL10* (B-cell CLL/lymphoma 10), *MALT1* (mucosa associated lymphoid tissue lymphoma translocation gene 1) and *XDH* (xanthine dehydrogenase) genes (Nakamura *et al.*, 2007; Go *et al.*, 2011).

Patau syndrome

Patau syndrome (PS), caused by trisomy of chromosome 13, is the third most common autosomal trisomy in newborns (Rasmussen *et al.*, 2003; *Duarte et al.*, 2004) after DS and ES, and has the greatest negative impact on survival of embryos. Rarely, the extra genetic material is attached to another chromosome (Robertsonian translocation), and there can be mosaic variations also. Trisomy 13 was first identified in 1960 by Klaus Patau as a cytogenetic disorder. The risk of PS rises with increasing maternal age but not as strongly as in case of DS and ES (Parker *et al.*, 2003). Many fetuses never survive until term and are stillborn or spontaneously abort. PS frequency is around 1/20000 live births (Brewer *et al.,* 2002). Average time of survival is fewer than three days.

On fetuses who survive to gestation and born alive, many of clinical feature widely vary, but severe mental impairment is a constant feature in infants born with trisomy 13. PS syndrome is recognized at birth by the presence of structural birth deficiency and poor neurologic performance. Other characteristic features include: low birth weight, heart defects, polydactyly and overlapping of fingers over thumb, abnormal palm creases, facial clefting and abnormal genitalia (Plaiasu *et al.*, 2010; Tsukada *et al.*, 2012, Caba *et al.*, 2013; Polli *et al.*, 2014).

Genes involved in Patau syndrome development

Patau syndrome is associated with *AFP* (alpha-fetoprotein) gene (Chen, 2007; Demirhan *et al.*, 2011); *PAPPA* (pregnancy associated plasma protein-A) (Bersinger *et al.*, 1994; Shiefa *et al.*, 2013) gene, *EIF2C2* (Protein argonaute-2) gene (Chen et al.,

2004), *ZIC2* (zic family member 2) gene (Lim *et al.*, 2008), *SIX3* (SIX homeobox 3) (Sergi *et al.*, 2012), *PTER* (phosphotriesterase related) gene (Tapper *et al.*, 2002) and *ASTML* (acetylserotonin O-methyltransferase-like) gene (Li *et al.*, 2012).

Among all trisomies, DS is the most common (1/900) viable autosomal trisomy in live born infants. Chromosome 21 is the shortest chromosome, and this trisomy is tolerated probably because the number of protein-coding sequences predicted for chromosome 21 is the smallest of any human autosomes. Hence, an extra copy of chromosome 21 would change the normal function of cells less than an extra copy of any other autosome (O'Connor, 2008).

Genome, transcriptome and proteome analysis of trisomies

Gene dosage studies revealed a set of 324 genes with considerable dosage effect from 45 different experiments related to DS. A high number of genes involved in neuro-development, synapsis and neuro-degeneration were identified (Vilardell *et al.*, 2011). Donnelly and Storchova described gene dosage compensation by altering the mRNA levels in aneuploidy cells. Gene dosage changes could be counteracted by up- or downregulation of gene transcription in aneuploids. Aneuploid cells dispose protein level changes, impaired protein folding and protein degradation. Pathways involved in protein degradation have a significant role in interceding the compensation of proteins from additional chromosomes and other key phenotypes of aneuploidy cells (Donnelly and Storchova, 2014).

In a transcriptome analysis study of trisomy 21 and trisomy 13, FitzPatrick suggest the function of a refined primary upregulation of genes on the trisomic chromosome, resulting in a secondary, generalized and more extreme transcriptional misregulation. The level of this misregulation defines the gravity of the phenotype in most aneuploidy (FitzPatrick *et al.*, 2002). Gene expression profiling of 317 differentially expressed genes in the brain of a mouse model for DS revealed that the overexpression of interferon receptor may lead to overstimulation of Jak-Stat signaling pathway which may contribute to the neuropathology DS brain (Ling *et al.*, 2014).

