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Contents

In Memoriam

LJUBOMIR BERBEROVIĆ

(1933 – 2019)

On February 12, 2019, our dear professor, colleague and a friend of many years, academician Ljubomir Berberović, passed away in the 86th year of life.

Ljubomir Berberović was born in 1933 in Sarajevo. He studied medicine, biology and philosophy at University of Ljubljana and University of Sarajevo. He graduated with a degree in Biology from Faculty of Philosophy in Sarajevo in 1958. One year later, he was elected a teaching assistant at the Department of Natural Sciences and Mathematics. His doctoral dissertation "Microevolution of *Eobania vermiculata* on Central Adriatic Coast and the Islands" was successfully defended at the same faculty in February 1964.

In June 1964 he was awarded a position of assistant professor for the course "Organic evolution". In January 1970 he was awarded the position of associate professor and in June 1975 he was granted tenure as full professor. He lectured in many subject areas such as Genetics, Evolution, Anthropology and other subjects at several faculties within the University of Sarajevo, as well as other universities in the country and abroad. During period 1985-1988 he served as a Rector of the University of Sarajevo. In 1978, he was awarded an honor of becoming a corresponding member of the Academy of Sciences and Arts of Bosnia and Herzegovina (ANUBIH), of which he became a full member in 1984. Late academician Berberović authored numerous books, textbooks, scientific, professional and scientific - popular publications and discussions. His opus may be ordered within three main thematic cycles: genetics of human populations, karyology and cytotaxonomy, as well as history and philosophy of natural sciences. The papers about genetic characteristics of Bosnian and Herzegovinian population, in which many phenotypic systems of morphological and biochemical variation of human individuals and groups were studied, represent significant contribution towards the elucidation of many questions in terms of contemporary genetics of recent human populations, such as issue of relationship between the levels of reproductive isolation and evolutionary diversification of population.

As a scientist, he dedicated much attention to studying genetic characteristics and significance of certain evolutionary factors such as random genetic fluctuation (genetic drift). Some of his work in the field of human genetics was dedicated to the improvement of medical genetics as a whole. Honorable professor Berberović was an advocate and protector of globalism, open-mindedness, and free information flow. He dedicated his last decades to emphasizing the threats to the freedom of scientific thoughts that lurk behind the narrow-minded ideas of ethno- and national groups. Furthermore, he constantly emphasized the futility and irrationality of fearing the new technologies such as genetically modified organisms or nuclear energy, which marked him a herald of progress, placing him many steps ahead of

entire generations of citizens of Bosnia and Herzegovina.

In both his public and private life, the late academician Berberović was very clear regarding the genocide in Srebrenica. His stance was that there is no thin line between what makes such a devastating event "only" a crime and what constitutes a genocide. As a member of Serb Civic Council of B&H, he intellectually opposed the idea of denying the existence of Bosnia and Herzegovina as a sovereign state. He also strongly opposed the idea of "one-nation" state, as well as any justification of tragedies that befell our country and region.

The honorable Berberović was the first president of the Association of Geneticists of B&H in a period from 1979 to 1990, as well as vice president (1979-1980) and president of the Union of Genetic Societies of Yugoslavia (1980-1982). He presided the Second Congress of Geneticists of Yugoslavia held in Vrnjačka Banja 1981, as well as many other scientific congresses. He was an honorary member of newly formed Genetic Association in Bosnia and Herzegovina (2011), and a member of many other scientific associations. He also served as a reviewer and an editor for several reputable journals. Academician Berberović was an honorary member of the editorial board of Genetics & Applications, the first scientific journal in the field of genetics published by the University of Sarajevo – Institute for Genetic Engineering and Biotechnology in B&H.

A faithful friend, and a reputable scientist, Berberović was polyvalent activist and intellectual presence in many fields of science and society as a whole. He was also a secretary for the Department of Natural and Mathematical sciences, a president of Board for International Relations as well as vice president of ANUBIH itself. Academician Berberović, an honorary member of PEN Center Sarajevo, was recipient of numerous acknowledgments for his scientific, professional and societal work.

ANUBIH and late Berberović had a significant role in initiating macro project known as "Genetic engineering and biotechnology", especially within the system of funding and implementation of so-called "societal goals" in B&H from 1986-1992. He was also an active member of Consortium for Genetic Engineering and Biotechnology, later called Center for Genetic Engineering and Biotechnology, which gave rise to internationally acknowledged Institute for Genetic Engineering and Biotechnology within the University of Sarajevo. He insisted on establishing this Institute even though some contested such idea based on the fact that similar institutes existed in former Yugoslavia. And in doing so, in his distinct manner, he revealed his patriotism which he later corroborated throughout the aggression on his homeland. He shared the destiny of his fellow citizens and actively joined intellectual defense of the idea of Bosnia and Herzegovina as his homeland. His energetic intellect initiated and created significant publications and projects. His passing has left a void in the field of genetics in B&H, as well as at the Faculty of Natural Sciences and INGEB that remain behind. His fellow students and coworkers must carry on his legacy and continue to advance and develop genetic and similar scientific research in Bosnia and Herzegovina.

With great sadness and love, we bid him final farewell.

Institute for Genetic Engineering and Biotechnology

Review

TRIGGERING APOPTOSIS IN TUMORS: AN OVERVIEW OF POTENTIAL APPROACHES IN TREATMENT OF LEUKEMIA

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Abstract

Apoptosis, as a well-studied process of a programmed cell death, is essential for the maintenance of cell homeostasis and integrity of organisms. This process occurs normally during development and aging and it is a balance of the sustainability of the tissue cell population. In addition, apoptosis also occurs as a defensive mechanism such as an immune response or after cell damage as a consequence of a pathological condition or the action of harmful agents. Apoptotic activation tends to be less responsive with aging, causing accumulation of non-functional cells and pathological changes such are degenerative diseases or tumor transformation. This overview aims to provide summarized facts about different approaches of apoptosis research, targeting and regulation in tumors especially in leukemic cells as a way of pharmacological manipulation with a potential therapeutic benefit.

Keywords

cell death, hematological malignancy, tumor cell elimination

Introduction

In a broad sense apoptosis is a mode of programmed cell death which is genetically controlled. Generally, the factors of apoptosis are one of the most thoroughly studied biological phenomena which are evolutionarily conserved and immanent to metazoan organisms. Primary physiological role of apoptosis is in physical sculpting of species specific body organs shape at early stage of development. Apoptosis is also the way of elimination of non-functional cells, for example cells that did not establish functional synaptic network in neural system. Normally, in humans billions of cells as being eliminated daily as a part of this "sweeping process". Although, there is a wide range of stimuli and conditions which can cause apoptosis, both physiologically and pathologically not all cells undergo elimination in response to the same stimulus. Radiation or chemotherapy drugs cause damage of DNA in some cells, which can lead to apoptosis induction (Elmore, 2007). Understanding the importance of the apoptosis process as well as

the way of its regulation can contribute to the clarification of key factors that affect cell proliferation and differentiation. Therefore, the overall cells survival has crucial importance in some pathological conditions, especially malignancy. In fact, defects of apoptotic pathways are believed to contribute to numerous human diseases, from neurodegenerative disorders to various types of malignancy (Lowe & Lin, 2000).

Apoptosis activation and mechanisms of control

Molecular machinery of apoptosis is strictly and genetically regulated. It engages BCL-2 (antiapoptotic B cell lymphoma 2) family of tens of genes, classified as pro-apoptotic or anti-apoptotic (pro-survival) genes. There are also many "independent" genes with critical role in regulation of cell-death such as TP53 (*tumor protein 53*) or other genes of cell-cycle regulation which is clearly shown in many genes knock-out studies (Chin & Fu, 1998; Salleh et al. 2004). Critical components of apoptotic process are also caspases, a unique family of cysteine proteases. The initiation of the apoptotic process activates a cascade series of regulatory proteins, caspases (*cysteine-aspartic proteases, cysteine aspartases or cysteine-dependent aspartate-directed proteases*). Depending on the way in which apoptosis is induced, as well as the caspase position in the apoptotic signal cascade, various caspases will be activated. Generally, caspases are divided into initiatory (e.g., caspase-9) and effectors' caspases (e.g. kaspaza-3 and -7). Due to inability of the cell to modulate caspase activity, the apoptosis process can be disturbed and potentially lead to carcinogenesis, autoimmunity, neurodegeneration and immunodeficiency (Parrish et al, 2013).

Mechanism of apoptosis is very complex and involves two main pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial mediated pathway. The extrinsic pathway is activated by ligation of different plasma membrane receptors such as FAS (*Fas cell surface death receptor*), TNF (*tumor necrosis factor*) or TRAIL (*TNF-related apoptosis-inducing ligand*) (Sheikh & Huang, 2004). The intrinsic pathway is activated by mitochondrial dysfunction and release of death promoting proteins: AIF (*apoptosis inducing factor*), EndoG (*endonuclease G*), Smac/DIABLO (*second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI*), Omi/HtrA2 (*mitochondrial serine protease*) (Green & Reed, 1998). Different proteins regulate these pathways including p53, PI3K (*phosphoinositide 3-kinase*), NF-κB (*transcription factor*) and the ubiquitin proteasome system (Ghobrial et al, 2005). There is an additional perforin/granzyme pathway mediated by cytotoxic T cell for induction of apoptosis through either granzyme B or granzyme A (Elmore, 2007).

Regardless of the biochemical differences of activation of apoptosis, the final result is cell death accompanied by the characteristic morphological changes of the plasma membrane, mitochondrial dysfunction, cell shrinkage, chromatin lysis and condensation, nuclear fragmentation, dissociation of cell organelles and formation of apoptotic bodies. In further development of this review we will focus to the research of apoptosis as a key reason for tumor development and progression and the proposed ways for their therapeutic restoration to normal level in leukemia.

Link between apoptosis and tumorigenesis with an emphasis on leukemia

In tumor cells, the normal cell cycle control is dysfunctional, causing over-proliferation of cells and/or decreased cells elimination (King & Cidlowski, 1998) that is fundamental principle of immanent anti-tumor activity. Apoptosis as a biological phenomenon has long been associated with elimination of tumor cells and tumor progression (Kerr et al, 1972). Different changes and defects in apoptotic mechanisms play important roles in tumor pathogenesis accumulating the genetic alterations (Hassan et al, 2014). Deregulation of apoptosis causes misbalance between cell proliferation, cell survival and cell death and plays a major role in the initiation and progression of solid and hematological tumors. Over-expression of apoptosis inhibitors as well as inactivation of apoptosis promoters is observed in human cancers (Kaufmann & Vaux, 2003; Hassan et al, 2014). According to Wong (2011) different

mechanisms contribute to tumor cell evasion of apoptosis and carcinogenesis (Figure 1). However, there is a growing interest for development of therapeutic strategies based on apoptosis regulation and induction in different tumors. Leukemia is heterogeneous group of haemopoietic cancers and represents the 11th and 10th most frequent cause of cancer occurrence and death worldwide (Miranda-Filho et al. 2018). Its development is a multistep process characterized by progressive genetic alterations that leads to the transformation of normal hematopoietic stem cells into leukemic derivates (Testa & Riccioni, 2007). One of the most widespread malignancies of myeloid cells is acute myeloid leukemia (AML) characterized by an accumulation of immature myeloid blasts in the bone marrow. Generally, AML is associated with a poor prognosis and overall survival, especially in the older population and current treatment has been improved only modestly for the past 30 years (Peña-Martinez et al, 2017). The leading approach in leukemia treatment is targeting leukemic cells via induction of apoptosis (Cassier et al, 2017). Currently, targeted therapies designed to induce apoptosis in leukemia are the most promising anti-leukemic strategies for targeting and elimination of the tumor cell, especially without or with limited collateral damage to normal hematopoietic progenitor cells (Testa & Riccioni, 2007).

Multidrug resistance (MDR) in leukemia

An important factor that limits the successful treatment of a wide range of malignancy is tumor resistance to chemotherapeutic agents or to multiple drugs. This phenomenon is known as multidrug resistance (MDR), which is a main reason why chemotherapy treatment outcome vary in cancers. Resistance to multiple drugs is clinically recognized as the development of tumor resistance to a wide variety of antitumour drugs after exposure to a single drug. This phenotype is well known in clinical practice and has been extensively studied, especially in AML (Hunault et al. 1997; Mahadevan & List, 2004). One of the basic mechanisms underlying this MDR phenotype is the active cellular extrusion of chemotherapeutic agents via p-glycoprotein, or resistance to multiple drug-coded MDR1 gene. Today, there are outstanding efforts to improve measuring of MDR in clinical samples, which is a critical step in the correct determination of MDR phenotypes in patients with malignancies (Leith, 1998). MDR mechanism may be developed by increased release of the drug outside the cells and reduced absorption of the drugs (Mansoori et al. 2017). Recent studies (Li & Dalton, 2006; Chapuy et al. 2008; Mansoori et al. 2017) suggest that cell-to-cell interactions, tumor microenvironment, signal transduction molecules or lysosomal drug sequestration could also be factors of MDR

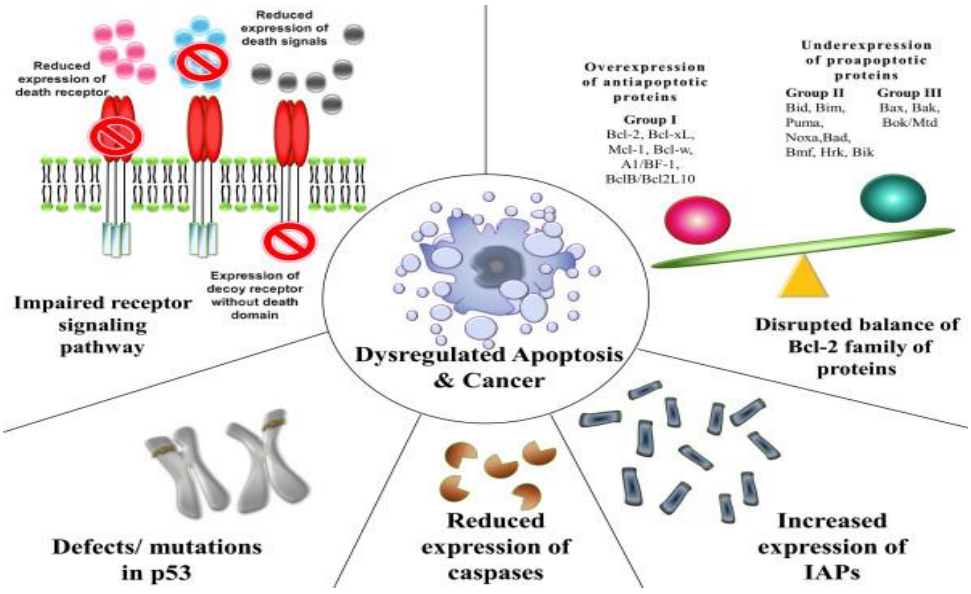

Figure 1. Various mechanisms that contribute to the deregulation of apoptosis and cancer development (Wong, 2011)

development. Interaction between leukemia and stromal bone marrow cells (fibroblasts, adipocytes, myocytes, chondrocytes, etc.) is associated with regulation of stem cell maintenance and localization that stimulate proliferation, self-renewal and differentiation. These cells are involved in the regulation of normal and tumor hematopoiesis due to production of wide range of stimulus and signaling molecules such as cytokines, chemokines, growth factors that activate hematopoiesis precursors. Therefore, it has been shown that this interaction is related and contributes to the development of chemo-therapy resistance in vitro and in vivo (Macanas-Pirard et al., 2017). All MDR mechanisms are potential targets in development of new personalized treatments. Great progress represent new scientific knowledge about the expression level of certain genes and/or proteins in various types of leukemia cells, especially those of primary cultures (Hlozková & Starková, 2018), which offers the new way of creating metabolic profiles and directions of therapeutic action even in individual cases.

In vitro and *in vivo* methods for apoptosis research

In researches of antitumor activity, different types of leukemia cells or cell lines with very heterogeneous characteristics are frequently used. All of these characteristics include differences in their morphology and expression for specific markers that make them universal models of research, especially considering the complexity of leukemia and all of its subtypes. Lymphoblastoid cell lines used in leukemia research are developed by infecting peripheral blood lymphocytes with Epstein Barr virus to immortalize B cells in vitro giving rise to an actively proliferating B cell population (Neitzel, 1986). Epstein Barr virus encoded crucial proteins for cell immortalization which is successful method from the last few decades with minimal amendments and provides an excellent in vitro model system as these cells are relatively easy to prepare and maintain, somatic mutation rate is low and represent an unlimited source of biomolecules (Hussain & Mulherkar, 2012). In addition to primary cell-lines, there is a wide range of commercially available leukemic cell lines and panels which are used in different studies (Table 1). The cytotoxic effects of potential new treatments in leukemia can be studied using several standardized methods for cell viability assessment: vital dye exclusion examines individual cells in a population, whereas the MTT assay for example, provide information about relative proportion of live and dead cells. The most used vital dyes are trypan blue, nigrosin, eosin Y, fast green and neutral red (McCarthy & Evan, 1998). However, these methods cannot distinguish the nature of cell death. Analysis of cyto-morphological alterations in the apoptosis monitoring implies studying of apoptosis by standard light microscopy using histological dyes that enable distinguishing between apoptosis and necrosis. Using fluorescence dyes (Hoechst 33258, acridine orange, Annexin V-FITC/PI) contributes to extended range of fluorescence microscopy, flow cytometric and immunofluorescence assays in apoptosis detection (Ji & Yu, 2015; Zhuo et al, 2015). Analysis of apoptosis can also be conducted using electron microscopy that clearly reveals DNA condensation in the nucleus and other morphologic changes characteristic for apoptosis (McCarthy & Evan, 1998). Also, they utilized a time-lapse video microscopy where cells are viewed microscopically over an extended period making them suitable for various forms of analysis. Czarnota et al. (1999) have described ultrasound imaging of apoptosis; the new non-invasive detection method in vitro, in situ and in vivo. In vivo detection of apoptosis may be very helpful and even crucial in monitoring tumor progression and prediction of the responses to antitumor treatment.

One of the useful methods for assessing apoptosis and monitoring drug response in hematological tumors is based on fluorodeoxyglucose-based positron emission tomography (FDG-PET) described by Newbold et al. (2014). Analysis of apoptosis is also possible using rhodamine 123 fluorescence dye intensity to measure mitochondrial membrane potential via flow cytometry (Ji and Yu, 2015). In addition, DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis. Observations of DNA fragments include the most commonly used TUNEL (Terminal dUTP Nick End-Labeling) method (Darzynkiewicz et al. 2008). Notably, molecular techniques are developed

to detect caspases, cleaved substrates, regulators and inhibitors and include various types of caspase activity assays, western blot analysis, immunoprecipitation and immunohistochemistry. Additionally, apoptosis PCR microarray methodology uses real-time PCR to profile thev expression of genes that encode key ligands, receptors, intracellular modulators, and transcription factors involved in the regulation of programmed cell death (Elmore, 2007).

Table 1. The most common human leukemic cell lines in cytotoxicity studies

Cell line	Type of leukemia	Antitumor approach	Reference
HL-60	Acute myeloid leukemia	cell cycle regulation, induction of apoptosis	Sanchez-Gonzales et al. 2006
		cytotoxic and pro-apoptotic activity	Stanojković et al. 2018
		induction of apoptosis	Nakamura et al. 2001
		cytotoxic and antiangiogenic activity	Jackson et al. 1998
		cytotoxicity induction	van der Weide et al. 2012
		histone methylation blocking, leukemogenic gene expression inhibition	Daigle et al. 2011
		inhibition of cell signal transduction	Kobune et al. 2009
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
		inhibition of proliferation and apoptosis induction	Shashi et al. 2006
Jurkat	Acute T cell leukemia	cytotoxicity induction	Ramage et al. 2003
		cell cycle arresting and induction of apoptosis	Spinozzi et al. 1994
		induction of apoptosis	Lee et al. 2009
		histone methylation blocking, leukemogenic gene expression inhibition	Daigle et al. 2011
K-562	Chronic myeloid leukemia	cell growth inhibition, induction of apoptosis	Zhang et al. 2006
		cytotoxic and pro-apoptotic activity	Stanojkovic et al. 2018
		cell growth inhibition, cell cycle arresting, apoptosis induction	Chun-Guang et al. 2010
		cytotoxic and antiangiogenic activity	Jackson et al. 1998
		apoptotic response and cell differentiation	Benito et al. 1996
		inhibition of cell viability, cell cycle arresting, apoptosis induction	Zhang et al. 2017
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
NB4	Acute promyelocytic leukemia	induction of apoptosis	Rubio et al. 2014
		cytotoxicity induction	van der Weide et al. 2012
		inhibition of cell signal transduction	Kobune et al. 2009
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
HEL	Erythroleukemia	apoptotic response and cell differentiation	Benito et al. 1996
		partial differentiation and apoptosis induction	Roboz et al. 2000
UT-7	Acute myeloid leukemia	inhibition of angiogenesis, growth arresting, apoptosis induction	Spiekermann et al. 2002
		regulation of cellular proliferation and differentiation	Drexler et al. 1998
		cytotoxicity induction	van der Weide et al. 2012
		cytotoxicity induction	Ramage et al. 2003
TF-1	Erythroleukemia	regulation of cellular proliferation and differentiation	Drexler et al. 1998
		cytotoxicity induction	van der Weide et al. 2012
		inhibition of cell signal transduction	Kobune et al. 2009
Kasumi-1	Acute myeloblastic leukemia	inhibition of angiogenesis	Zhang et al. 2013
		histone methylation blocking, leukemogenic gene expression inhibition	Daigle et al. 2011
		inhibition of cell signal transduction	Kobune et al. 2009
MOLT-4	Acute lymphoblastic leukemia	cell growth inhibition and apoptosis induction	Broggini et al 2003
		inhibition of proliferation and apoptosis induction	Shashi et al. 2006
		induction of apoptosis, reduction of cell growth	Mertens-Talcott and Percival 2005
TPH-1	Acute monocytic leukemia	inhibition of cell viability, cell cycle arresting, apoptosis induction	Zhang et al. 2017
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
KG-1	Acute myelogenous leukemia	inhibition of cell signal transduction	Kobune et al. 2009
		inhibition of cell viability, apoptosis induction	Pan et al. 2017
RS4;11	Acute lymphoblastic leukemia	apoptosis induction	Uckun et al. 1995
		apoptosis induction	Dörrie et al. 2001

Development of new therapeutic models based on modulation of apoptosis

Understanding of the apoptotic process has led to the development of new therapeutic strategies in the treatment of various types of cancer. Creating innovative methodology and approaches in apoptosis induction in leukemia and other malignancies, increase the possibility of discovering more effective anticancer therapeutics.

In order to develop and implement more efficient anticancer therapeutics, previous studies have provided a lot of useful information about apoptosis targeting in lymphoid and myeloid leukemia cells by various compounds of different origin. Induction of apoptosis in myeloid cells via activation of caspases under different conditions and treatments has been described (Huang et al, 1999; Yinjun et al, 2004). In this type of tumor cells, regulation of apoptosis by activation or suppression of some signaling pathways is also observed (Pomares et al, 2016; Zhou et al, 2017). There are many natural and synthetic products known as apoptosis inducers in leukemias (Huang et al, 1999; Shashi et al, 2006; Billard 2014; Rubio et al, 2014; Zhou et al, 2017) with different mode of action. Leukemia cells undergo apoptosis in the presence of some plant product, such as some flavonoids (Jihed et al, 2012; Ruela-de-Sousa et al., 2010) and algae extracts (Bechelli et al., 2011). Generally, numerous natural products are recognized as apoptosis inducers in different cancers (Taraphdar et al, 2001).

However, the most antitumor approaches that aim to induce apoptosis are mainly based on regulation of apoptotic pathways in leukemic cells. There are various strategies via activation of the extrinsic apoptotic pathway. Some members of the TNF-family (*three ligands TNF-α, FasL and TRAIL*) directly trigger apoptosis and together with their respective four receptors (TNF-R1, Fas, TRAIL-R1 and TRAIL-R2) have been considered as potential anticancer therapeutics (Testa & Riccioni, 2007). According to Samudio et al. (2009) the majority of leukemia cells express TRAIL receptors, but these samples are notoriously resistant to apoptosis induction by TRAIL. However, in different types of leukemia, especially in AML, many directions in apoptosis mediation and induction are described. That includes regulation of apoptotic process through p53-dependent manner and targeting MDM2/p53 pathway (Cassier et al. 2017) as well as development of XIAP (*X-linked inhibitor of apoptosis protein*) inhibitors (Testa & Riccioni, 2007). XIAP is the most potent endogenous inhibitor of caspase activity which overexpression confers resistance to both mitochondrial and death receptor pathway of apoptosis activation (Samudio et al, 2009). Mitochondrial or intrinsic apoptotic pathway is regulated by pro- and antiapoptotic members of the BCL–2 family. It is confirmed that the most genotoxic chemotherapeutics activate this pathway of apoptosis via activation of p53 signaling, resulting in the expression of proapoptotic target genes (Chipuk et al, 2004). An additional difficulty is affecting the specific fusion proteins, characterized in approximately 50–55% of AML cases that play a key role in the development of leukemia through their effect on cell proliferation, survival and apoptosis (Frohling et al, 2005).

Most cytotoxic drugs kill cells by modulating the process of apoptosis based on the fact that apoptotic markers may be indicators of tumor chemosensitivity. This concept has been investigated in AML by Ong et al. (2000) suggesting that high bax expression was associated with significantly improved survival, emphasizing bax to be an independent predictor of survival. Del Poeta et al. (2008) also confirmed impact of the bax/bcl-2 ratio, determined by flow cytometry, on AML prognosis. Clinical studies of Bcl-2 antisense drug in combination with cytotoxic therapy of AMLs, showed promising results.

BH3 mimetics in modulation of apoptosis

Targeting inhibition of anti-apoptotic factors, especially the membres of BCL-2 family proteins, is one of the leading approaches to activating the apoptosis in tumors. This family of proteins includes pro- and anti-apoptotic crucial factors in apoptosis regulation and control of cell death primarily by interacting with direct binding that regulates the permeabilization of the mitochondrial outer membrane. This leads to irreversible release of intermembrane spatial proteins and caspases thus causing apoptosis (Kale et al, 2018).

Understanding the regulation of apoptosis by BCL-2 factors has led to the development of new class of antitumor drugs targeting anti-apoptotic members by mimicking their natural BH3 proteins (*BCL-2 homologous proteins, BH3-only proteins*), also called BH3 mimetics. BH3 mimetics represent a new class of small molecule blockers with a promising potential in developing targeted therapies based on apoptotic regulation (apoptosis-based targeted therapy). These drugs directly activate apoptosis by binding and inhibiting selected anti-apoptotic protein members from the BCL-2 family (Delbridge & Strasser, 2015). Example of synthetic peptide venetoclax that belongs to a class of BH3 mimetics (Figure 2) is currently approved by the

Malignant stem cells as potential therapeutic target

In the last 50 years, intensive studies have confirmed that one of the leading antitumor strategies in many malignancies, especially in AML, is targeted therapy, or the elimination of malignant stem cells (Dick, 2005). Leukemia stem cells (LSCs) are defined as cells that can initiate the disease when they are transplanted into immunodeficient animals and can be self-renewed to maintain leukemia in series of transplantation. These cells may be partially differentiated into non-leukemic stem cells that resemble progenitor cells and original disease but are not able to self-regenerate (Thomas &

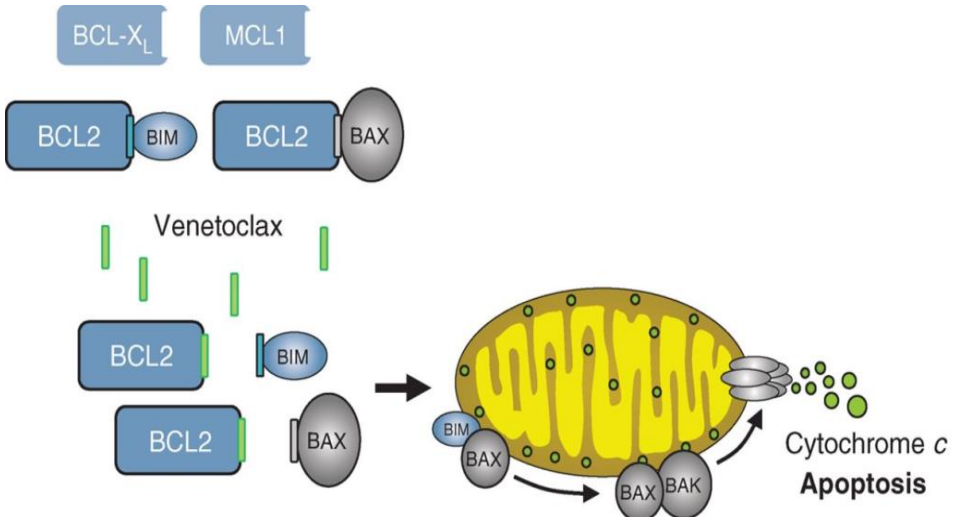

Figure 2. Example of BCL2 inhibition by venetoclax and apoptosis induction via release of proapoptotic proteins (Konopleva et al. 2016)

Food and Drug Administration for the treatment of relapsed/refractory chronic lymphocytic leukemia (CML) (Chung, 2018). Activation and/or re-establishment of apoptosis offer the potential for elimination of tumor cells in all stages of tumorigenesis. As BH3-mimetics become a part of clinical practice, they could significantly improve the outcome of therapy and overall survival (Campbell & Tait, 2018). Moreover, preclinical studies announce the potential for development of BH3 mimetics that target other BCL-2 members, especially in leukemia of myeloid cell. Thus, BH3 mimetics appear to be destined to become a powerful new weapon in the tumor arsenal (Cory et al, 2016).

