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CONTENT

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THE COMPLEX ORGANIZATION OF EUKARYOTIC CELL NUCLEUS (III): THE NUCLEAR MATRIX AND THE NUCLEAR LAMINA

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Abstract

A large variety of nuclear fibrous proteins (such as actin, myosin, lamin B, transcription factors, topoisomerases, etc) represent constitutive elements of complex structures present in the eukaryotic nuclei: the nuclear matrix and the nuclear lamina, repectively. These nuclear compartments, with fibrous network-like structure, play crucial roles in structural organization of nuclei, chromatin remodeling, DNA transcription, signals transduction, cell cycle regulation, embryonic development and other nuclear basic processes.

Introduction

In the first two parts of this minireview we synthesized the present knowledge regarding the structure and functions of some essential nuclear compartments: the nuclear bodies, the chromosome territories and the interchromatin domains. Yet, these nucleoplasmatic compartments could not properly function without the participation of other nuclear components. Among these components, the *nuclear matrix* and *the nuclear lamina* act, primary, as a nuclear framework for chromatin, nucleols, different proteinaceus bodies and the inner nuclear membrane; their structural and functional characterization will be discuss further in the part III of this minireview.

THE NUCLEAR MATRIX

The nuclear matrix appears as a concept capable to explain, at least in a theoretical manner, the existence of a nuclear compartment with fibrous network-like structure, which can organize other nuclear compartments (such as chromatin and nucleoli) and can also serve as attachment frame for some of the nuclear bodies.

Although a cell fraction equivalent to what we designate today as the **nuclear matrix** was first obtained and described long time ago (1948) by Russian researchers (Pederson, 2000), it’s just in 1974 this fraction was rediscovered, isolated and characterized as a nuclear framework (Berezney and Coffey, 1974).

The nuclear matrix represents the major residual part of isolated nuclei (by centrifugation); after combined treatment of these nuclei with DNase and RNase and extraction of 90% of the nuclear proteins and phospholipids results an internal protein predominant structure, termed the *nuclear protein matrix.*

The electron microscopy images of this final nuclear matrix reveals that it forms an internal framework composed of remaining nucleolar structures, connected to a granular and fibrous internal matrix structure, which appear to be associated with the inner layer of the surrounding nuclear envelope containing residual nuclear pore complexes; biochemically, the nuclear matrix consists of large amounts of proteins and smaller amounts of DNA, RNA, phospholipids and carbohydrates (Berezney and Coffey, 1977).

Later, other structures, more or less similar with the nuclear matrix have been isolated by different research laboratories, using various experimental methods. These structures were called: *scaffold*, *nucleoskeleton*, *karyoskeleton* and *nuclear endoskeleton* by different authors (Tsutsui et al. 2005).

Whether its particular structure and cell related aspects, the nuclear matrix appears as a complex proteinaceous structure, similar with the fibrous architecture of the cytoskeleton, when observed in electron microscopy, after its isolation from other nuclear components.

Various researchers have been shown that a large variety of proteins could be associated with the nuclear matricial network (e.g. in the mature spermatic cells): actin, myosin, lamin B and also transcription factors or

topoisomerases (Moss et al. 1993, Carrey et al. 2002, Ocampo et al. 2005, Har-Vardi et al. 2007, cited by Johnson et al., 2011).

The existence of *nuclear actin* was contested at first, its presence in the nuclei being considerated as a contamination with cytosolic actin. Nowadays, the supramolecular organization, intranuclear locations and functions of actin constitutes major issues (and no longer its nuclear component status, which is a fact).

Beyond its resemblances with cytoplasmatic actin forms (monomeric, globular - G-actin and polymeric, filamentous - F-actin), the nuclear actin presents a larger range of conformations and oligomeric and polymeric forms (Pederson and Aebi, 2002).

The actin and the *actin - related proteins* (Arps) found in nuclei are members of the actin superfamily and are highly conserved throughout evolution, similar to their cytoplasmatic counterparts (Chen and Shen, 2007).

Nuclear actin plays various and crucial roles in nuclear structure organization, chromatin remodeling, DNA transcription and signal transduction.

As a part of *chromatin - remodeling complexes*, nuclear actin participates in gene expression.

In a large variety of organisms β-actin and different Arps have been identified as components of various chromatin remodeling and histone acetyltransferase (HAT) complexes, although the mechanisms by which such molecules carry out these functions are not fully understood. On the other hand, not all chromatin - modifying complexes contain actin or ARPs (Visa and Percipalle, 2010).

One of the most important functions of nuclear actin consists in its participation both in *DNA transcription* and *regulation of transcription*. Actin is associated with all three RNA polymerases (Pol I, II, and III) and togheter with *nuclear myosin 1* (NM1) acts in driving the transcription (Grummt, 2006).

The involvement of actin molecules in RNA transcripts synthesis manifests in several ways: they can bind transcription factors, determining their localization in cells, they can associate directly with the RNA polymerases and they couple with with nascent mRNPs, in order to select the histone modifiers to transcribed genes (Miralles and Visa, 2006).

In Pol I transcription, actin-NM1 interactions act as molecular motors; the same interactions play also a role in the transition of the initiation complex into the elongation complex (Grummt, 2006).

During the process of transcription controlled by Pol II, β-actin participates at the formation of the preinitiation complex; in Pol III transcription, β-actin functions as a subunit of this enzyme.

Two possible models which explain the involvement of actin in transcription elongation stage were conceived.

One model states that the actin-NM1 complex allows the transcription machinery to slide along the DNA molecule, the elongation molecular motor being in permanent contact with both the polymerases and the DNA (chromatin). The second model assumes that actin concurs at the recruitment of histone modifiers to protein-coding genes; the actin binds to hnRNP proteins and finally becomes a component of the nascent pre-mRNPs (Visa and Percipalle, 2010).

Some studies demonstrates the involvement of nuclear actin in the *nuclear export* of some molecules (e.g. retroviral RNAs and proteins such as protein kinase inhibitor (PKI) because of its association with the nucleoplasmic filaments of nuclear pore complexes (Hofmann et al., 2001).

THE LAMINS AND THE NUCLEAR LAMINA

The *lamins* are fibrous proteins and represent elements of cytoskeleton – Class V intermediate filaments, present in most animal cell nuclei.

Inside nucleus the lamins are distributed and concentrated in two main regions: in close contact with the inner nuclear membrane (INM) of the nuclear envelope, where they form the **nuclear lamina** – a layer with proteinaceous structure, and throughout the nucleoplasm where, together with other proteins (such as actin , myosin, etc.) compose the nucleoplasmic "veil" of the nuclear matrix.

The nuclear lamina is a dense structure, with a fibrillar network oganization and ~30 to 100 nm thickess.

In the early seventies, the nuclear lamina was a matter of uncertainties concerning its architecture, functions as well its presence in different cells; these uncertainties were also reflected in the names gave by different cell biologists to the concept of nuclear lamina: “dense lamella”, “fibrous lamina” , “zona nucleum limitans” or just plain “lamina” (Leslie, 2005).

Furthermore, the studies carried out in the same period (immunofluorescent microscopy and electron microscope immunoperoxidase staining of antibodies to each of the three predominant pore complex-lamina bands, applied on a centrifugal fraction from rat liver nuclei consisting of nuclear pore complexes associated with the proteinaceous lamina which underlies the INM) showed that the major polypeptides of these structures are localized at the periphery of the interphase nucleus and have little or no cytoplasmic presence (Gerace et al. 1978).

Besides lamins, the nuclear lamina comprises other important components - *the lamin-associated membrane proteins.*

According some criteria (such as the sequence homology, biochemical properties and cellular localization during the cell cycle) the lamins can be classified in two major types: the *A-type* (lamin A, C), which are splicing variants of the *LMNA* gene, present at 1q21 and the *B-type* (lamin B_1, B_2), coded by *LMNB1* and *LMNB2* genes, situated on 5q23 and 19q13, respectively.

The lamin intermediate filaments weight from 60 to 80 kDa and display an almost complete α-helical conformation, with numerous α-helical domains which are separated by non-α-helical linkers; both the C-terminus (with a domain containing the nuclear localization signal (NLS) and the N- terminus have not an α-helical conformation.

The *lamin-binding proteins* are among the most important lamin-associated membrane proteins. They are either integral or peripheral membrane proteins of INM, bound to the fibrilar elements of the lamina and many of them are well known and characterized nowadays: the *nesprin*, the *emerin*, *lamina-associated proteins 1 and 2* (LAP1 and LAP2), the *lamin B receptor* (LBR) and the *LEM domain-containing protein 3* (MAN1). These proteins mediate the attachment of the nuclear lamina to the nuclear envelope.

Other lamin-binding proteins are transcription factors: the *retinoblastoma transcriptional regulator* (RB), *germ cell-less* (GCL), *sterol response element binding protein* (SREBP1), FOS and MOK2. The *barrier to autointegration factor* (BAF) is a chromatin-associated protein linked to the nuclear lamina and to several of the nuclear envelope proteins mentioned before. *Heterochromatin protein* 1 (HP1) binds both chromatin and the LBR (Coutinho et al., 2009).

Due to its position inside nucleus, its structure, spatial organization and the complex relations with other molecular components (lamin-binding proteins and chromatin), the nuclear lamina plays multiple and essential roles in the accurate run of nuclear functions, such as: chromatin organization, cell cycle regulation, DNA replication, cell differentiation and apoptosis.

The involvement of lamina in *chromatin organization* is strongly supported by the evidence of the non-random organization of the nuclear genome. The lamins and the lamin-binding proteins - lamina-associated polypeptide (LAP2 beta) and the lamin B receptor bind to DNA or interact with chromatin via histones, BAF-1 and HP1 chromodomain proteins, respectively; thus, they may provide anchorage sites for chromatin fibers at the nuclear envelope (Gotzmann and Foisner, 1999).

The lamin polypeptides have an affinity for binding chromatin at specific DNA sequences called *matrix attachment regions* (MAR), through their α-helical domains.

The crucial role of nuclear lamina in results in its participation at the onset of mitosis (prophase, prometaphase), when disassembly of various cellular structures (such as nuclear envelope, nuclear lamina and nuclear pore complexes) occurs.

These events are triggered by lamin phosphorylation by the cyclin B/Cdk1 protein kinase complex (Mitosis-Promoting Factor, MPF), which drives the disorganization of the lamina and the nuclear envelope. At the end of mitosis, (anaphase, telophase), after chromosome segregation, dephosphorylation of nuclear lamins induces the reassembly of the nuclear envelope and thus, the reformation of nucleus.

The role of nuclear lamina in *embryonic development* and cell differentiation was studied on various animal models (*Xenopus laevis*, the chicken and mammals).

These studies demonstrate that the presence and proportion of B-type and A-type lamins in higher vertebrates (or their counterparts in *Xenopus*) vary during development, according to a similar pattern: in the early embryonic stages, the B-type lamins are the only (or prevailing) lamins present; in further stages, as different tissues differentiate, the expression of lamin B decreases (or is relatively constant), whereas an increase in the levels of lamin A and lamin C is observed.

A possible inference of these findings is that a functional nuclear lamina, in its most basic form, could contain only B-type lamins.

The nuclear lamina also participates in the elongation phase of *DNA replication.*

This function is accomplished by lamins because they act as a structural scaffold which support the correct assembly of the elongation complexes in nucleus; another hypothesis postulates that the lamina could be the initiation point for the assembly of this scaffold.

The disorganization of nuclear lamina is one of central events which occurs in early stages of *apoptosis.* Unlike the disassembly of lamina during mitosis, which is a phosphorylation-induced process, the degradation of lamina (both of lamins and nuclear lamin-associated membrane proteins) at the beginning of apoptosis is carried out by proteolytic cleavage. This proteolytic activity is performed by enzymes who belongs to caspase-protein family.

The participation in *anchoring the nuclear pore complexes* (NPC) in the nuclear envelope is another important function of nuclear lamina.

The interference of nuclear lamina in *cell movements* is now an evidence: the lamina provides a supporting structure for the 3D migrations of single animal cells, via linker complexes (LINC). These linkers connect the

nucleoskeleton (the nuclear lamins A/C) to the cytoskeleton (the actin bundles which form a perinuclear cap) allowing the formation of lamellipodia and determining the successive protrusion and pulling of cells; the disruption of LINC complexes disorganizes the actin cap and affect the 3D cell migration.