Recent studies demonstrated that aneuploidy inhibits cell proliferation, induces alterations in the transcriptome and proteome and perturbs cellular proteostasis. Aneuploidy impairs induction of the heat shock response, thus activity of the transcription factor heat shock factor 1 (HSF1) is compromised; HSF1 is a critical factor underlying the phenotypes linked to aneuploidy (Donnelly *et al*., 2014).

Future research should explain the exact influence of aneuploidy on the activity of proteostasis network, and if impaired proteostasis is the basis of reduced cell proliferation and damaged metabolism.

REFERENCES

Baekgaard Laursen, H. (1976) Congenital heart disease in Down's syndrome, *British Heart Journal,* **38**, 32-38

Barkai, G., Goldman, B., Ries, L., Chaki, R., Zer, T., Cuckle, H. (1993) Expanding multiple marker screening for Down's syndrome to include Edward's syndrome, *Prenat. Diagn*., **13(9)**, 843-850

Baty, B.J., Blackburn, B.L., Carey, J.C. (1994) Natural history of trisomy 18 and trisomy 13, Growth, physical assessment, medical histories, survival, and recurrence risk, *Am. J. Med. Genet.*, **49**, 175–188.

Benke P.J., Carver V., Donahue R. (1995) Risk and recurrence of Down syndrome, http://www.nas.com/downsyn/benke.html

Bersinger, N.A., Brizot, M.L., Johnson, A., Snijders, R.J., Abbott, J., Schneider, H., Nicolaides, K.H. (1994) First trimester maternal serum pregnancy-associated plasma protein A and pregnancy-specific beta 1-glycoprotein in fetal trisomies, *Br. J. Obstet. Gynaecol.*, **101(11)**, 970-974

Brewer, C.M., Holloway, S.H., Stone, D.H., Carothers A.D., FitzPatrick, D.R. (2002) Survival in trisomy 13 and trisomy 18 cases ascertained from population based registers, *Journal of Medical Genetics*, **39(9)**, e54

Bull, M.J. & the Committee on Genetics (2011) Health supervision for children with Down syndrome, *Pediatrics*, **128**, 393–406

Burt, D.B., Loveland, K.A., Lewis, K.R. (1992) Depression and the onset of dementia in adults with mental retardation, *American Journal of Mental Retardation,* **96(5)**, 502-511

Caba, L., Rusu, C., Butnariu, L., Panzaru, M., Braha, E., Volosciuc, M., Popescu, R., Gramescu, M., Bujoran, C., Martiniuc, V., Covic, M., Gorduza, E.V. (2013) Phenotypic variability in Patau syndrome, *Rev. Med. Chir. Soc. Med. Nat. Iasi*, **117(2)**, 321-327

Carey, J.C. (2010) Trisomy 18 and trisomy 13 syndromes, In: *Management of genetic syndromes*, *3rd edition*, Cassidy, S.B., Allanson, J.E. (ed) New York: John Wiley & Sons; pp 807–823

Carothers, A.D., Hecht, C.A. and Hook, E.B. (1999) International variation in reported livebirth prevalence rates of Down syndrome, adjusted for maternal age*, J. Med. Genet.*, **36**, 386–393

Cereda, A. and Carey, J.C. (2012) The trisomy 18 syndrome, *Orphanet Journal of Rare Diseases*, **7**, 81

Chen, C.P. (2007) Meckel syndrome: genetics, perinatal findings, and differential diagnosis, *Taiwan J. Obstet. Gynecol.*, **46(1)**, 9-14

Chen, M., Yeh, G.P., Shih, J.C., Wang, B.T. (2004) Trisomy 13 mosaicism: study of serial cytogenetic changes in a case from early pregnancy to infancy, *Prenat. Diagn.*, **24(2)**, 137-43

Cho, R.C., Chu, P., Smith-Bindman, R. (2009) Second trimester prenatal ultrasound for the detection of pregnancies at increased risk of Trisomy 18 based on serum screening, *Prenat. Diagn.*, **29**, 129–139

de Graaf, I.M., Pajkrt, E., Bilardo, C.M., Leschot, N.J., Cuckle, H.S., van Lith, J.M. (1999) Early pregnancy screening for fetal aneuploidy with serum markers and nuchal translucency, *Prenat. Diagn.*, **19(5)**, 458-462