Majeti, 2017). The earliest conceptual idea that leukemia is hierarchically organized, returns to the fields of this type of research to identify clonogenic progenitors of AML in vitro.

It has been proven that AML, similar to hematopoiesis, is hierarchically organized in vivo (Bonnet & Dick, 1997). Although analogy with stem cells of normal hematopoiesis can be very instructive and give some research guidance, it is important to highlight the very dynamic and unstable nature of LSCs. This is crucial in the development of improved and effective therapy (Pollyea & Jordan, 2017). From a clinical point of view, the tumor stem cell model implies that, in order to eradicate the disease and achieve long-term remissions, potential

treatments must be aimed to eliminate the LSCs population (Reya et al. 2001). In previous studies, the detailed characterization of LSCs has shown the properties of self-renewal, relative rest, resistance to apoptosis and reduced susceptibility to conventional therapy. Certainly, genetic analyzes and profiling of DNA methylation has contributed to expanding knowledge about the epi/genetic profile of this disease (Papaemmanuil et al. 2016). However, the application of these data and their association with the *in vivo* biology of LSCs is still in the intensive research. Two basic strategies for the target treatment of malignant stem cells in leukemia are divided into: therapies that selectively remove LSCs, also called specific therapies and therapies that remove the widespread population of leukemia cells and part of active stem cells. Target therapy may have multiple approaches, among which the most commonly strategies are based on immunological, antigen-specific or metabolic modulation as well as epigenetic regulation (Pollyea & Jordan, 2017).

In conclusion, targeting apoptosis process is one of the promising antitumor strategies, that specifically refers to hematological tumors and its resistance to conventional chemotherapy occurs. Today, induction and modulation of the apoptosis, especially in the cells of various types of leukemia, is applicable as one of the leading anti-leukemic strategies with high potential for developing and promoting targeted therapy.

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Research article

MtDNA VARIATION IN THREE MAIN ETHNIC GROUPS IN TUZLA CANTON OF BOSNIA AND HERZEGOVINA

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Abstract

This study was based on the analysis of mtDNA polymorphisms in three ethnic groups of Tuzla Canton of Bosnia and Herzegovina (Bosniaks, Croats and Serbs). The main aim of this study was to analyze the influences of the maternal gene flow on the genetic profile of ethnic groups. The analysis of mtDNA variation based on relevant restriction fragment length polymorphisms (RFLP) in combination with HVSI variations of the control region enabled the identification of the Western-Eurasian haplogroups (H, I, J, T, W, U, HV, HVO, K, V, X), African/Near East lineages N1a and Asian haplogroup M. Our data indicate a close gene similarity among maternal gene pools of the ethnic groups of Tuzla Canton as well as similar influence of the maternal gene flow on genetic structure of those populations. The presence of important maternal determinants of the Late Glacial expansion (U5a), postglacial re-colonisation of Europe from refugia of southwestern Europe (H, V, U5b1), central-eastern European Plain (U4), Italian Peninsula (U5b3) and Neolithic expansion (U3, N1a, J, T) was noted in the genetic structure of the ethnic groups in Tuzla Canton. Conclusions in our study are consistent with the results of previous studies based on the distribution of mtDNA haplogroups and Y-chromosome haplogroups in three main ethnic groups of modern Bosnia and Herzegovina, suggesting similar effects of the paternal and maternal gene flows on genetic structure of the three main ethnic groups of modern Bosnia and Herzegovina.

Introduction

Mitochondrial DNA (mtDNA) is very informative genetic tool of the population studies in estimation of the genetic differentiation of populations, as well as genetic origin and diversification of the maternal lineages (Chiara et al., 2017; Behar et al., 2012; Malyarchuk et al., 2002, 2003, 2006, 2008, 2010; Palanichamy et al., 2010; Malyarchuk & Derenko 2001; Richards et al., 1998, 2000, 2002; Macaulay et al., 1999; Pala et al., 2009, 2012; Torroni et al., 1996, 2001; Cvjetan et al., 2004; Kivisild et al., 1999; Grzybowski et al., 2007). Classification of the mtDNA variations indicates temporal and spatial

haplogroup/haplotype affiliation of different maternal lineages. Therefore, the European populations predominantly contain haplogroups of the Western-Eurasian mtDNA phylogeny (H, I, J, K, T, U, V, W and X) (Macaulay et al., 1999; Richards et al., 1998; Torroni et al., 1996). Most of mtDNA lineages in Europe are marked as paleolithic (U5a, U5b, U5b1b, U5b3, U4, U2, U1, HV, H, H1, H3, H5, V) whose expansion in Europe is correlated with the Upper Palaeolithic and postglacial re-colonization of Europe after the Last Glacial Maximum (LGM) (Behar et al., 2012; Pala, et al., 2009, 2012; Malyarchuk et al., 2008, 2010; Richards et al., 2000). Other significant haplogroups in Europe are of Neolithic origin from the Near East (N1, J, T1, U3, subhaplogroups of the H and the W) (Derenko et al., 2013; Pala et al., 2012; Palanichamy et al., 2010; Richards et al., 2000).

The region of Tuzla Canton belongs to northeastern part of Bosnia. This region is very specific due to the presence of different archeological sites from ancient and recent past, rich deposits of mineral ores as well as different influence of the migrations of human populations. Many archaeological investigations demonstrate the presence of the prehistoric period of the civilization from the Neolithic in this region (Perić, 2002). During history, the Tuzla Canton, the rest of Bosnia and Herzegovina as well as Balkan region were settled by different Illyrian and Thracians tribes, then Avars, Slavs (Wilkes, 1992; Živković, 2002), new tribes such as the Croats and the Serbs (Marjanović et al., 2005). The expansion of the Ottoman Empire in the fifteenth century greatly influenced the structure of the populations of Bosnia (Malcolm, 1994). Due to its wealth in minerals, the Tuzla region was attractive to immigrants from all over the world. Namely, it has a very important geographic position which is connected to wider region of the Southeast and Central Europe. Therefore, Tuzla region has always been marked by demographic changes, which probably had an important impact on genetic structure of the human populations. Nowadays, this region is multinational and multireligious with three main ethnic groups (Bosniaks, Bosnian Croats and Bosnian Serbs). According to the demographic data of the register of the Federal Office for Statistics of Bosnia and Herzegovina for 2013, the Tuzla Canton has the total population of 445,028 individuals, of which 392,356 Bosniaks, 23,592 Croats, 7,058 Serbs and a small percentage of other ethnic groups (http://fzs.ba/).

The previous population genetic studies of ethnic groups in Bosnia and Herzegovina were conducted by Kovačević et al. (2014) and Marjanović et al. (2005). The study by Kovačević et al. (2014) was based on the analysis of autosomal and haploid lineages of the populations from the Western Balkans, including three ethnic groups from Sarajevo and Zavidovići (Bosnian Bosniaks), Central Bosnia (Bosnian Croats), southern Bosnia and Herzegovina (Bosnian Croats) and Central Bosnia and Banja Luka (Bosnian Serbs). Study by Marjanović et al. (2005) based on the analysis of the Y-chromosome haplogroups in the three ethnic groups in Bosnia and Herzegovina suggests that paternal gene pool of these three groups is predominantly marked with a contribution of the post-glacial expansions from different refuges (the Balkans, Ukraine and Franco-Cantabrian) with lesser impact of the neolithic and postneolithic expansions from the Middle East. Our study is based on the preliminary analysis of mtDNA data in the three main ethnic groups of Tuzla Canton, in order to test the hypothesis on a similar influence of the maternal gene flow on genetic profile of these groups in Tuzla Canton and similar contribution of the paternal and maternal gene flow on gene pool of the three main ethnic groups in Bosnia and Herzegovina.

Materials and methods

Data of 241 mtDNA samples from northeastern Bosnia (Ahmić et al., 2014) were used in the analysis of the haplogroup affiliation of individuals from different ethnic groups of Tuzla Canton (Bosnian-Bosniaks N=201; Bosnian-Croats N=22; Bosnian-Serbs N=18).

The ratio of the number of samples for the sampled groups reflects ethnic structure of Tuzla Canton population according to the data of the Federal Office of the Statistics of the Federation Bosnia and Herzegovina (web:www.fzs.ba). The haplogroup identification of the mtDNA in the three ethnic groups of Tuzla Canton was based on the analysis of

relevant restriction fragment length polymorphisms (RFLP) in combination with variations in HVSI of the control region of the mtDNA (Table 1). HVSI sequences were aligned using BioEdit software through ClustalIW multiple alignment (Hall, 1999) and compared with the revised Cambridge reference sequence (Anderson et al., 1981; rCRS, Andrews et al., 1999). For the haplogroup classification, mitomaster software (www.mitomap.com) and the PhyloTree build 17 were applied (van Oven and Kayser, 2009). The analyses of the mtDNA variations in the three ethnic groups of Tuzla Canton and its comparison with neighboring European populations from narrower geographic region were done using the methods implemented in the Arlequin ver.3.11 software (Excoffier et al., 2005). The haplogroup diversity was calculated according to Nei (1987). Estimation of the genetic differentiation between the comparative groups was based on pairwise FST analysis (Weir & Cockerham, 1984). Multi-dimensional scaling plot was performed on pairwise Fst values using SPSS Statistics 17.0 (Chicago, IL,USA).

Table 1. Haplogroups, method of detection of the coding region polymorphisms and HVSI haplotypes, and frequency (%) of haplogroups in three ethnic groups of Tuzla Canton

Haplogroup	Method detection of the haplogroups	Coding region polymorphisms and restriction enzymes	HVSI (+16000)	Bosnian-Bosnincs (N=201)	Bosnian-Croats (N=22)	Bosnia-Serbs (N=18)
HV	RFLP,sequencing	+7025 *Alu*I	217-243-261	0.50		
HVO	RFLP, sequencing	+7025 *Alu*I	298		4.55	
HVO	RFLP,sequencing	+ 7025 *Alu*I	216-298	0.50		
H	RFLP	-7025 *Alu*I		54.73	45.46	44.45
V	RFLP	-4577 *Nla*III		6.46		5.56
I	RFLP	-1715 *Dde*I, +8249 *Ava*II, +10028 *Alu*I		1.49	4.55	
J	RFLP	-13704 *BstO*I		8.96	4.55	11.11
T	RFLP	+13366 *Bam*HI, +15606 *Alu*I		4.48	4.55	11.11
W	RFLP	+8249 Ava*II*, -8994 Hae*III*		3.48	4.55	
X	RFLP	- 1715 *Dde*I		4.48	4.55	5.56
K	RFLP	+12308 *Hinf*I,- 9052 *Hae*II		3.48	4.55	5.56
U7	RFLP, sequencing	+12308 *Hin*fI	309 – 318	0.50		
U5a1	RFLP, sequencing	+12308 *Hin*fI	256-270		4.55	
U5a1	RFLP, sequencing	+12308 *Hin*fI	75-256-270	0.50		
U5a1	RFLP, sequencing	+12308 *Hin*fI	192-256-270	1.49		
U5a1	RFLP, sequencing	+12308 *Hin*fI	189-192.1-195-234-256-270-311-330-362-384	0.50		
U5b1b1	RFLP, sequencing	+12308 *Hin*fI	16189-192-270	1.00		
U5b1	RFLP, sequencing	+12308 *Hin*fI	93–189–270			5.56
U5b3	RFLP, sequencing	+12308 *Hin*fI	192–270–304	1.00		
U4a2	RFLP, sequencing	+12308 *Hin*fI	356	1.00		5.56
U4a2	RFLP, sequencing	+12308 *Hin*fI	189–242–288–356-362	0.50		
U4c1	RFLP, sequencing	+12308 *Hin*fI	179–356	0.50		
U3	RFLP, sequencing	+12308 *Hin*fI	343	0.50		
U3	RFLP, sequencing	+12308 *Hin*fI	261-343		4.55	
U3a	RFLP, sequencing	+12308 *Hin*fI	343–390	0.50		
U2e	RFLP, sequencing	+12308 *Hin*fI	51-129-182-183-189-362	1.00		
U2e	RFLP, sequencing	+12308 *Hin*fI	51-129-183-189-362			5.56
U2e	RFLP, sequencing	+12308 *Hin*fI	129-179-182-183-189-362		4.55	
U1a	RFLP, sequencing	+12308 *Hin*fI	183-189-249	0.50	4.55	
N1a	sequencing		147G-172-223-248-355	0.50		
N1a	sequencing		147G-172-223-248-295-355	0.50		
M	RFLP, sequencing	-10871 *Mnl* I, +10397 *Alu*I	126-148-309-318	1.00		

Results and Discussion

MtDNA variation in the analyzed groups of Tuzla Canton

The analysis of mtDNA variation in the three main ethnic groups from Tuzla Canton enabled the identification of the typical Western-Eurasian haplogroups (H, I, J, T, W, U, HV, HVO, K, V, and X), African/Near Eastern lineages N1a and Asian haplogroup M (Table 1). The identified haplogroups in the groups of the Bosnian Bosniaks, the Croats and the Serbs of Tuzla Canton represent nearly entire mtDNA variation found in other European populations.

In subcluster diversification of the haplogroups, the mitochondrial haplogroups H, U5, U4, U2, U1, W, X, K and I whose origin is related to different periods of the Late Glacial and postglacial re-colonization of Europe (Pala, et al., 2009; Malyrchuk et al., 2008, 2010; Richards et al., 2000) as well as the Neolithic haplogroups U3, N1a, J and T (Palanichamy et al., 2010; Pala et al., 2009, 2012; Richards, et.al. 2000) were detected. All three ethnic groups of Tuzla Canton have a relatively high frequency of the macro-haplogroup H (haplogroup H): Bosniaks (54.73%), Croats (45.46%) and Serbs (44.45%). The observed frequencies of haplogroup H in the analyzed ethnic groups in Tuzla Canton are in accordance with the frequency of this haplogroup (including all the subhaplogroups) in the analyzed ethnic groups from the other regions of Bosnia and Herzegovina(Central Bosnia, the region of Sarajevo, Banja Luka) (Kovačević et al., 2014).

Namely, haplogroup H is a characteristic haplogroup of almost all European populations, and about 30-50% of the European mtDNA lineages are classified as the haplogroup H (Roostalu et al., 2007; Richards et al., 2002, 2000;). The haplogroup H entered Europe from the Near East ∼20,000–25,000 years ago and expanded during the postglacial re-colonisation of Europe (∼15,000 years ago) from southwestern European refuges (Hernandez et al., 2017; Pereira et al., 2005; Achilli et al., 2004; Richards et al., 2000; Torroni et al., 1998). The observed data of slightly increased frequency of the mitochondrial palaeolithic haplogroup H in all three ethnic groups in our study and the presence of the R-M269 Y chromosome mutation in all three ethnic groups from Bosnia-Herzegovina (Kovačević et al., 2014; Marjanović et al., 2005), which according to the classical theory Semino et al. (2004) spread from the Franco-Cantabrian refuge, can be useful additional information in the consideration of the post-glacial expansion of populations from southwestern European refuges to the Balkan region. According to the data that the paternal haplogrup R and maternal haplogroups H1 and H3 share similar patterns of distribution and evolutionary history (Achilli et al., 2005), additional studies on diversification within haplogroup H in our study are necessary. The study by Kovačević et al. (2014) indicates the presence of sublineages H1 and H3 in all the three analyzed ethnic groups. The second most frequent haplogroup in the total population is the haplogroup U (represented with all known subhaplogroup). Subcluster diversification of the haplogroup U indicates that all three ethnic groups contain the oldest European haplogroup U5, (coalescence time of about 37,000 years) (Soares et al., 2010; Richards, et al., 1998), whose expansion started in central and southern Europe (Malyrchuk et al., 2010). Beside the haplogruop U5, U-haplotypes of the ethnic groups belong to U4a, U4c, U2e and U1 lineages, which spread with Paleolithic migrations in different periods of the Late Glacial and post-glacial re-colonization of Europe, as well as haplogroup U3 whose arrival in Europe is correlated with Neolithic expansion. In the Bosniak group, both clusters U5a and U5b are repesented. The ancestral founder haplotypes U5a1 (with HVSI motif 16192-16256-16270) (Richards et al., 2000), U5b1 (which reached northern Europe from Franco Cantabria in the post-LGM times) (Pala et al., 2012) as well as U5b3 (which expanded along the Mediterranean coast in the Holocene) (Pala et al., 2009) were observed. Also, the Bosniak group contains almost all sublineages of the haplogroup U (U1, U2e, U3, U3a, U4a, U4c, U7). Interestingly, gene pool of the analyzed group of Bosniaks from the region of Sarajevo and Zavidovići (Kovačević et al., 2014) does not contain any of the sublineages of the subhaplogroup U. Bosnian Croat U-haplotypes, beside haplogroup U51a, belong to the U3, U2e and U1 lineages, which have been represented in the

population of the Croats in an earlier study by Cvjetan et al., (2004) while the study by Kovačević et al. (2014) indicates the presence of only U2e haplogroup in both groups of Bosnian Croats. Bosnian Serbian U-haplotypes, beside haplogroup U51b, belong to the components of Middle Upper Palaeolithic U4 and U2 (Richards et al., 2000). In our study, the frequency of the haplogroup U4a2 (5.56%) in Bosnian–Serbs is in accordance with the frequency of this haplogroup in the previous studies of the Serbian populations, 8.63% (Davidović et al., 2015) - 6.80 % (Cvjetan et al., 2004). Also, the ethnic group of Bosnian Serbs from central Bosnia and Banja Luka (Kovačević et al., 2014) indicates an increased frequency of this haplogroup (10.53%). This haplogroup is recognized as an indicator of postglacial re-colonization of Europe from the central-eastern European (after 15,000 YBP ; Pala 2012). The Y hromosom haplogroup R-M17, a possible paternal signal of the expansion of populations from the Ukrainian glacial refuge, (Semino et al., 2000), was observed in all three ethnic populations in modern Bosnia and Herzegovina (Kovačević et al., 2014; Marjanović et al., 2005). Other significant indicators of the population expansions and postglacial re-colonization of Europe from glacial refuge, such as the haplogroup V, which reach this region from Franco Cantabria refuge (Torroni et al., 2001; Richards et al., 2000) have been identified in analyzed ethnic groups from Tuzla Canton (excluding Bosnian-Croats). In our study, the observed mtDNA signals of the postglacial expansions from different glacial refuges are in accordance with the presence of specific paternal signals of the post-LGM event from refuges: Balkan, (I-P37), Ukraine (R-M17) and Franco-Cantabrian (RM269) in three ethnic groups of Bosnia and Herzegovina (Kovačević et al., 2014; Marjanović et al, 2005). The previous study by Dogan et al. (2016) based on the analysis of Y-chromosomal haplogroup distribution in Tuzla Canton indicates that the general population of Tuzla Canton contains about 50% of the I-P37, whose paleolithic origin is related to the expansion along the Balkan Peninsula (Marjanovic et al., 2005) and about 23% of the R1a which characterizes the populations of East Europe (Semino et al., 2000). All three ethnic populations from northeastern Bosnia contain significant mitochondrial determinants of expansion of populations from the Near East to Europe during the Neolithic, such as haplogroups J, T and U3 (excluding U3 in the case of Bosnian Serbs). In our study, the haplogroup J is the third prevalent mitochondrial determinant in total population, with greater frequency in the population of Serbs (11.11%), than Bosniaks (8.96%) and Croats (4.55 %). Also, haplogroup J (subhaplogroups J1b and J1c) was observed in all the three analyzed ethnic groups from central Bosnia and the region of Sarajevo and Banja Luka (Kovačević et al., 2014). The studies by Kovačević et al. (2014) and Marjanović et al. (2005) reported the presence of neolithic Y–chromosome haplogroups E and J whose spread from Near East and Africa occurred during the Neolithic and post-Neolithic period (Cruciani et al., 2004). Interestingly, higher frequency of neolithic Y–chromosome haplogrup E-M78 was noted in the group of Bosnian-Serbs (19.8%) (Marjanović et al., 2005). In our study, gene pool of the Bosniak group contains African/Near East lineages N1a with mutation 16147G (Palanichamy et al., 2010), which occurred in Europe 7500 years ago (Haak et al., 2005). This haplotype is observed in the neighboring population from Serbia, in individuals who originated from Bosnia (Davidović et al., 2015).

Other lineages of the European/Middle Eastern origin (T, K, X and W) with frequency < 5.00% in the ethnic populations were observed. Of interest is the presence of non-European haplogroup M in the sample of Bosniaks, which is also represented in gene pool of populations from Macedonia, Croatia and Serbia (Cvjetan et al., 2004; Malyrchuk et al., 2003) with low frequency.

MtDNA variation in the analyzed groups of Tuzla Canton

Table 2 shows the values of haplogroup diversity of the observed ethnic groups of Tuzla Canton. The value of the haplogroup diversity in the Bosnian-Bosniaks (0.6829) was lower in relation to the Bosnian-Croats and Bosnian-Serbs groups. The analysis of genetic differentiation between pairs of the observed groups (pFST) based on frequencies of the haplogroups is given in table 3.

Table 2. Diversity of the haplogroups in the analyzed ethnic groups

Populations	Genetic diversity
Bosnian-Bosniaks	0.6829 +/- 0.0353
Bosnian-Croats	0.8009 +/- 0.08790
Bosnian-Serbs	0.8039 +/- 0.09070

Table 3. Matrix of genetic differentiation (pFst) between analyzed ethnic groups

Populations	Bosnian-Bosniaks	Bosnian-Croats	Bosnian-Serbs
Bosnian-Bosniaks			
Bosnian-Croats	-0.00391		
Bosnian-Serbs	-0.01274	-0.03163	

Table 4. Haplogroup diversity in the analyzed populations and the compared ethnic groups

Populations	Diversity
Bosniaks (this study)	0.6814 +/- 0.0351
Bosnia (Malyarchuk et al., 2003)	0.7483 +/- 0.0358
Bosnia (Cvjetan et al., 2004)	0.7566 +/- 0.0269
Bosnian Croats (this study)	0.8052 +/- 0.0892
Croatia (Cvjetan et al. 2004)	0.7589 +/- 0.0230
Bosnian Serbs (this study)	0.8039 +/- 0.0907
Serbia (Cvjetan et al., 2004)	0.8078 +/- 0.0330
Serbia (Davidvić et al., 2015)	0.7515 +/- 0.0359
Herzegovina (Cvjetan et al., 2004)	0.7931 +/- 0.0319
Slovenia (Malyrchuk et al., 2003)	0.7534 +/- 0.0406
Slovenia (Šarac et al., 2014)	0.7966 +/- 0.0289
Macedonia (Cvjetan et al., 2004)	0.8009 +/- 0.0290

No significant pairwise Fst differences ($p>0.05$) have been observed among three main ethnic populations from northeastern Bosnia. The Fst values indicate close genetic similarity, which may suggest a common mtDNA pool of the ethnic groups. The results obtained in our study confirm the results of genetic differentiation (< 0.1%) based on the analysis of 15 morphological and physiological parameters (Pojskić et al., 2003). Increased differentiation (5.8%) between ethnic groups of the Bosnian-Croats and Bosnian-Bosniaks, as well as Bosnian-Croats and Bosnian-Serbs observed in the study by Marjanović et al. (2005), based on analysis of the Y-chromosome haplogroups, is probably a consequence of internal immigration of the Croats from the region of Herzegovina. For clearer positioning of the analyzed ethnic groups of Tuzla Canton in the context of populations of former Yugoslavia, we compared the obtained data at the level of frequencies of haplogroups with those obtained in previous studies of the populations: Croatia, Herzegovina (Cvjetan et al., 2004), Serbia (Davidović et al., 2015; Cvjetan et al., 2004), Slovenia (Šarac et al 2014 Cvjetan et al., 2004) and the previously studies populations from Bosnia and Herzegovina (Cvjetan et al., 2004; Malyrchuk et al., 2003). Lower values of the haplogroup diversity were observed between the Bosnian Bosniaks and the comparative European populations (table 4). Lower values of this parameter may be due to slightly higher frequencies of a specific haplogroup such as H in Bosniak group with regard to the compared populations. Table 5 shows the analysis of genetic differentiation between pairs of the observed populations (pFST). Significant pairwise Fst differences ($p<0.05$) between the Bosnian-Bosnianks and populations from Croatia, Serbia, Macedonia and Herzegovina were observed. Multi-dimensional scaling plot was created based on pairwise Fst values (Figure 1). Genetic division between groups of Bosnian-Croats and Bosnian-Serbs and their neighboring populations is probably a consequence of a small sample size for these populations (Figure 1).

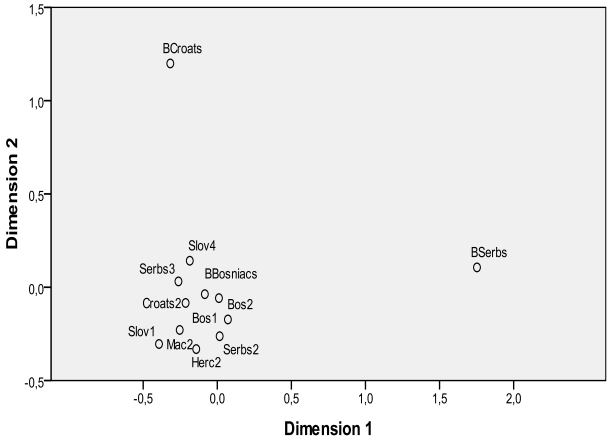

Figure 1. Multi-dimensional scaling plot of populations according to pairwise F_{ST}, constructed from the following data: BBosnianks - Bosnian Bosniaks; BCroats - Bosnian Croats; BSerbs - Bosnian Serbs (this study); Bos1 - Bosnian population; Slo1- Slovenian population (Malyrchuk et al., 2003); (Bos2 - Bosnian population; Croats2 - Croatian population; Serbs2-Serbian population; Her2 - Herzegovinian population; Mac2-Macedonian population (Cvjetan et al., 2004); Serbs3 – Serbian population (Davidović et al., 2015); Slov4 - Slovenian population (Šarac et al., 2014)

Table 5. Matrix of genetic differentiation (pFst) between the compared populations

	B-Bos	Bos[1]	Bos[2]	B-Cro	Cro[2]	B-Ser	Ser[2]	Ser[3]	Her[2]	Slo[1]	Slo[4]	Mac[2]
B-Bos												
Bos[1]	0.0025											
Bos[2]	0.0050*	0.0011										
B-Cro	-0.0115	0.01412	-0.0172									
Cro[2]	0.0084*	0.0029	0.0037	-0.0108								
B-Ser	-0.0149	-0.0194	-0.0161	-0.0378	-0.0235							
Ser[2]	0.0128*	0.0005	0.0002	-0.0152	0.0012	-0.0213						
Ser[3]	0.0059	-0.0007	0.0003	-0.0145	0.0047	-0.0214	-0.0013					
Her[2]	0.0103*	0.0004	0.0009	-0.0170	0.0044	-0.0236	-0.0014	-0.0018				
Slo[1]	0.0001	-0.0053	-0.0010	-0.0168	-0.002	-0.0248	-0.0033	-0.0026	-0.0015			
Slo[4]	0.0291*	0.0164*	0.0174*	-0.0010	0.0010	-0.0227	0.0076	0.0165*	0.0090	0.0088		
Mac[2]	0.0129*	0.0045	0.0044	-0.0166	-0.0004	-0.0260	-0.0007	0.0032	0.0016	0.0004	0.0008	

** p<0.05; B-Bos - Bosnian Bosniaks; B-Cro - Bosnian Croats; B-Ser - Bosnian Serbs (this study); Bos1-Bosnian population (Malyarchuk et al., 2003); Bos2-Bosnian population; Cro2-Croatian popualation; Ser2-Serbian population; Her2-Herzegovinian population; Mac2-Macedonian population (Cvjetan et al., 2004); Ser3-Serbian population (Davidović et al., 2015); Slo1-Slovenian population (Malyrchuk et al., 2003); Slo4- Slovenian population (Šarac et al., 2014).*

Conclusions

The results of our study suggest that mitochondrial DNA pool of the three main ethnic groups of Tuzla region was shaped by early and late migration events which marked the process of settlement of the Balkans. The presence of important maternal determinants of the Late Glacial expansion (U5a), postglacial re-colonisation of Europe from glacial refuges of southwestern Europe (H, V, U5b1), central-eastern European Plain (U4), Italian Peninsula (U5b3) as well as of neolithic expansion (U3, N1a, J and T) was noted in the genetic structure of the ethnic groups. Conclusions in our study are consistent with the results of previous study based on the distribution of Y-chromosome haplogroups in three main ethnic groups of modern Bosnia and Herzegovina (Kovačević et al., 2014; Marjanović et al., 2005), suggesting that the post-glacial expansion of populations from different refuges had a great influence on gene pool of the analyzed populations, while Neolithic and post-Neolithic gene flow had a less important role. There are no significant pairwise Fst differences observed between the three main ethnic groups from Tuzla Canton. These data can indicate common maternal pool of the ethnic groups, as well as similar contribution of the paternal and maternal gene flow on the genetic structure of the three main ethnic groups of modern Bosnia and Herzegovina.