The structural and functional defects of lamins (or lamin-associates membrane proteins) which lead to emergence of abnormal nuclear lamina behaviour are designated as **laminopathies**; they belong to a more generic nuclear pathology class termed *envelopathies* and represent a group of rare genetic disorders produced by multiple causes: mutations (nonsense and missense mutations or point mutations) in genes encoding proteins of the lamina, splicing defects of LMNA mRNA, processing defects, gene dosage defects (e.g. the duplication of lamin B gene LMNB1) and autoimune antibodies.

Because the normal lamina form a nuclear scaffold which confer mechanical strenght to nuclear structures it appears to be an adaptation to motility of animal cells; thereby, the animals manifest muscle defects diseases (myophaties) as symptoms of different envelophaties, whereas other organisms (such as plants or fungi), which are sessile, do not move and lack the nuclear lamina.

At present, a large number of different types of laminophaties is known: muscular distrophies, cardiomyopathies, dysplasies, progeria, etc.

For instance, the Emery-Dreifuss muscular dystrophy is a muscular wasting disease, progeria causes premature aging, the restrictive dermopathy is a disease associated with extremely tight skin and other severe neonatal abnormalities.

Conclusion

Various studies carried on during the last decades have revealed the biochemical, structural and functional complexity of the nuclear matrix and the nuclear lamina; their features explain the roles of these components in supporting and connecting the other nuclear compartments. The specific disorders of nuclear cytoskeleton (especially the envelopathies) which manifest not only at micoscopic level, but also at whole body level, emphasize the importance of the highly organized proteinaceus nuclear network in the economy of eukaryotic cells.

In the last part (IV) of this minireview a presentation of boundary nuclear structures (the nuclear envelope whith nuclear pores) will be made, in order to complete the informations about the complex organization of eukaryotic cell nucleus.

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NEW MOLECULES USEFUL IN THE MIGRAINE TREATMENT

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Abstract: Migraine is a neurovascular condition characterized by episodes of severe headache with inter-individual variability. Inflammation of neurogenic origin contributes to the mechanism of occurrence of migraine and other primary headaches. Neurovascular headache is a condition in which neural events have as a result dilation of blood vessels and the appearance of painful sensation. CGRP (calcitonin-gene-related peptide) is a neuropeptide widespread both in the central and peripheral nervous system, being one of the most potent vasodilator substances with important role in controlling blood pressure in both normal and abnormal conditions. The releasing of perivascular peptides relaxes cerebral arteries while stimulating cAMP accumulation or release of EDRF (endothelium derived relaxing factor). An alternative to acute treatment of migraine used so far is the CGRP receptor blockade with selective antagonists. They represent potential therapeutic molecules with superior advantages to triptans and a longer duration of action.

INTRODUCTION

Primary headache include migraine, tension-type and the cluster type headache and can be defined as independent diseases which are not caused by other diseases or trauma.
Migraine is a neurovascular disorder with a high prevalence which is characterized by severe headache, unilateral, pulsating type associated with anorexia, nausea, vomiting, phono/photophobia and in some cases of diarrhea (Goadsby P. et al., 2002).
The mechanism of headaches appearance consist in activation of nociceptors, nerve endings located in the wall of intracranial blood vessels, which respond by antidromic release of vasoactive neuronal messengers (the main source of pain in headache). The nociceptors are in connection with second-order neurons in the trigeminal sensory complex nuclei in the brainstem and upper cervical spine. Local or antidromic stimulation of sensory nerve endings causes dilation of the peripheral blood vessels via substance P and of the isomer peptide corresponding to calcitonin gene (CGRP - calcitonin gene related peptide) from trigeminovascular system in humans.
Recent theories indicate that an additional component of neurogenic inflammation, neurogenic vasodilation mediated by CGRP plays an important role in the pathogenesis of migraine. Neurogenic vasodilation, mediated by nociceptors activation from the trigeminal C fibers (depolarization) or by agents that induce migraine attacks is completely abolished by CGRP receptor antagonists (Goadsby P. et al. 1988).

MIGRAINE MANAGEMENT

Antimigrain treatment consists in the usage of two large classes of compounds that concern even the crisis treatment or the background treatment designed for the interval between the crises. The first category of drugs is designed for the most of the migraineurs, while the second category is used by a narrower category. Thus, the substances that have proven the efficacy in migraine crisis are:

- nonspecific treatments: antalgic drugs and NSAIDs (nonsteroidal antiinflamatory drugs)
- specific treatments: triptans and ergot derivatives.

Other substances, such as caffeine, antiemetic drugs and psychotropic substances have proven useful as adjuvants (Massion H. 2010).
Specific antimigraine compounds, including older generations of ergot derivatives and recently triptans inhibits sensory neuropeptide release from trigeminal neurons. (Figure 1).

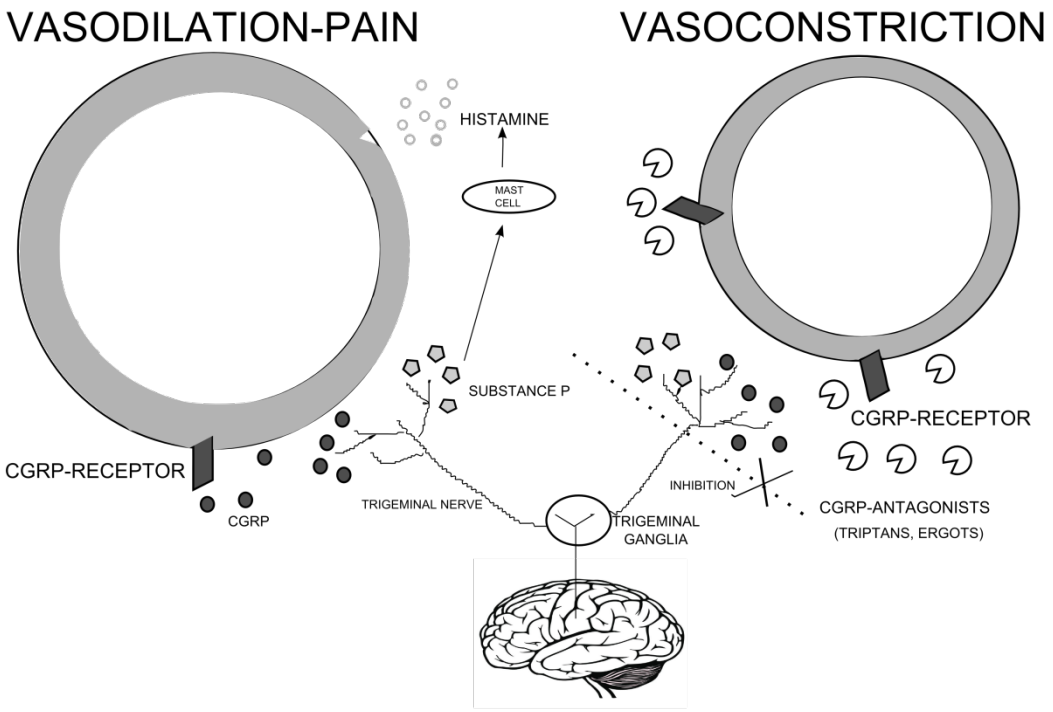

Figure 1. The action of CGRP antagonists

NO donors, who are known to be inducers of migraine attacks, release CGRP from the trigeminal nerve fibers. It was demonstrated that infusion of CGRP in volunteers can induce to a significant proportion of migraine patients, along with a number of moderate cardiovascular effects (hypotension and tachycardia), the emergence of a migraine type headache (Benemei S. et al. 2009).

Among the drugs used in migraine attacks, triptans represented a big step forward with effects that have greatly improved the lives of patients but their side effects, vasoconstrictor type, limit a lot their usage in patients with multiple vascular risk factors. (Dahlhof C. 2002).

Despite major therapeutic advances represented by triptans, there are some patients who do not respond in crisis to any treatment or do not tolerate it. These patients should benefit in future of new therapies, such as currently open perspectives for the development of a new class of drugs, the CGRP receptor antagonists.

CGRP (CALCITONIN GENE RELATED PEPTIDE)

Calcitonin gene encodes two different forms of mRNA (α-CGRP and β-CGRP) depending on the anatomical location. While transcription product of the calcitonin gene predominates in the thyroid, α-CGRP prevails in nerve tissue, these being a key molecule in the occurrence of headaches. It was emphasized also a second form, β-CGRP which is expressed in the intestine and internal organs. In humans, these two forms differ in their structure by three amino acids, but have similar effects on blood vessels motricity (both forms are powerful natural vasodilators) (Mulderry P.K. et al 1988).

The peptides of this family which includes adrenomedullin, amylin and calcitonin possess various biological functions, both at the CNS level and the peripheral one.

CGRP is a neuropeptide consisting of 37 amino acids present in the central and peripheral nervous systems and is often co-located with other peptides in the of the C type nervous fibers. CGRP is a potent vasodilator although the role it plays in the cardiovascular system remains incomplete known. It is known, however, that on the peripheral blood vessels act on smooth muscle cells, causing myorelaxation (non-endothelial mechanism) by activating adenylate cyclase (Quayle J.M et al., 1994).

The releasing of perivascular peptides relaxes cerebral arteries in the same time with stimulation of cAMP accumulation or releasing of EDRF (*endothelium derived relaxing factor*). Clinical and experimental studies have shown that injection of CGRP is responsible for inducing migraine headache (in migraine sufferer subjects).

CGRP RECEPTORS

CGRP receptor family shows a great complexity because their activity is regulated by receptor activity modifying protein, RAMP (*receptor activity-modifying protein*) and RAMP1 type is a compulsory subunit of CGRP receptors, being involved in determining their functional phenotype. High levels of RAMP-1 may sensitize certain individuals to the action of CGRP in migraine (Zhang Z.et al., 2007). For the beginning, CGRP receptors have been divided into two subclasses, CGRP-1 and CGRP-2, but recently is a tendency towards accepting a single type of receptor, namely CGRP (Hay D.L. et al., 2008).

The mRNA for CGRP receptor is expressed throughout the human intracranial arteries. CGRP functional receptors are located in the vascular smooth muscle cells of cranial arteries in particular, this being supported by the fact that in the cerebral veins territory CGRP response is very weak (Petersen K.A. et al. 2005). The basic mechanism of vascular headache involves CGRP receptor components present in brain and middle meningeal arteries. Thus, it was revealed that these arteries with cerebral capillaries express mRNA for RAMP-1.3 (Edvinsson L. et al. 2010). Induced vascular biological action in response to the activation of CGRP receptors is mediated by an increase of the intracellular levels of the second messenger cAMP (Benemei S. et al., 2007).

The answer of the muscular wall of various cranial blood vessels to stimulation of CGRP receptors is different. Pharmacological studies performed on brain and meningeal arteries revealed that CGRP receptors have a dominant role of inducing a cerebral artery dilatation, stronger effect compared with the middle meningeal artery. At the same time, responses to amylin and adrenomedullin are much lower, but are themselves mediated by CGRP receptor (Sams A. et al., 1998).

CGRP ROLE IN NOCICEPTION

As emphasise the evidence obtained so far, CGRP neurotransmitter confirm it widespread in the central and peripheral nervous system which modulates the function of other neurotransmitters (Messlinger K. et al. 1995). Satellite glial cells in the gasserian trigeminal ganglion can also express CGRP receptors, these cells having an important role in the modulation of neuronal metabolism through gap junction (Ceruti S. et al., 2011). Regarding clinical data on peripheral actions of this peptide, they are related to neurovascular inflammation which appears to have a major importance in migraine. The release of CGRP from trigeminal nerve endings is not limited only to inducing the vasodilatation occurrence, but implicitly to degranulation of mast cells and edema, associations that contribute to neurogenic inflammation.