Demirhan, O., Pazarbaşı, A., Güzel A.I., Taştemir, D., Yilmaz, B., Kasap, M., Özgünen, F.T., Evrüke, C., Demir, C., Tunç, E., Kocatürk-Sel, S., Onatoğlu-Arikan, D., Koç, S., Özer, O., İnandiklioğlu, N. (2011) The reliability of maternal serum triple test in prenatal diagnosis of fetal chromosomal abnormalities of pregnant Turkish women, *Genet. Test. Mol. Biomarkers*, **15(10)**, 701-707

Donnelly, N., Passerini, V., Dürrbaum, M., Stingele, S., Storchova, Z. (2014) HSF1 deficiency and impaired HSP90-dependent protein folding are hallmarks of aneuploid human cells*, EMBO J.*, **33(20)**, 2374-2387

Donnelly, N., Storchova, Z. (2014) Dynamic karyotype, dynamic proteome: Buffering the effects of aneuploidy, *Biochim. Biophys. Acta,* **1843(2)**, 473-481

Down syndrome (2012) In: *A.D.A.M. Medical Encyclopedia*, *http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0001992*

Duarte, A.C., Menezes, A.I.C., Devens, E.S., Roth, J.M., Garcias, G.L., Martino-Roth, M.G. (2004) Patau syndrome with a long survival. A case report, *Genetics and Molecular Research*, **3(2)**, 288–292

Edwards, J.H., Harnden, D.G., Cameron, A.H., Crosse, V.M., Wolff, O.H. (1960) *A new trisomic syndrome,* Lancet, **1**, 787-789

Embleton, N.D., Wyllie, J.P., Wright, M.J., Burn, J., Hunter, S. (1996) Natural history of trisomy 18, *Arch. Dis. Child.*, **75**, 38–41

Fisch, H., Hyun, G., Golden, R., Hensle, T.W., Olsson, C.A., Liberson, G.L. (2003) The influence of paternal age on Down syndrome, *J. Urol.,* **169**, 2275-2278

FitzPatrick, D.R., Ramsay, J., McGill N. I., Shade M., Andrew D. Carothers, A.D., Hastie, N.D. (2002) Transcriptome analysis of human autosomal trisomy, *Human Molecular Genetics,* **11(26)**, 3249–3256

Ghosh, S., Feingold, E., Dey, S.K. (2009) Etiology of Down syndrome: Evidence for consistent association among altered meiotic recombination, nondisjunction, and maternal age across populations, *Am. J. Med. Genet. Part A,* **149A**, 1415–1420

Giaccardi, A., Sardi, R., Priora, U., Vivalda, M., Domeneghetti, G., Girone, P. (1991) Trisomy 18 or Edwards' syndrome. A report of 4 clinical cases, Minerva Pediatr. **43(4)**, 343-349

Go, H., Cho, H.J., Paik, J.H., Park, C.M., Oh, Y. H, Jung, K.C., Kim, C.W., Jeon, Y.K. (2011) Thymic extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue: a clinicopathological and genetic analysis of six cases, *Leuk. Lymphoma*, **52(12)**, 2276-2283

Goldstein, H., Nielsen, K.G. (1998) Rates and survival of individuals with trisomy 18 and 13, *Clin. Genet.*, **34**, 366–372

Granholm, A.C., Sanders, L.A., Crnic, L.S. (2000) Loss of cholinergic phenotype in basal forebrain coincides with cognitive decline in a mouse model of Down's syndrome, *Exp. Neurol.*, **161**, 647–663

Hassold, T. and Chiu, D. (1985) Maternal age specific rate of numerical chromosome anomalies with special reference to trisomy, *Hum. Genet.,* **70**, 11–17

Hassold, T., Abruzzo, M., Adkins, K., Griffin, D., Merrill, M., Millie, E., Ilnicka, A., Pawłowska, B., Czyzewska, J., Głogowska, I., Korlak, J., Rodo, M., Bieganowska, K., Szirkowiec, W., Zdzienicka, E., Zaremba, J. (1999) The alpha- fetoprotein level analysis in open neural tube defects and chromosomal aberrations in the fetus, *Neurol. Neurochir. Pol.*, 30 (1), 65-75