Conflict of interest

The authors report no conflicts of interest.

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Research article

ASSESSMENT OF LISTERINE COOL MINT MOUTHWASH INFLUENCE ON POSSIBLE DNA DAMAGE MEASURED BY BUCCAL MICRONUCLEUS CYTOME ASSAY - PRELIMINARY RESULTS

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Abstract

Listerine is a brand of mouthwash most used worldwide in oral hygene maintainance. Due to its antimicrobic and antifungal characteristics, it can stop/diminish the development of plaque and gingivitis. Among different types of this mouthwash, all 5 ingredients of Listerine Cool Mint, 21.6% ethanol and 4 herbal extracts-thymol, menthol, eucalyptol and methyl salicylate, have shown capacity to cause cell damage and buccal epithelial cells are in direct contact. Buccal micronucleus cytome assay (BMN) measures changes in differentiation as the frequency of basal/differentiated, binuclear, and cells in different phases of cell death-apoptosis/necrosis (cells with condensed chromatin, karriorhectic, pycnotic and karyolitic cells) and changes in genomic stability measured as micronuclei or nuclear buds/broken eggs frequency. Samples from 10 healthy individuals using Listerine Cool Mint mouthwash twice/day during two-weeks treatment were analyzed before and after the treatment. There was no significant influence on cell differentiation and genomic stability on the group level, although micronuclei frequency (MN) of entire group was higher after the treatment (1 vs. 1.5). We also found interindividual differences and showed that hard liquor consumers had higher MN frequency. Future studies should include more individuals, especially those that regularly consume alcohol for the analysis of possible synergistic influence and consequential increase in risk of changes in genomic tability. Genetic polymorphisms in enzymes responsible for metabolism of ethanol should also be considered, since they may drastically influence the duration of ethanol exposure and its metabolite acetaldehyde and also influence genomic instability and possible development of oral squamous cells cancer.

Introduction

Mouthwashes are oral hygienic products used to maintain oral cavity hygiene (Croatian Chamber of Dental Medicine, 2013), usually divided in: alcoholic/aqueous solutions with different active substances such as chlorhexidine, triclosan, hexethidine, hydrogen peroxide, fluorine or essential oils (menthol, eucalyptus ...); or into cosmetic/therapeutic solutions and their combinations. Therapeutic mouthwashes include ingredients with antiseptic/anti-plaque characteristics that can prevent the onset or development of oral diseases, gingivitis and bad breath and inhibit organisms in the oral cavity that cause plaques (Fine et al., 2007) or caries development by preventing demineralization and stimulating the remineralisation of dental enamel and teeth strengthening (Boyle et al., 2014).

In the USA, a product that reduces the appearance of plaque and gingivitis should undergo two clinical trials to obtain a marketing authorization, and these studies must show 15% (estimated proportional reduction) or 20% (arithmetic mean of the estimated proportional reduction) reduction in plaque and gingivitis incidence in patients with mild gingivitis symptoms during testing for at least 4 weeks, taking into account the placebo group (American Dental Association ADA, 2011). In the European Union, mouthwashes are treated as a category of antibacterial products under the supervision of the European Medical Agency (EMA). If such a product is a blend of herbal preparations, it is expected that each individual ingredient must either increase clinical efficacy or alleviate side effects and does not exhibit toxic activity, which is tested in toxicological studies on each component of the blend separately and the blend itself (for more details see EMA webpages about herbals products and WHO, 1996). The most common brand name of mouthwash in the world is Listerine with proven anti-plaque (Johnson & Johnson, 2014a,b) and gingivitis suppression capabilities (Lamster et al., 1983; Gordon et al. 1985), together with strong antimicrobial and antifungal activity (Kubert et al., 1993; Yamanaka et al., 1994; Kasuga et al., 1997; Okuda et al., 1998). Listerine was originally a cosmetic herbal preparation consisting of 4 essential oils, peppermint, Eucalyptus, winter-green and thyme oil (Lambert Pharm Company, 1912; Vlachojanis 2015, 2016). Its composition has changed over the centuries and finally extracted oil mixture consists of methanol (0.042%), eucalyptol (0.092%), methyl salicylate (0.06%) and thymol (0.064%) in a 27% ethanol solvent (21.6-26.9%) (FDA, 2003; Vlachojanis, 2015, 2016). Today methanol and methyl salicylate are synthesized, so they are no longer natural herbal extracts. Listerine also contains inactive ingredients: water, ethanol (21.6%), sorbitol, flavor, poloxamer 407 (polymeric stabilizer that increases the solubility of poorly water-soluble compounds), benzoic acid, sodium saccharin, sodium benzoate and a protected compound of Listerine brand FD & C Green No. 3.

Since Listerine contains 21.6% of alcohol, which is known to cause oxidative damage and is already metabolised in the mouth, which of itself can cause an increased amount of DNA damage, the hypothesis of this paper was that Listerine Cool Mint stimulates apoptosis in the buccal cells and that due to the potential genotoxic effect on DNA it may have a lasting effect in changes of cells genomic stability.

Long-term exposure to high alcohol content should also favor genotoxic effect and stimulate programmed cell death due to increased membrane permeability and cell dehydration (Manzo-Avalos & Saavedra-Molina, 2010). The two general aims of the study were: (a) to determine whether two-week treatment with a specific mouthwash containing 21.6% ethanol causes changes in the rate and frequency of buccal cell differentiation, and whether it induces apoptosis (fused chromatin, karryocytic cells, pycnotic and kariolytic cells) and (b) to determine the effect on the amount and form of genomic defects measured as differentiated cells with micronucleus, and with the nuclear bud/broken egg structure.

Materials and methods

Volunteers and questionnairrie

Ten healthy subjects, 5 male and 5 female, from the Zagreb area participated in the study. All respondents were less than 60 years old due to the fact that the parameters for micronucleus test on buccal cells change for the age group over 60 years.

After the interviewers had explained the purpose of the research and the manner of conducting the treatment, volunteers completed questionnaires and gave written consent. The questionnaires included questions about the lifestyle, general diet and habits and a detailed food and drink description consumed

Table 1. Answers from questionnaire on lifestyle and habits of respondents.

QUESTIONS	ANSWERS (N)			
SEX	Male	Female		
	5	5		
AGE				
	Mean	Standard deviation	Range	
	32	10.64	18-51	
EXPOSURE TO TOXIC AGENTS	Pesticides, herbicides, fungicides	Paints, varnishes, adhesives	Processing of wood, metal	Cytotoxic drugs, organic solvents
	0	0	0	3
ALCOHOL CONSUMPTION	Few times/week	Few times/month	Never	
	3	6	1	
SMOKING	Yes	No		
	5	5		
DIAGNOSTIC/THERAPEUTIC TREATMENTS IN HOSPITAL	Ionizing radiation	Surgery		
	3	0		
DISEASES	Chronic	Tumour		
	2	0		
THE USE OF DRUGS	Antibiotics	Analgetics	The rest	
	2	0	0	
THE USE OD SUPPLEMENTS IN DIET	Vitamins	Minerals	Fish Oil	
	3	2	0	

24h before sampling. If the volunteers used Listerine or other mouthwashes, they were not supposed to take them at least three months before the study started. The summary of the main questions with answers is given in Table 1.

Ethics Committee

This research was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb.

Treatment of subjects

The subjects used Listerine Cool Mint mouthwash twice a day, in the morning and evening. Each time 20 mL of solution was swished in the mouth during 30 seconds without rinsing, as suggested by the producer for regular use.

The treatment lasted for 2 weeks. After the treatment examinees were asked for a subjective impression and they all noted the burning sensation while swishing the solution in the mouth.

Sampling, slide preparation and scoring

Unless otherwise stated, the reagents were purchased from Sigma Aldrich, USA, plastics from Eppendorf, Germany and the glass slides and coverslips from Biognost, Croatia. The samples were taken before and after the two weeks of Listerine use. All respondents gave their samples after washing the mouth three times with water to remove bacteria and dead cells. The samples were collected with a sterile hard tooth brush with a small head so that the subjects made 10 circles on the inside of each cheek, after which the brush was immersed in a conical polypropylene centrifuge tube (50 mL) containing 20 mL buffer for buccal cells. All the solutions were prepared and samples processed according to the protocol by Thomas et al. (2009) with few modifications (Milić et al., 2018; Pastorino et al., 2018). The samples were processed on the day of collection. After centrifugation, supernatant was removed and the cells resuspended in 20 mL buccal buffer and aspirated with 18G needle. The procedure was repeated three times. The step involving homogenization was omitted because it caused excessive cell loss. In order to increase the number of clearly separated cells, after the third centrifugation of the suspension, the cells were aspirated and expelled 6 times through 18G needle.

The suspension was then filtered through a 100 μm nylon filter. The nylon filter was placed in the filter holder along with the rubber seal to prevent fluid leakage near the filter. The cell filtrate was collected in 15 mL centrifuge tubes. The sample was centrifuged for 10 minutes at 1600 rpm. Upon completion of centrifugation the supernatant was removed and the cells resuspended in 1 mL of buffer.The average number of cells in suspension was determined using the Burcker-Turk Cell Counting Chamber. Depending on the result obtained, the cell suspension was diluted with the buccal cell buffer to a desired concentration of 80,000 cells/ml.Since the cells were already well separated, it was not necessary to add dimethylsulfoxide to the pellet (used when the cells are aggregated, Thomas et al., 2009). Microscope slides pre-cleaned with ethanol and cytocentrifuge sample funnels were prepared. For each volunteer, 4 slides were prepared. Sixty μL of the buffer was added to each the centrifuge funnel and centrifuged for 3 minutes at 600 rpm. Upon completion of centrifugation, 120 μL of cell suspension was added to the same funnel and centrifuged for 6 minutes at 600 rpm. The prepared slides with samples were detached from the funnel and were left to be air-dried at room temperature. The dry slides were fixed for 10 minutes at 4°C in a Coplin vessel filled with 200 mL of the fixation solution (cold glacial acetic acid: cold methanol, 1:3, Kemika, Croatia) and left to dry at room temperature.

Afterwards, the slides were kept for 1 minute in 50% ethanol and then in 20% ethanol and allowed to dry. The dried slides were immersed in freshly prepared 5 M HCl (Kemika, Croatia) for 30 minutes and washed in water for 3 minutes thereafter.

After drying, the slides were placed in a Coplin's container with Schiff's reagent for 1.5 hour, at room temperature, protected from the light. After washing in water for 5 minutes, the slides were stained by immersion in a 50% water solution of Fast Green for 2 seconds and thoroughly washed with distilled water (Yasenka Vukovar, Croatia). When the slides were dried , 1-2 drops of DePex adhesive was applied to the sample area by means of which a coverslip wasmounted, ensuring that the adhesive was evenly distributed so that no bubbles remain. The slides were left overnight in the hood to dry and stored in a slide box at room temperature. The cells were counted on a fluorescence microscope at a 400-fold magnification with oil immersion. 1000 cells were counted to determine the frequency of each cell type: basal, differentiated mononuclear, binuclear, cells with condensed chromatin, karryorhectic, picnotic and karyolitic cells. Thereafter, 2000 differentiated mononuclear cells were screened to determine the presence of micronuclei, nuclear buds and broken eggs (Thomas et al., 2009; Tolbert et al., 1992). The data were processed using STATISTICA 13 (StatSoft, Dell) software. The data from the questionnaire itself and the data analyzed by the buccal micronucleus cytome assay were analyzed by descriptive statistics. Mann Whitney's U-test was used to compare the groups before and after the treatment. Spearman rank correlation analysis was used to correlate the damage in the whole group and in each individual, before and after the treatment. ANOVA variance analysis was used to check variation within the group. Each of the examined categories in the micronucleus test was analyzed by a chi square for each individual comparing the results before and after the treatment. Nuclear buds and broken eggs cathegories were merged into one variable.

As for the micronuclei, they were analysed in 2000 differentiated cells but they were expressed as MN frequency per 1000 differentiated cells. Statistical significance was set at ≤ 0.05.

Results and Discussion

Buccal micronucleus cytome assay, unlike the micronucleus assay on lymphoctyes, is a non-invasive and simple technique, and, requiring no cell culture establishment, it can give the results on the day of sampling. Since the cells, after differentiation from the basal cells, do not have active repair mechanisms and they are sufficiently large, any morphological changes after the exposure to a harmful agent can be easily recognized with little doubt. The technique comprises measuring the incidence of micronuclei, small circular retentions in cell cytoplasm after initial cell division (basal cells) which is a sign of the loss of an entire chromosome or its part, the nuclear bud as an indicator of the future micronucleus not yet separated from the

nucleus or amplification of genes, and structures called broken eggs that were merged with nuclear buds, although it is still uncertain whether buds and broken eggs are of the same origin. The technique can also measure changes in the frequency of apoptotic/necrotic events and changes in cytokinesis. Since buccal cells are constantly distributed and regenerated, if they are exposed to an agent such as mouthwash, in two to three weeks the damage to cells in the form of micronuclei or nuclear buds/broken eggs can be detected (Paetau et al., 1999; Gillespie 1969).

For this reason, the selected treatment of 2 weeks of exposure may show the changes measured by this test. In this preliminary study we wanted to examine the effect of a two week treatment of oral cavity with Listerine Cool Mint on the proliferation and differentiation cycle of the buccal epithelial cell and their genomic stability in a group of ten healthy randomly selected individuals.

The results of the buccal micronucleus test on the whole group were compared before and after the treatment (Table 2).

Table 2. Descriptive analysis of entire group before and after the treatment

Variable	Mean	Median	Min	Max	SD	SE	
BC	2.80	1.50	0.00	9.00	3.12	0.99	
DF	700.60	789.50	298.00	887.00	198.75	62.85	
CC	55.80	56.50	27.00	87.00	19.98	6.32	
KARRC	21.40	14.50	8.00	75.00	19.85	6.28	
PC	5.70	4.50	2.00	16.00	4.45	1.41	
KYC	207.60	134.00	45.00	583.00	184.16	58.24	
BN	6.10	2.50	0.00	34.00	10.16	3.21	**BEFORE**
MN	2.00	2.00	0.00	5.00	1.56	0.49	
BE	2.70	0.50	0.00	10.00	3.86	1.22	
NB	1.30	0.50	0.00	6.00	2.06	0.65	
MN freq	1.00	1.00	0.00	2.50	0.78	0.25	
BE+NB total	4.00	2.50	0.00	16.00	5.23	1.65	
BE+NB freq	2.00	1.25	0.00	8.00	2.61	0.83	
BC	3.80	3.00	0.00	11.00	3.68	1.16	
DF	771.00	789.00	678.00	858.00	71.84	22.72	
CC	65.40	62.00	24.00	121.00	32.61	10.31	
KARRC	18.90	15.50	0.00	69.00	20.10	6.36	
PC	4.60	3.50	0.00	15.00	4.48	1.42	
KYC	130.40	114.50	58.00	260.00	69.74	22.05	
BN	5.90	3.00	1.00	21.00	6.69	2.12	**AFTER**
MN	3.00	2.50	0.00	7.00	2.40	0.76	
BE	2.90	2.50	0.00	10.00	2.88	0.91	
NB	1.80	0.50	0.00	9.00	2.90	0.92	
MN freq	1.50	1.25	0.00	3.50	1.20	0.38	
BE+NB total	4.70	3.50	1.00	10.00	3.33	1.05	
BE+NB freq	2.35	1.75	0.50	5.00	1.67	0.53	

B-basal cells, DF-differentiated cells, CC-cells with condensed chromatin, KARRC-karriorhectic cells, PC-pycnotic cells, KYC-karyolitic cells, BN-binucleated cells; counted in 1000 cells; MN-micronucleus, BE-broken egg, NB-nuclear bud; counted in 2000 DF, frequency at 1000; Min- Minimum, Max- maximum, SD-standard deviation, SE-standard error, freq-frequency

Mann Whitney-U test for the entire group did not show statistically significant differences before and after the treatment. Cell differentiation results were similar to Thomas et al. (2009) for the younger population. Spearman correlation demonstrated that the incidence of differentiated cells was in negative correlation with the occurrence of karyolitic cells (R=-0.732682) and karryorhectic cells (R=-0.574289), indicating that two weeks treatment causes higher percentage of cell departure in later phases of apoptosis.

Although three studies conducted *in vivo* with the intention of studying the cytotoxicity of Listerine demonstrated no statistical significance in buccal cells, Tsourounakis et al. (2013) demonstrated that the use of Listerine Hydroxide induced apoptosis of almost the entire population of human gingival fibroblasts and periodontal ligament fibroblast24 hours after treatment for 60 s. Ros-Llor and Lopez-Jornet (2014) in a similar two-week treatment of 80 people divided into 4 groups of 20 showed that there was no significant difference in nuclear rupture between different mouthwash groups. They used chlorhexidine (no alcohol), triclosan, mouthrinse with oil extracts in ethanolic solution, with control group receiving the placebo mouthwash-physiological saline. The problem of the Listerine basic formula is that it consists of: 21.6% ethanol, a substance that is associated with the risk of developing oral tumors (Wight & Ogden, 1998; Fioretti et al., 1999; Schlecht et al., 1999; IARC Monographs, 2012). Then there is2.69 mM menthol that showed a cytotoxic activity on cell line A-375 at a concentration of 0.012 mM with 50% survival of cells (LC50) (Kijpornyongpan et al., 2014), and that acts on the lipid phase of plasma membrane (Kupisz et al. 2015). Listerine thymol concentration is4.3 mM, and besides LC50 at 0.7 mM (Stammati et al., 1999), thymol demonstrated in those lower concentrations its antibacterial capabilities (Shapiro et al., 1994; Didry et al., 1994; Botelho et al., 2007; Karpanen et al. 2008) in the Hep-2 cell line (Pemmaraju et al., 2013; de Vasconcelos et al., 2014). It also demonstrated that it affects cell membrane damage and hence the release of intracellular substances and changes in transmembrane potential (Shapiro & Guggenheim, 1995), probably not acting on calcium TRP channel receptors but through mitochondrial damage and stimulation on apoptosis. Then there is 3.94mM methyl salicylate for which a pilot document for the determination of the initial *in vitro* dose for acute toxicity testing has been found to have an LC50 value of 1.7mM (Website, 2001; Vlachojannis, 2015). Eucalyptol in Listerine has the least toxic effect (Ribeiro et al., 2006; Wang et al., 2012), but it has also been shown by SEM microscopy it can cause damage the cells (Dörsam et al., 2014; Zengin & Baysal, 2014).

The frequency of micronuclei after treatment was slightly higher (1.5 vs. 1 before treatment), but still within the limits of normal values recommended by Bonassi et al. (2011) (upper limit of 1.7 micronuclei per 1000 differentiated cells).

Due to statistical analysis, we have combined the category of nuclear buds and broken eggs into one. Spearman correlation showed that the occurrence of micronuclei after the treatment was in a positive correlation with the frequency of binuclear cells (R=0.693673), condensed and kariorrhectic cells (R = 0.748022). This means that individuals with higher DNA damage also had higher percentage of cells moving into early and late apoptosis.

Although the whole group after treatment did not differ significantly from the results beforethe treatment considering the genomic stability parameters, in some individuals the treatment caused greater number of micronuclei and with them a greater number of binuclear cells, and a decrease in the number of differentiated with the increase in the number of cells in the late phase of apoptosis. Such results show the existence of interindividual differences in the group and the presence of individuals that are more sensitive to Listerine exposure.

ANOVA analysis has shown that there are differences in the incidence of different types of differentiated cells and the frequency of genomic damage within groups, and therefore each individual was analyzed separately by means of hi-squares. Although the group was small, the three people who stated that they consumed larger amounts of strong alcoholic beverages had higher frequency of genomic damage (micronuclei and nuclear buds) after the treatment (Table 3). Smokers also showed higher incidence of micronuclei, but as the two

Table 3. Results of the buccal micronucleus cytome assay for each individual before and after the treatment

samples		Cell differentiation changes							Genomic instability freq.		
		B	DC	CC	KARRC	PC	KYC	BN	MN	*BE*	NB
I	before	0	754	87	8	5	139	7	3	0	0
	after	1	829	71	12	4	79	4	2	1	0
hi-square, p		1,P=0.3173	17.04,P=<.0001	1.76,P=0.1846	0.81,P=0.3681	0.11,P=0.7401	18.53,P=<.0001	0.82,P=0.3652	0.2,P= 0.6547	1,P=0.3173	
II	before	0	298	70	29	16	583	4	3	0	4
	after	0	685	121	20	2	157	15	2	3	0
hi-square, p		0, P = 1	299.62,P=<.0001	15.06,P=0.0001	1.69,P=0.1936	10.99,P=0.0009	389.27,P=<.0001	6.43,P=0.0112	0.2,P=0.6547	0.14,P = 0.7083	
III	before	1	831	72	11	2	49	34	1	0	0
	after	1	840	57	8	0	73	21	7	0	1
hi-square, p		0, P = 1	0.29, P = 0.5902	1.86,P= 0.1726	0.48,P = 0.4884	2,P = 0.1573	5.03,P = 0.0249	3.16,P=0.0755	4.52,P=0.0335	1,P=0.3173	
IV	before	2	887	38	20	7	45	1	3	3	1
	after	6	691	24	2	15	260	2	1	4	4
hi-square, p		2.01,P = 0.1563	115.38,P=<.0001	3.26,P = 0.071	14.89,P=0.0001	2.94,P = 0.0864	178.83,P=<.0001	0.33,P=0.5657	1,P = 0.3173	1.34,P = 0.247	
V	before	9	460	69	75	4	375	8	5	4	0
	after	5	762	113	20	6	92	2	3	0	9
hi-square, p		1.15,P = 0.2835	191.86,P=<.0001	11.7,P= 0.0006	33.43,P= <.0001	0.4,P = 0.5271	223.74,P=<.0001	3.62,P=0.0571	0.5,P = 0.4795	1.93,P = 0.1648	
VI	before	7	793	55	13	2	129	1	1	9	1
	after	3	678	79	19	3	216	2	3	4	0
hi-square, p		1.61,P = 0.2045	33.99,P = <.0001	4.61,P= 0.0318	1.14,P = 0.2857	0.2,P = 0.6547	26.51,P = <.0001	0.33,P=0.5657	1,P = 0.3173	2.58,P = 0.1082	
VII	before	5	852	27	9	10	97	0	0	0	1
	after	0	858	62	7	9	62	2	1	2	1
hi-square, p		5.01,P = 0.0252	0.15,P = 0.6985	14.41,P=0.0001	0.25,P = 0.6171	0.05,P = 0.8231	8.37,P = 0.0038	2,P = 0.1573	1,P = 0.3173	1,P = 0.3173	
VIII	before	1	786	55	13	3	141	1	2	1	0
	after	8	831	29	69	1	58	4	4	10	0
hi-square, p		5.47,P = 0.0193	6.54,P = 0.0105	8.4,P = 0.0038	39.88,P= <.0001	1,P = 0.3173	38.44,P = <.0001	1.8,P = 0.1797	0.67,P= 0.4131	7.38,P = 0.0066	
IX	before	2	530	27	16	2	420	3	2	10	6
	after	3	816	36	0	2	137	6	0	3	3
hi-square, p		0.2,P = 0.6547	185.84,P=<.0001	1.33,P= 0.2488	16.13,P= <.0001	0,P = 1	199.29,P=<.0001	1,P = 0.3173	2,P = 0.1573	4.57,P = 0.0325	
X	before	1	815	58	20	6	98	2	0	0	0
	after	11	720	62	32	4	170	1	7	2	0
hi-square, p		8.38,P = 0.0038	25.29,P = <.0001	0.14,P= 0.7083	2.84,P = 0.0919	0.4,P = 0.5271	22.34,P = <.0001	0.33,P=0.5657	7.02,P= 0.0081	2,P = 0.1573	

B-basal cells, DF-differentiated cells, CC-cells with condensed chromatin, KARRC-karriorhectic cells, PC-pycnotic cells, KYC-karyolitic cells, BN-binucleated cells; counted in 1000 cells; MN_micronucleus, BE-broken egg, NB-nuclear bud; counted in 2000 DF, frequency (freq.) at 1000 DF; hi-square and Pearson with p

subjects reported higher alcohol consumption, this small group could not be examined further. If the cumulative effect and the synergistic effect of all components of Listerine are considered together, these results would mean devastating consequences for tissues exposed to Listerine (Bassole & Juliani, 2012). The same components of Listerine have shown toxic effects *in vitro* and *in vivo*, and the greatest contributor may be due to the large amount of ethanol in Listerine Cool Mint, which is considered to be the most responsible for the possible toxic effects of prolonged exposure to Listerine. Although consumed ethanol should only be metabolised in the liver, there is evidence that the microorganisms in the oral cavity can also metabolize ethanol and the first metabolite derived from the alcohol dehydrogenase enzyme activity is acetaldehyde that is as toxic as ethanol itself in *in vitro* conditions and cellular models (Homann et al., 1997; Obe & Ristow, 1977). Acetaldehyde remains longer in the the oral cavity saliva and can affect the decrease in basal cell count, and thus cause epithelial atrophy (Mascres et al., 1984). In the oral cavity there are microorganisms that can metabolize ethanol and convert it into acetaldehyde, which also has toxic effects on cells *in vitro* and *in vivo* and according to carcinogen classification is placed in group 2B. Ethanol metabolism starts already in the mouth by bacteria (Homann, 1997) and its first metabolite acetaldehyde exhibits even more intense toxicity, as demonstrated in some cellular and animal models (Homann et al., 1997; Obe & Ristow, 1977). A one-time use of mouthwash containing ethanol resulted in increase in the acetaldehyde level in the saliva to the level normally present after consuming alcoholic beverages (Lachenmeier et al., 2009). After adding 0.5 grams of alcohol per kilogram of body weight corresponding to the consumption of half a liter of wine, the acetaldehyde level was between 50-100 μM, which is the range of concentrations that can cause mutagenic effects, such as inherited changes in the cell genome. However, Seitz and Stickel (2007) have shown that after using alcohol-containing mouthwash for two weeks, the acetaldehyde concentration is reduced by approximately 30-50%, suggesting that the reduction of the presence of oral bacteria decreases the concentration of toxic substances in the mouth. Mechanisms of acetaldehyde genotoxic activity are adduct formation in DNA molecules, cross-linking of DNA chains, DNA-protein crosslinking, and increased frequency of sister chromatids exchange (Seitz & Stickel, 2007). The IARC (International Agency for Cancer Research) proclaimed the aldehyde as a possible human carcinogen and placed it in group 2B (IARC Monographs, 1999). Vlachojannis et al. (2016) reviewed 19 studies on Listerine mouthwash. Of these 19 studies, 16 focused on the efficacy of Listerine, and only 3 investigated the potential harm of the solution. Although the FDA (Health and Human Services) rated Listerine as safe and effective in 8 of the 16 clinical studies (efficacy, non-harm) conducted until then, doubts still exist because, according to the findings of this study, the guideline hardly exceeds the presumed margin of harm, and only 8 of the above confirmatory studies were performed at clinically significant conditions for 6 months (Vlachojannis et al., 2016).