Peripheral neurons (1^{st} order neurons) containing CGRP, both in trigeminal ganglia and other areas, correspond to multimodal nociceptors that are present in all peripheral tissues and send primary afferent signals to the 2^{nd} order neurons in the cervical-spinal horn (C1-C2), trigeminal caudalis subnucleus and the nucleus of the solitary tract (Bigal M.E., 2013). At the 2nd order neurons on the way of cephalic sensitivities (somatic and visceral) CGRP acts postsynaptic mediating the transmission of pain signals from the brain stem sensory nuclei of the cranial nerves, mainly of the trigeminal cranial nerve, to the thalamus (3^{rd} order neurons) (Eftekhari S. et al., 2011).

Brainstem trigeminal sensory nuclei and especially through the area of cervical spinal C1-C2 corresponding to trigeminal caudalis subnucleus (subcomponent of the spinal trigeminal nucleus which also contains at bulbo-pontine level oralis and interpolaris subnuclei which also have significant actions in the integration of the pain in cephalic territory) plays a major role in the pathophysiology of migraine. It is shown that stimulation of these structures with certain intensities of the excitant, duration and frequency of its application, determines both the activation of trigemino-neuralgic, and trigemino-vascular system, which will trigger the peripheral release of CGRP and the development of neurogenic type inflammation. Furthermore, the prolonged stimulation and in repeated volleys is frequently associated with alteration of the sensory perception (exteroceptive, interoceptive, proprioceptive) called allodynia, in which painless stimulation is perceived as painful, as well as hypersensitisation of second order neurons (cervical spinal and brain stem) and third order (thalamus) on sensory pathways (Sun R.Q. et al. 2003). If migraine is considered to be the combined result of altered perception of stimuli that are not normally painful, plus activation the neurovascular dilator mechanism in the territory of the trigeminal nerve ophthalmic, appears probable the involvement of CGRP in migraine pathophysiology, both at central and peripheral level.

CGRP appears to play an important role in determining neuronal plasticity and the formation of synapses, both through direct action on neurons and by indirect action on the glial cells mediated by its modulatory effects (Galeaza M.T. et al., 1995).

Current therapeutic management strategies try reducing painful sensation by blocking CGRP both centrally and peripherally bringing into question the possibility that diffusion of blocking substances in the brain is not essential to the

analgesic properties of CGRP antagonists. The penetration of therapeutic active molecules at this level can have a direct influence on photophobia and other neurological symptoms of migraine with importance in its acute treatment, but not necessarily in the preventive treatment.
A reverse of penetration of CGRP antagonists in the meningo-cerebral vascular territory could be the disturbance of homeostatic physiological role of CGRP existing naturally in neurons, including its action on neuroplasticity. Another problem is unequal actions as importance and magnitude played by CGRP in different nerve structures suspected to participate in the circuitry of migraine.
To this aspect is relatively easier to answer because the identification of markers of CGRP presence induces a heightened possible functional significance. On this line, is first mentioned as recent immunohisto-reactivity research in confocal microscopy have revealed using labeled antibodies, the presence of both components of CGRP receptor, and RAMP1 protein highly expressed in cerebellar Purkinje cell soma and their branching endings. It is so believed such a strong involvement of the cerebellum in modulating the nociception process, involvement that make him even a potential therapeutic target to prevent complications of migraine such as migraine stroke. (Moulton E.A. et al., 2010)
Sporadic administration of CGRP antagonists which can penetrate the brain in acute treatment of migraine, most likely would not affect homestasis however at this level, but chronic administration in order to achieve a preventive treatment requires more studies to assess both benefit and secure their administration.

CGRP RECEPTORS ANTAGONISTS

It is known that the adverse effect of triptans in migraine therapy but also limiting their use in patients with cardiovascular disease derives from their ability to maintain a vasoconstrictor activity. Under these circumstances a judicious alternative in the treatment of migraine by restriction on inflammatory neurogenic vasodilatation consists of CGRP receptor blockade by selective antagonists. (Geppetti P. et al., 2005).
Effectiveness of two CGRP receptor antagonists, *olcegepant* and *telcagepant* (the new gepants class) has recently been demonstrated, but the precise location of their action remains to be revealed. There are three possible targets, namely: intracranial vessels, trigeminal nerve endings in the central or peripheral and central nervous structures (Massion H., 2010). It seems that both antagonists act on the RAMP1 elements from central and peripheral sites alleviating signaling at trigeminal pathway level. Antagonist molecules have a high specificity for CGRP receptors, clinical studies already having some positive results (Olesen J. et al., 2004). However, although *in vitro* results showed a high potency *in vivo* has revealed that large quantities are required to obtain a significant antimigrain effect (Edvinsson L. et al., 2007).
The studies also showed that increased levels of RAMP1 protein increase CGRP receptor activity and moreover, may sensitize trigeminal ganglia of migraine patients to CGRP actions. RAMP1 effects include increasing CGRP synthesis and increased neurogenic inflammatory process, which enhances the nociceptive action of CGRP in migraine.
The antagonist *olcegepant* appears to have similar efficacy in reducing acute migraine pain similar to triptans, but has a longer duration of action and also fewer side effects (Edvinsson L. et al., 2010).
Regarding the place of *gepants* class action, were delimited four possible areas - target relevant to the treatment antimigrain:

- cerebral and extracerebral blood vessels, the area where CGRP receptors induce an vasodilator response which can be blocked by *olcegepant* and *telcagepant in vitro* and *in vivo*;
- dural mast cells can be degranulate by CGRP (because they contain CGRP receptors) triggering the release of histamine, bradykinin and serotonin which will cause the release of cytokines and inflammatory agents involving neurogenic inflammation appearance;
- second order sensory neurons in the trigeminal nucleus of the brainstem, but particularly appropriate in trigeminal neural caudalis subnucleus areas present in the marrow spino-cervical C1-C2 levels (otherwise involved in major trigeminal neuralgia), which were proved that postsynaptic CGRP receptors can be blocked by *olcegepant* and *telcagepant*;
- nociceptive sensory neurons of the Gasser trigeminal ganglion, where CGRP stimulates the release of the gaseous neurotransmitter nitric oxide (NO) and several pro-inflammatory cytokines (Edvinsson L. et al., 2012).

Possible consequences of CGRP antagonists in the treatment of migraine
Taking in consideration the physiological role of CGRP and its cardiovascular effects are taken into account the following consequences they might have the use of CGRP antagonists in therapeutic anti-migraine management:

- *the risk of vasoconstriction appearance*: the inhibition of CGRP, vasoconstriction may occur mainly in the small blood vessels. However, clinical trials in humans have revealed that *olcegepant* has no effect on global blood flow or the cerebral or over the blood flow velocity in the middle cerebral artery. It has also been revealed that these anatagonists seem to restore the normal tone at the already dilated arteries without causing an abnormal constriction;
- *the risk of suppressing the actions of the associated antihypertensive medications:* to see whether CGRP antagonism may affect vasodilation produced by certain antihypertensive agents has been conducted a series of clinical studies which revealed that the administration of *telcagepant* after nitroglycerin, these had no vasoconstrictor effect;

- *the risk of inhibiting the compensatory vasodilation by ischemia*: in clinical studies, the supra-therapeutic doses of *telcagepant* in patients with angina, it was revealed that compensatory vasodilator response is preserved during myocardial ischemia even in presence of CGRP receptor antagonists. (Bigal M.E. et al., 2013).

CONCLUSIONS

The possibility as CGRP receptor antagonists to be beneficial in the treatment of migraine leads to the hypothesis that migraine pain has a unique mechanism, which in part is responsive to common analgesics (eg. non-steroidal anti-inflammatory analgesics) but mainly it seems that respond to medications which are not used in other types of pain.
After drugs making based on triptans and ergot derivatives, the development of a third generation of antimigraine drugs make great progress. Preclinical and clinical data obtained so far show that CGRP and its receptor represent a major route into the mechanism of migraine, but certain conclusions about the real benefits of this new class of CGRP receptor antagonists will be set only after achieving clinical trials phase III type which is currently underway. With all important data obtained so far, it is prudent to conclude that studies can not specify more precisely the complete role of CGRP and specific place of action for antagonists in the complex mechanisms of migraine.
In terms of CGRP antagonists, in the future, remains to be seen whether the inhibition of peripheral release at the sensory nerves level is sufficient for their anti-migraine action or contrary, the CGRP inhibition at central level will play a major role in their clinical effectiveness.

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SNP GENOTYPING BY TAQMAN ALLELE DISCRIMINATION TECHNIQUE

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Abstract : Breast cancer is the most frequent neoplasm in women worldwide and the principal cause of deaths by cancer, the majority being by metastatic disease. About half of breast tumors are hormone dependent, and in post-menopause women the preferred first line treatment uses third generation aromatase inhibitors. Aromatase is encoded by CYP19 gene on 15q21.1, and there is strong evidence that mutations in this gene affect its expression, with direct consequences on cancer phenotype and response to treatment. Several single nucleotide polymorphisms have been studied on CYP19A1 transcription variant, notably rs727479, rs10046, rs4646 and rs700518. We implemented a Taqman-based allele discrimination assay for the rapid investigation of the 4 SNPs in CYP19A1. We genotyped 22 metastatic breast cancer patients by the technique described.

INTRODUCTION

Breast cancer is one of the leading causes of cancer morbidity and mortality worldwide (Bray et al, 2004). It is the second most common cancer in the world and, by far, the most frequent cancer among women with an estimated 1,67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Ferlay et al, 2013). In Romania, the incidence of breast cancer in women was 66,2/100000/year, with a mortality rate of 21,6/100000/year (Steliarova-Foucher et al, 2015), the vast majority of deaths by breast cancer being by metastatic disease. The treatment options in metastatic breast cancer are mainly chemotherapy, targeted biological agents for HER-2/neu-positive tumours, and hormone therapy for hormone-dependent tumours (Cardoso et al, 2009). Up to 60% of breast tumours are positive for Estrogen Receptors alpha and beta, therefore being sensitive to estrogen binding. Estrogens induce target cells proliferation, such as breast epithelial cells and estrogen-dependent malignant cells (Weigel et al, 2001). For estrogen receptor positive breast cancer in postmenopausal women, the preferred first-line treatment is based on the administration of third generation Aromatase Inhibitors (Goldhirsch et al, 2009). Inhibitors that are in current clinical use include anastrozole, exemestane, and letrozole. Because estrogens play an important role in carcinogenesis and progression of breast cancer, genes encoding for enzymes involved in estrogen biosynthesis and metabolism are plausible candidates as breast cancer susceptibility genes.

Aromatase, also called estrogen synthetase or estrogen synthase, is an enzyme responsible for a key step in the biosynthesis of estrogens. It is a member of the cytochrome P450 superfamily (EC 1.14.14.1), which are monooxygenases that catalyze many reactions involved in steroidogenesis. In particular, aromatase is responsible for the aromatization of androgens into estrogens (Simpson et al, 2002). Aromatase is a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This protein localizes to the endoplasmic reticulum and catalyses the last steps of estrogen biosynthesis. Mutations in this gene can result in either increased or decreased aromatase activity; the associated phenotypes suggest that estrogen functions both as a sex steroid hormone and in growth or differentiation (Chen et al, 2004). In humans, the gene CYP19, located on chromosome 15q21.1, encodes the aromatase enzyme (OMIM 108330). CYP19 gene has nine coding exons and a number of alternative non-coding first exons that regulate tissue specific expression (Czajka-Oraniec et al, 2010). The gene expresses two transcript variants, the most studied being CYP19A1 (Entrez Gene ID 108330). Germline mutations in CYP1A1 could have either inhibition or gene expression stimulation effects and association of polymorphisms in the CYP19 with breast cancer risk has previously been studied (Raskin et al, 2009).

Non-pathogenic single nucleotide polymorphisms (SNPs) of CYP19A1 showed to modify aromatase activity and to influence inhibitory medication in post-menopausal women (Miron et al, 2012). Most of the studies correlated CYP19A1 SNPs with lower or higher breast cancer risk (Sobczuk et al, 2009), or in prognostic of response to aromatase inhibitors treatment (Colomer et al, 2008). The furthergoing study of the presence, distribution and prevalence of CYP19A1 SNPs should bring insights in tumoral cell development, and could also allow individualization of personalized therapies based on host genetics. Therefore, developing, adapting and implementing robust and cost-effectiveness tools for SNP genotyping is a continuous challenge of cancer molecular biology.