Kosho, T., Nakamura, T., Kawame, H., Baba, A., Tamura, M., Fukushima, Y. (2006) Neonatal management of trisomy 18: clinical details of 24 patients receiving intensive treatment, *Am. J. Med. Genet.*, **140A**, 937–944

Lee, D.E., Kim. S.Y., Lim, J.H., Park, S.Y., Ryu, H.M. (2013) Non-Invasive Prenatal Testing of Trisomy 18 by an Epigenetic Marker in First Trimester Maternal Plasma, *PLoS ONE,* **8(11)**, e78136

Leshin, L. (1997) Trisomy 21: the story of down syndrom, http://www.ds-health.com/trisomy.htm

Li, W., Wang, X., Fan, W., Zhao, P., Chan, Y.C., Chen, S., Zhang, S., Guo, X., Zhang, Y., Li, Y., *et al.* (2012), Modeling abnormal early development with induced pluripotent stem cells from aneuploid syndromes, *Hum. Mol. Genet.*, **1**, 32-45

Lim, A.S., Lim, T.H., Kee, S.K., Chia, P., Raman, S., Eu, E.L., Lim, J.Y., Tien, S.L. (2008) Holoprosencephaly: an antenatally-diagnosed case series and subject review, *Ann. Acad. Med. Singapore*, **37(7)**, 594-597

Ling, K.H., Hewitt, C.A., Tan, K.L., Cheah, P.S., Vidyadaran, S., Lai, M.I., Lee, H.C., Simpson, K., Hyde, L., Pritchard, M.A., Smyth, G.K., Thomas, T., Scott, H.S. (2014) Functional transcriptome analysis of the postnatal brain of the Ts1Cje mouse model for Down syndrome reveals global disruption of interferon-related molecular networks, BMC Genomics, **15**, 624

Määttä, T., Tervo-Määttä, T., Taanila, A., Kaski, M., Iivanainen, M. (2006) Mental health, behaviour and intellectual abilities of people with Down syndrome, *Down Syndrome Research and Practice,* **11**, 37-43

Martin, G.E., Klusek, J., Estigarribia, B., Roberts, J.E. (2009) Language characteristics of individuals with Down syndrome, *Topics in Language Disorders,* **29(2)**, 112–132

Matthews, A.L. (1999) Chromosomal abnormalities: trisomy 18, trisomy 13, deletions, and microdeletions, *J. Perinat. Neonatal. Nurs.*, **13(2)**, 59-75

Morris, J.K., Savva, G.M. (2008) The risk of fetal loss following a prenatal diagnosis of trisomy 13 or trisomy 18, *Am. J. Med. Genet.*, **146A**, 827–832

Nakamura, S., Ye, H., Bacon, C.M., Goatly, A., Liu, H., Banham, A.H., Ventura, R., Matsumoto, T., Iida, M., Ohji, Y., Yao, T., Tsuneyoshi, M., Du, M. Q. (2007) Clinical impact of genetic aberrations in gastric MALT lymphoma: a comprehensive analysis using interphase fluorescence in situ hybridization, *Gut*., **56(10)**,1358-1363

O'Nuallain, S., Flanagan, O., Raffat, I., Avalos, G., Dineen, B. (2007) The prevalence of Down syndrome in County Galway, *Ir. Med. J.*, **100**, 329–331

O'Connor, C. (2008) Chromosomal abnormalities: Aneuploidies, *Nature Education,* **1(1)**, 172

Papp, Z. (1995) Klinikai Genetika, Ed. Golden Book, Budapest

Parker, M.J., Budd, J.L., Draper, E.S., et al (2003) Trisomy 13 and trisomy 18 in a defined population: epidemiological, genetic and prenatal observations, *Prenat. Diagn.*, **23(10)**, 856-60

Pellestor, F., Anahory, T., Hamamah, S. (2005) Effect of maternal age on the frequency of cytogenetic abnormalities in human oocytes, *Cytogenet. Genome. Res.*, **111**, 206– 212