Listerine, unlike other mouthwash formulations such as 0.2% chlorhexidine, the gold standard among the mouthwashes that is always used as comparator, shows no toxicity in short term (a period of a few days) but reaches its maximum after two weeks, when the effect 0.2% of chlorhexidine and Listerine is equal, as shown by Haerian-Ardakani et al. (2015) and this reduction of bacterial count goes down to a factor of 2. The authors have shown that Listerine eliminates harmful effects of bacteria during this period to a sufficient extent, thus preventing the formation of acetaldehyde.Therefore a treatment of at least two weeks allows the evaluation of the effects of ethanol itself on the buccal cells.

There are studies that show that oral exposure to ethanol increases the risk of developing oral cavity cancer (Wight & Ogden, 1998; Fioretti et al., 1999; Schlecht et al., 1999) and that additional exposure to smoking increases the risk of developing malignant neoplasms (Schlecht et al., 1999).

The results obtained in our study showed no significant genotoxic effect of Listerin exposure, although the values for micronuclei were higher after the treatment, but also showed that individuals who consume larger amounts of alcohol are more sensitive and this group also includes smokers. Reis

et al. (2002, 2006) demonstrated that chronic exposure to alcohol causes an increased incidence of micronuclei even in non-smokers, but this change was not statistically significant (Reis et al., 2002, 2006).

Concerning smokers, in our research, no particular conclusions or important correlations could be reached-because heavy smokers (more than 40 cigarettes per day) were at the same time the consumers of strong alcoholic beverages so that the genotoxic effects could not be attributed solely to the influence of tar and nicotine. According to this logic, alcohol would also be unacceptable as genotoxin, but other authors' research provided enough evidence that ethanol is taken as a major factor, and is also present in the Listerine content in sufficient proportion to be taken into account. Since ethanol is not carcinogenic, the mechanisms are yet to be clarified how ethanol influences genomic instability, and thus the development of tumor lesions. Although our research has shown that after the treatment the whole group had slightly elevated frequencies of micronuclei, these results were within the limits of normal values.

However, there is a large individual variation in the resulting lesions and cell differentiation, indicating that there are probably some other mechanisms that cause such great differences. People who consume regular quantities of strong alcoholic beverages showed higher values for micronuclei after the treatment (Pastorino et al., 2018), indicating that an additional source of ethanol other than that of Listerine two times daily may increase the effect on genomic stability.

Vlahojannis et al. (2015) showed that 27% ethanol has higher antimicrobial activity than Listerine. Alcohol in Listerine was also responsible for the cytotoxic effect of Listerine on gingival fibroblast (Eick et al., 2011) and stem cells (Park et al., 2014) and reduction in the number of primary human gingival fibroblasts and primary human nasal epithelial cells (Schmidt et al., 2016).

Vlachojannis et al. (2016) reviewed the results of the research on all types of Listerine mouthwashes and found that in 16 studies Listerine improved health and maintainance of oral hygiene, but that this still does not mean that Listerine is safe from the toxicological view in short and especially long term use. Assays for long term exposure should include factors affecting metabolism and prolonged exposure to harmful ethanol metabolites, such as the genetic polymorphisms. Namely, there are 5 types of alcohol dehydrogenase enzyme in humans, of which two enzymes (ADH2 and ADH3) may have polymorphic (non-mutated) forms that can affect faster or slower metabolism of ethanol and thus shorter or longer exposure to this harmful agent. Polymorphic ADH3 strongly affects the metabolism of ethanol in acetaldehyde, and ADH3 1 allele carriers can metabolise ethanol faster than ADH3 2 allele carriers. Also, people with this enzyme deficiency have an increased risk of developing oral cancer associated with heavy alcohol ingestion (Carretero et al., 2004).

Due to the lack of understanding of cancer mechanisms, the scientific community has not yet ruled out the use of alcoholic antiseptics as an actor in the development of oral cancer (American Dental, 2009; Boyle et al., 2014), although there have been studies that provide evidence for this link (Currie & Farah, 2014). In our study, interindividual differences could mask actual relationship. In the future studies, the volunteers should be chosen with similar life styles in order to reduce the effect of variables that contribute to cytometry variations before exposure to the selected substance. This logic has even greater weight when it comes to the buccal mucosa, which is highly adaptive tissue when it comes to environmental pressures, and exhibits the most diverse profiles in healthy persons. But even if subjects are classified as healthy, the styles and the place of life dictate the whole line transition from "healthy" to "prone", for example by using a water-based Listerine which components themselves have proven to have an adverse effect.

However, considering that the correlation between the use of alcoholic antiseptics and oral cancer development has not been unambiguously and fully demonstrated, but not completely rejected as yet, dentists should not recommend long-term use of alcohol-based antiseptics.

Some vulnerable groups of people such as the smokers, people with alcohol intolerance and alcohol dehydrogenase deficiency and other people with higher risk of developing oral cancer should limit such use if needed.

Conclusions

The buccal micronucleus cytome test proved to be a sensitive method for analysing changes in cell differentiation, the frequency of apoptotic/necrotic cells, and changes in genomic stability in a 2 week exposure to Listerine Cool Mint mouthwash according to the manufacturer's instructions. The results of this small study conducted on ten individuals did not demonstrate statistically significant effect of this mouthwash on the differentiation and genomic stability of the buccal cells, although the entire group had higher micronuclei frequency and showed a significantly higher incidence of apoptosis. Inter-individual differences have shown some indications and guidelines for similar research on a large number of people. Individuals who regularly enjoy hard liquor had higher number of microanalysis and nuclear buds but did not express distinct differences in differentiated cells, indicating that alcohol did not affect rapid apoptosis, but Listerine along with additional concentrations of alcohol from alcoholic beverages demonstrated a synergistic effect. Also, greater values of genomic instability were observed in smokers. This knowledge should be verified on a large number of people with similar habits (regular consumers of hard liquor with and without smoking habits) to assess the extent to which lifestyle affects genomic stability. Genome-specific SNP (single nucleotide polymorphisms) variants associated with metabolism of ethanol to acetaldehyde should also be included in the following studies.

Conflict of interest

Authors declare no conflict of interest.

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Research article

ROLE OF HOST AND VIRAL FACTORS AND GENETIC VARIATION OF IL28B ON THERAPY OUTCOME IN PATIENTS WITH CHRONIC HEPATITIS C GENOTYPE 1B FROM SERBIA

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Abstract

Viral and host factors in hepatitis C virus (HCV) infection can influence on therapy outcome to pegylated interferon/ribavirin (PEG-IFN/RBV) and progression of liver fibrosis. Although novel direct-acting antivirals (DAAs) represent promising successful treatment of hepatitis C infection, the majority of patients are deprived of this therapy because of its expensiveness and therefore they remain untreated. Also, the efficacy of this novel treatment may be affected by the presence of resistance-associated substitutions (RASs). This study was designed to describe associations between baseline host and viral factors, progression of liver fibrosis and response to therapy with pegylated interferon/ribavirin (PEG-IFN/RBV) in patients with chronic hepatitis C (HCV) genotype 1b. Pre-treatment of 100 patients with chronic hepatitis C genotype 1b was analyzed, and related to outcome of therapy. TaqMan assay was used to determine SNP rs12979860 in all patients. In our study there was significant correlation between age and response to therapy. Also, we found associations between a known route of transmission and age, gender, stage of liver fibrosis and therapy outcome. With respect to SNP rs12979860, the frequency of the CC genotype in the group with a sustained virologic response (SVR) was significantly higher than in the group of non-responders (NR). In contrast, there was no correlation between IL28B polymorphism and progression of liver fibrosis.

Introduction

Hepatitis C virus (HCV) is a global health problem affecting more than 150 million people worldwide and approximately 8 to 11 million people in Europe (WHO, 1999; Cornberg et al., 2011). Patients with chronic hepatitis C infection have a higher risk of developing liver cirrhosis and hepatocellular carcinoma (Maasoumy and Wedemeyer, 2012). Pegylated interferon (PEG-IFN) and ribavirin (RBV) is the standard of care for treating HCV infection in Serbia. New DAA therapy, such as sofosbuvir, daclatasvir and the sofosbuvir/ledipasvir combination, has increased the rate of SVR and eliminated persistent HCV infection in > 90% of patients. Although, current DAA therapy significantly increased the SVR, and showed better tolerance over a shorter time, it is still limited by viral resistance and high cost, especially in countries with limited resources (Bartenschlager et al., 2018). Since the treatment response is influenced by genetic variations in patients, as well as in the HCV genome, recognition of biological markers that may predict the response and hence treatment outcome would be helpful also in the era of DAA. Therefore, identification of the determinants of response to treatment has high priority, even though many viral, genetic and host factors interact in the prediction of therapy outcome in patients with HCV infection (Hadziyannis et al., 2004). In this study we analyzed relationships between age, gender, alanine aminotransferase (ALT) concentration, stage of liver fibrosis, route of transmission and pre-treatment viral load with therapy outcome in patients with chronic hepatitis C genotype 1b. Previously it was shown that single nucleotide polymorphisms (SNPs) near the interleukin (IL) 28B gene were strongly associated with response to PEG-IFN/RBV therapy in patients with genotype 1 (Ge et al., 2009; Suppiah et al., 2009) as well as the risk of fibrosis progression, cirrhosis and hepatocellular carcinoma (HCC), especially in NR (Fabris et al., 2011; de la Fuente et al., 2017). The aim of this study was to evaluate the effects of host and viral factors during antiviral treatment with PEG-IFN/RBV in patients chronically infected with HCV genotype 1b with special emphasis on the effects of potentially important prognostic factors such as IL28B rs12979860 polymorphism on therapy outcome.

Materials and methods

Samples were collected from a total 100 patients with chronic hepatitis C genotype 1b, before the start of PEG-IFN/RBV therapy. A virologic response was defined as SVR (absence of HCV RNA in serum 6 months after end of treatment) and non-response (NR) (detection of serum HCV RNA 6 months after cessation of treatment). Histological activity grade and fibrosis stage were evaluated according to the METAVIR scoring system (Bedossa and Poynard, 1996) as follows: F0 (no fibrosis), F1 (mild fibrosis without septa), F2 (moderate fibrosis with few septa), F3 (severe fibrosis with numerous septa without cirrhosis) and F4 (cirrhosis). Our study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade, and all patients provided written consent.

Total ribonucleic acid (RNA) was extracted from 100 μL of serum using the Ribo-Sorb-100 (HCV Quant) RNA/DNA Extraction Kit (Sacace Biotechnologies, Como, Italy) according to the manufacturer's protocol. Concentration of HCV RNA was determined by real-time PCR (Applied Biosystems 7500, Foster City, United States) using the commercially available R-TMQ HCV Kit (Sacace Biotechnologies, Como, Italy) according to the manufacturer's instructions (sensitivity limit-250 IU/mL). Genotyping of HCV was performed with a combination of type-specific primers as described previously (Okamoto et al., 1992, 1994).

Genotyping of IL28B

Genomic DNA was extracted from serum using a commercially available kit QIAamp UltraSens (Qiagen, GmbH, Germany) according to the manufacturer's instructions. The IL28B SNP rs12979860 was analyzed using Custom® SNP Genotyping Assays (Applied Biosystems). The allele specific primers were designed as reported previously (Alestig et al., 2011). Genotyping was performed on ABI-7500 real-time PCR (Applied Biosystems, Foster City, United States) in 25 μL reaction volume containing 10 ng DNA, 12.5 μL

TaqMan® Universal PCR Master Mix and 1.25 μL (40x) Custom® SNP Genotyping Assays.

Statistical analysis

The results are presented as mean ± standard deviation (SD) or number (percentage). Differences in the frequency distribution between two or more categorical variables were evaluated by Pearson's $\chi 2$ test. Means of normally distributed continuous variables between sustained virologic responders and non-responders were compared by the unpaired t-test, while the non-parametric Mann-Whitney U Test was used for means of skewed continuous variables. Relations between ALT levels and RNA viral load were evaluated by the Spearman rank order correlation test. In all tests, p-values of less than 0.05 were considered statistically significant. All statistical analyses were performed using the Sigma Plot 10.0 licensed statistical analysis software package.

Results and Discussion

Baseline characteristics for the 100 patients divided according to therapy outcome (SVR, n = 47, NR, n = 53) are shown in Table 1. There was no significant difference between the genders and response to the therapy (Table 2). The mean age of all our patients was 44.3 ± 12.7. Patients with SVR were significantly younger than those with NR (Table 1). Both groups had elevated (ALT) levels (upper limit of normal is 40 U/L) with a statistically insignificant difference (Table 1). Moreover, the ALT concentration was not correlated with pre-treatment viral load (Spearman's, p = 0.653, data not shown), stage of fibrosis or therapy outcome (Table 1).

The major route of HCV transmission was unknown (48%), subsequent to blood transfusion (23%) or intravenous drug use (IVDU, 21%). The correlations between the known route of transmission and response to therapy, gender and stage of liver fibrosis were statistically significant. Thus, patients with a positive history of blood product transfusion were more frequently NR than with SVR, while IVDU was more common among SVR than NR patients ($p < 0.001$, data not shown). Additionally, in the group of IVDU males were significantly more common than females (18% vs. 3%; $p < 0.001$, data not shown), while women were significantly more frequent than men in the group with a positive history of blood product transfusion (16% vs. 7%; $p < 0.001$, data not shown). With respect to IL28 rs12979860 genotype distribution, more than half of patients (56%) carried the CC genotype and the remainder (44%) had CT/TT genotypes. Therapy response rates divided according to the IL28B genotype are presented in Table 2. The frequency of the CC genotype in the group with SVR was significantly higher than for the NR group, while patients with the other genotypes (CT/TT) were more frequently NR (Table 2).

The genotype CC was associated with the patient's age. Thus, subjects with the rs12979860 CC genotype were markedly younger than those with CT/TT genotypes (Table 2). Moreover, by multiple logistic regressions, age (below 40 years) and rs12979860 CC genotype were shown to be the strongest prediction factors for SVR ($p < 0.001$, data not shown). In contrast, the rs12979860 genotype was not correlated with level of ALT, pre-treatment viral load and fibrosis stage. Currently, PEG-IFN/RBV is the standard routine treatment for chronic hepatitis C infection in Serbia. We found a significant correlation between age and response to therapy. Moreover, older patients had a lower SVR rate to PEG-IFN/RBV therapy than younger ones which confirms previous results (Antonucci et al., 2007; Jovanovic-Cupic et al., 2014, 2016).

Connections between HCV transmission and therapy outcome were analyzed in the recent studies. Our results showed a significant relationship between a known route of transmission and response to the therapy. The major known route of HCV transmission was blood transfusion (23/100), followed by intravenous drug use IVDU (21/100) and post-operative (4/100). The high percentage of patients infected through blood transfusion may be the consequence of migration, inadequate screening or poor prophylaxis, which is characteristic of countries with limited resources. Moreover, there was a significant association between age and route of transmission. Thus, younger patients were more frequent among the IVDU group than among those infected by blood transfusion. As in previous investigations, men were more common in the

Table 1. Baseline clinical characteristics of HCV genotype 1b patients according to therapy outcome

Clinical and pathological characteristics of patients	Sustained responders (n=47)	Non-responders (n=53)	HCV patients overall value ± SD	HCV patients overall p
Age (years) [a]	41.0 ± 13.9	47.3± 10.8	44.3 ± 12.7	0.013* [d]
Gender, n (male/female)	24/23	28/25	52/48	0.864 [d]
ALT level (U/L) [a]	90.3±62.7	48.64±6.68	84.8±55.6	0.315 [d]
HCV RNA level (log IU/mL) [c]	9.5±32.7	23.4±97.4	16.7±75.5	0.973 [d]
Stages of fibrosis [b]	n (%)	n (%)	All n (%)	
F0-F2	27 (27)	29 (29)	56 (56)	0.788 [d]
F3-F4	20 (20)	24 (24)	44 (44)	

[a] Data expressed as mean ± SD; ALT alanine aminotransferase; [b] Stage of fibrosis expressed by METAVIR score (fibrosis 0-2 and 3 to 4); [c] Expressed HCV RNA level x 10^5; * Statistically significant; p-values < 0.05 were considered statistically significant; [d] Mann-Whitney U-test

Table 2. The IL28 rs12979860 polymorphism distribution according to response to therapy and baseline characteristics of patients with chronic hepatitis C infection and genotype 1b

Clinical and pathological characteristics of patients	Sustained virological responders IL28B rs12979860 (n=47)		Non-responders IL28B rs12979860 (n=53)		HCV patients overall value ± SD	p
	CC (n=44)	CC/TT (n=3)	CC (n=12)	CT/TT (n=41)		
Age (years) [a]	40.7 ± 13.6	46.0±20.9	48.7 ± 11.0	47.0±10.9	44.3 ± 12.7	0.013*
Gender, n (male/female)	24/20	0/3	5/7	23/18	52/48	0.641
ALT level (U/L) [c]	91.1±64.4	63.3±13.3	72.4±40.3	82.1±51.0	84.8±55.7	0.560
HCV RNA level (log IU/mL) [c]	9.9±33.8	3.5±7.7	9.8±35.4	23.0±10.5	16.7±73.5	0.947
Stages of fibrosis [b]	n (%)	n (%)	n (%)	n (%)	n (%)	
F0-F2	26 (26)	2 (2)	7 (7)	22 (22)	57 (57)	0.183
F3-F4	18 (18)	1 (1)	5 (5)	19 (19)	43 (43)	

[a] Data expressed as mean ± SD; ALT alanine aminotransferase; [b] Stage of fibrosis expressed by METAVIR score (fibrosis 0-2 and 3 to 4); [c] values expressed as median-range; * Statistically significant; p-values < 0.05 were considered statistically significant; [d] Mann-Whitney U-test

IVDU group, while women were significantly more frequent among the patients with a positive history of blood transfusion (Pawlotsky et al., 1995; Cornberg et al., 2011). With respect to previous results SVR rate was reported to be significantly higher in patients with the favorable (CC) genotype of IL28B than in those with unfavorable (CT/TT) genotypes (Halfon et al., 2011, Lazarevic et al., 2013) as confirmed here. Therefore, 44% of our patients with the CC genotype achieved SVR compared to 2% of those with the CT genotype and only 1% of those with the TT genotype. The main limitations of this study are the relatively small number of patients and a study design matched for genotype and therapy outcome. Considering host factors, we have demonstrated that patient age (below 40 years) and the CC genotype of IL28B could be important predictive factors for SVR. According to our knowledge, this is the second study to provide detailed results on the effect of polymorphism on therapy outcome of patients with chronic infection genotype 1b from Serbia. Combined therapy with PEG-IFN/RBV, remains an important and relevant therapy option. Thus, patients with genotype 1 achieved SVR rates up to 79%, while the rate was 89% for those with genotype 2 or 3. This rate increases for patients with favorable IL28B genotypes (Huang et al., 2017). Previous data have shown that IFN-induced SVR is associated with a lower incidence of hepatocellular carcinoma compared with DAA therapy. Also, the effects of novel DAAs are still unclear, especially in patients with cirrhosis and/or hepatocellular carcinoma. In this interferon-free era, the previous combined therapy remains a safe and effective option for selected HCV patients (Huang et al., 2017). The efficacy of treatment using new DAA therapy for patients with chronic infection may be affected by the presence of resistance-associated substitutions (RASs) (Bartenschlager et al., 2018).

Conclusions

This study represents an overview of the current state of patients with chronic hepatitis C genotype 1b in Serbia and their response to the combined therapy with PEG-IFN/RBV. The response to novel DAA therapy remains to be explored, especially in developing countries. One of the main issues is that a vast number of patients do not respond to this therapy and show the progression of liver fibrosis. On the other side, decreasing the cost of DAA therapy is an important challenge for its introduction in basic clinical routine.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Research article

INTERMEDIATE FREQUENCY MAGNETIC FIELD AT 250.8 KHZ DOES NOT INDUCE DNA DAMAGE OR "ADAPTIVE RESPONSE" *IN VITRO*

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Abstract

The aim of this study was to examine whether intermediate frequency (IF) magnetic field (MF) induces genotoxic effects or play a role in the induction of adaptive response after combined exposure to IF MF and ionizing radiation in leukocytes and in adrenocortical carcinoma cell line H295R. Cells were exposed to 250.8 kHz at the magnetic field strength of 80 A/m (equivalent to 100 μT magnetic flux density) for 20 hours, or exposed to IF MF for 20 hours and 24 hours later challenged with ionizing radiation (1.5 Gy X-ray). Evaluation of the DNA damage was performed with alkaline comet assay. Our results showed that there was no significant genotoxic effect of IF MF exposure compared to the controls in both cell types. Furthermore, results did not indicate a statistically significant change in DNA strand breaks in IF MF pre-exposed cells when they were subsequently exposed to 1.5 Gy. Consequently no adaptive response was detected.

Keywords

genotoxicity, alkaline comet assay, intermediate frequency, magnetic field, adaptive response

Introduction

Electromagnetic fields (EMF), especially extremely low frequency (ELF) electric and magnetic fields (0-300 Hz) and radiofrequency (RF) fields (10 MHz – 300 GHz) have been thoroughly studied through the decades. The World Health Organization defines the intermediate frequency (IF) magnetic field (MF) as a frequency range between the ELF and RF fields, from 300 Hz to 10 MHz (WHO, 2005)(Figure 1). Many different consumer and industrial equipment used in public and working environment produce intermediate frequency magnetic fields, varying widely in frequency and strength. IF MFs are generated in household devices such as compact fluorescent lamps (CFL), induction cookers, devices

that use inverter technology to gain better control of speed or temperature at higher energy efficiency (microwave ovens, refrigerators, laundry machines, air conditioning systems)(Litvak et al. 2002, Aerts et al. 2017). Electronic article surveillance systems (EAS) or anti-theft devices are commonly installed in shops and libraries and operate over a wide range of frequencies (20 Hz - 2.45 GHz) (Roivainen et al. 2014). Electric and hybrid vehicles produce IF MF in the range of few kHz - 1 MHz (Percebon et al. 2016, Tell et al. 2014, Vassilev et al. 2015). Proximity Radio Frequency Identification (RFID) readers operate at 125 kHz or 13.50 MHz for remote reading of magnetic badges of personnel passing through control gates. Wireless power transmission (WPT) a new and rapidly developing technology is aimed to supply power to electronic equipment without wires. Household appliances, mobile phones, and even electric cars can be charged by

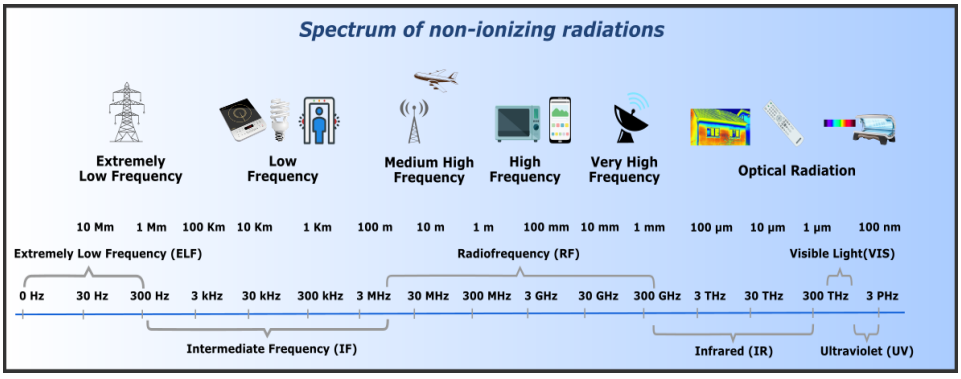

Figure 1: Diagram of non-ionizing radiation spectrum, showing intermediate frequency across the range of frequencies and wavelengths.

WPT. So far, research focus was mainly on IF field emitting sources while the number of studies concerning exposure to IF MF on biological systems is very low (SCENIHR, 2015). There are just a few *in vitro* studies investigating genotoxic endpoints (Bodewein et al., 2019). One of the most investigated frequencies within the spectrum of intermediate frequency is that emitted from inductive cook top heaters, 23 kHz. Miyakoshi et al. (2007) and Sakurai et al. (2009) exposed CHO-K1 cells at 23 kHz for 2 hours with the magnetic flux density of 532 µT and 6.05 mT, respectively and investigated the cell growth, comet assay, micronucleus formation and hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutation. They concluded that IF magnetic field for 2 hours does not cause cellular genotoxicity. A few years later the same working group (Sakurai et al. 2012, 2013) exposed human fetus-derived astroglia cell line for 2, 4 and 6 hours at 23 kHz (100 µT and 2 mT) and found that IF MF did not induce detectable changes in gene expression profile. The other investigated intermediate frequency is the frequency of wireless power transmission. Shi et al. (2014) conducted experiments on human lens epithelial cells at 90 kHz (93.36 µT) for 2 and 4 hours and found, that there was no effects on cell proliferation, apoptosis, comet assay and γH2AX foci formation. Sun et al. (2017) exposed the retinal pigment epithelial cells to 100 kHz for 24 hours and found no statistical differences at the comet assay parameters.

The term adaptive response (AR) describes the phenomenon where a relatively low dose of ionizing radiation (IR) as adaptive dose (AD) induces a kind of adaptation (sometimes referred to as resistance) to genetic damage induced by a subsequently higher dose of IR (challenge dose, CD). Adaptive response induced by the low dose ionizing radiation depends on several factors, e.g. cell types, but also the cell metabolic state. Jiang et al. (2008) studied the adaptive response in different non-cancer (healthy) and cancer cell lines and showed that adaptive response was induced only in normal human fibroblast, but not in lung carcinoma cell line, glioma cell line, erythroleukemia cell line, or acute promyelocytic leukemia cell line. It seems that cancer cells show less sensitivity to AR than healthy cells. AR is not only inducible via the same agent, but there is a kind of “cross-resistance” to similar

genotoxic agents (Vijayalaxmi et al. 2014). Combined exposures to different agents such as non-ionizing radiation (as AD) and chemicals/pharmaceuticals or IR (as CD) induce the AR phenomenon also. For example, Sannino et al. (2013) studied the AR in human lymphocytes *in vitro* after exposure to RF at 1950 MHz as AD and later to 1.0 or 1.5 Gy as CD. It was shown that the IR induced significant decrease of micronuclei incidence rate when the lymphocytes were pre-exposed to RF-EMF. In earlier studies of Sannino and colleagues (2009; 2011), AR was also detected in human lymphocytes using 900 MHz RF EMF and the chemical mutagen mitomycin C.

Accordingly, the question arises: do other frequencies of the electromagnetic field spectrum induce the AR as well? Since adaptive response has not yet been studied after IF exposure, we investigated if IF MF could cause an adaptive response against ionizing radiation and if IF MF by itself could induce genotoxic effect. We selected one frequency in the middle of the IF frequency range at few hundred kHz, where several medical, industrial and household devices operate (e.g. proximity readers, inverter technology). We used two different cell types, leukocytes from healthy human blood and a human cancer cell line to test whether these two cell types act differently in the adaptive response protocol.

Materials and methods

Chemicals and reagents

The normal melting point agarose, KCl, DMSO, Triton-X, 0.25% Trypsin-EDTA, Dulbecco's Modified Eagle's Medium/Nutritient Mixture F-12 HAM were purchased from Sigma (St. Louis, USA), low melting point agarose from ICN Biomedicals Inc. (Ohio, USA), glass slides and coverslips from VWR International (Pennsylvania, USA), alcohol, Sodium Hydroxide (NaOH), Sodium Cloride (NaCl), Na_2EDTA, Tris from Reanal (Budapest, Hungary), RPMI-1640 medium from Thermo Fisher Scientific (Massachusetts, USA), ITS+Premix, Nu-Serum from BD Bioscience. Gel Red stain was purchased from Csertex Kft (Budapest, Hungary), T25 flasks from Nunk (Denmark), Petri dishes from Corning (NY, USA).