In this study we describe a simple and efficient tool developed and implemented in order to quickly genotype the CYP19A1 SNPs rs727479, rs10046, rs4646 and rs700518, by Taqman-based allele discrimination assay.

PATIENTS AND METHODS

Samples were obtained from post-menopausal metastatic breast cancer female patients. All patients agreed to participate by written informed consent. Genomic DNA was extracted in duplicate from 300 µl peripheral blood, using an adapted protocol of the Wizard® Genomic DNA Purification Kit, Promega™ (Negura et al, 2011). DNA extractions were performed the same day with blood collection, or blood samples were kept at 4°C for a maximum of 48 hours. Extracted DNA was resuspended in TE 1x buffer. DNA was stored at 4°C for immediate evaluation, or at -20°C in the biobank. DNA quantity and quality was estimated by spectrophotometry, by measuring absorbance at 260 and 280 nm, on appropriate dilutions, using a Biowave DNA Spectrophotometer, Biochrome™.

Primers and probed (Life Technologies™) were adapted to flank polymorphic regions of CYP19A1. Sequences are detailed in the Results section. PrimerExpress® software (Applied Biosystems™) was used to verify specificity, Tm and cross-annealing of oligonucleotides. PCR reactions were optimized for 25 µl total volume, containing 20 ng genomic DNA, 0.4 mM each dNTP, 0.8 µM of each primer, 0.25 µM of each probe, and one unit of AmpliTaq® Polymerase with appropriate Buffer, Life Technologies™. For optimizing PCR reactions without Taqman probes, the PCR program comprised an initial denaturation step at 94ºC for 5 min followed by 30 cycles of 94ºC for 20 s, 54ºC for 20 s and 72ºC for 30 s, and a final extension of 7 min at 72ºC. 10 µl PCR product were mixed with 2 µl loading dye and deposed in a 1% agarose gel stained with 0,5 µg/ml ethidium bromide. Once the reactions optimized and specific, the Taqman PCR assay including the Taqman probes was performed using a program with an initial denaturation step at 94ºC for 5 min, followed by 50 cycles of 94ºC for 15 s denaturation and 60ºC for 90s annealing/extension, in a two-step PCR. The run were performed in a 7500 Fast DX Real-Time System, Life Technologies, and the fluorescence results were interpreted and converted to genotypes with the 7500 software.

RESULTS

The genotyping of CYP10A1 SNPs were realized by allelic discrimination in a Taqman system. Briefly, each region of interest containing the SNP was amplified in a Real-Time PCR system. Each reaction contained two **sequence-specific** primers for amplifying the polymorphism of interest (forward and reverse), as well as two **allele-specific** TaqMan probes for detecting the alleles for the specific polymorphism of interest. Each allele-specific TaqMan probe has a non-fluorescent quencher (NFQ) at its 3´ end. The quencher molecule quenches the fluorescence emitted by the fluorophore. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. Also, each allele-specific TaqMan probe has a reporter dye at its 5´ end (VIC dye is linked to the 5´ end of the Allele 1 probe, and FAM dye is linked to the 5´ end of the Allele 2 probe), which allows the allele-specific fixation of each probe. During the extension PCR step, the 5'-exonuclease activity of the Taq polymerase will degrade the annealed probe, liberating the associate fluorescence. In homozygous patients, one only fluorescence will be liberated, depending on the annealed probe. In heterozygous samples, each probe is fixed on each chromosome, and both fluorescences will appear. Each sample will be then distributed, based on the emitted fluorescence, in a allelic discrimination plot, within one of the 3 genotypes. Background noise is corrected and sample genotype is established.

We designed and verify a primers/probes system for genotyping each SNP (Table 1).

The sequences of the regions of interest are presented below. Each amplicon is shown in the genomic context. Primers target sequences are underlined. Probes target sequences are double-underlined. SNP site is bold-red coloured.

Table I. Sequence of primers and probes used for the Taqman allele discrimination assays. For the Taqman probes, the Allele 1 VIC dye-labeled probe corresponds to the first nucleotide inside the square brackets. The Allele 2 FAM dye-labeled probe corresponds to the second nucleotide inside the square brackets. Both nucleotides are red-coloured.

CYP19A1 SNP	Primers		Probe
rs727479	F	5’ – GCTCAAGATGGGGTGGAGTAAAG – 3’	5’ – TTCTTCTCCCTTTCACTTTGTTTCC[**G/T**] CCATGCCCCCTCTTTGTCTTGTATT – 3’
	R	5’ – CCACTATCACCACATTCCCAAGAA – 3’	
rs10046	F	5’ – CCATCCTCGTTACACTTCTGAG – 3’	5’ – TCTGGTGTGAACAGGAGCAGATGAC[**G/A**] AATAGCACCTAGCTTGGTGACAACC – 3’
	R	5’ – CTTCACCGACTATTTCTCCCTC – 3’	
rs4646	F	5′ – CCTTGCACCCAGATGAGAC – 3′	5′ – CTACTGATGAGAAATGCTCCAGAGT[**T/G**] GGTACTGACCAGCCTTCTCTAGTGT – 3′
	R	5′ – CCTCAAACTCTTGGCCTCTG – 3′	
rs700518	F	5′ – CTACTGCATGGGAATTGGAC – 3′	5’ – GCCTGCAACTACTACAACCGGGT[**A/G**] TATGGAGAATTCATGCGAGTCT – 3’
	R	5′ – GAACAGACTCACTTGCTGATA – 3′	

rs727479 – (CYP19A1 NM_000499.3:c.145+418G>T, NG_007982.1:g.101249G>T)
Amplicon – 229 bp. Probes anneal on reverse strand. Allele 1 is G (VIC probe). Allele 2 is T (FAM probe).
5’ATGAAAGCACTTAAAAAATATCTTAGATAAACCCTTCCACTCTGTGCTGTGCTTTCGTAAAGTAGACTTGG
CACAAGTACCCTTTAAAACAAGCTCAAGATGGGGTGGAGTAAAGTAAAATTCCAGGGTTTTCTGGTTAACT
TTTAAAAAATAGATTGCAATTTTGCTCTGAAGGTGAAAATGTCTCTGGAACATCTTCTTCACTGCTTTTCTTC
TCCCTTTCACTTTGTTTCCGCCATGCCCCCTCTTTGTCTTGTATTTATCCCTTCTCTTTATTCCACTCATCATTT
TCTCTTATTCTTGGGAATGTGGTGATAGTGGTCCCCATTTTTCTCCCACTGTCTGGATCCCATTCGACCCTTA
TGGGAGCCTGGTAGTGGGAGAAAAATGGGGACCACTCCAGTGCTCTTGGAACATCATACCCCGGGAGAAT
CTTCAACAAGATCT 3’

rs10046 – (CYP19A1 NM_000499.3:c.1531C>T, NG_007982.1:g.132810C>T)
Amplicon – 414 bp. Probes anneal on forward strand. Allele 1 is C (VIC probe). Allele 2 is T (FAM probe).
5’AGCCATTTGGCTTTGGGCCCCGTGGCTGTGCAGGAAAGTACATCGCCATGGTGATGATGAAAGCCATCCT
CGTTACACTTCTGAGACGATTCCACGTGAAGACATTGCAAGGACAGTGTGTTGAGAGCATACAGAAGATAC
ACGACTTGTCCTTGCACCCAGATGAGACTAAAAACATGCTGGAAATGATCTTTACCCCAAGAAACTCAGAC
AGGTGTCTGGAACACTAGAGAAGGCTGGTCAGTACCCACTCTGGAGCATTTCTCATCAGTAGTTCACATAC
AAATCATCCATCCTTGCCAATAGTGTCATCCTCACAGTGAACACTCAGTGGCCCATGGCATTTTATAGGCAT
ACCTCCTATGGGTTGTCACCAAGCTAGGTGCTATTCGTCATCTGCTCCTGTTCACACCAGAGAACCAGGCTA
CAAGAGAAAAAGCAGAGGCCAAGAGTTTGAGGGAGAAATAGTCGGTGAAGAAACCGTATCCATAAAGAC
CCGATTCCACCAAATGTGCTTTGAGAAGGATA 3’

rs4646 – (CYP19A1 NM_000499.3:c.1673T>G, NG_007982.1:g.132952T>G)
Amplicon – 309 bp. Probes anneal on forward strand. Allele 1 is T (VIC probe). Allele 2 is G (FAM probe).
5’TTCCACGTGAAGACATTGCAAGGACAGTGTGTTGAGAGCATACAGAAGATACACGACTTGTCCTTGCACC
CAGATGAGACTAAAAACATGCTGGAAATGATCTTTACCCCAAGAAACTCAGACAGGTGTCTGGAACACTAG
AGAAGGCTGGTCAGTACCAACTCTGGAGCATTTCTCATCAGTAGTTCACATACAAATCATCCATCCTTGCCA
ATAGTGTCATCCTCACAGTGAACACTCAGTGGCCCATGGCATTTTATAGGCATACCTCCTATGGGTTGTCAC
CAAGCTAGGTGCTATTTGTCATCTGCTCCTGTTCACACCAGAGAACCAGGCTACAAGAGAAAAAGCAGAGG
CCAAGAGTTTGAGGGAGAAATAGTCGGTGAAGAAACCGTATCCATAAAGACCCGATTCCACCAAATGTGCT
TTGAGAAGGATAGGCCTTCATTAACAAAATGTATGTCTGGTTCCC 3’

rs700518 – (CYP19A1 NM_000499.3:c.240A>G, NG_007982.1:g.106684A>G)
Amplicon – 156 bp. Probes anneal on reverse strand. Allele 1 is A (VIC probe). Allele 2 is G (FAM probe).
5’AATTTTTGGTGTAACTAAGATGTTGCTTATGCTCTGACACCTGTCCTAGGTCCTGGCTACTGCATGGGAAT
TGGACCCCTCATCTCCCACGGCAGATTCCTGTGGATGGGGATCGGCAGTGCCTGCAACTACTACAACCGGG
TATATGGAGAATTCATGCGAGTCTGGATCTCTGGAGAGGAAACACTCATTATCAGCAAGTGAGTCTGTTCA
TAATCGAAGACATACTTTTTA 3’

We firstly optimized each PCR reaction, prior to the addition of the Taqman probes, in order to harmonize the reaction conditions to all polymorphisms. In figure 1, one can see specific unique bands corresponding to PCR products of rs727479 (Fig. 1a), rs10046 (Fig. 1b), rs4646 (Fig. 1c) and rs700518 (Fig. 1d). Figure 1e correspond to a multiplex-PCR assay we performed with all 8 primers, in order to verify cross-annealing and primer dimers absence.

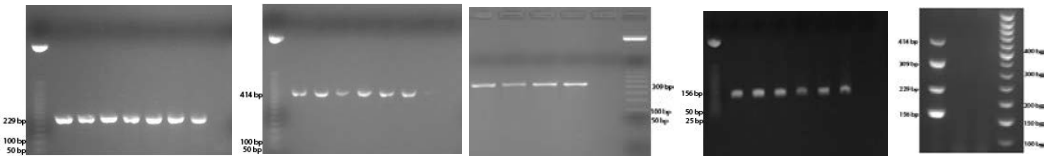

Figure 1. Electrophoresis of PCR products corresponding to amplifications in the regions of interest of rs727479 (1a), rs10046 (1b), rs4646 (1c) and rs700518 (1d). Multiplex-PCR of all regions is shown in 1e.

Subsequently, we performed the Taqman assay in a Real-Time PCR system. 22 metastatic breast cancer patients were genotyped by the technique described, in order to verify reproducibility and efficiency. The results are presented in Figure 2 to 5.