Plaiasu, V., Ochiana, D., Motei, G., et al (2010) Clinical relevance of cytogenetics to pediatric practice. Postnatal findings of Patau syndrome - Review of 5 cases, *Maedica (Buchar)*, **5(3)**, 178-85

Polli, J.B., Groff, D.P., Petry, P., Mattos, V.F., Rosa, R.C., Zen, P.R., Graziadio, C., Paskulin, G.A., Rosa, R.F. (2014) Trisomy 13 (Patau syndrome) and congenital heart defects, *Am. J. Med. Genet. A.*, **164(1)**, 272-275

Pradhan, M., Dalal, A., Khan, F., Agrawal, S. (2006). "Fertility in men with Down syndrome: a case report". *Fertil. Steril.,* **86(6)**, 1765.e1–3

Rasmussen, S.A., Wong L.-Y. C., Yang, Q., May, K.M., Friedman, J.M. (2003) Population-based analyses of mortality in trisomy 13 and trisomy 18, *Pediatrics*, vol. 111, no. 4, pp 777–784

Ricki, L. (2013) A Very Special Birthday for a Young Man with Trisomy 18, DNA Science Blog, http://blogs.plos.org/dnascience/2013/09/05/a-very-special-birthday -for-a-young-man-with-trisomy-18/

Rosa, R.F.M., Rosa R.C.M., Lorenzen, M.B., Zen P.R.G., Oliveira, C.A.V., Graziadio, C., Paskulin, G.A. (2013) Trisomy 18 (Edwards syndrome) and major gastrointestinal malformations, *Sao Paulo Med. J.,* **131**, 133-134

Saker, D., Shen, J., Zaragoza, M. (1996) Human aneuploidy: incidence, origin, and etiology, *Environ. Mol. Mutagen.*, **28**, 167–175

Salehi, A., Delcroix, J.D., Belichenko, P.V., Zhan, K., Wu, C., Valletta, J.S., Takimoto-Kimura, R., Kleschevnikov, A.M., Sambamurti, K., Chung, P.P., *et al*. (2006) Increased App expression in a mouse model of Down's syndrome disrupts NGF transport and causes cholinergic neuron degeneration, *Neuron*, **51**, 29–42

Savva, G.M., Walker, K., Morris, J.K. (2010) The maternal age-specific live birth prevalence of trisomies 13 and 18 compared to trisomy 21 (down syndrome), *Prenat. Diagn.*, **30**, 57–64

Scriven, P.N., Flinter, F.A., Braude, P.R., Mackie Ogilvie, C. (2001) Robertsonian translocations – reproductive risks and indications for preimplantation genetic diagnosis, *Human Reproduction,* **16**, 2267-2273

Seo, H. and Isacson, O. (2005) Abnormal APP, cholinergic and cognitive function in Ts65Dn Down's model mice, *Exp. Neurol.*, **193**, 469–480

Sepulveda, W., Wong, A.E., Dezerega, V. (2010) First-trimester sonographic findings in trisomy 18: a review of 53 cases, *Prenat. Diagn.*, **30**, 256–259

Sergi, C., Gekas, J., Kamnasaran, D. (2012) Holoprosencephaly - polydactyly (pseudotrisomy 13) syndrome: case report and diagnostic criteria, *Fetal Pediatr. Pathol.*, **31(5)**, 315-318

Sethupathy, P., Borel, C., Gagnebin, M., Grant, G.R., Deutsch, S., Elton, T.S., Hatzigeorgiou, A. G., Antonarakis, S.E. (2007) Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3'untranslated region: a mechanism for functional single nucleotide polymorphisms related to phenotypes, *Am. J. Hum. Genet.*, **81**, 405–413

Sherman, S.L, Allen, E.G., Bean, L., Freeman, S.B. (2007) Epidemiology of Down syndrome, *Mental Retard. And Dev. Disab.,* **13**, 221–227

Sherman, S.L., Takaesu, N., Freeman, S.B., Grantham, M., Phillips, C., Blackstone, R.D., Jacobs, P.A., Cockwell, A.E., Freeman, V., Uchida, I., Mikkelsen, M., Kurnit, D.M., Buraczynska, M., Keats B.J.B., Hassold, T.J. (1991) Trisomy 21: association between reduced recombination and nondisjunction, *Am. J Hum. Genet.,* **49**, 608–620