Exposure system

The exposure system consisted of a solenoid coil cylinder (inner diameter 12 cm, height 19 cm), a function-generator, and an RF power amplifier. The solenoid coil cylinder was constructed by double wrapped coils made from electrically isolated copper wires of 2x30 loops (outer layer), and was cooled by water tubes (inner layer) (Fig. 2A). Between the water tubes and the inner wall of the cylinder a referent coil loops were located in order to record the magnetic field continuously. The temperature of the inner wall of the coil was controlled and cooled by water flow. The entire coil system was placed in a CO_2 incubator (HETO-HOLTEN A/S, Cellhouse 154, Allerød, Denmark) where the background power frequency (50 Hz) stray magnetic field was the lowest, between 0.5-1.5 μT (Gresits et al. 2015)(Fig. 2B). The cell flasks or the Petri dishes were placed in the coil, using a plastic holder (Fig. 2C). The coil was operated at resonant mode. The magnetic field strength was 80 A/m (equivalent to 100 μT magnetic flux density) at 250.8 kHz. The magnetic flux density in the exposure space (at all levels where the flask or Petri dishes were placed) was measured using EM Field Analyzer EFA-3 (Wandel & Goltermann, Germany). The inhomogeneity of the magnetic flux density inside the coil and within the exposure area at different levels was less than 10%. The measurement of the temperature in samples was performed by four channel non-perturbing optical temperature probes (Luma Sense Technologies Inc., USA) and monitored during exposures. The temperature of the sample was maintained at 37°C by the water flow system. At the same time the control samples (negative control) were kept in another CO_2 cell incubator at 37°C.

Cell cultures and experimental protocol

Two cell types were used in this study: leukocytes from whole blood and H295R human adrenocortical carcinoma cell line. Blood samples were taken from three healthy human donors, one for each experiment. The volunteers had not been exposed to pharmaceuticals, drugs and ionizing radiation three months prior to venous puncture and gave informed consent previous to experiments. Permission for the

experimental work on human blood tissues of volunteers was received from the Hungarian Scientific and Research Ethics Committee of the National Scientific and Medical Council (7478-1/2012/EKU 110/PI/12.). Approximately 10 ml blood was drawn by venous puncture into sterile heparinised Vacutainer vials by authorized personnel and on the same day diluted with RPMI-1640 medium to three times of its original volume. The diluted blood was distributed in 35 mm Petri dishes (2 ml/Petri-dish) for the exposure treatments.

H295R cells (ATCC® CRL-2128™) were cultured in DMEM/F-12 supplemented with 1 % ITS+ Premix, 2.5 % Nu-Serum and 0.5 % penicillin-streptomycin at 37ºC in a humidified atmosphere at 5 % CO_2 and 95 % air. Cells were routinely maintained as monolayers and sub-cultured twice a week by trypsinization when the confluency of the cells reached 70%. For our experiments, cells were used between the fifth and tenth passage and seeded in T25 flasks for the exposure treatments. Diluted blood samples in Petri dishes were exposed either to IF MF (250.8 kHz, 80 A/m, 100 μT) or to control conditions (incubation in CO_2 thermostat at 37°C) for 20 hours. For the AR experiments blood cells were exposed to IF MF as an adaptive dose (250.8 kHz, 80 A/m) for 20 hours followed by 24 hour incubation period before it was exposed to challenge dose of 1.5 Gy ionizing radiation (X-rays) (Fig. 3A). The control samples were sham exposed cells that were held in same conditions as exposed ones except for the exposures: during IF MF they were incubated in CO_2 thermostat at 37°C and during X-ray irradiation were taken to the irradiation facility but were not irradiated. For positive control 4 Gy X-ray ionizing irradiation was used at a dose rate of 1.23 Gy/min (200 kV, 20 mA and 1 mm Cu filter with a RTG THX-250 device). The same protocol was used for both cell types except

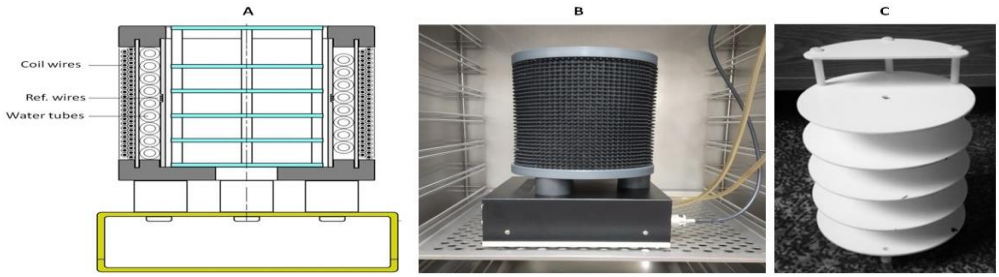

Figure 2: The intermediate frequency exposure system. A: scheme of the solenoid IF exposure system. B: the solenoid coil exposure system placed in the CO_2 incubator. C: sample holder within the solenoid coil.

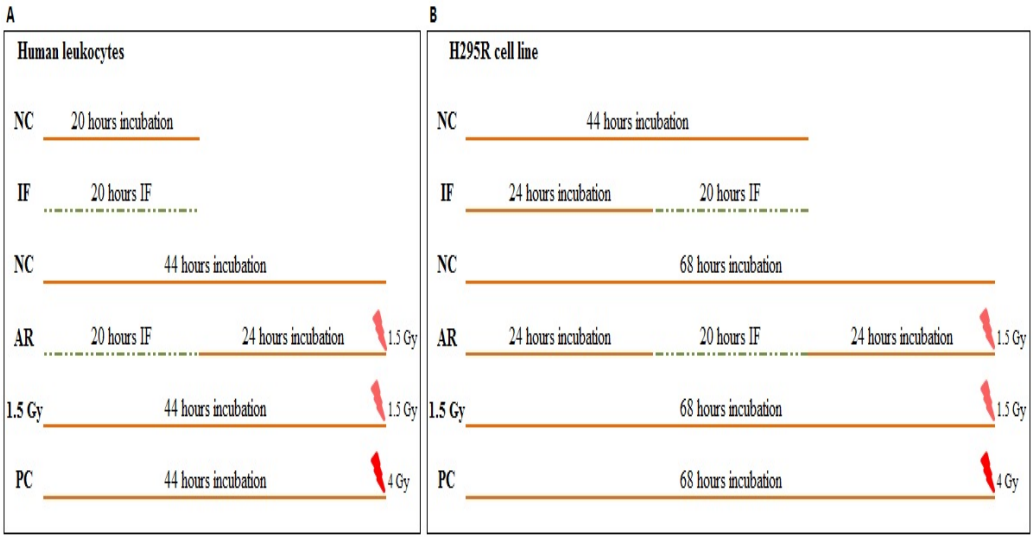

Figure 3: Scheme of experimental protocol to investigate the genotoxicity and the adaptive response of IF MF exposure in leucocytes (A) and H295R cells (B)

that H295R cells were pre-incubated for 24 hours before exposures to allow cells to attach to the flasks bottom (Fig. 3B). Immediately after the end of exposure protocol they were collected with trypsinization and centrifugation (30 - 40 min procedure) for the comet assay testing. There were three independent experiments for both cell types.

Single cell gel electrophoresis assay

The comet assay (a single-cell gel electrophoresis technique) was used as a method to detect the DNA damage. A slightly modified alkaline comet assay protocol of Singh et al. (1988) was used. Cell suspension was mixed with 0.5 % low-melting point agarose (37ºC) and pipetted to 1 % normal-melting point agarose pre-coated slides. This suspension was immediately covered with cover glass, and kept at 4ºC until agarose solidified. After 5 minutes the cover glass was gently removed, and the slides were immersed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH; 1% Triton X-100, 10% DMSO added freshly) and lysed overnight at 4ºC. Slides were rinsed in cold electrophoresis solution (1mM Na_2EDTA, 300 mM NaOH, pH 13), and placed in a horizontal gel electrophoresis tank (APELEX, Maxigel Eco2, France) which was filled with cold, fresh electrophoresis solution to a level approximately 0.5 cm above the slides. Duration for DNA unwinding was 30 minutes, and for electrophoresis 20 minutes. The voltage was constant at 25V, 0.83 V/cm (300 mA). After electrophoresis, the slides were washed 3 times with Tris buffer (0.4M Tris, pH 7.5), once with ethanol, air dried and stored at room temperature until analysis. Staining was performed with GelRed and slides were examined at 40x magnification objective using a Zeiss AxioPlan fluorescence microscope (Oberkochen, Germany). Microphotographs of comets were taken by CCD camera and evaluated using the CaspLab image analysing software (University of Wroclaw, Poland). For each exposure condition 100 comets were examined per slide and the tail DNA % parameter was calculated. Three independent experiments were performed (n=3). Data analysis was done using R statistical software version 1.1.463 (2009-2018 RStudio, Inc). Effects of the treatment on tail DNA% was investigated by linear mixed-effects models (LMMs). Post-hoc Tukey test was used for treatment comparison. Results for each exposure condition are presented as mean (±SD).

Results and Discussion

The aim of this study was to evaluate the possible genotoxic effects of intermediate frequency magnetic field exposure (250.8 kHz) in leukocytes and in adrenocortical carcinoma cell line, and to examine if IF MF as a physical agent could induce adaptive response.

The results of 20 hour 250.8 kHz exposure alone on leukocytes and in H295R cells are presented as tail DNA % in Figure 4A, 4C and 5A, 5C, respectively. The 20 hours exposure to IF MF did not alter the level of DNA damage significantly, compared to the control samples in both cell types. The mean of tail DNA % of IF exposed blood samples was 5.97 ± 2.8, while for control samples 5.43 ± 2.9 (Fig. 4/C). Similar result was for the adrenocortical carcinoma cell line: in IF exposed cells 5.02 ± 3.57 and for negative control cells 4.47 ± 2.21 (Fig. 5/C). Our result supports the findings that intermediate frequency do not induce genotoxicity *in vitro* (Miyakoshi et al. 2007, Sakurai et al. 2009, 2012, and 2013). Although these results referred to a short term exposure for only few hours, the same was stated for the 24 hours exposure by Sun et al. (2017). The other objective of this study was related to adaptive response. To provoke the adaptive response with non-ionising radiation, mainly radiofrequency radiation (on which mobile phones operate) was used as AD. Ji et al. (2016) and He et al. (2017) applied 900 MHz for few days on mouse bone-marrow stromal cells (BMSC) *in vitro* and introduced 1.5 Gy as the challenge dose. A consistently and significantly decreased DNA damage was detected in cells exposed to RF + 1.5 Gy accompanied by a faster DNA repair kinetics. Sannino et al. (2013) exposed human blood lymphocytes to 1950 MHz RF for 20 hours and used 1.0 or 1.5 Gy X-ray as CD. The authors reported a significantly decreased incidence of micronuclei in RF pre-exposed cells when they were subsequently exposed to IR. In our study we used the intermediate frequency (250 kHz) for investigating the adaptive

response. Human leukocytes were pre-exposed to IF MF for 20 hours and challenged with 1.5 Gy X-ray radiation (treatment named AR) after 24 hour time interval (Fig. 4B, 4D). There was a noticeable tendency of decrease in DNA damage, but no significant difference (P=0.086) was detected compared to the samples that were only irradiated with 1.5 Gy X-rays (treatment named 1.5 Gy) (Fig. 4D). However, the samples irradiated with 1.5 Gy X-ray contained significantly more DNA damage (P<0.001) than the sham exposed control samples (treatment named NC). The results of positive control (4 Gy) showed statistically significant increase in DNA damage in leukocytes. Although not indicated on the Figure 4D, values of the 4 Gy treatment group were significantly higher than the NC, AR and 1.5 Gy group values (P < 0.001). Regarding the H295R cell line the combined exposure of IF and 1.5 Gy (AR group) did not alter the level of DNA strand breaks significantly (P=0.901) compared to its respective control (Fig. 5B, 5D). In experiments with H295R cell line the positive control value was lower than expected but still differed significantly from the AR (P=0.037) and NC groups (P=0.012).

We got such low values because after the end of the exposure protocols 30-40 minutes have passed (collection of attached cells with tripsinization and centrifugation procedure) and by that time the repair of DNA damage partially developed. This is the difficulty and the challenging task when using attached cells for comet assay in exploring the effects of non-ionising radiation. We presume, that if exposure to non-ionizing radiation would have had any effect, it would probably be insignificant, and even smaller after cell collection due to DNA repair.

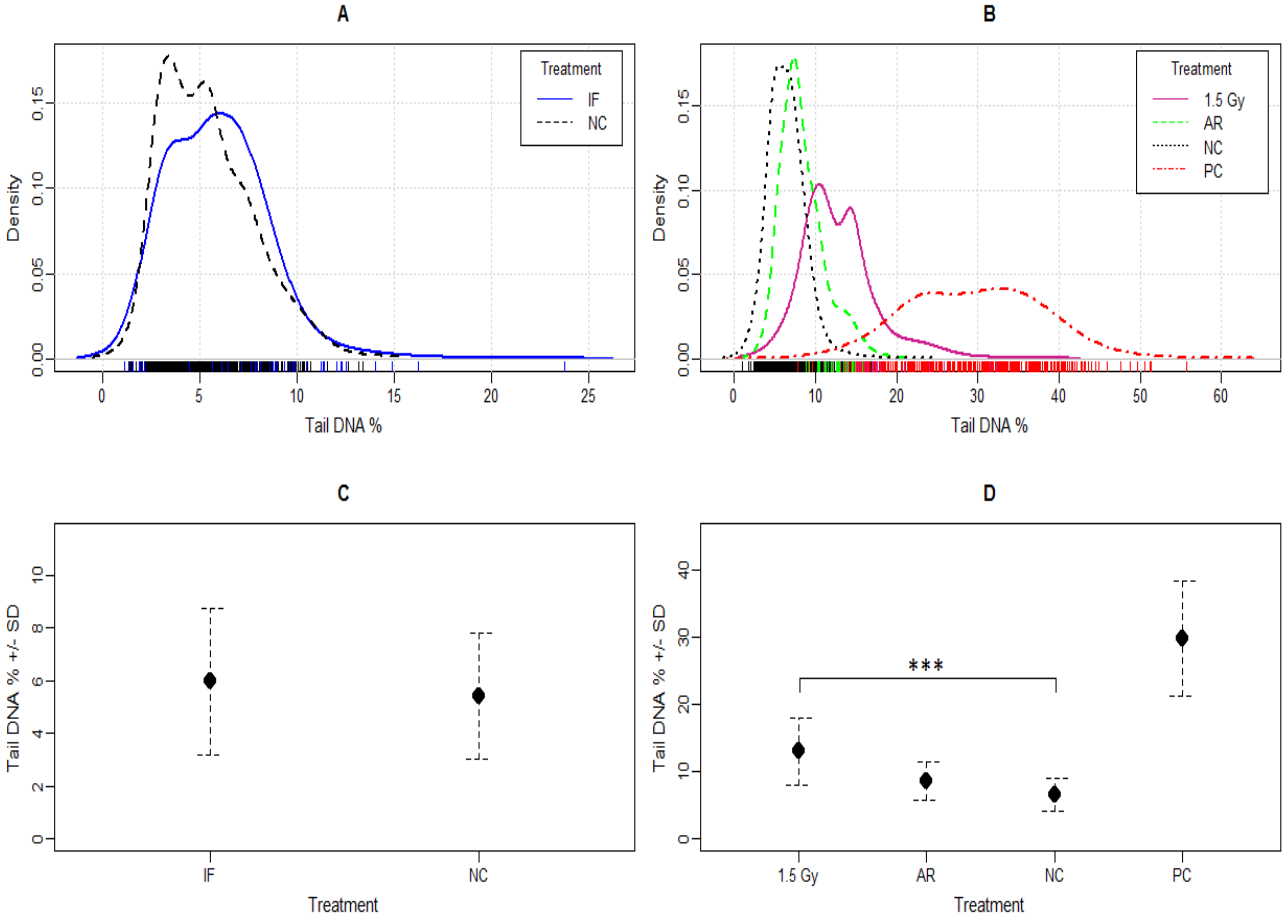

Figure 4: Density distribution of tail DNA % of human blood cells in IF exposed genotoxic (A) and adaptive response (B) experiments. Plot of mean values of human blood cells in IF exposed genotoxic (C) and adaptive response (D) experiments. Dots represent the mean ± SD of three independent experiments. The asterisk indicates that P < 0.001.

What might be the reason that in our experimental setup the AR was not detected? One of the reasons could be that the 250 kHz intermediate frequency is not effective in this manner. To our knowledge this frequency was not used in genotoxicity testing so far, so no results are available for comparison. The other explanation is related to work of Sannino et al. (2009). The authors discussed the presence of the heterogeneity in the induction of the adaptive response between individuals exposed to RF radiation, and that there was variability between the donors in RF-induced AR. Some individuals are "responders" and others "non-responders" when challenged to elicit the adaptive response. According to the literature the adaptive response induced by EMF were presumed to occur only in non-cancer cells (Jiang et al. 2008). To test this theory we used leukocytes from whole blood as healthy cells and the H295R adrenocortical carcinoma cell line to compare if there is difference regarding the IF MF exposure or the AR. There were no significant differences in DNA damage (tail DNA %) of IF MF exposed groups when compared to the respective control group. Thus, our results did not indicate any significant adaptive response.

Conclusions

Our results show that the applied IF MF alone is not inducing any genotoxic effects in any of the two cell types (human leukocytes from whole blood and H295R adrenocortical carcinoma) and has not modified the effects of X-ray irradiation in terms of AR. It should be mentioned here, that in this study we used only one biological assay to analyze the DNA damage and the number of donors were limited. In further studies we should cover other frequency ranges (like inductive cook top heater frequencies), different combined exposure protocols using other assays and endpoints to detect potential effects of IF MF.

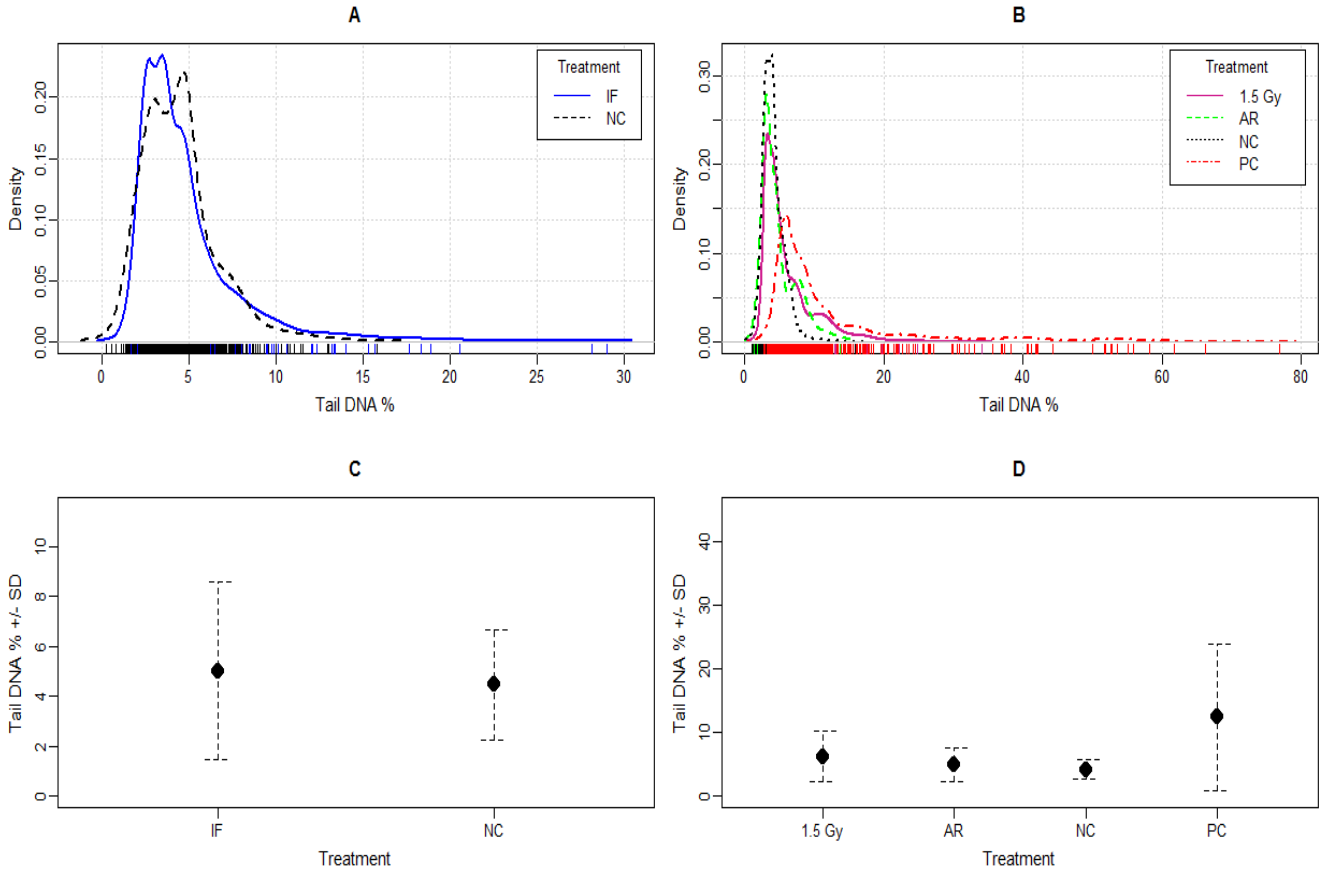

Figure 5: Density distribution of tail DNA % of adrenocortical carcinoma cells in IF exposed genotoxic (A) and adaptive response (B) experiments. Plot of means of adrenocortical carcinoma cells in IF exposed genotoxic (C) and adaptive response (D) experiments. Dots represent the mean ± SD of three independent experiments.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Research article

CYTOGENETIC ANOMALIES IN MULTIPLE MYELOMA PATIENTS: A SINGLE CENTER STUDY

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Abstract

Conventional karyotyping in the patients with Multiple myeloma (MM) is very important because the detected chromosomal abnormalities have diagnostic and prognostic value. In this retrospective study we aim to evaluate cytogenetic abnormalities in 133 MM patients whose diagnosis was established at the Hematology Department of Hacettepe University. The bone marrow samples were treated with trypsin and stained with Giemsa (GTG banding). For each patient, 20 metaphases were examined and karyotypes were formed. Cytogenetic results of the patient's bone marrow samples were obtained in 114 patients. Of 114 examined karyotypes, 80 patients had normal karyotype while 34 patients had abnormal karyotypes. Both numerical and structural chromosomal anomalies were detected in patients with abnormal karyotype. The most frequent numerical and structural anomalies were detected in chromosomes 1, 9, 16 and 13. The anomalies we found in our patient group were consistent with the literature.

Keywords

Multiple myeloma, cytogenetic, chromosomal abnormalities

Introduction

Multiple myeloma which is a highly heterogenous clonal disease of plasma cells (Kapoor & Rajkumar, 2011; Avet-Loiseau & Facon , 2018) There is a clinical heterogeneity among myeloma patients due to the underlying genetic heterogeneity. Genetic heterogeneity can be observed at chromosomal level as well. Multiple myeloma patients exhibit both numerical and structural chromosomal abnormalities (Nemec et al., 2012; Carrasco et al., 2006). Generally, patients may have hyperdiploid karyotypes or non-hyperdiploid karyotypes. In the hyperdiploid karyotypes, trisomies of 3, 5, 7, 9, 11, 15, 19, 21 chromosomes and fewer structural aberrations are seen. On the other hand, in non-hyperdiploid karyotypes there may exist some translocations which involve the

immunoglobulin heavy chain (IGH) locus. The most frequently observed immunoglobulin heavy chain translocations are t(14;16)(q32;q23), t(11;14)(q13;q32) and t(4;14)(p16;q32) (Ooi et al. 2016) . Conventional karyotyping is significant in MM as ploidy number has prognostic value. Hyperdiploid karyotpe signifies good prognosis while hypodiploid karyotype signifies the bad one. The chromosome 13 abnormalities, its deletion signifies bad prognosis. Anomalies of 14q32 region which has a various translocations partners in MM could be high risk for MM depending on the translocation partner (Rajan & Rajkumar , 2015; Levin et al., 2018; Lonial, 2010; San-Miguel et al., 2009; Zhan et al., 2006). Deletion of 1p and amplification of 1q are the most common cytogenetic abnormalities seen in MM patients. They are associated with bad prognosis and especially anomaly of 1q is taken as genomically unstable tumor marker (Sawyer , 2011; Szalat & Munshi, 2015; Brioli et al.,2014; Cagnetta et al.,2015).

In this retrospective study, cytogenetic data of MM patients are evaluated. We aimed to compare chromosomal anomalies, found in MM patients data with the literature findings.

Materials and methods

Patients

A total of 133 MM patients who were referred to Hacettepe University Department of Basic Oncology cytogenetic laboratuvary for routine karyotype analysis between 2008 and 2015 years were included in this study. Of the 133 patients with MM, 74 (55.6%) were male and 59 (44.3%) were female (Figure 1).

Method

Cytogenetic analysis of MM patients was performed on metaphase cells derived from 24-h unstimulated bone marrow aspirate cultures. All the procedures were performed in accordance with the Helsinki declaration and approved by the local ethics committee (Approval no: GO 15/544-10) to collect human bone marrow for diagnosis. For complete karyotyping of samples a minimum of 20 metaphases were analyzed. Briefly, the cells were synchronized using uridine, fluorodeoxyuridine and thymidine. Colcemid was added to culture (0.05 g/ml) for half an hour before harvesting. After 30 min incubation in hypotonic solution (0.075 M KCl), the cells were fixed with Carnoy's solution (3 parts methanol to 1 part glacial acetic acid). After one night aging at 65 oC, the samples were treated with trypsin and stained with Giemsa (GTG banding). The preparations ready for analysis were examined using the image analysis system (Metasistem/Germany). The karyotypes were interpretted according to the 2015 International System for Human Cytogenetic Nomenclature (Haffer LG et al.,2013).

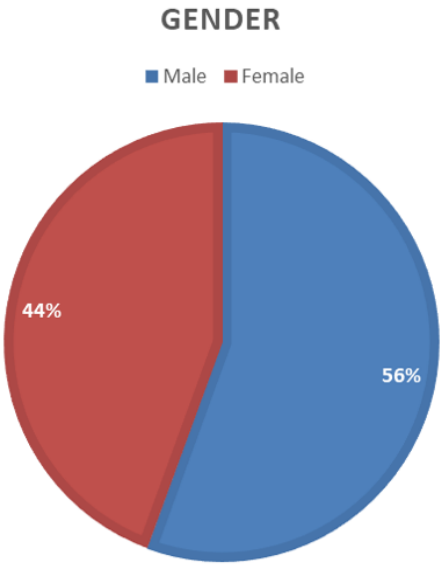

Figure 1 Graph includes 133 patients suffering from Multiple Myeloma. Of these, 55.6% are male and 44.3% are female.

Results and discussion

Cytogenetic analyses of the bone marrow samples were not successful in 19 (14.2%) patients while in 114 (87.2%) patients complete karyotype was obtained. Conventional cytogenetic results of these 114 patients showed that 80 patients had normal karyotypes while 34 patients had abnormal karyotypes (Figure 2).

As demonstrated in table 1, both numerical and structural chromosomal anomalies were detected in patients with abnormal karyotype.

Hyperdiploid karyotypes were found in 13 (38.23%) and non-hyperdiploid karyotypes (hypodiploid, pseudodiploid and near-tetraploid) were found in 18

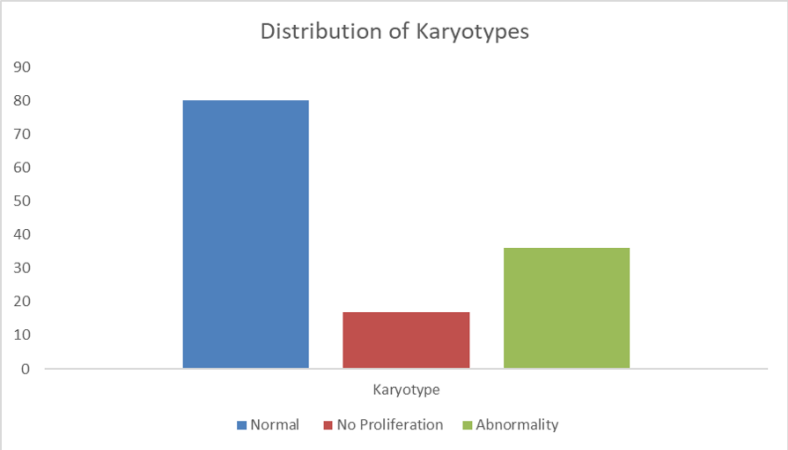

Figure 2. Among 133 Multiple Myeloma cases, 80 patients with normal karyotpe (blue), 34 patients with abnormal karyotype (green) were observed. 19 patients had no karyotype results (red).