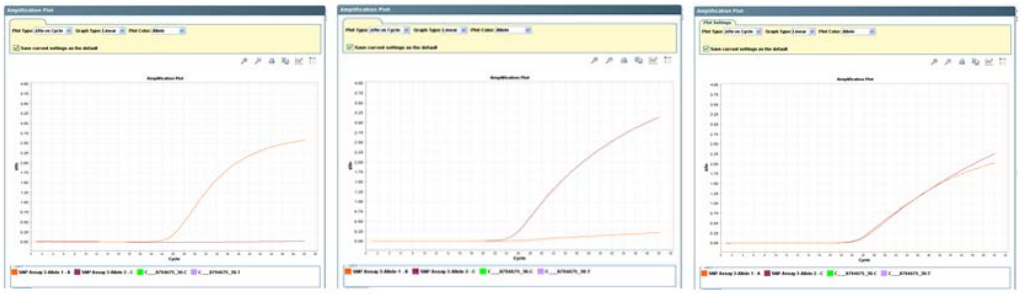

Figure 2. Genotyping of rs727479 revealing GG homozygous (left), TT homozygous (right) and heterozygous (middle)

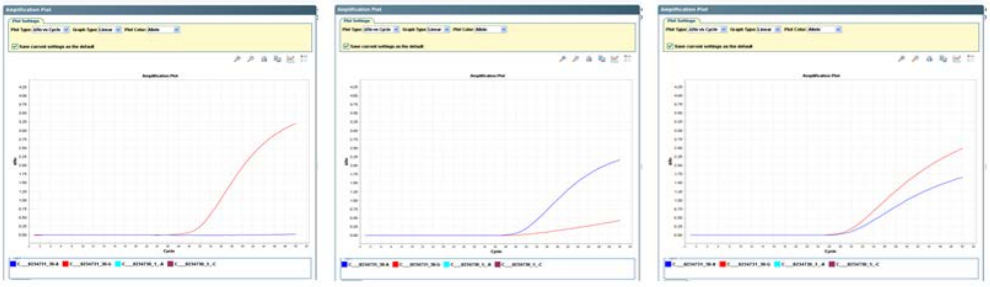

Figure 3. Genotyping of rs10046revealing CC homozygous (left), TT homozygous (right) and heterozygous (middle)

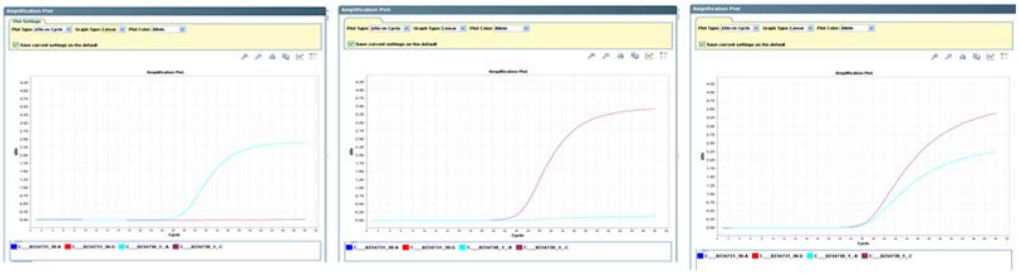

Figure 4. Genotyping of rs4646 revealing TT homozygous (left), GG homozygous (right) and heterozygous (middle)

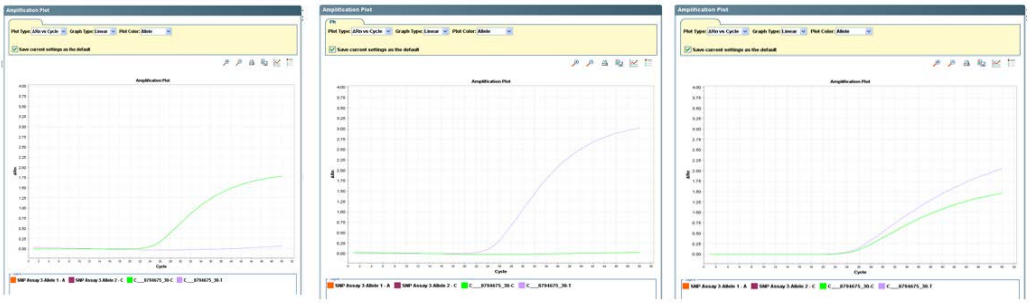

Figure 5. Genotyping of rs700518 revealing AA homozygous (left), GG homozygous (right) and heterozygous (middle)

DISCUSSION AND CONCLUSIONS

Single Nucleotide Polymorphisms occur once in every 300 nucleotides on average, which means there are roughly 10 million SNPs in the human genome. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease by affecting the gene function. Some of these genetic differences have proven to be very important in the study of human health, being involved in the predisposition to the disease or in the response to medication and treatment. Given the huge amount of SNPs possibly involved in cancer diseases, developing robust and efficient techniques for quickly genotyping an important number of samples is the present challenge of molecular medicine. Here we describe a rapid, cost-effectiveness method of genotyping CYP19A1 SNPs by Taqman allele discrimination assay. By analyzing a consecutive series of 22 metastatic breast cancers, we verified the reproducibility of the method, therefore recommending it for large scales genotyping in the evaluation of the response to breast cancer treatment.

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THE ASSESSMENT OF BIOLOGICAL MARKERS IN PATIENTS WITH PREECLAMPSIA WHEN AN INFLAMMATORY PROCESS APPEARS

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Abstract. Preeclampsia represents a pathological state that is specific to pregnancy, is characterized by high blood pressure *de novo* and significant proteinuria and appears after 20 weeks of pregnancy. The continuously increasing mortality caused by preeclampsia in our country totally justifies the fact that all efforts are directed towards primary and secondary prevention of the disease and underlines the necessity of urgent intervention at population level, together with the implementation of a screening programme that is able to reduce the impact of this condition on the mother and the baby. The cases were gathered between 2003 and 2014. The patients were selected by studying the observation charts of the pregnant women hospitalized in ”Cuza Vodă” Clinical Hospital of Obstetrics and Gynecology Iaşi, having a pregnancy over 20 weeks, who came for a specialized consult and who were harvested CRP, without an infectious context or prematurely and spontaneously ruptured membranes. The comparison of the lab markers for the pregnant women with severe preeclampsia, depending on the plasmatic level of CRP over 12 mg/l, showed significantly higher values of fibrinogen, LDH, GOT, GPT, serum blood urea nitrogen, creatinine and urine proteins, while the mean number of white cells was significantly reduced ($p<0,05$). The study confirms the change in the inflammatory process markers, the hepatic and kidney function, associated with a high plasmatic level of CRP for pregnant women with severe preeclampsia.

INTRODUCTION

Preeclampsia is characterized by generalized vascular endothelial dysfunction, which appears long before the first clinical signs. Pathophysiological changes in preeclampsia are the result of pathological changes of placenta and induce a generalized alteration of the endothelium, which results in vasoconstriction, hypovolemia and thrombus formation. But systemic endothelial damage manifests itself differently in different organs and tissues: foetus-placenta, brain, liver, kidney or haematological. Endothelial dysfunction in preeclampsia is also associated with an exaggerated systemic inflammatory response from the mother and this is why these phenomena are considered to have a crucial role in the physio-pathological mechanism of preeclampsia (Roberts, 2005).

The inflammatory maternal response in preeclampsia and especially in severe preeclampsia is an exaggerated systemic inflammatory response. There must be noted that some cytokines increase in preeclampsia like: IL 6, TNF alpha, IL 1ß, IL 8 just like in SIRS (Systemic Inflammatory Response Syndrome), but they are differentiated by NO which, unlike SIRS, has low values in preeclampsia. So preeclampsia, at a certain moment in its evolution, borrows a link from the systemic inflammatory response, which gets intricate with the endothelial dysfunction. The following fact has to be remarked: IL 6 and TNF alfa significantly increase in preeclampsia in comparison with normal pregnant women; IL 6 and IL 1ß significantly increase in severe preeclampsia in comparison with the normal pregnant women, but for IL 6, IL 8, IL 1ß, TNF alfa there are no significant differences between mild and severe preeclampsia. The conclusion of these studies is that the increase in the value of IL 6 and TNF alfa can play the role of trigger from mild to severe preeclampsia, but further studies need to be made in order to use these markers in clinical practice (Merih, 2012).

CRP marker is closer to our practice and routinely used and is also a marker belonging to the group of acute phase proteins. It is synthesized by the liver and RHS (reticulo-histiocytic system) and does not cross the placenta. CRP increased levels usually reflect the existence of an inflammatory process, which can be destructive, infectious or non-infectious, but unspecific. The normal plasmatic concentration (<6 mg/l) increases slightly with age and gets to a level that increases progressively from pregnant women, to mild inflammations, viral infections, bacterial infections and severe sepsis.

Thus, the study of different biochemical potential markers for prediction in the first and second trimester of pregnancy and of the detection markers in case of manifested preeclampsia, and also their association with ultrasound exploration, shows that although there are different potential markers for preeclampsia, their validity as predictors appears differently and uncertain in different stages of pregnancy. In the end the conclusion is that preeclampsia, which is a multifaceted

disorder, is not actually a disease, but an association of 'different diseases' and, in order to propose these markers for the routine medical practice, further studies are necessary (Can, 2011; Tjoa et al, 2004).
In Romania, the annual incidence of reported preeclampsia cases varies between 6 and 14%. An early detection of preeclampsia, not only based on the inventory of risk factors, but also with the help of some markers, could indicate more correctly the moment when treatment should start, be in progress or the birth moment as the only effective treatment. Motivated by the fact that these changes appear long before the first clinical signs of the disease, the research in this field has intensified and the recent studies focus on the endothelial dysfunction as a physio-pathological mechanism of preeclampsia.

MATERIAL AND METHODS

The retrospective case-control study focused on studying the clinical-progressive aspects of preeclampsia, by checking the correlation between the inflammatory parameters and arterial blood pressure, and also the relation between the plasmatic level of C reactive protein and other biological markers, on a group of 54 pregnant women with preeclampsia and a pregnancy over 20 weeks, hospitalized in 'Cuza Vodă' Clinical Hospital of Obstetrics and Gynaecology Iași, in the period of time between 2003 and 2014. The data was systematized and centralized in a SPSS 18.0 data base and it was processed with the appropriate statistical functions, with a significance threshold of 95%.

RESULTS AND DISCUSSION

The age over 30 represented a relative risk 2.5 higher of developing a severe form of preeclampsia, as 85,7% of the pregnant women with severe preeclampsia were over 30 years old.
The profile of the pregnant woman with preeclampsia is characterized by values of arterial blood pressure over 160/100 mmHg. These values induce a relative risk about 1,4-1,8 higher towards a severe evolution. The mean arterial blood pressure over 120 mmHg showed, in the present study, a relative risk of severe that is 2,2 higher and this aspect does not have correspondence in other studies.
The cases we studied showed a significant increase of fibrinogen, LDH, GOT, GPT, blood urea nitrogen, urine proteins, but a significantly reduced level of thrombocytes ($p<0,05$) for the patients with preeclampsia.

Table I. Predictive markers of severe preeclampsia

Parameter	Preeclampsia				Statistical significance		RR	IC95%
	severe (n=7)		mild (n=47)					
	N	%	N	%	χ^2	P		
Age ≥ 30 years old	6	85.7	16	34.0	4.74	**0.029**	2.52	1.53÷4.15
Body mass index (kg/m^2)	4	57.1	1	2.1	15.89	**0.001**	6.71	4.47÷9.58
Systolic blood pressure ≥ 160 mmHg	7	100.0	33	70.2	1.48	0.224	1.42	1.18÷1.72
Diastolic blood pressure ≥ 100 mmHg	7	100.0	26	55.3	3.41	**0.024**	1.81	1.40÷2.34
Mean arterial pressure >100 mmHg	7	100.0	21	44.7	5.42	**0.019**	2.24	1.63÷3.08
CRP ↑	7	100.0	45	95.7	0.27	0.605	1.04	0.98÷1.11
Haemoglobin ↓	5	71.4	30	63.8	0.15	0.975	1.12	0.67÷1.87
Haematocrit ↓	7	100.0	42	89.4	0.82	0.836	1.12	1.01÷1.24

Parameter	Preeclampsia				Statistical significance		RR	IC95%
	severe (n=7)		mild (n=47)					
	N	%	N	%	χ^2	P		
Thrombocytes ↓	4	57.1	26	55.3	0.10	0.751	1.03	0.52÷2.06
Fibrinogen ↑	6	85.7	38	80.9	0.05	0.831	1.06	0.76÷1.48
LDH ↑	6	85.7	39	83.0	0.13	0.717	1.03	0.74÷1.44
GOT ↑	6	85.7	20	42.6	4.55	**0.033**	2.01	1.29÷3.16
GPT ↑	6	85.7	19	40.4	5.03	**0.025**	2.12	1.34÷3.36
Uric acid ↑	5	71.4	40	85.1	0.13	0.717	0.89	0.52÷1.36
Serum blood urea nitrogen ↑	5	71.4	35	74.5	0.08	0.771	0.96	0.58÷1.58
Creatinine	5	71.4	34	72.3	0.16	0.688	0.99	0.60÷1.63
Proteinuria	4	57.1	21	44.7	0.04	0.833	1.28	0.62÷2.62
Edema	0	-	6	12.8	0.13	0.720	-	-

In the present study we demonstrated that the plasmatic level of C reactive protein is significantly correlated with systolic, diastolic and mean arterial pressure, and this aspect is in agreement with many studies (Pricop et al, 2001; Carcia, 2007; Eiland et al, 2012).