Shiefa, S., Amargandhi, M., Bhupendra, J., Moulali, S., Kristine, T. (2013) First Trimester Maternal Serum Screening Using Biochemical Markers PAPP-A and Free β-hCG for Down Syndrome, Patau Syndrome and Edward Syndrome, *Indian Journal of Clinical Biochemistry*, **28(1)**, 3-12

Spano, B., Balata, A., Sanna, M.G., Fogu, G., Angius, A.G., Sanna, R., Congiu, A.M. (1990) The gene-dose effect for peptidase A on the erythrocytes of a patient with Edwards' syndrome, *Pediatr. Med. Chir.*, **12(4)**, 393-394

Sumar K., Lee B. (2011) Down Syndrome and Other Abnormalities of Chromosome Number, In: *Nelson Textbook of Pediatrics*, 19th Ed., Kliegman R.M., Stanton B.F., St. Geme J.M., Schor N.F., Behrman R.E. (eds.) Elsevier Saunders, Philadelphia, pp 400-403

Szemere, G. (2001) A Klinikai Genetika Alapjai, Ed. Szote, Szeged

Tapper, J.K., Zhang, S., Harirah, H.M., Panova, N.I., Merryman, L.S., Hawkins, J.C., Lockhart, L.H., Gei, A.B., Velagaleti, G.V. (2002) Prenatal diagnosis of a fetus with unbalanced translocation (4;13)(p16;q32) with overlapping features of Patau and Wolf-Hirschhorn syndromes, *Fetal Diagn. Ther.*, **17(6)**, 347-351

Tsui, D.W., Lam, Y.M., Lee, W.S., Leung, T.Y., Lau, T.K., Lau, E.T., Tang, M.H., Akolekar, R., Nicolaides, K.H., Chiu, R.W., Lo, Y.M., Chim, S.S. (2010) Systematic identification of placental epigenetic signatures for the noninvasive prenatal detection of Edwards syndrome, *PLoS ONE*, **30**, e15069

Tsukada, K., Imataka, G., Suzumura, H., et al (2012) Better prognosis in newborns with trisomy 13 who received intensive treatments: a retrospective study of 16 patients, *Cell. Biochem. Biophys.*, **63(3)**, 191-8

Ulrich, D.A., Burghardt, A.R., Lloyd, M., Tiernan C., Hornyak J.R. (2011) Physical activity benefits of learning to ride a two-wheel bicycle for children with Down syndrome: A randomized trial, *Physical Therapy*, **91**, 1463–1477

Vilardell, M., Rasche, A., Thormann, A., Maschke-Dutz, E., Perez-Jurado, L.A. Lehrach, H., Herwig, R. (2011) Meta-analysis of heterogeneous Down Syndrome data reveals consistent genome-wide dosage effects related to neurological processes, *BMC Genomics*, **12**, 229

Warren, A.C, Chakravarti, A., Wong, C., Slaugenhaupt, S.A., Halloran, S.L., Watkins, P.C., Metaxotou, C., Antonaraki, S.E. (1987) Evidence for reduced recombination on the nondisjoined chromosomes 21 in Down syndrome, *Science,* **237**, 652–654

Wiseman, F.K., Alford, K.A., Tybulewicz, V.L.J., Fisher, E.M.C. (2009) Down syndrome — recent progress and future prospects, *Human Molecular Genetics*, **18**, 75-83

Won, R.H., Currier, R.J., Lorey, F., Towner, D.R (2005) The timing of demise in fetuses with trisomy 21 and trisomy 18, *Prenat. Diagn.*, **25**, 608–611

Yamanaka, M., Setoyama, T., Igarashi, Y., Kurosawa, K., Itani, Y., Hashimoto, S., Saitoh, K., Takei, M., Hirabuki, T. (2006) Pregnancy outcome of fetuses with trisomy18 identified by prenatal sonography and chromosomal analysis in a perinatal center, *Am. J. Med. Genet.*, **140**, 1177–1182