Table 1. Karyotypes of the Multiple Myeloma Patients

Patient No	Karyotypes
1	49,XY,+4,+7,+9,+20,-2[7]/46,XY[43]
2	47-57,XY[9]/46,XY[31]
3	59,XY,+2,+3,+4,+5,+7,+8,+9,+11,+11,+15,+19,+21,+21[5]/46,XY/[45]
4	46,XY,-9,-11,-14,-16,+mar1,+mar2,+mar3,+mar[46]/46,XY[14]
5	46-50,XX,-8,-9,+14,+15,-16,+18,+18,-19,+19,-22,+22,+mar[12]/46,XX[8]
6	49-55,XX,+X,del(1)(p31),-1,-4,+5,-6,+7,+8,-11,+12,-12,-13,-14,+17,-17,+18,+19,+20,+21,+22,+mar1,+mar2;+mar3,+mar4,+mar5,+mar6,+mar7,+mar8[52]/46,XX/[38]
7	53,X,-X,-2,+3,+5,-8,+9,+11,+15,+19,+21,+mar1,+mar2,+mar3[4]/46,XX[46]
8	46,XX,+3,-4,-4,-7,der(8),-9,del(12)(p13),-14,-14,+15,-16,-19,-22,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7[23]/46,XX[77]
9	46,XY,der(1)[20]
10	46,XY,der(9)[20]
11	45,X,-Y,[3]/46,XY[47]
12	50,X,-X,+del(1)(p35p21),+5,+5,-11,-16,+19,+mar1,+mar2,+mar3[8]/46,XX[52]
13	60,XX,+3,+4,+5,+6,+7,+9,+11,+11,+15,+16,+19,+19,+21,+21[15]/46,XX[40]
14	45,X,-Y,[4]/46,XY[46]
15	46,XY,der(16)[20]
16	44,X,-X,-1,-8,-8,del(9)(p24p21),-13,+mar1,+mar2,+mar3[22]/46,XX[23]
17	45,XY,i(1)(q10),-2,-13,-14,del(16)(q24),+mar1,+mar2[15]/45,XY,i(1)(q10),-2,-13,-14,-14,del(16)(q24),+mar2,+mar3,+mar4,[10]/84-98,[4n],XY,i(1)(q10),-2,-13,-13,-14,-14,del(16)(q24)x2,+mar1,+mar2,+mar5[40]/46,XY[5]
18	46,XY,dic(1;21)(p11;p11),del(3)(q25q29),del(6)(q24q26),t(11;14)(q13;q32),del(13)(q14q21)[20]
19	44-46,XY,-3,-5,del(7)(q31q36),-9,-10,-12,-14,-15,-17,-18,+mar1,+mar2,+mar3,+mar4,+mar5[18]/46,XY[2]
20	45,X,-Y[14]/46,XY[56]
21	50-53,-X,-Y,del(3)(q21q26),+5,+9,+11,-13,der(14),+15,-16,+18,-22,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7[20]
22	46,XX,del(11)(q23q25),-18,+mar1[15]/46,XX[5]
23	90,XY,+X,der(1)x2,del(1)(p13p16)x4,+2,+3,+3,+5,+6,+7,+8,+8,+9,+10,+10,+11,+12,+13,+13,+14,+15,+16x4,+17,+18,+19,+19,+20 x4,+21,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7[2]/46,XY[18]
24	46,XX,der(3),t(11;14)(q13q32)[16]/58-81[4]
25	68-82,XY,+X,-Y,+1,+1,+2,+2,+3,+3,+4,+5,+6,+6,+7,+7,+8,+8,+9,+10,+11,+11,+12,+12,+13,+13,+der(14),+15,+15,+16, +18,+18,+19,+20,-21,+22,+mar1,+mar2,+mar3,+mar4[10]/46,XY[70]
26	46,XY,del(1)(q31q36),-12,-13,-17,+19,+mar1,+mar2[9]/46,XY[11]
27	40-42,XY,-1,-5,-6,-7,-13,-15,-16,-17,-20,-20,-21,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6[35]/46,XY[15]
28	45,XX,-2,-8,-11,-16,-16,+mar1,+mar2,+mar3,+mar4[18]/46,XX[2]
29	46,XX,-5,-12,-22,+mar1,+mar2,+mar3[3]/46,XX,-2,-5,-12,-22,+mar1,+mar2,+mar3,+mar4[3]/46,XX[14]
30	44,X,-Y,del(1)(q32q43),-11,-14,-17,+mar1,+mar2(2}/46,XY[18]
31	47-95,X,-X,-3,-4,-4,-8,-9,-11,-11,-13,+21,+mar1,+mar2,+mar3,+mar4,+mar5,+mar6,+mar7,+mar8[13]/46,XX[7]
32	46-48,XY,+Y,-1,-2,-3,-11,+20,+mar1,+mar2,+mar3,+mar4[7]/46,XY[13]
33	58-59,XY,+3,+5,+5,+6,+9,+9,+11,+11,+15,+17,+19,+20,+21[20]
34	44,XY,der(1),+del(1)(p13p36),+t(9;9)(p22;p24),-13,dert(14),-15,-16,-22,+mar1[17]/46,XY[3]

(52.94%) of 34 patients with abnormal karyotypes. Among the patients with hyperdiploid karyotypes, 5 had only trisomies and monosomies. The other 4 hyperdiploid karyotypes had marker chromosomes beside trisomies and monosomies and 4 patients with hyperdiploid karyotypes had deletion of 1p and deletion of 3q in addition to trisomies, monosomies and marker chromosomes. Among 15 non-hyperdiploid karyotypes 5 of them were pseudodiploid and 10 of them were hypodiploid karyotypes. Pseudodiploid karyotypes were complex karyotypes which had more than three chromosomal abnormalities. These complex pseudokaryotypes had structural and numerical abnormalities. Monosomies and trisomies of many chromosomes were found in these karyotypes. Monosomies of chromosomes 9, 14, 16, and 12 were common in all pseudokaryotypes. Additionally, trisomies of chromosomes 3, 15 and 9 were found. Beside these numerical deviations, various structural anomalies were determined in pseudodiploid karyotypes as derivate 8, deleletion of chromosome 12p13, deletion of chromosomes 11q23q25 and deleletion of chromosome 1q31q36. Marker chromosomes were also determined in all pseudodiploid karyotypes. In hypodiploid karyotypes having chromosome numbers between 40-45, various monosomies were detected. Among them, monosomies of chromosomes 1, 5, 13, 14, 15 and 17 were particularly common in most hypodiploid karyotypes. Del(16)(q24), del(7)(q31q36), del(1)(p13p36), del(9)(p24p21), derivate 1, derivate 14, i(1)(q10), t(9;9)(p22;p24) were also seen in these hypodiploid karyotypes as structural abnormalities. In 4 patients with abnormal karyotypes only structural abnormalities were detected. In three of them derivate 1,9 and 16 chromosomes were seen as sole genetic abnormalities in all metaphases. Also, dic(1;21) del(13), del(6) and t(11;14) were detected in one patient. When we retrospectively look at the cytogenetic results of 133 MM patients, it was observed that 114 of the patients were karyotyped. While normal karyotype was determined in 80 of these 114 patients, abnormal karyotype was found in 34 patients. No results were obtained in 19 patients. Of the 34 patients with abnormal karyotype, 28 had complex karyotype which means karyotypes were carrying 3 or more chromosomal anomalies. There were also diploid karyotypes with anomalies. Loss of chromosomes Y was detected in 2 of these non-complex karyotypes. In the other 3 metaphases with non-complex karyotype, derivate 1, derivate 9 and derivate 16 were found.

Both numerical and structural anomalies were determined in complex karyotypes. Numerical and structural anomalies of chromosomes 1, 9, 16 and 13 were detected most frequently among these complex karyotypes (Figure 3). Chromosome 1 anomalies identified can be summarized as; derivate 1,

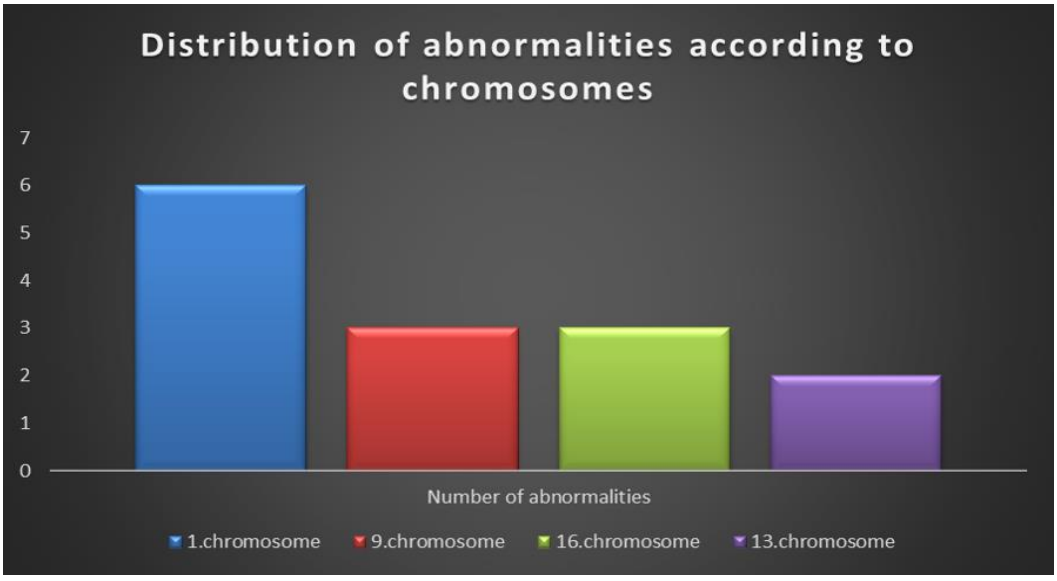

Figure 3. Numerical and structural anomalies of chromosomes 1, 9, 16 and 13 were detected most frequently among Multilple Myeloma patient

t(1;21)(p11;p11), del(1)(p) and del(1)(q). Chromosome 1 anomalies are the most common anomalies in MM. Anomalies associated with chromosome 1 in MM are deletion in p arm and increase in q arm (Marzin et al., 2006; Chang et al., 2010). In a study of Wu et al. 2016 chromosome 1 and chromosome 13 abnormalities were the most frequent abnormalities in MM patients. In our study we observed correlated results, displaying chromosome 1 and chromosome 13 anomalies as the most frequent ones. Trisomies of chromosomes 5, 9, 11, 13, 15, 19 and 21 and monosomies of chromosomes 11, 13, 14, 16 and 17 were detected most frequently in patients with hyperdiploid karyotype. On the other hand, chromosomes 1, 4, 5, 13, 14, 15 and 17 monosomies were found most frequently in patients with hypodiploid karyotype. When we examined the karyotypes in our study group, we observed that structural anomalies were more frequent in hypodiploid karyotypes rather than hyperdiploid karyotypes. In hyperdiploid karyotypes trisomies of various chromosomes and marker chromosomes also, del(1)(p31), del(3)(q21q26), derive 14 and +del(1)(p35p21) were detected. This last anomaly leads to the 1q amplification seen frequently in MM cases. According to our results, although hypodiploid karyotypes have more structural anomalies than hyperdiploid karyotypes, anomalies seen in both groups were common anomalies in MM. For example patients with hypodiploid karyotype have chromosome 1 anomalies such as i(1)(q10), del(1p) like patients with hyperdiploid karyotypes.

In one patient with pseudodiploid karyotype, dic(1;21)(p11;p11) was found. Translocation between chromosomes 1 and 21 in these break points was reported for the first time by Okay M et al., 2019. This patient also had t(11;14)(q13;q32) which is one of the most frequent translocation seen in MM.

Conclusions

Prediction of survival and management of risk classification in MM patients is crucial. Cytogenetic data are important prognostic factors in MM. Therefore, detection of cytogenetic anomalies is very important in MM patients. In this study, we examined the karyotypes of MM patients retrospectively and found that the anomalies we found were compatible with the literature. As in other hematological malignancies, MM needs more genetic information for better understanding the pathogenesis of the disease. Therefore, it is thought that more cytogenetic data of MM patients will serve to shed light on new treatment options.

Conflict of interest

Authors declare no conflict of interest.

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Research article

LEAD AND CADMIUM INDUCED CYTOTOXIC AND GENOTOXIC EFFECTS ON HL-60 AND JURKAT CELL LINES

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Abstract

Humans are exposed to a mixture of toxic heavy metals in the environment. Because of the lack of information on the toxicity of their mixtures, in this study, two common heavy metals, lead and cadmium, were introduced individually and as mixtures in HL-60 and JURKAT cell lines for 24 hours. Our experimental results have shown that these two heavy metals induced cytotoxic and genotoxic effects in both cell lines. Also, cadmium exhibited a higher cytotoxic and genotoxic potential than lead. The cytotoxicity data of single metals were used to determine the mixtures interaction profile by using the effect additivity method. Metal mixtures showed synergistic effect in HL-60 cells and antagonistic effect in JURKAT cells, compared to individual metals. The combined effects should be considered in the risk assessment of heavy metal co-exposure and potency.

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Introduction

Heavy metals are released by natural events and human activities into the environment. They are used in multiple technological, domestic, medical and agricultural applications, which led to their wide distribution, raising concerns over their potential effects on human health. In developed countries, human exposure to toxic metals has lately been decreasing, but in other parts of the world, it is increasing (Jarup, 2003). Heavy metals are systemic toxicants that induce multiple organ damage, even at lower levels of exposure (Duffus, 2002). In general, lead (Pb), cadmium (Cd), methylmercury (MeHg) and arsenic (As) are some of the most toxic metals that humans are exposed to. They target essential organs like brain, liver and kidney, causing neurotoxicity, hepatotoxicity and nephrotoxicity

(WHO, 2007). These four elements rank among the priority metals that World Health Organization has marked as great public health concerns (WHO, 2010). Lead has unique physical and chemical properties that make it suitable for great variety of applications. Because of its numerous industrial applications, lead is a common environmental and occupational contaminant widely distributed around the world. Even though the toxic effects of lead and its compounds have been extensively investigated in a variety of systems the existent data regarding its mutagenic and carcinogenic properties are contradictory. Lead has been classified as possible human carcinogen (group 2B) (IARC, 1987) and its inorganic compounds as probable human carcinogens (group 2A) (IARC, 2006). According to Agency for Toxic Substances and Disease Registry (ATSDR, 1999), one of the major mechanisms of lead toxicity is through biochemical processes that include lead's ability to inhibit or mimic the actions of calcium and to interact with proteins. Acute and chronic exposure to lead may influence immune response, which may lead to an increase in the incidence of allergies, infectious diseases, autoimmune processes or cancer (Dietert et al., 2004; Hsiao et al., 2011). Numerous experimental studies have shown that lead can influence the levels of immunoglobulins, numbers of lymphocytes, peripheral blood mononuclear cells and macrophages (Başaran & Undeğer, 2000; Mishra et al., 2003). Also, it may cause impaired responses to mitogens and depression of neutrophil functions (Undeğer & Başaran, 1998). Due to its prolonged biological half-life, low rate of excretion and high accumulation capacity in soft tissues, cadmium is one of the most toxic heavy metals. It is a widespread toxicant because environmental levels have steadily risen with an increase in the production of cadmium for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings, and alloys (Lalor, 2008; Daud et al., 2013). Humans are exposed to cadmium through occupational (industries) and non-occupational activities (cigarette smoking and consumption of contaminated foods and water; Lalor, 2008; Daud et al., 2009; Daud et al., 2013). The toxic effects of cadmium have been extensively studied in *in vivo* and *in vitro* systems. It has been classified as a group I carcinogen (IARC, 1993) and as a probable human carcinogen (group B1) by Environmental Protection Agency (EPA; Merrill et al., 2001). Cadmium affects metabolic processes including membrane transport, energy metabolism and protein synthesis. It may also act on DNA, directly or indirectly, by interfering with gene control and repair mechanisms (Beyersmann & Hechtenberg, 1997; Beyersmann & Hartwig, 2008). Numerous studies have shown that cadmium damages mammalian organs including the kidneys, testes, lungs, and the hematopoietic, cardiovascular, and nervous systems (Siu et al., 2009; Sabolić et al., 2010). It can induce apoptosis via the mitochondrial pathway in cell lines (Long et al., 2008; Szuster-Ciesielska et al., 2000; Jiang et al., 2014). Cadmium causes apoptosis in cell culture systems at low and moderate concentrations (e.g., 0.1-10 μmol/L). At higher concentrations (>50 μmol/L), necrosis becomes evident (Templeton & Liu, 2010). More than 95% of toxicological research studies are focused on single chemicals and almost completely neglect the mixtures (Kortenkamp et al., 2009). The available toxicity data for the mixtures of metals are very limited (Karri et al., 2018). Although many studies offer evidence that lead and cadmium are multi-target toxicants, little is known about the effect of these two metals in mixture, especially of their joint effect on the immune cells (Yedjou et al., 2003; García-Lestón et al., 2010; Bernhoft, 2013; Rukhsanda et al., 2014) . Hence, in the present study human leukemia HL-60 cells and Jurkat T cells were used as models to explore the single and combined cytotoxic effect of lead and cadmium on immune cells. Also, the genotoxic potential of each individual metal was analyzed.

Materials and methods

Chemicals, reagents and antibodies

All analyses that include heavy metals were performed using analytical grade chemicals, reagents and standards. For all solution preparations and dilutions only double-distilled deionized water was used. Primary antibody anti-phospho-histone H2A.X was obtained from Merck Millipore. Secondary antibody conjugated with Alexa Fluor 488 and PrestoBlue™ cell viability reagent was obtained from Thermo Fisher Scientific.

In vitro culture of the cell lines

Promyeloblastic HL-60 cell line was cultured in RPMI 1640 medium (Sigma) supplemented with 20% heat inactivated (HI) FBS (Sigma) and 1% penicillin/streptomicin antibiotics (Sigma), in humidified atmosphere containing 5% CO_2 at 37°C. Jurkat human T lymphocyte cell line was cultured in RPMI 1640 medium (Sigma) containing 10% FBS (Sigma) and 1% penicillin/streptomycin antibiotics (Sigma). For each experiment cells were grown to 80% confluence in 75cm2 culture flasks.

Analysis of cell viability/cytotoxicity by Presto blue

For each experiment HL-60 and Jurkat cells were seeded (3 X 104 cells/well) in 96 well plates and grown for additional 24 h after exposure to a metal. Triplicate wells were treated with different levels of Pb (10-100 μM), Cd (2.5-10 μM) or their mixtures. Pb and Cd standards were prepared with reagent-grade chemicals. Untreated cells were used as negative control while cells treated with 30% dimethyl sulfoxide (DMSO) in RPMI nutrient medium provided positive control. The cells were cultivated in RPMI culture medium supplemented with 1/10 volume of Pb or Cd metal solutions to achieve final concentrations of Pb (10-100 μM) or Cd (2.5-10 μM) in culture medium. Pb and Cd stock solutions were prepared in double-distilled deionized water and solutions were sterilized by filtration through 0.2 μm sterile syringe filters. The Presto blue assay was carried out according to the manufacturer's instructions (Thermo Fisher Scientific). After 24 h treatment with RPMI culture medium supplemented with 1/10 volume of Pb or Cd metal solutions, cells were incubated with Presto blue reagent. The absorbance was measured after 2 h of incubation at 570 nm wavelength using microplate reader. The measured absorbance values were converted to cell viability percentage with negative control as a reference.

Assessment of interaction using the effect method "effect additivity"

The concentrations of Pb and Cd for the analysis of mixture interaction were selected from their individual cytotoxic effects. The effect additivity method, described by Lau et al. (2006) was utilized, in which concentration of each compound equivalent to its IC20 (concentration that induced 20% cytotoxic effect) was calculated from single metal response curve. In order to assess the effect of metals mixture, the cells were treated with either a single metal at a concentration of IC20 or with a mixture calculated to produce the same effect of 20% inhibition if their effects were simply additive (i.e., zero interaction). The cells were exposed to pairs of the metals in the following proportions: 100% Pb: 0% Cd, 75% Pb: 25% Cd, 50% Pb: 50% Cd, 25% Pb: 75% Cd, and 0% Pb: 100% Cd. Each experiment was performed in independent triplicates. In this model of synergy (Axelrad et al., 2002), the combined effect of two agents is considered to be equal to the sum of the effects of single compound; thus the cytotoxic effect, produced by any mixture, can be predicted. Deviations from this expected constant inhibition (zero interaction) produced by the calculated IC20 are either synergistic (above expected zero effect), or antagonistic (below expected zero effect). The statistical analysis was performed according to Student's two-tailed t-test; p values below 0.05 were considered to be significant.

Genotoxicity analysis

Indirect immunofluorescence method with primary anti-phospho hystone H2A.X antibody was used to determine genotoxicity of Pb and Cd in cell lines. For the assessment of genotoxicity, HL-60 and Jurkat cells were seeded in 24 well plates and treated for 24 h with different levels of Pb or Cd water standards. Cells were treated with RPMI culture medium supplemented with 1/10 volume of Pb or Cd metal solutions Cells were treated with 10% metal solutions and 90% RPMI culture medium. Untreated cells were used as negative control. After 24 h of metal treatment, HL-60 and Jurkat cells were washed with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature (RT). The cells were permeabilized by 0.5% Triton X-100 in PBS, pH 7.4 for 5 minutes at RT and nonspecific staining was blocked with 5% BSA (Sigma) in PBS, pH 7.4 for 30 minutes. The cells were labeled with primary mouse monoclonal anti γH2A.X antibody (Merck Millipore) in 0.5% BSA in PBS for 1 hour at 37°C. The cells were then washed and incubated with secondary goat anti-

mouse antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific) for 1 hour at RT. After final wash, the cells were mounted on slides with ProLongTM Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). The images were taken using fluorescence microscope Olympus IX81 with Prior ProScan11 (Olympus Q Color 5 imaging system, Slide Book 5.0 software) at a 400x magnification. The genotoxic effects were evaluated by the chi square test; p values below 0.05 were considered to be significant.

Results and Discussion

To characterize the individual effects of Pb and Cd on HL-60 and Jurkat cells, Presto blue viability assay was performed. As expected, these metals caused cytotoxic effects in both cell lines (Figure 1).

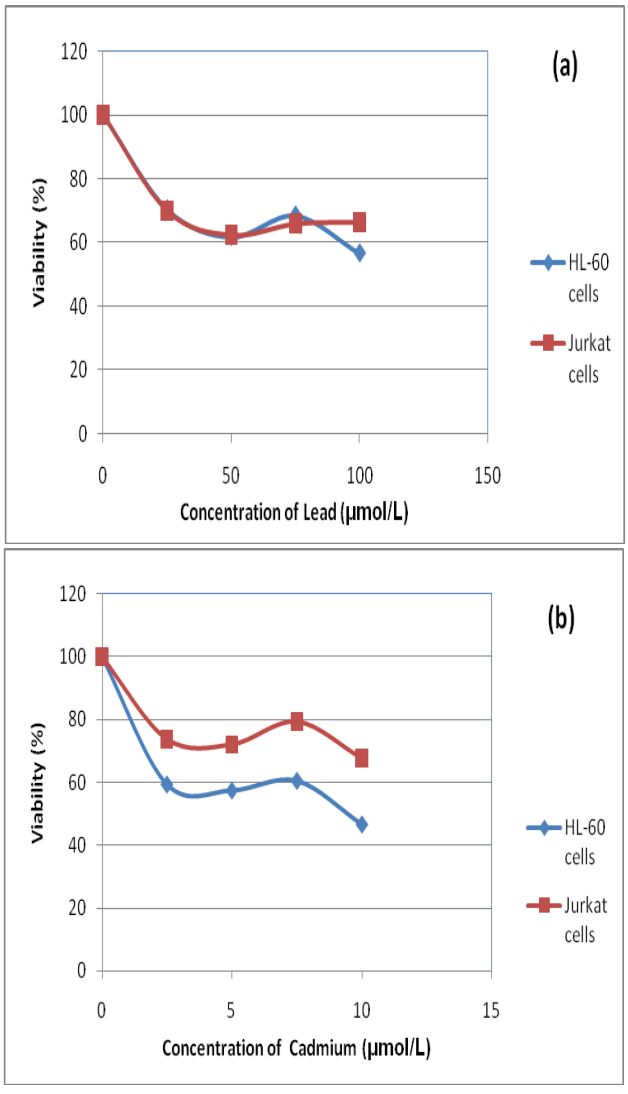

Figure 1. Viability of HL-60 and Jurkat cells after 24 hours exposure to increasing concentrations of Pb (a) and Cd (b)

Our results are in accordance with the results of Yedjou et al. (2003), who found that lead induced cytotoxicity in HL-60 cells, and with the results of Nemmiche et al. (2011) who found that cadmium significantly decreases viability of Jurkat cells. Cadmium exhibited higher cytotoxic potential, which means that the same percent of cytotoxicity was observed at significantly lower concentration of cadmium compared to lead. It was interesting that in both cell lines the most pronounced cytotoxic effect was observed at the lowest concentrations of metals (2.5 µmol/L for cadmium and 25 µmol/L for lead). Further increase in metal concentration did not lead to proportional decline in cell viability, in both lines, respectively. The studies on metal mixtures toxicity have revealed that the effects of mixtures are hard to predict as all possible outcomes have been observed (Norwood et al., 2003; Vijver et al., 2011). These interactions can be conflicting across various experiments (Norwood et al., 2003), or can be concentration dependent (Sharma et al., 1999; Liu et al., 2015). The concentrations of Pb and Cd for the analysis of their interaction were selected from their individual cytotoxic effects. In the absence of interactions, two compounds in combinations at fixed concentrations have the same effect as the individual compounds at those same concentrations; this was taken as the "theoretical expected values"." Significant synergistic effect was observed in HL-60 cells for Pb and Cd in the 50:50 mixture; the cytotoxic effect (28.05%) of mixture was significantly different ($p=0.0471$) from the expected value (Figure 2a). On the contrary, in Jurkat cells, the antagonistic effect was observed, the 50:50 mixture did not cause cytotoxicity ($P<0.0001$) (Figure 2b). These results could be explained by the fact that two examined cell lines originate from different progenitor cells and thus have different patterns of protein expression, some of which could be in interaction with heavy metals and ultimately result in counter-effects. Damage of genetic material after exposure to Pb and Cd was analyzed with indirect immunofluorescence. For the assessment of genotoxicity of lead, both cell lines were exposed for 24 hours to increasing concentrations of lead nitrate water standards, ranging from 25 µmol/L to 4000 µmol/L. For the assessment of genotoxicity of cadmium, both cell lines were exposed to increasing

concentrations of cadmium nitrate water standards, ranging from 10 µmol/L to 160 µmol/L.

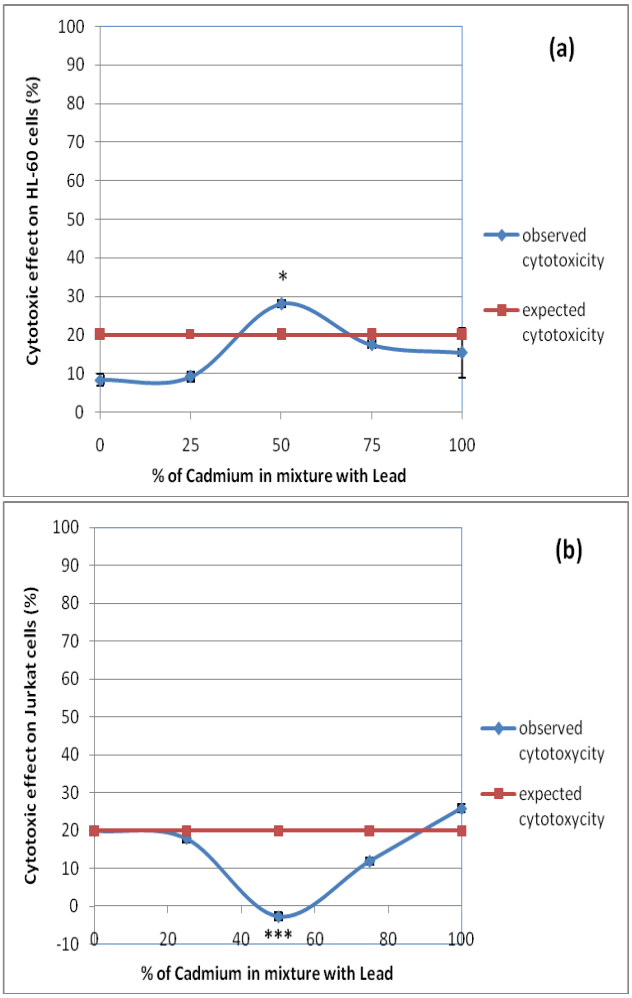

Figure 2. The relationship between cytotoxic effect and the percentage of Pb and Cd in a mixture in HL-60 cells (a) and Jurkat cells (b). Error bars represent standard error of mean (n=3). The 50:50 mixture of Pb and Cd produced significantly higher cytotoxic effect than expected in HL-60 cells and no cytotoxic effect in Jurkat cells, *p<0.05 and ***p<0.0001.