In his study reported in 2011, Can Murat uses mean arterial pressure as indicator of severity in preeclampsia and proves the direct association with the inflammatory reaction (Can, 2011). This result follows the same trend as the studies that claim that C reactive protein is an efficient marker of preeclampsia and is significantly correlated with the severity of the disease.

Carl A. and collaborators prove in a recent study the fact that a value over 3 mg/l is a good predictor of cardiovascular and inflammatory risk for women with a history of preeclampsia/eclampsia (Carl et al, 2008).

Also, Mihu D. and collaborators, published a work in 2008, where they establish that CRP is a marker for the severity of preeclampsia and low weight of the new-born at birth (Mihu et al, 2008).

A prospective study, initiated by Behboudi G. and collaborators (2012) on a group of 778 pregnant women, establishes a benchmark of 4,5 mg/dl for the C reactive protein in the first trimester of pregnancy as a predictive factor for preeclampsia (Behboudi et al, 2012), while Bita M. (2010), studies a group of 400 pregnant women and establishes the threshold for predicting preeclampsia in the first trimester of pregnancy at over 5 mg/l (Bita, 2010).

With the purpose of establishing CRP benchmarks for the normal pregnant women and also for the ones with preeclampsia, Hwang HS and collab. also prove the possibility of using CRP as a marker of severity in preeclampsia (Hwang, 2007).

Kumru S. highlights the positive correlation between CRP, mean arterial blood pressure and proteinuria. This study also highlights the relations between C reactive protein and the clinical and biochemical parameters in preeclampsia; the increased values of haemoglobin, creatinine, GOT, GPT, LDH, blood urea and proteinuria were associated with increased values of C reactive protein (Kumru, 2006).

C reactive protein is used by Azizia MM and collab. to monitor chorio-amniotitis in the ruptured membranes. Also, preeclampsia and gestational diabetes were related to inflammation (Azizia, 2006).
In the recent research, Stefanovic M. has focused on endothelial dysfunction as anomaly in preeclampsia and has concluded that there is also an increased resistance to insulin in preeclampsia, but regarding CRP as an inflammation marker he concludes that its level is not increased and cannot be associated with the severity of preeclampsia (Stefanovic, 2009).
The close relationship between adiposity and CRP can be a possible explanation for CRP's lack of predictability in the studies that do not consider this variable. An increased CRP is a useful parameter in assessing the severity risk of preeclampsia in pregnant women with an increased body mass index in the third trimester of pregnancy (Ertas, 2010). According to the cases we studied, we noticed a risk 6, 71 times higher of severe preeclampsia in obese patients.
Our study showed that the plasmatic level of C reactive protein is directly correlated with the individual values of haemoglobin and haematocrit and this aspect is also confirmed in Kumru S.'s study (2006), but our result is not significant statistically speaking ($p>0,05$).
According to the cases we studied, we noticed a low level of thrombocytes (55-57%) and an increased level of fibrinogen (81-86%) for the pregnant women with preeclampsia, without significant percentage differences depending on severity ($p>0.05$). This aspect is also underlined by other authors (Kupferminc, 2003; Lin, 2005).
The changing of the hepatic enzymes, especially GOT and GPT, induces a relative risk of severe preeclampsia twice higher ($p<0.05$).
The pathological urinalysis appeared in 57.1% of the pregnant women with severe preeclampsia and in 44.7% of the pregnant women with less severe forms of preeclampsia and also insignificant distributions of frequency from the statistic point of view ($p=0.833$). The parameters of the renal function showed increased levels in over 70% of the pregnant women with preeclampsia, without significant percentage differences depending on severity ($p>0.05$), which aspect was also showed by Gabble SG et al, in Obstetrics, 5th Ed, 2007.
The cases studied did not show oedemas for the patients with severe preeclampsia, but they appeared in 6 patients (12.8%) with mild preeclampsia, without significantly associating the presence of the oedema with the severity of preeclampsia.

CONCLUSIONS

The cases studied showed the association of preeclampsia with a high value of haematocrit, with low thrombocytes and fibrinogen, increased uric acid (an important element in the assessment of renal damage in preeclampsia, the high levels being correlated with the severity of the disease and the foetal prognostic), increased transaminases, low proteinemia, increased creatinine.
In preeclampsia, CRP is correlated with systolic blood pressure, diastolic blood pressure and average blood pressure. The increase in CRP in severe preeclampsia can be attributed to other complications of the disease, too, not only to preeclampsia alone.

CRP is an inflammation marker, but in preeclampsia there is no response of acute phase that can be detected only by CRP that can be used in clinical practice as a singular element.

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INFLUENCE OF POLYPHENOLIC COMPOUNDS ON *OCIMUM BASILICUM L.* DEVELOPMENT

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Abstract. The activities and role of phenolic compounds in the plant kingdom are well known. They are especially recognized for their function as plant growth regulators, but also for the important role in the biosynthesis process. Based on that, the aim of this work is to establish the influence of polyphenolic compounds, on the main physiological processes involved in basil cultivation under controlled conditions. Studies were carried out on sweet basil seeds (*Ocimum basilicum L.*) treated with different spruce bark polyphenolic extracts (aqueous extract and ultrasound assisted aqueous extract) on several concentrations. The germination energy and germination capacity, plants vegetative organelles development and photoassimilatory pigments content were investigated. The results show that the *Picea abies* extracts, rich in phenolic compounds, have an influence on the global development of plantlets. An increased value for the growth parameters and pigments concentration was observed, compare with a control sample. Also it was shown that the effect of phenolic compounds on plants development significantly depends on their concentration.

INTRODUCTION

Basil (*Ocimum basilicum* L.) is a popular aromatic culinary herb, belonging to the Laminaceae family, native to Iran, Afghanistan and India as described previously (Asghari *et al.*, 2012). Commonly it is used in food industry as a flavoring, being one of the major essential oils producing species from the *Ocimum* genus as described previously (Koca and Karaman, 2014). It's also used in cosmetics, perfumery and medicine, as cosmetic and pharmaceutical products, presenting great antioxidant, antimicrobial and antitumor activities due to their aromatic compounds content as described previously (Taie *et al.*, 2010). Basil can be as well used as a stomachic, anthelmintic, antipyretic, diaphoretic or as a carminative.

Based on these properties, there is an increase of interest on conservation and development of basil plants, many studies being carried out recently with the aim of establishing new and simple methodologies both to increase polyphenols content in plant, to further enhance of their overall nutritional value, and to ensure a high reproducibility and viability.

Polyphenols are one of the major groups of plant secondary metabolites being responsible for pigmentation, astringency, flavor, attraction of pollinators and offer protection against UV light and pests and well recognized for their antioxidant activity. Beside human health treating polyphenols are use in commercial applications as food and soft drinks colorants and additives, in cosmetics and pharmaceuticals products, as bioaccumulation agents, allelochemicals, chelating agents or as plants grown up regulators as described previously (Ignat *et. al.*, 2009; Popa *et al.*, 2008; Tanase *et al.*, 2011; Volf *et al.*, 2012).

In the present study spruce bark was chosen as raw material. The choice was based on resource availability, spruce bark coming as a waste resulted in large amounts from the wood processing. In our research group many studies have been done on this raw material. Taking in to account the obtained results regarding the reported total polyphenols content, the tannins content, anthocyanins, flavones, flavonols and other, it could be concluded that the spruce ritidom represent an important source of phenolic compounds as gallic acid, vanillic acid or catechine with a polyphenolic content up to 5.2 mg GAE/ g spruce bark, as reported previously (Tanase *et al.*, 2014).

Application of polyphenols as a biostimulator on plant growth is an innovative concern in the plant science world and shows a real success as described previously (Popa *et al*., 2002). Their proprieties have been gratefully tested on a large group of plants as soybean and sunflower, bean, oat, rape, maize and tomatoes as described previously (Bălaș and Popa, 2007; Ignat *et al*., 2009; Ignat *et al*. 2011; Tanase *et al.*, 2011; Tanase *et al*., 2014).

The main goal of this study was to compare the action of different aqueous polyphenolic extracts obtained using hot water extraction and ultrasound assisted extraction techniques, and to establish the influence of these compounds on *Ocimum basilicum* L. seeds. Thus, using different concentration and reported to a control, germination test were carried out and also radicles elongation, fresh biomass accumulation and photoassimilatory pigments content was determined.

MATERIALS AND METHODS

Extraction method

Extractions were performed by conventional extraction method (hot water extraction) and by ultrasound assisted extraction in order to establish the influence of the extraction method on the recovered polyphenolic content and the reactions induced by this on the main biosynthesis processes during seeds germination and plants development.

The hot water extraction was performed using two different solid/solvent ratios (5g/L (SB1) and 10 g/L (SB2)) of ground and dried spruce bark placed in a Erlenmeyer flask over which 125 mL distilled water were added. The mixture was incubated for 45 min on a water bath at 85-90 ^{0}C, shaking from time to time. Collected extracts were filtered and the extracted material was subjected to a second extraction with fresh distilled water. This operation was repeated 2-3 times until the spruce bark was fully exhausted (colorless extract). All extracts were pooled in a 1000 mL volumetric flask and mark up to volume mark with distilled water.

Ultrasound assisted extraction was performed using 5 g of spruce bark, immersed in 125 mL distilled water in a Erlenmeyer flask using a ultrasonic bath (Bandelin Sonorex), at 35 kHz frequency, 70 ^{0}C for 15 min, the extraction time being selected based on the literature data and previous preliminary studies. The operation was four times repeated, extracts being filtered and collected in a 500 mL volumetric flask and volume was completed with distilled water. In case of the SB3 solution the obtained extract was diluted 1:1 (v/v) with distilled water.

Aqueous extracts were used in four working solutions: SB1 - aqueous extract obtained by hot extraction with 5 g/L spruce bark concentration and 0.06 mg GAE/mL phenolic content, SB2 - aqueous extract obtained by hot extraction with 10 g/L spruce bark concentration and 0.13 mg GAE/mL phenolic content, SB3 - aqueous ultrasonic extract with 5 g/L spruce bark concentration and 0.32 mg GAE/ml phenolic content and SB4 - aqueous ultrasonic extract with 10 g/L spruce bark concentration and 0.65 mg GAE/mL phenolic content.

Total polyphenols content determination

Total polyphenolic content was determined by Folin-Ciocalteu method, using a UV-VIS GBS AVANTA spectrophotometer. The total phenols content was given by reading the absorbance at 765 nm wavelength and taking into account the calibration curve of gallic acid standard solution, results being expressed in milligrams gallic acid equivalents per mL spruce bark extract (mg GAE/mL).

Plant characterization

The germination tests were performed on *Ocimum basilicum* L. seeds obtained from SC. Agrosel SRL, Campia Turzii, Romania. 15 basil seeds were placed in Petri dishes covered with filter paper at approximately equal distances over which 10 mL of different extracts concentrations were added. The reference samples were prepared in same conditions using distilled water. Germination tests were performed in controlled conditions at 30^{0}C in a thermostatic room for 7 days, and wetted with 10 mL fresh solutions of polyphenolic extracts and distillated water, maintaining the specific working concentrations at every three days. On the third day after starting the experiments the germination energy (speed of the seeds germination) was determinate as a report between the germinated seeds number and total seeds number taken for experimental tests. 7 days after germination the germination capacity was determined as a numeric percentage of the total germinated seeds. For each experiment four repetitions were performed, the results being reported as percentages differences from control (Tanase *et al.*, 2014).