After the exposure, the cells were labeled with anti-phospho- histone H2A.X antibody which binds to the sites of DNA double strand breaks and DNA damage was observed by fluorescence microscopy. Based on the intensity of green fluorescence, positive cells with fractures in the DNA strands (strong intensity) and negative cells (low intensity, auto fluorescence) were distinguished. For each sample, approximately 200 cells were analyzed and the percentage of positive cells (those with damaged genetic material) was calculated.

Representative images of genotoxic effects are shown in Figure 3. In general, lead exhibited fewer genotoxic effects relative to cadmium. Significant genotoxic activity of lead in HL-60 cells was observed at a concentration of 4000 µmol/L (7.11% of cells were labeled) compared to untreated cells (3.45% labeled cells due to various stress factors, cell division), calculated by chi square test ($\chi 2=8.48$, $p<0.05$).

Genotoxic activity of lead in Jurkat cells was observed at a concentration of 1000 µmol/L (9.69% of cells were labeled). When compared to untreated cells (2.82% labeled cells), this percent of positive cells was significantly higher ($\chi 2=6.65$, $p<0.05$). Our results are consistent with the results of other numerous studies that have demonstrated the genotoxic potential of lead in different cell cultures (Garcia-Leston et al., 2010).

However, the results obtained using different detection methods, different cell lines as experimental models and different lead salts, were diverse with respect to the concentration of lead that causes genotoxic effects.

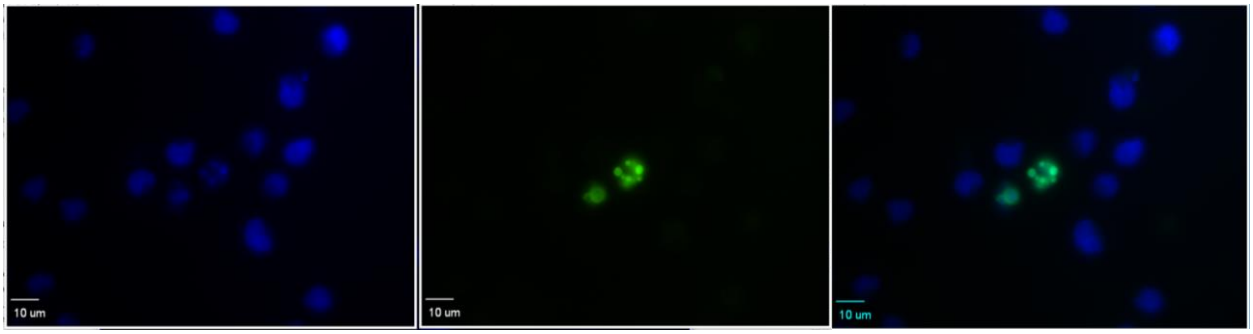

Figure 3. Genotoxic effects of 10µmol/L Cd on HL-60 cells after 24 hours exposure. Cells stained with DAPI are visible in violet-blue color and show the total number of all cells in the vision field of the microscope (left). Positive cells or cells with DNA damage that are stained with anti-phospho-histone H2A.X antibody and secondary antibody conjugated with Alexa Fluor 488 are visible in green (center). Merge (right) obtained by a ImageJ software. Each dot in the cell nucleus that emits a strong green fluorescence represents DNA strand break.

After extensive research of available publications, we concluded that the genotoxic activity of lead has not been investigated earlier by detecting the double strand breaks with anti-phospho-histone H2A.X antibody. On the other hand, cadmium exerted greater genotoxic activity, which showed dose dependence in both cell lines (Figure 4).

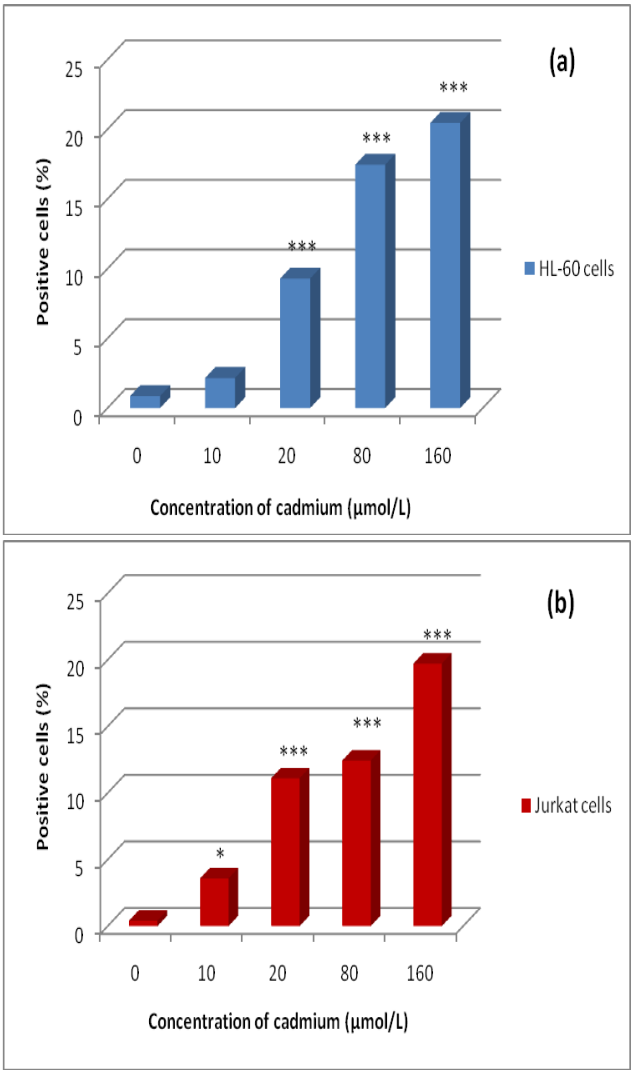

Figure 4. Genotoxic effects of cadmium on HL-60 cells (a) and Jurkat cells (b). *p<0.05 and ***p<0.0001.

The number of stained (positive) cells and unstained (negative) cells (in both cell lines) after the exposure to cadmium concentration gradient are presented in Table 1. Statistical significance between the frequencies of stained cells after exposure to certain Cd concentration and negative control (untreated cells) was tested by a Chi-square test whose values are also presented in the Table.

Although the toxic effects of lead and cadmium compounds have been extensively studied, inconsistent results have been obtained about their mutagenic, clastogenic and carcinogenic properties. Valverde et al., (2001) suggested that these metals cause genotoxicity and carcinogenicity not by direct interaction with DNA, but by indirect interactions, such as oxidative stress. The authors found an induction of lipid peroxidation and an increase in free radical levels in the different organs of mice after inhalation of lead acetate or cadmium chloride, suggesting the induction of genotoxicity and carcinogenicity by indirect interactions with DNA.

Conclusions

Heavy metals are ubiquitous and occur as heterogeneous mixtures in the environment. Our results indicate that mixtures of Pb and Cd cause synergistic cytotoxic effects in HL-60 cells. In general, the combined effects should be considered in the risk assessment of heavy metal co-exposure and potency. In order to reduce the environmental and public health effects of heavy metal pollution, governments and health agencies need to pay additional attention to the environment and anthropogenic activities.

Table 1. Cadmium induced genotoxic effects

	Number of HL-60 cells			Number of Jurkat cells		
Cd (µM)	positive	negative	χ^2	positive	negative	χ^2
0	4	242		1	245	
10	5	226	0.66	8	215	6.29^{*}
20	21	195	14.73^{***}	23	184	25.67^{***}
80	41	194	35.47^{***}	28	197	29.47^{***}
160	45	175	43.76^{***}	43	175	50.25^{***}

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Conflict of interest

Authors declare that they have no conflict of interests.

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Research article

NEW INSIGHTS INTO THE ANTIFUNGAL ACTIVITY OF *Taxus baccata* L.

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Abstract

Taxus baccata L., yew, is dioecious, small to medium-sized evergreen tree, native to Europe, Africa and Asia, but it is cultivated worldwide because of its ornamental features. It is long known that all parts of yew (except for aril) are toxic, due to the alkaloid taxine. Nevertheless, some parts of yew tree can be used to treat cancer and as antimicrobial agents. Unlike its antibacterial properties, antifungal activity of *T. baccata* is poorly investigated. In this research, antifungal activity of yew methanolic and aqueous extracts was tested against *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404, and *Ascosphaera apis* MUCL 30769, through the agar well diffusion method. Leaves, bark and reproductive structures extracts were prepared separately for male and female plants. Unlike the aqueous, methanolic extracts caused variable degree of fungal growth inhibition. The strongest inhibition was observed in the activity of the aril extract against *A. brasiliensis* and *C. albicans*, with the activity of the male bark extract against *A. apis* following close. Considering the emerging multidrug resistance in *C. albicans*, an interesting finding is the inhibition of this species by all tested methanolic extracts, which is significantly stronger in comparison to the inhibition by standard antimycotic solution. According to the available data, male reproductive structures of this species were not tested earlier for their antifungal activity, and our study showed high level of antifungal activity of methanolic microstrobili extract. The realized research indicates great antifungal potential of *T. baccata*, which should be further confirmed by more comprehensive studies.

Introduction

Genus *Taxus* L. (Taxaceae), yew, is widely distributed in Europe, North America, Eastern Asia and Asia Minor, and it comprises eight species and two hybrids worldwide (Farjon et al., 2019). Great attention is focused on the genus *Taxus*, due to the isolation of paclitaxel, a unique diterpene taxoid from the bark of the *T. brevifolia* Nutt. (Wani et al., 1971), that is widely used as anticancer agent (Erdemoğlu et al., 2003). *Taxus baccata* L. is a dioecious, evergreen and widespread shrub, commonly used for ornamental landscaping (Kucukboyaci & Şener, 2010). *T. baccata* is well

known for its poisonous properties. All plant parts with the exception of the aril, which envelopes seeds, contain toxic taxine alkaloids (Anadón et al., 2018). This is the main reason why this species has rarely been documented as a remedy in traditional medicine. Nevertheless, there are some reports that yew leaves have been used as abortifacient, antimalarian, antirheumatic and for bronchitis, while dried leaves and bark have been used against asthma (Küpeli et al., 2003). Recent studies show that lignans, specific secondary metabolites isolated from *T. baccata*, possess several biological activities: antiviral, antifungal, antibacterial, anticancer, antioxidant and anti-inflammatory (Kucukboyaci & Şener, 2010). Different parts of *T. baccata* are rich in bioflavonoids such as: sciadopitysin, ginkgetin, kayaflavone and amentoflavone (Krauze-Baranowska & Wiwart, 2003), and while dimeric flavones possess antiviral, antibacterial and antimycobacterial properties (Erdemoğlu et al., 2003), antifungal activity is still poorly investigated. Literature survey revealed some data regarding antifungal activity of *T. baccata* (Erdemoğlu & Şener, 2001; Krauze-Baranowska & Wiwart, 2003; Kucukboyaci & Şener, 2010), as well as of *T. cuspidata* Sieb. et Zucc. var. *nana* Rehder. (Tachibana et al., 2005) and *T. wallichiana* Zucc. (Nisar et al., 2008). Emergence of new, multidrug-resistant pathogens, as well as undesirable side effects of antimicrobial drugs have triggered tremendous interest in the search for new antimicrobial agents of plant origin (Bernaitis et al., 2013). The main goal of this investigation was to evaluate antifungal activity of methanolic and aqueous extracts of *T. baccata*, obtained from different vegetative and reproductive plant structures.

Materials and methods

Plant material

The plant material of female and male yew individuals was collected in Sarajevo, Bosnia and Herzegovina and determination was carried out in Laboratory for Plant Systematics, Department of Biology, Faculty of Science, University of Sarajevo. The plant material was separated into leaves, bark and reproductive structures (arils and microstrobili) and such separated material was dried in dark, in ventilated room at the ambient temperature.

Preparation of extracts

For this investigation of antifungal activity, methanolic and aqueous extracts were prepared from dried, finely ground plant material. For methanolic extracts 100 mg of dry plant material was soaked in 10 ml of 80% methanol, and the extraction lasted for 24 hours. Aqueous extracts were prepared by boiling the 100 mg of herbal powder in 10 ml of distilled water. The obtained extracts were dried, weighted and dissolved to the final concentration of 1 mg/mL for antifungal assays. All extracts were stored in dark, at +4 °C.

Investigation of antifungal activity

Antifungal activity of *T. baccata* methanolic and aqueous extracts was tested against three reference strains of fungi: *C. albicans* ATCC 10231, *A. brasiliensis* 16404 and *A. apis* MUCL 30769, using agar-well diffusion method (Balouiri et al., 2016). Fungal species were cultured at 37 °C on Sabouraud Dextrose Agar (HiMedia Laboratories Pvt.Ltd., India) for 24-48 hours. The inoculums harvested from agar plates were prepared in sterile saline solution and according to the National Committee for Clinical Laboratory Standards (2015), adjusted to 0.5 McFarland scale (~1.5 x 108 CFU/mL).
Prepared fungal inoculums (100 µl) were spread over the entire surface of plates with growth medium and left for 15 minutes at room temperature to achieve total absorption. Investigated extracts and control samples (50 µl) were then transferred into the wells of inoculated agar plates. Plates were incubated for 24-48 hours at 37 °C. Antifungal activity of the tested extracts was evaluated based on diameter (mm) of inhibition zones, which result from extract diffusion in the medium and inhibition of fungal growth. Antibiotic-antimycotic solution (10.000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL; Sigma-Aldrich) was used as positive control, while methanol and distilled water were used as negative controls. Positive control was prepared according to the manufacturer's recommendations. All the tests were performed in three replications and the mean values (±SD) were calculated.

Statistical analysis

All values are presented as mean ± standard deviation (SD). Descriptive statistical analyses were conducted using Microsoft Office 2013 Excel (Microsoft Corporation, Redmond, USA). Data were further analysed by using one-way ANOVA and post hoc LSD test (STATISTICA 10; StatSoft. Inc.), with significance level set at $p < 0.05$.

Results and Discussion

In this study, antifungal activities of methanolic and aqueous extracts obtained from leaves, bark and reproductive structures of *T. baccata* were tested against three fungal species. Antifungal activity of negative controls: 80% methanol and distilled water was not observed. Aqueous yew extracts did not inhibit fungal growth while methanolic extracts exhibited varying degree of antifungal activity (Table 1). It is known that methanol as a solvent gives better results in extraction of bioactive compounds, when compared to other solvents (Altemimi et al., 2017). Also, in antimicrobial surveys, methanolic extracts usually exhibit stronger antimicrobial activity then aqueous extracts (Ghosh et al., 2008, Mahesh & Satish, 2008).

The widest zones of inhibition were observed in *A. brasiliensis* (23.33±2.08 mm) and *C. albicans* (22.67±0.58 mm) treated with aril methanolic extract (Table 1.). Arils are soft, fleshy, bright red, berry-like structures that represent a specialized outgrowth of a seed, and are the only non-toxic parts of the yew plant.

Recent study (Siegle & Pietsch, 2018) reported that arils contain bioactive compounds: 3,5-dimethoxyphenol, 10-deacetylbaccatin III, baccatin III, cephalomannine, taxol A and taxinine M. Considering that these compounds are precursors of drugs derived from *T. baccata*, it is possible that they are also responsible for antifungal activity of aril methanolic extracts. Following an extensive literature survey, no data on the study of antifungal activity of *T. baccata* microstrobili extracts were found. Microstrobili are male cones of *T. baccata*, which are yellow, globose and pedunculate. Each male cone is composed of 6-14 peltate, radially extending microsporophylls, with 6-8 microsporangia i.e. pollen sacs (Predan & Toniuc, 2009). It has been proven that *T. baccata* pollen contains bioactive diterpenic alkaloids belonging to taxine and taxoids (Vanhaelen et al., 2002). Of all investigated fungi, the most sensitive species to the microstrobili methanolic extract was *A. brasiliensis*, with inhibition zones of 19.00±3.46 mm (Table 1.). In this research *C. albicans* was the most sensitive species in terms of yew extracts antifungal activity. All investigated extracts showed strong antifungal activity against *C. albicans* (Table 1.), with inhibition zones ranging from 14.67±1.15 mm (microstrobili) to 22.67±0.58 mm (aril). This result is particularly intriguing, since *C. albicans* is a multidrug-resistant pathogen (Arendrup & Patterson, 2017), and even in this study, positive control caused significantly narrower inhibition zones (Table 1.). In addition, the tested antibiotic-antimycotic solution showed no inhibitory effect

Table 1. Antifungal activity of investigated *Taxus baccata* extracts

Methanolic extracts	Tested fungal species		
	Aspergillus brasiliensis	*Candida albicans*	*Ascosphaera apis*
♀ Leaf	14.33±3.21 ^{b,c}	18.33±3.79 ^{b,e}	11.00±2.64 ^{b}
♀ Bark	20.67±2.08 ^{a,e}	18.33±2.31 ^{b,c,e}	11.66±2.08 ^{b}
Aril	23.33±2.08 ^{a}	22.67±0.58 ^{a}	NI ^{c}
♂ Leaf	10.33±0.58 ^{c}	18.67±1.53 ^{b,c,d}	19.67±4.93 ^{a}
♂ Bark	15.00±2.00 ^{b,d}	19.33±1.53 ^{a,b,c,d}	20.83±0.29 ^{a}
Microstrobili	19.00±3.46 ^{d,e}	14.67±1.15 ^{e}	18.00±0.50 ^{a}
Positive control	0.00±0.00 ^{f}	4.90±0.36 ^{f}	17.00±0.50 ^{a,b}

Results are mean diameter in mm ± SD (n=3). NI = No inhibition. 80% methanol = NI.
Values not sharing the same letters differ significantly at $p < 0.05$ after *post-hoc* LSD test.

against *A. brasiliensis*, while all investigated methanolic extracts of *T. baccata* successfully inhibited growth of this fungal species (Table 1.). Antifungal drugs in general can be divided into four groups: polyenes (1), nucleic acid synthesis inhibitors (2), ergosterol biosynthesis inhibitors (3) and echinocandins (4), but clinical resistance has been observed for all classes of antifungals (Marie & White, 2009). Microbiological resistance of a fungi to an antifungal agent can be primary (intrinsic) or secondary (acquired). While primary resistance is found naturally among certain species without prior exposure to antifungal agents, secondary resistance develops among previously susceptible strains after exposure to antifungal agent, and is usually dependent on altered gene expression (Kanafani & Perfect, 2008). Mechanisms of antifungal drug resistance include resistance genes, transcriptional regulation of drug resistance and some alternative mechanisms such as: overexpression of PDR16 gene (Liu et al., 2007; Znaidi et al., 2007), aneuploidy (as a way of increasing gene copy number by whole chromosome duplication or other genomic rearrangements) which serves as an adaptive response when cells are stressed (Selmecki et al., 2008), and formation of biofilms (Nett et al., 2007; Seneviratne et al., 2008). The resistance to antifungal agents is mainly the result of specific gene mutations, and many of them have so far been identified. Among the most investigated are point mutations in Erg11p that result in amino acid substitutions in lanosterol demethylase (Mellado et al., 2007); several mutations in the glucan synthase gene FKS1 (Cleary et al., 2008; Garcia-Effron et al., 2008); and mutation of the genes involved in 5-FC toxicity (Papon et al., 2007). When discussing transcriptional regulation of resistance, mechanisms that should be mentioned are overexpression of ERG11 and other ergosterol biosynthetic genes due to the Upc2p - the major regulator of ergosterol biosynthesis (White & Silver, 2005). Furthermore, fungal efflux pumps could mediate drug resistance through different inducible and constitutive pathways (Sanglard & White, 2006). In general, *A. apis* was the most resistant fungal species in this investigation, which could be partly explained by its morphology. *A. apis* is spore-forming, filamentous fungus, with mature spores tightly packed inside the spherical spore balls. More than ten spore balls could be wrapped and formed spherical, nearly hyaline spore cyst. The spore wall is two-layered, and chitin is the major component of the spore wall. Furthermore, in addition to the typical fungal spore organelles, an unknown structure positioned close to the inner spore wall, and covering almost the entire wall area, was recently discovered in this fungus (Li et al., 2018). *A. apis* was the most susceptible to the male bark extract (20.83±0.29 mm), while the aril extract showed no antifungal activity (Table 1.). *T. baccata* methanolic leaves extracts were efficient against all three investigated fungal species, especially in case of the *A. apis* inhibition (19.67±4.93 mm) by the male leaves extract (Table 1.). Investigation of Krauze-Baranowska & Wiwart (2003) showed that methanolic extracts made from *T. baccata* leaves contain biflavones: bilobetin, amentoflavone, 4-O methyl amentoflavone, 7-O methyl amentoflavone, sciadopitysin and ginkgetin. These compounds significantly inhibit the growth of fungal sporae and germ tubes. Furthermore, Patel et al. (2009) reported lignans and flavonoids in the yew leaves extract, which are the potential antimicrobial agents. Extracts made from leaves, bark and heartwood of *T. baccata* successfully inhibited some other fungal species such as: *Trichophyton longifusus, Micosporum canis,* and *Fusarium solani*, but failed to inhibit of the growth of investigated *Candida* and *Aspergillus* species (Nisar et al., 2008). Contrary to these findings, the realized study accomplished significant inhibition of *C. albicans* and *A. brasiliensis* by all investigated yew extracts. Erdemoğlu & Şener (2001) reported successful inhibition of *Nigrospora oryzae, Epidermophyton floceasum, Curvularia lunata* and *Pleuralus astreatus* with *T. baccata* extracts, which is attributed to the presence of the lignan derivative taxiresinol and 3'-demethylisolariciresinol (Kucukboyaci & Şener, 2010). Overall results of this investigation showed that methanolic extracts obtained from female yew plant exhibited greater antifungal potential when compared with the extracts made from male individuals (except in *A. apis*). It is known that the sex of yew trees may have a significant impact on the content of bioactive compounds taxanes. Females of dioecious plants usually have greater reproductive effort in

comparison to males, and this phenomenon was found in the case of yew. The greater reproductive effort by females results in the intensification of gas exchange and consequently significantly higher concentrations of carbon-based secondary metabolites, including taxanes (Iszkuło et al., 2013).

Conclusions

Taxus baccata is well known plant, mainly for its poisonous and ornamental features. Although it is rarely mentioned in traditional (folk) medicine, some studies do confirm its bioactive properties. However, antifungal effects of yew are poorly investigated, and this study highlighted significant antifungal activity of *T. baccata* methanolic extracts, obtained from leaves and bark of female and male individuals, as well as from reproductive structures: arils and microstrobili. The most sensitive fungal species was *C. albicans*, while the most resistant was *A. apis*. Realized investigation represents an original approach in obtaining extracts of diecious plants with potential antimicrobial activity. Also, to the best of our knowledge, this is the first time that *T. baccata* was investigated in a way of separating extracts per plant part and sex. Considering the fact that tested fungal species exhibit multidrug-resistance, successful inhibition by obtained yew extracts is noteworthy. With regard to the poisonous activity of *T. baccata*, these results should be further confirmed by more comprehensive studies, especially in terms of toxicity and genotoxicity. Also, wider use of *T. baccata* extracts inevitably implies determination of adequate therapeutic concentrations.

Conflict of interest

Authors declare no conflict of interest.

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Short communication

VALIDATION OF CRC AND NSCLC SOMATIC MUTATIONS DETECTED WITH NGS USING ddPCR

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Abstract

Next Generation Sequencing (NGS) has become powerful tool in molecular oncology. It allows multiparallel targeted sequencing that enables comprehensive assessment of tumor heterogeneity. Detection of mutations in colorectal cancer (CRC) and non-small cell lung cancer (NSCLC) defines patients diagnosis, therapy and prognosis. Multiple genes, their somatic mutations to be precise, carry different degrees of importance for each of these aspects. Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2, which was used in this study, allows detection of hotspot mutations in 22 genes in a single reaction. Droplet digital PCR (ddPCR) has a unique advantage in low frequency mutation detection and it has been used as a validation tool for mutations that were detected with NGS. It has high sensitivity and enables accurate detection of a mutant allele against a background of abundant wild type alleles. For this study 35 samples of CRC and NSCLC were sequenced and selected samples were analysed with ddPCR for KRAS, NRAS, EGFR and BRAF genes. All the processed samples were successfully sequenced and had average base coverage >500X. NGS sequencing proved itself to be cost effective, has shorter turnaround time and is highly sensitive. Out of 35 samples, 25 had genetic alterations, while 10 samples were reported as wild type but were still tested with ddPCR as controls. In three samples low frequency somatic mutations were detected with NGS and mutation frequencies were verified using ddPCR.

Introduction

In the past few years, next generation sequencing (NGS) played an important role in understanding altered molecular pathways in cancer. First of all, it is a massive parallel sequencing process that consists of simultaneous sequencing of multiple targets in the same run. Having said that, it is cost effective, it has reduced turnaround time and it requires low DNA input which makes the method perfect for low copy DNA samples (cfDNA) (Serrati et al., 2016). Sequencing by synthesis is performed in

microscopic wells on semiconductor chip. In this study, Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 was used to screen CRC and NSCLC cancers. NGS sensitivity is high (2-6%) and it allows quantitative evaluation of the mutated allele (Quail et al., 2012; Ross et al., 2013). Detection of gene alterations in solid tumors define patients diagnosis, treatment plan and prognosis. Droplet digital PCR offers a number of unique advantages, especially when it comes to rare mutation detection and precise quantification of DNA. Compared to real-time PCR, ddPCR is considerably more sensitive and reproducible (Huggett et al., 2015). DdPCR enables accurate detection of a mutant allele in a background of highly abundant wild type allele. Bio-Rad QX200 ddPCR system combines water-oil emulsion droplet technology. Droplet generator partitions samples into maximum of 20 000 droplets and PCR amplification is carried out inside each droplet. Fluorescence is detected in each droplet using QX200 droplet reader. Using a well designed experiment setup, ddPCR can detect 1 mutant in a 10 000 wild type alleles. That kind of sensitivity makes ddPCR perfect for validation of low frequent somatic mutations detected by NGS. Colorectal cancer (CRC) is the second most frequent cancer in Europe and is responsible for 12% of cancer deaths (Ferlay et al., 2013). KRAS is predictive biomarker for the efficiency of anti-EGFR therapy in colorectal cancer (CRC) because KRAS mutant CRC patients are resistant to treatment with EGFR inhibitors. NSCLC accounts for 80-85% cases of lung cancers. Clinical molecular diagnosis of lung cancer consists of identifying druggable alterations (EGFR, RAS, BRAF, MET, ERBB2, ALK etc) (Garinet et al., 2018). New NCCN (The National Comprehensive Cancer Network) Guidelines (Benson et al., 2018) for CRC strongly recommend broader molecular testing and identifying rare driver mutations for which effective treatments already exists. In this study we validated ddPCR as a confirmation method for somatic mutations detected by NGS.

Materials and methods

For this study 35 samples of CRC and NSCLC were obtained from different medical centers from Bosnia and Herzegovina. All of the analyzed samples were FFPE blocks previously reviewed by a pathologist. Pathologist determined type and stage of cancer, area with the highest neoplastic cell content was marked and percentage of neoplastic cells was determined.