After germination the Petri dishes were kept for 3 days in daylight to allow the seedlings to synthesize assimilatory pigments as described previously (Tanase *et al.*, 2011). The young plantlets were analyzed by biometric and quantitative measurements in term of elongation of vegetative organs, biomass accumulation and photoassimilatory concentration determination.

For pigments content quantification 0.05 g of fresh vegetal material was milled in a mortar with quart sand and extracted in acetone (80%). The carotenoids and chlorophyll content (chlorophyll a and chlorophyll b) were than spectrophotometrically determined by reading absorbance at specific wavelengths (470, 646 and 663 nm) using mathematical formulas as described previously (Lichtenthaler and Wellburn, 1983).

Statistical analysis

The results are expressed as mean ± SD, where n = 3. Comparison of the means was performed by the Fisher least significant difference (LSD) test ($p \leq 0.05$) after ANOVA analysis using program PAST 2.14. Sampling and chemical analyses were examined in triplicate in order to decrease the experimental errors and to increase the experimental reproducibility.

RESULTS AND DISCUSSIONS

Analyzing the experimental data, it can be seen that the presence of aqueous polyphenolic extract has an stimulating effect both on energy and germination capacity (Fig.1, Fig. 2) influencing the germination process of basil seeds. Remarking is the incentive effect of polyphenolic extract obtained by ultrasonic extraction with 0.65 mg GAE/mL phenolic content (SB4), where the stimulation percentage of germination energy is 26.5% (±2.6) and 18.5% (±2.2) for germination capacity, compared to control.

Elongation and biomass accumulation in plant tissue were another parameters included in the study. As it can be seen from Figure 3, no significant differences were reported between experimental variants in terms of vegetative organs elongation.

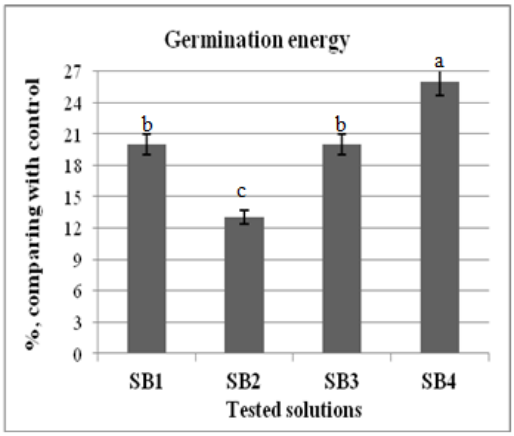

Fig. 1. The influence of spruce bark polyphenolic aqueous extracts on seed germination energy of *Ocimum basilicum* L. Bars show that the same letter is not significantly different at $p \leq 0.05$. Error bars represent the standard deviation of means (n = 3).

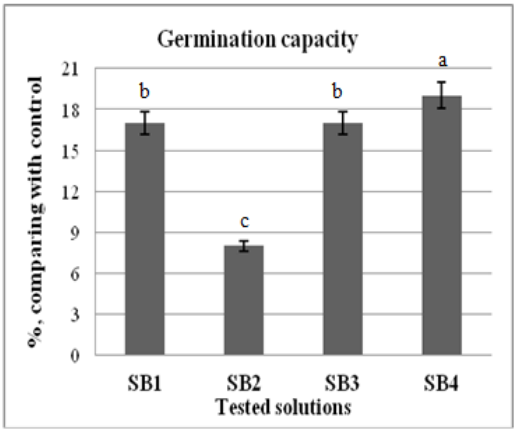

Fig. 2. The influence of spruce bark polyphenolic aqueous extracts on seed germination capacity of *Ocimum basilicum* L. Bars show that the same letter is not significantly different at $p \leq 0.05$. Error bars represent the standard deviation of means (n = 3).

It can be noted the fact that in case of using SB2 and SB3 extracts there is a decrease in growthness of the roots. This can be attributed to the increasing of extract concentration wich was applied. Regarding the biomass accumulation (Fig. 4) it was recorded an increase for all experiments. The highest value was recorded for SB1 version, where the applied extract concentration was 0.06 mg GAE/mL of phenolic content.

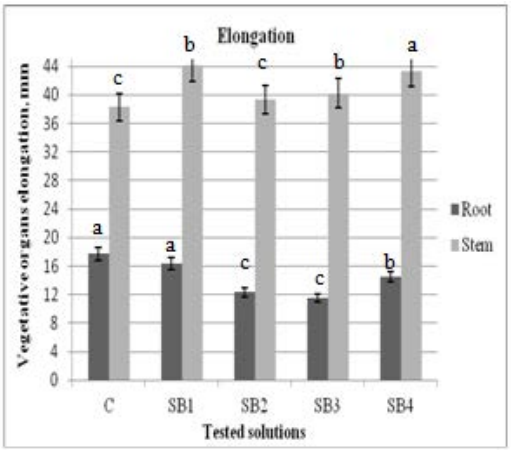

Fig. 3. The influence of spruce bark polyphenolic aqueous extracts on vegetative organs elongation of *Ocimum basilicum* L. Bars show that the same letter is not significantly different at $p \leq 0.05$. Error bars represent the standard deviation of means(n = 3).

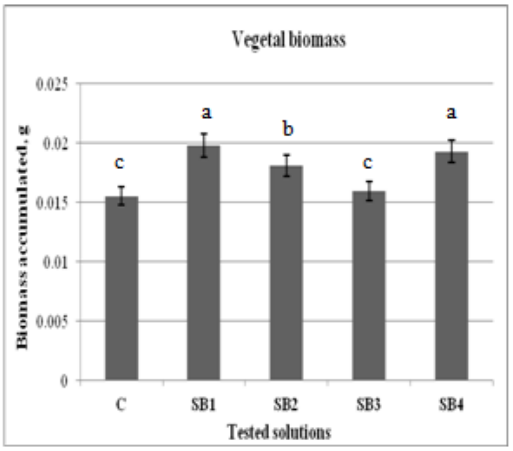

Fig. 4. The influence of spruce bark polyphenolic aqueous extracts on vegetal biomass accumulation of *Ocimum bas* Bars show that the same letter is not significantly different at $p \leq 0.05$. Error bars represent the standard deviation of means(n = 3).

In Table 1 are presented the values obtained for photo-assimilatory pigments content which were synthesized in basil primary leaves. Analyzing these recorded values it can be observed an increase of chlorophyll and carotenoids pigments biosynthesis for SB1 and SB3 variations.

In case of SB2 and SB4 variants we can notice a decrease in chlorophyll pigments accumulation. Thus, compared with the control, on SB1 there was a decrease by 10% (±1.1) and 12% (±1.6) for chlorophyll *a* chlorophyll *b*. Also SB4 show a decrease by 12% (±1.4) for chlorophyll *a* and 16% (±1.6) for chlorophyll *b*. A particular situation was reported in SB2 where the amount of carotenoids is higher compared to control (41%, ±3.2). This situation can be attributed as being a defense reaction of plant to high concentration of the extract applied in the growth medium.

Table 1. The amount of photo-assimilatory pigments synthesized in primary leaves

	Chl a µg/g	***Chl b µg/g***	***Carotens µg/g***	***Chl a+b***	***Chl a/b***
C	123.68±2.23	29.96±0.51	62.22±0.73	153.64	4.128906
SB1	133.98±2.47	38.72±0.63	80.42±0.86	172.69	3.460537
SB2	96.63±1.45	26.08±0.22	88.46±0.81	122.71	3.704961
SB3	127.94±2.65	34.84±0.55	83.41±0.79	162.78	3.672757
SB4	108.76±2.01	25.11±0.21	61.83±0.53	133.87	4.331877

CONCLUSIONS

Results showed that aqueous polyphenolic extract obtained by spruce bark heating extraction has influence on *Ocimum basilicum* L. plant acting as a stimulating on seed germination energy and capacity, root and stem growth, biomass accumulation and photoassimiling pigments synthesis. A higher extract concentration was found to inhibit the synthesis of chlorophyll *a* and *b*.

Polyphenolic aqueous extract obtained by spruce bark ultrasonic extraction increase seeds germination capacity and energy, stimulates biomass accumulation and encourages photoassimiling pigments synthesis. At higher concentrations synthesis of chlorophyll and carotenoids pigments is inhibited.

Our data show that applying aqueous ultrasonic extracts have an overall accentuated positive influence on most of the investigated parameters (as germination capacity, seeds activation energy, elongation and biomass accumulation) comparing with the hot aqueous extracts, given the fact that the ultrasounds lead to a more complete and selective extraction, extracts being more rich in a large group of polyphenols. This fact is sustained by the literature data, as described (Santos *et al*., 2013). However we do not exclude the possibility that other constituents present in the extracts, recovered during the extraction processes could also have an influence the main studied physiological processes.

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THE COMPARISON OF ACID ASCORBIC CONTENT DURING PROCESSING OF SOME VEGETABLES AND FRUITS

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Abstract. In this work there was searched the ascorbic acid content variation in four plant species during processing. The biological material was represented by: green onion, spinach leaves, pear fruits (from vegetable farms around Suceava town) and kiwi fruits (from supermarket). The ascorbic acid content was determined through a method based on reduction (by the ascorbic acid) of 2.6-Dichlorphenol-indophenol to the corresponding leucoderivate. Through grinding and one hour exposure to air at 20-22°C, the largest reduction in ascorbic acid content was in pear fruits (50%), and the lowest one in spinach leaves (11.7%). By means of thermal processing (boiling) the ascorbic acid content of the analyzed species has changed in different proportions. Thus, as compared to fresh samples, in boiled ones the vitamin C content has decreased most in pear fruits (by 52.6%) and least in spinach leaves (by 38.5%). Under the same conditions, in green onion sample the ascorbic acid mean content has increased by 10.8%, compared to fresh sample. In the fruit species, under thermal processing conditions, the ascorbic acid mean content was different as against heat-unprocessed samples. Thus, at kiwi fruits both in samples poached with sugar and without sugar, the total content of ascorbic acid (from product and boiling liquid) was superior to the heat-unprocessed samples. In contrast, at pear fruits, under the same experimental conditions, the total content of ascorbic acid was lower than in heat-unprocessed samples.

INTRODUCTION

Spread in fruits and vegetables, vitamin A, C, and E are bioactive compounds with antioxidant activities, which have a high antioxidant capacity (Hassimoto et al., 2005; Sanchez-Moreno et al., 2006). According to some authors (Dillard and German, 2000; Vinson et al., 2001; Cano et al., 2003; Chaovanalikid and Wrolstad, 2004), the phenolic compounds are also good contributors to the total antioxidant capacity of the foods containing them, but both vitamin C, carotenoids and phenolics may be poorly absorbed and rapidly metabolized, thus limiting their antioxidant ability in vivo (Gardner et al., 2000; Zulueta et al., 2007).

The storage conditions, on the one hand, and processing technology, on the other hand, can influence the content of bioactive compounds and their antioxidant capacity. Thus, the freezing process can cause, sometimes, significant decrease in the level of vitamin C in legumes and fruits (Banu et al., 2003). According to Ball (2006), drying methods, exposing the food to air lead to the loss of vitamin C because of its oxidation.

The thermal treatments are the main cause of the depletion of natural antioxidants (Anese et al., 1999). By Zia-Ur-Rehman et al. (2003); Zhang and Hamauzu (2004), cooking, pasteurization and the addition of chemical preservatives guarantee vegetables and fruits safe, but bring not always desirable changes in their physical characteristics and chemical composition (ascorbic acid, phenolics, carotenoids etc.).

The aim of this paper was to search the ascorbic acid content variation in four plant species during processing, to see to what extent the content of this vitamin is influenced by processing mode, by the type of plant material, or by the both.

MATERIALS AND METHODS

The biological material used in this work was represented by samples belonging to the following plant species: green onion (leaves and bulbs), spinach (leaves) and pear (peeled fruits), coming from vegetable farms around Suceava town, as well as kiwi (peeled fruits) purchased from supermarket. The choice of material was based on increased consumption, in this period of early spring, of these vegetables and fruits containing different amounts of ascorbic acid.