Recommendations for molecular genotyping were made based on pathohistological report because of the targeted therapy prospects. Samples were transported with on standard room temperature. The manufacturer's protocols were followed without modification. Genomic DNA was extracted from all clinical tissue samples (FFPE) using QIAamp® DNA FFPE Tissue Kit (Qiagen, 2012) according to the manufacturer's protocol followed by quantification using Qubit 3 Fluorometer® and Qubit® dsDNA HS assay kit (Life Technologies, 2014). NGS libraries were prepared applying Ion AmpliSeq™ Library Kit 2.0 and Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 primers according to the manufacturer's instructions (Life Technologies, 2019). This is a hotspot panel designed to target clinically important regions of 22 genes covering 504 mutational hotspot regions of: AKT1 (NM_05163), ALK (NM_004304), BRAF (NM_004333), CTNNB1 (NM_001904), DDR2 NM_006182), EGFR (NM_005228), ERBB2 (NM_004448), ERBB4 (NM_005235), FBWX7 (NM_033632), FGFR1 (NM_000604), FGFR2 (NM_000141.2), FGFR3 (NM_000142), KRAS (NM_004985), MAP2K1 (NM_002755), MET (NM_000245), NOTCH1 (NM_017617.2), NRAS (NM_002524), PIK3CA (NM_006218.1), PTEN (NM_000314.4), SMAD4 (NM_005359.3), STK11 (NM_000455) and TP53 (NM_000546). Libraries are quantified with Ion Library TaqMan™ Quantitation Kit (Life Technologies, 2019).

Each library was diluted to 100 pM before final library mixing for chip templating on Ion Chef™ Instrument using Ion 510 & Ion 520 & Ion 530 Chef Kit. NGS sequencing was performed on Ion GeneStudio™ S5 instrument. Raw data was analysed using Torrent Suite Software 5.8.0 where the sequences were aligned to the h19 human reference genome. Expected coverage of 500X for each sample was acquired before further analysis of sequencing data. Mutations were analyzed using Variant Caller plug-in version v5.8.0.21. Parameter settings that were used are Generic-S5/S5XL

(510/520/530)- Somatic- Low Stringency. All detected variants were manually reviewed using ClinVar as a referent database. All 35 samples were also tested with ddPCR for specific KRAS, BRAF, NRAS and EGFR gene mutations. ddPCR™ KRAS G12/G13, NRAS Q61, BRAF V600, EGFR T790M, EGFR L861Q, EGFR L858R and EGFR del19 Screening Kits were used for detection of mutations. All of wild type controls were analyzed with ddPCR for each mutation. The multiplex assay was 20X concentrated and ddPCR supermix for probes was 2X concentrated. Samples were placed into a QX200 droplet generator which uses mirofluidics to portion each sample into 20 000 droplets. Formed droplets are then transferred to a 96-well plate and placed into Bio-Rad T100 thermal cycler. Following DNA amplification, the plate containing the droplets was placed in QX200 droplet reader which analyzes each droplet individually using two color system (FAM and HEX/VIC) (Bio-Rad, 2017). Chi-squared test was used to determine correlation between detected mutation frequencies acquired by NGS and ddPCR. For expected values, we used mutation frequencies detected by NGS and as observed values, we used mutation frequencies aquired by ddPCR. For chi-squared test 25 mutations were analyzed.

Results and Discussion

Out of 35 analysed samples 18 were from patients diagnosed with CRC and 17 with NSCLC. All 35 samples were successfully sequenced and had average base coverage of more than 500X. Number of samples which have one of analyzed mutations was 25, while 10 tested samples were wild type. Wild type samples were further tested with ddPCR to verify precision of NGS somatic mutation detection. Regarding sequencing performance, average number of reads for 35 samples was 490580. In CRC samples, none of the EGFR mutations were detected by NGS or ddPCR. BRAF V600 mutation was detected in 3/13 patients (23,07%), KRAS G12/13 mutation was detected in 8/13 patients (61,53%) and NRAS Q61 mutation was detected in 2/13 patients (15,38%). NRAS G12/13 mutation was not detected in the CRC patients. The similar result was reported for Belgian CRC patients where the highest mutation frequency was for KRAS mutation (46%) and the lowest (0.3%) for EGFR mutations (D'Haene et al., 2018).In NSCLC, none of the NRAS mutations were detected by NGS or ddPCR. EGFR L861Q mutation was detected in 1/12 NSCLC patient (8,33%), EGFR L858R mutation was detected in 2/12 patients (16,66%), EGFR T790M mutation was detected in 3/12 patients (25%), EGFR del19 mutation was detected in 2/12 (8,33%), BRAF V600 mutation was detected in 2/12 patients (16,66%) and KRAS G12/13 was detected in 4/12 patients (33,33%) (Table 1.). The prevalence of *EGFR* mutations in NSCLC patients ranges from 40% in Asian patients to 11–17% in Caucasian patients (Garinet et al., 2018).

Table 1. Percentage of specific mutations found in CRC and NSCLC

Mutation	CRC (%)	NSCLC (%)	Total (%)
EGFR L861Q	0	8,33	4
EGFR L858R	0	16,66	8
EGFR T790M	0	25	12
EGFR del19	0	16,66	8
BRAF V600	23,07	16,66	20
KRAS, G12/13	61,53	33,33	48
NRAS, G12/13	0	0	0
NRAS Q61	15,38	0	8

In our study, the most frequent mutation in both CRC and NSCLC was KRAS G12/G13 mutation. In three samples low frequency somatic mutations were detected by NGS and verified using ddPCR. In sample number 3, KRAS Gly13Asp mutation was detected by NGS with frequency of 3,5%; sample 3 was also tested with ddPCR and it showed the same mutation with frequency of 2,6% (Figure 1. and Figure 2.). Sample number 15 showed EGFR L861Q mutation with frequency of 4,3% on NGS and with ddPCR the same mutation was detected with the frequency of 4,1%. Sample number 21 had KRAS Gly12Val mutation with frequency of 3,9% with NGS and wtith ddPCR the same mutation was detected with the frequency of 3,1%. Our study is in correlation with other studies that suggest that the most frequent mutations in CRC are in KRAS gene (Dinu et al., 2014; D'Haene et al., 2018).

Value of $\chi 2$ for 27 observed mutations was 30,474 which is lower than critical value of 38,885. p-value was lower than 5% which proves that the results are

statistically significant and there is no difference between observed NGS and expected ddPCR frequencies. A major advantage of NGS is its ability to detect multiple mutations on multiple targets simultaneously. NGS sequencing has the advantage over other conventional methods in terms of cost, sensitivity and turnaround time which is of high importance for oncological patients. Earlier reports (D'Haene et al., 2015) validated NGS for surgical resection, biopsies and cell blocks on 90 CRC and NSCLC patients using Colon and Lung Cancer panel. For our laboratory, NGS is found to be cheaper and faster when multiple alterations must be screened. The number of genetic alterations associated with targeted treatment is increasing, so genetic profiling that includes more than few genes is imperative (Tsoulos et al., 2017). In the present study, targeted NGS panel was used for simultaneous analysis of alteration in 22 genes that are commonly mutated in CRC and NSCLC. This panel is cost effective, has good sequencing performance, high sensitivity and is compatible with low amounts of DNA which are expected from FFPE samples.

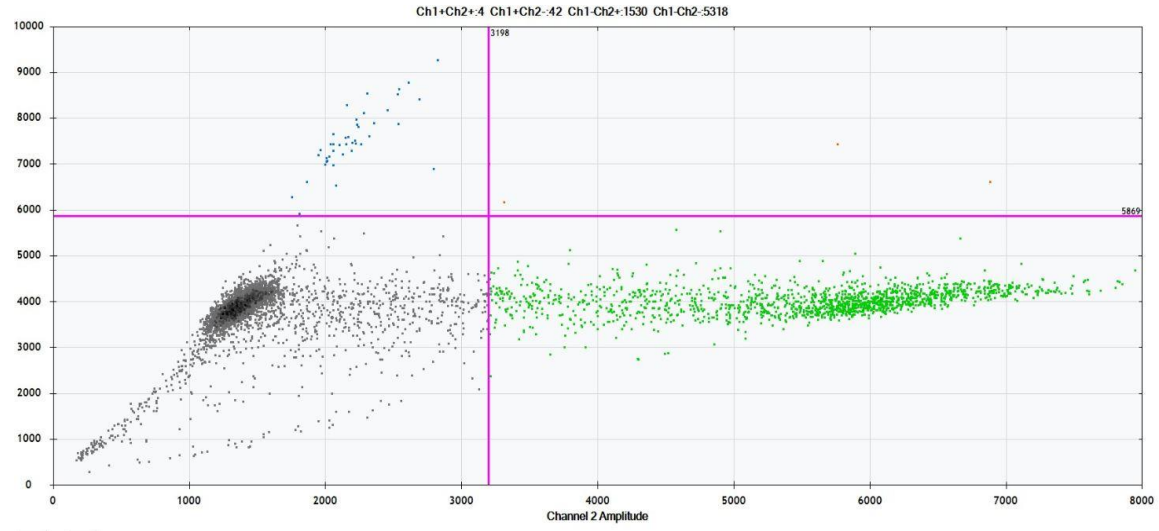

Figure 1. Generated droplets for KRAS G13 mutation in sample 3

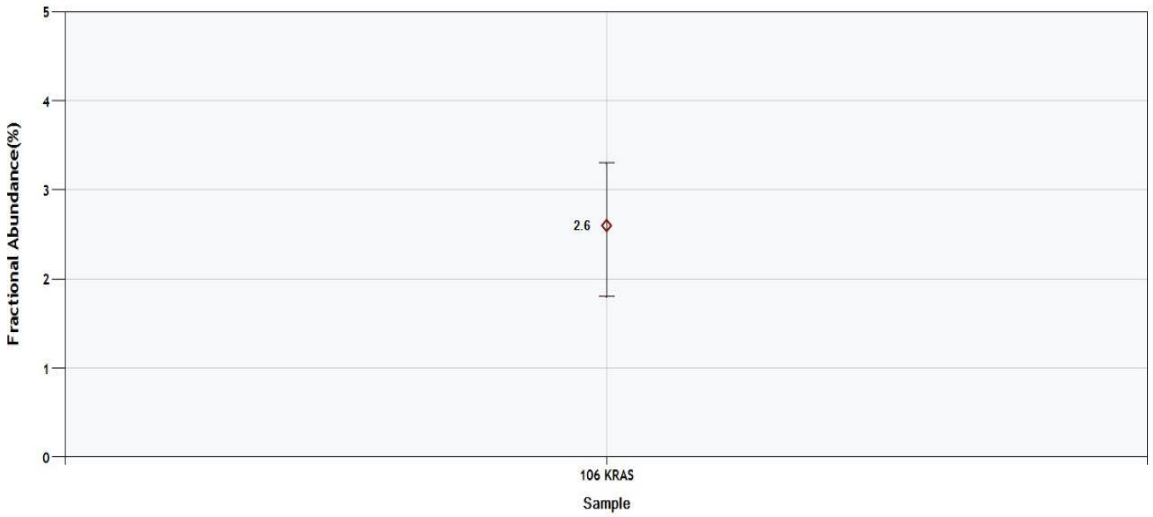

Figure 2. KRAS G13 mutation with the frequency of 2,6% detected by ddPCR in sample 3

Conclusions

Targeted sequencing by NGS is a method of choice for CRC and NSCLC molecular profiling due to the capability of providing necessary informations which guides physicians to an informative decision regarding targeted therapy. Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2, used on a Ion S5 platform, proved itself as highly informative and sensitive, and henceforth valuable in clinical practice. This study showed that the most frequent mutation in CRC and NSCLC is KRAS G12/G13 mutation. The study showed that there is no difference between observed genotypes obtained with NGS or ddPCR (Figure 3.). ddPCR can be used as a validation tool for mutations detected by NGS.

Conflict of interest

The authors have declared no conflicts of interest.

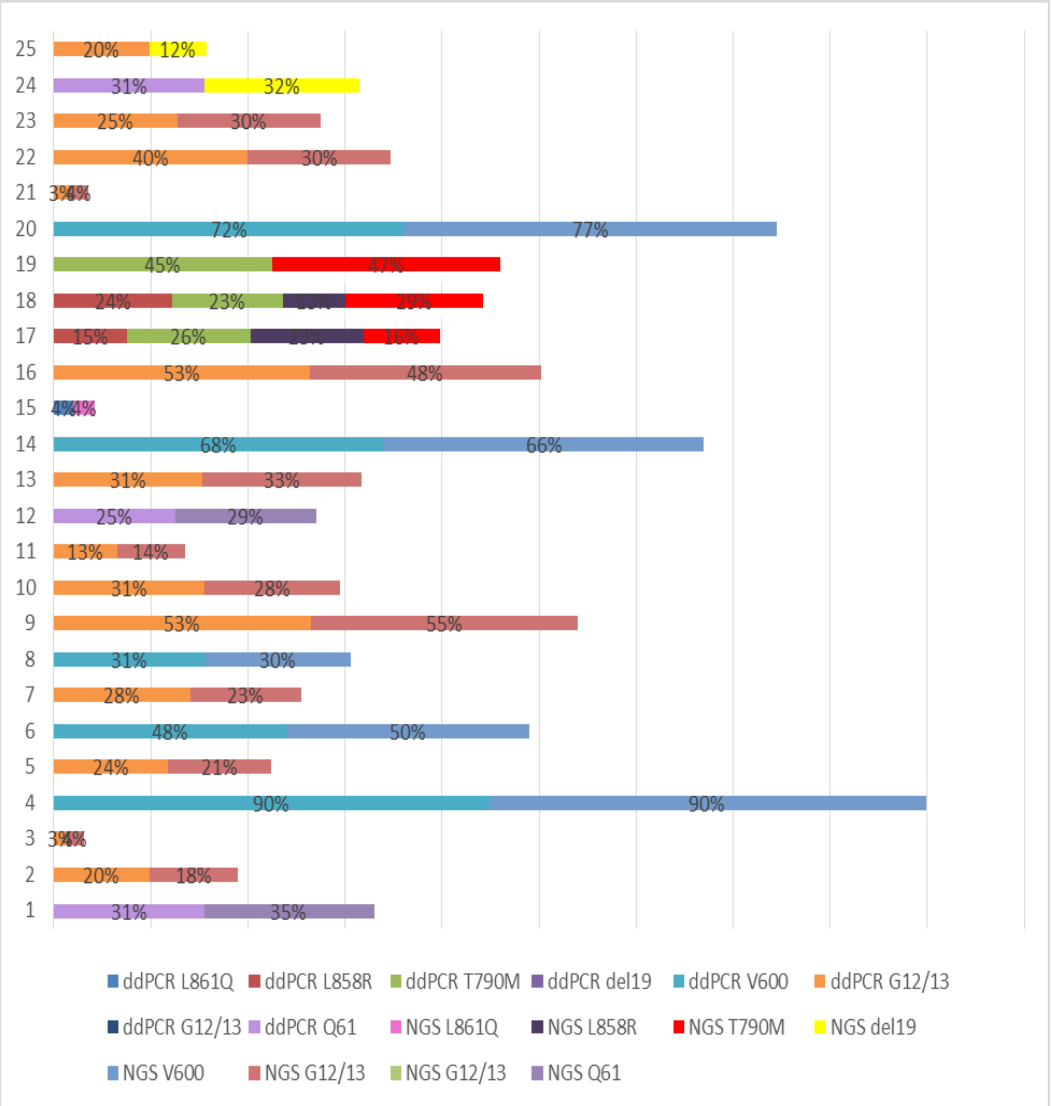

Figure 3. Frequency of mutations detected using ddPCR and NGS

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Notes

ALRATIO - R SCRIPT FOR THE ANALYSIS OF RELATION BETWEEN THE EFFECTIVE AND THE DETECTED NUMBER OF ALLELES

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It is widely accepted that understanding the heterogeneity of a population is important in assessment of the vulnerability of a conservation unit (Frankham et al., 2002). Standard measures such as estimation of heterozygosity, deviations from Hardy–Weinberg equilibrium, effective population size, inbreeding coefficients are widely used. Minor, but very important elements of these measures are allelic diversity, effective number of alleles and allelic richness which characterize the extent of genetic diversity. Allelic diversity (A_n) represents an average number of alleles per locus determined by direct count. When more than one locus is considered, it is calculated as a number of alleles averaged over loci expressed as *k/l* where *k* is the total number of alleles determined at all the observed loci and *l* is the number of loci (Frankham et al., 2002). The effective number of alleles (A_e) is a measure that shows the number of alleles required to ensure the same level of heterozygosity under the assumption of balanced allele frequency and low influence of rare alleles. It is expressed as $1/\Sigma p_i^2$ where p_i is the frequency of the i^{th} allele (Allendorf et al., 2013).

Assessing allelic richness (A_E) implies using the rarefaction method to estimate the number of alleles expected in samples of specified size (Foulley et Ollivier, 2006). It is based on the assumption that higher number of alleles is expected in large samples and rarefaction solves this problem (Kalinowski, 2004). This measure of genetic diversity is indicative of a population's long-term potential for adaptation (Greenbaum et al., 2014).

These three measures are used less often than heterozygosity as a genetic diversity measure, but their usefulness for identifying populations in need of for conservation is asserted. When we consider the loss of genetic variation in small populations we usually refer to lower levels of heterozygosity and allelic diversity. Even bottleneck of low intensity may have significant impact on loss of allelic diversity. Generally, bottlenecks have a stronger effect on allelic diversity than on heterozygosity (Allendorf et al., 2013).

In light of the above, in the estimates of the genetic diversity of a conservation unit, the predictions based on alleles count calculations are essential. The estimates based solely on the number of alleles may lead to inaccurate conclusions. Allelic diversity shows the number of alleles, while effective number

of alleles provides information about contribution of alleles to heterozigosity. The difference between the detected and effective number of alleles indicates the presence of rare alleles which with high probability, will be lost in next few generations. Pojskic et Kalamujic (2015) in their study discuss the practical applications of such approach in the analysis of feral populations of brown trout in the Neretva river and its tributaries as a model. Significance of allele number reduction (effective number of alleles) can contribute to the assessment of the genetic status of a conservation unit. In accordance with the above, we propose allele ratio as adequate measure of relation between effective (A_e) and detected number of alleles (A_n). It is expressed as $R=A_e/A_n$ where A_e is effective number of alleles and A_n is the number of the detected alleles. The range of this ratio is 0-1, where lower values represent larger difference, while higher values indicates smaller difference between effective and detected number of alleles. A Z-score of $P<0.01$ is considered as statistically significant. The abovementioned calculations can be made using ALRATIO R script http://www.ingeb.unsa.ba/popgen/R/alratio/alratio.html (Figure 1.). When R code is executed with previously introduced input data as .csv extension file (Figure 2.), three files are created (Figure 3.).

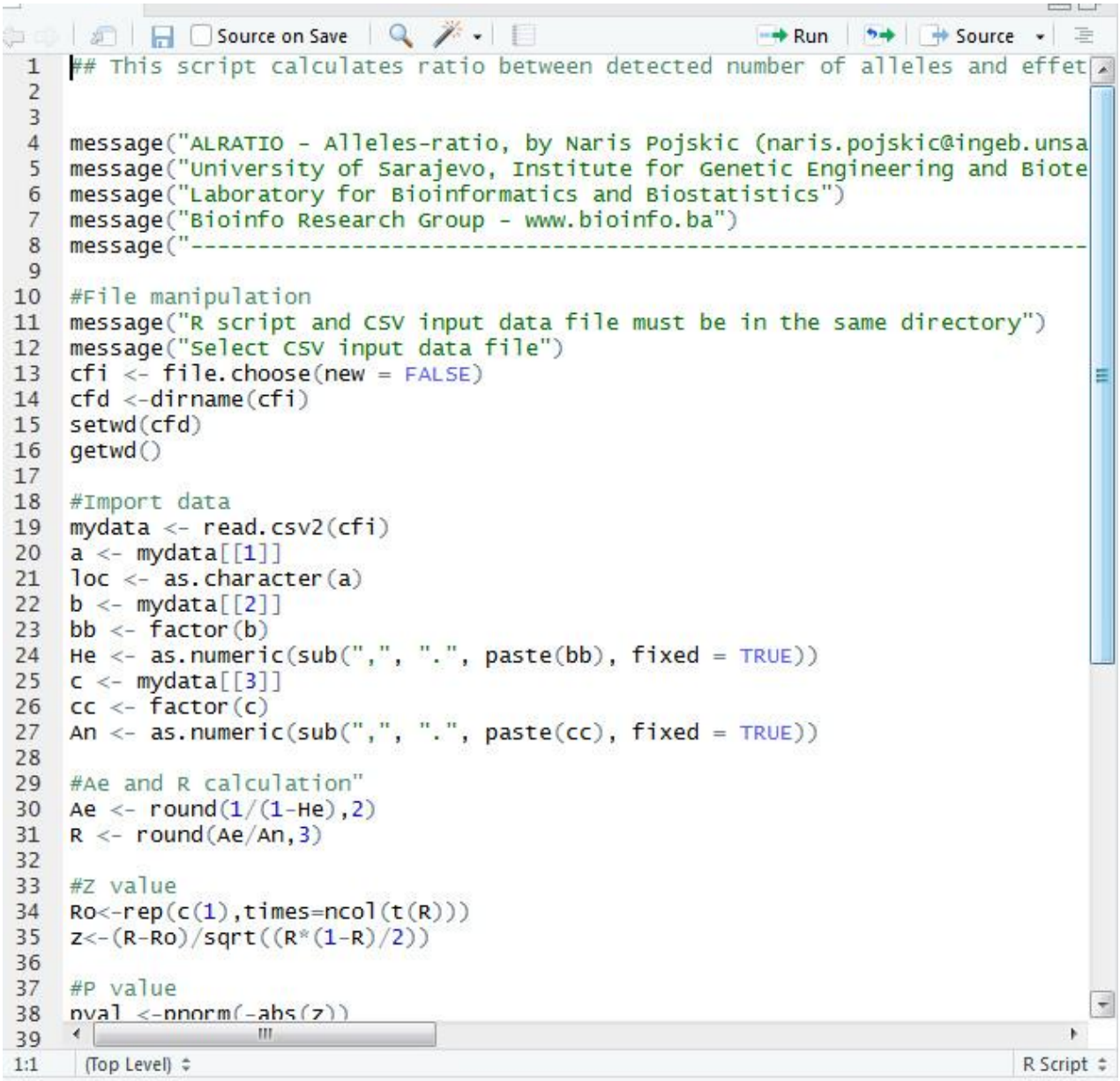

Figure 1. Presentation of ALRATIO R script code

```
> source("Z:/programiranje/Rcode/alratio/alratio.r")
ALRATIO - Alleles-ratio, by Naris Pojskic (naris.pojskic@ingeb.unsa.ba)
University of Sarajevo, Institute for Genetic Engineering and Biotechnology
Laboratory for Bioinformatics and Biostatistics
Bioinfo Research Group - www.bioinfo.ba
------------------------------------------------------------------------------
R script and CSV input data file must be in the same directory
Select CSV input data file
     Loc       He        An    Ae     R        Z         P
[1,] "CypG30"  "0.3784"  "11"  "1.61" "0.146"  "-3.42"   "0.00031"
[2,] "CypG24"  "0.828"   "15"  "5.81" "0.387"  "-1.78"   "0.03755"
[3,] "Ca1"     "0"       "1"   "1"    "1"      "NaN"     "NaN"
[4,] "Rru2"    "0.4624"  "4"   "1.86" "0.465"  "-1.517"  "0.06464"
[5,] "Z21908"  "0.1464"  "2"   "1.17" "0.585"  "-1.191"  "0.1168"
[6,] "Mean"    "0.363"   "6.6" "1.57" "0.238"  "-2.53"   "0.0057"
[7,] "Mean*"   "0.4538"  "8"   "1.83" "0.229"  "-2.595"  "0.00473"
Statistical significance level at P<0.01
Mean* = values without monomorphic loci
[1] "File (output.txt) and graphs (plot1.jpg; plot2.jpg) are saved in Z:/programiranje/Rcode/alratio"
>
```

Figure 3. View of RStudio console with results

Loc	He	An
CypG30	0,3784	11
CypG24	0,828	15
Ca1	0	1
Rru2	0,4624	4
Z21908	0,1464	2
Mean	0,363	6,6
Mean*	0,4538	8

Figure 2. Format of input data (Loc- locus; *He* - expected heterozygosity; A_n - number of alleles at given locus)

The first one is text file with the result of calculation (Figure 4.). It has tabular view with name of locus, values of expected heterozygosity, detected (A_n) and effective number of alleles (A_e), ratio (R) with Z and its P estimation. The others two are graphic files, which contain barplot graph (Figure 5), as well as scatter diagram (Figure 6). The ALRATIO script was tested using data from microsatellite loci analyzed in Dalmatian barbelgudgeon (Aulopyge huegelii Heckel, 1841; Kalamujic Stroil et al., 2019). The results are shown in Figure 3. and Figure 4. The *CypG30* locus has statistically significant deviation of effective number of alleles in comparison with detected number of alleles. The ratio for *Ca1* locus could not be estimated since it is monomorphic. Other loci did not show statistically significant deviation.

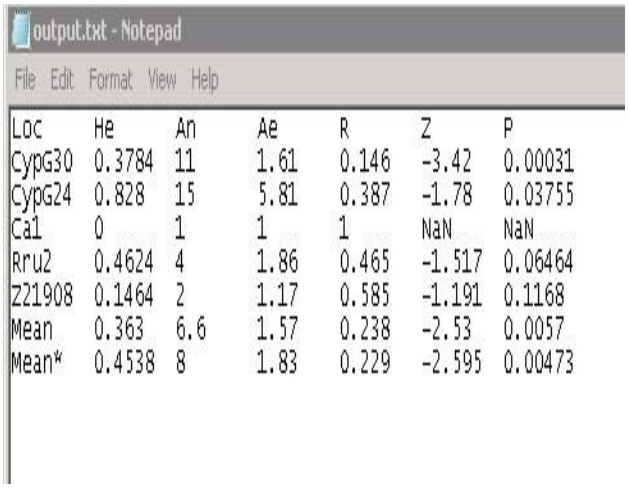

Figure 4. Format of output results (Loc- locus; *He* - expected heterozygosity; A_n - detected number of alleles at given locus; A_e - effective number of alleles; R - ratio between effective and detected number of alleles; Z - Z statistics; P - P value for ratio)

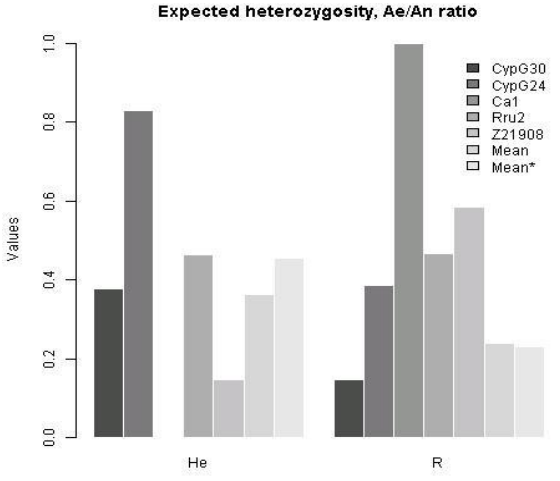

Figure 5. View of ratio plot

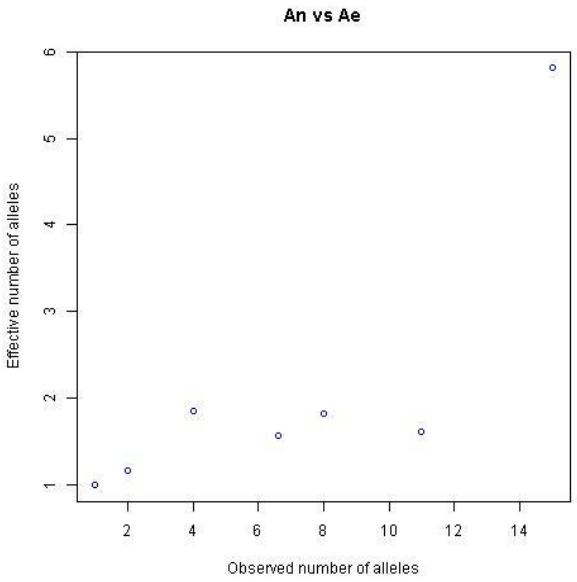

Figure 6. Scatter diagram between effective and detected number of alleles

We can conclude that the ratio between the effective and detected number of alleles can reveal the significance of the differences between them. The statistically significant value of such a measure indicates the proportion of alleles that do not really participate in genetic diversity of a population, which can be used for the prediction of genetic status of feral populations or agricultural varieties.

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Conference Invitation

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