Because both vegetables and fruits species, used in this research, are eaten either fresh (salads) or cooked, their ascorbic acid content was assessed in the following working variants: raw material, chopped material and left for 60 min. at room temperature, boiled material 30 min., with sugar and sugar free, and boiling liquid, with sugar and sugar free. As to boiling conditions, the ratio of vegetable material-to-water was 1:10 w/v, and the concentration of sugar used was 10%.

The ascorbic acid content was determined through a method based on reduction (by the ascorbic acid) of 2.6-

Dichlorphenol-indophenol (2.6-DCFIF) to the corresponding leucoderivate (Artenie and Tănase, 1980; Indyk and Konings, 2000). The result was expressed as mg ascorbic acid per 100 g or 100 ml (mg%) product.

The data obtained from four replications (for each sample) were analyzed using Statistical Package for Social Science software, version 16.0. The correlation analyses were performed at the probability levels of 95% and 99%. The differences between mean values of ascorbic acid were tested using Analysis of Variance ANOVA One-Way. In order to highlight the degree of influence of different factors, such as material status, the way of processing, and interaction between them on ascorbic acid content in each studied vegetable material, or upon ascorbic acid content from boiling liquid, there was applied factorial Analysis of Variance in the condition specified (Tabachnick and Fidell, 2007).

RESULTS AND DISCUSSIONS

In Fig. 1 are reproduced, comparatively, the ascorbic acid mean values from raw material (fresh) and processed (by crushing), and left at room temperature for one hour.

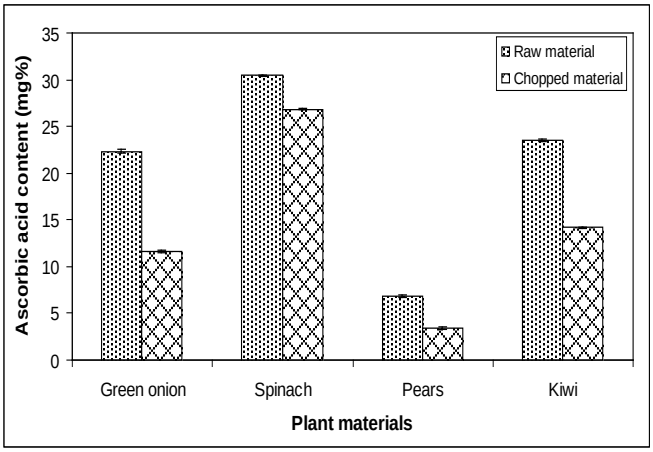

Fig. 1. The ascorbic acid mean content in raw and chopped plant materials

As shown in the graph, in the crude plant material the highest value of ascorbic acid was recorded in spinach (30.45±0.1 mg%), followed, in order, by kiwi (23.51±0.09 mg%) and green onion (22.35±0.15 mg%), the lowest one being in pears (6.87±0.11 mg%).

By grinding and exposure to air at 20-22°C, the ascorbic acid content was reduced in all 4 species examined, but with different percentages (Fig. 1). The F test result has shown a significant main effect (p=0.000), both of the variable material type, and of the variable material status on ascorbic acid content. Also, the value of F test has indicated a significant cumulative effect (p=0.000) of the factors material type and material status on ascorbic acid content. The ascorbic acid mean values in vegetable raw samples differ significantly (p=0.000) of those ones from grinded plant samples, for all kinds of analyzed materials, the material status significantly influencing the ascorbic acid content.

By grinding and in contact with air, the ascorbic acid content was reduced by 50% (in pears), by 47.8% (in green onion), by 39.7% (in kiwi) and by 11.7% (in spinach). According to Banu et al. (2003), cabbage and carrots simple cutting has lead to loss of vit. C up to 75%.

Fig. 2 shows, comparatively, the ascorbic acid mean values from unprocessed plant material and from thermally processed (cooked without sugar).

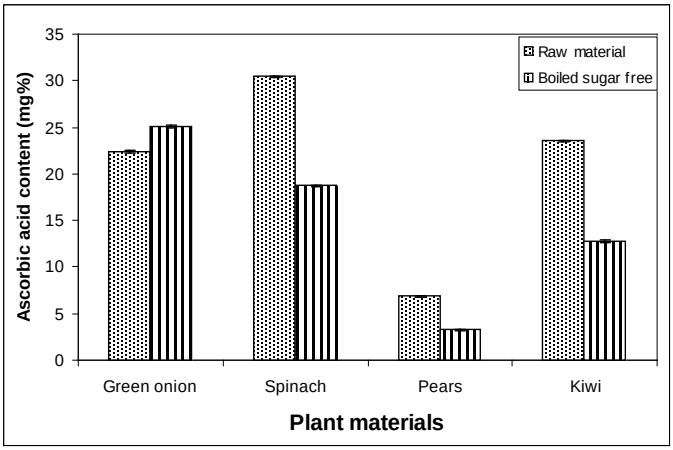

Fig. 2. Mean values of the ascorbic acid content in plant materials cooked sugar free, as compared to the raw material

As seen in the graph, the ascorbic acid values of the boiled material sugar free were: 25.05±0.12 mg% (green onion), 18.71±0.09 mg% (spinach leaves), 3.24±0.06 mg% (pear fruits) and 12.77±0.1 mg% (kiwi fruits). The unprocessed material, except green onion, indicates higher mean values for ascorbic acid content, as compared with the ascorbic acid content of the material boiled sugar free.

The average values of the ascorbic acid content in raw samples have differed significantly ($p<0.05$) of those ones in the samples boiled sugar free, for all types of plant materials studied: green onion ($r=0.968$), spinach ($r=–0.985$), pears ($r=–0.996$), kiwi ($r=–0.973$). As compared with fresh samples, in boiled samples the ascorbic acid content has decreased by 38.5% (in spinach), by 45.6% (in kiwi) and by 52.6% (in pears).

Under the same conditions (boiling), in green onion sample the ascorbic acid content has increased by 10.8%. This increasing, observed in heat-processed green onions could be explained by the fact that water, at high temperatures, has facilitated the destruction of cell walls and the release of ascorbic acid in an greater amount, than was achieved by grinding of the raw material. It is also possible that, in the case of green onion, the ascorbic acid to be present in cells as a complex with other molecules (protecting it from oxidation), and from that it is slower released, possibly through water action at these high temperatures.

In a study of an orange-carrot juice mixture, Torregrosa et al. (2006), cited by Cortés et al. (2007), observed that after pasteurization the remaining concentration of vitamin C was 83% of the concentration of the untreated juice.

In Fig. 3 are rendered the ascorbic acid mean values in pears (2.42±0.05 mg%) and kiwi (13.63±0.09 mg%) cooked with sugar, compared with unprocessed plant material.

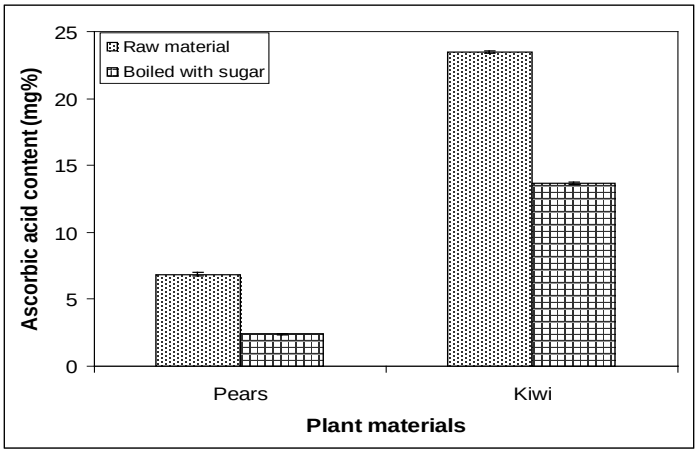

Fig. 3. Mean values of the ascorbic acid content in pears and kiwi cooked with sugar, as compared to the raw material

As seen from the graph, compared to the raw material (unprocessed), in the samples boiled with sugar, the percentage decreases of ascorbic acid were 64.9% (in pears) and 46.3% (in kiwi).

The material status (raw or boiled with sugar) and the type of material have significantly influenced ($p<0.001$) the ascorbic acid content in plant material. The results of ANOVA have revealed significant differences ($p<0.001$) between mean values of ascorbic acid from the material processed with sugar, and the raw material for the two types of plant material.

Comparing the values of ascorbic acid from samples cooked in the presence of sugar, with those ones from the samples processed without sugar it observes that, reported to raw material, a greater reduction in the content of this vitamin has occurred in pear samples in the presence of sugar (64.9%) as against 52.7% (in the absence of sugar), while in kiwi samples the percentages were close (46.3% in the presence of sugar, as against 45.6% in the absence of sugar).

Fig. 4 renders the mean values of ascorbic acid in the boiling liquid from pear and kiwi cooked with sugar, as compared to the boiling liquid from the same plant material cooked without sugar.

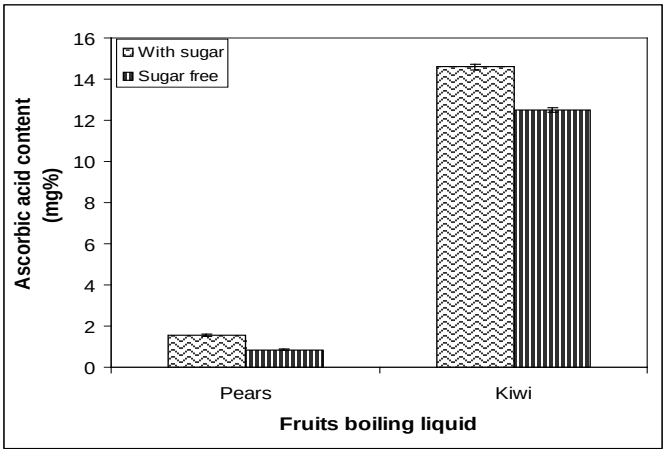

Fig. 4. Mean values of the ascorbic acid content in the boiling liquid from sugar and sugar-free materials

From Fig. 4 it can observe that in the boiling liquid with sugar, the ascorbic acid values were 14.59±0.12 mg% at kiwi, and 1.55±0.06 mg% at pears. In the boiling liquid sugar free, the ascorbic acid values were 12.5±0.09 mg% at kiwi, and 0.86±0.05 mg% at pears.

Making the amount of the ascorbic acid content from samples cooked with sugar and the boiling liquid with sugar, respectively from samples cooked without sugar and corresponding boiling liquid, is found a total of: 28.22±0.1 mg% in kiwi samples poached with sugar, respectively 25.27±0.09 mg% in kiwi samples poached without sugar ($p<0,05$), and 3.97±0.06 mg% in pears samples poached with sugar, respectively 4.1±0.06 mg% in pears samples poached without sugar ($p>0,05$). At kiwi fruits, both in samples poached with sugar and without sugar, the total content of ascorbic acid (from product and boiling liquid) was superior to the heat-unprocessed samples, and even to raw material. Like in onion, it seems that during boiling of kiwi fruits, the hot water has facilitated a more powerful destruction of cell walls and the release of a greater amount of ascorbic acid within the boiling fluid. Experimental version with added sugar (10%) has recorded higher values of ascorbic acid both in plant tissue and in boiling liquid as compared to sugar free version.

Large losses of vitamin C occur during blanching, boiling, when the water used for heat treatment is not used (mainly losses by solubilization), protective action on vitamin C having anthocyanins, sugar, starch (Banu et al., 2003).

CONCLUSIONS

The ascorbic acid content in green onion (leaves and bulbs), spinach leaves, pear and kiwi (peeled fruits) was influenced by processing mode and the type of plant material analyzed.

Compared to fresh (unprocessed) samples, grinding and exposure to air caused the greater decrease of ascorbic acid content in pears and onion, and the lowest one in spinach leaves.

The thermal processing (boiling) caused the greater decrease of ascorbic acid content in pear fruits and in spinach leaves. The presence of sugar (10%) in the boiling liquid made as

ascorbic acid to decrease less than in its absence, both in plant tissue and in the boiling fluid.

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