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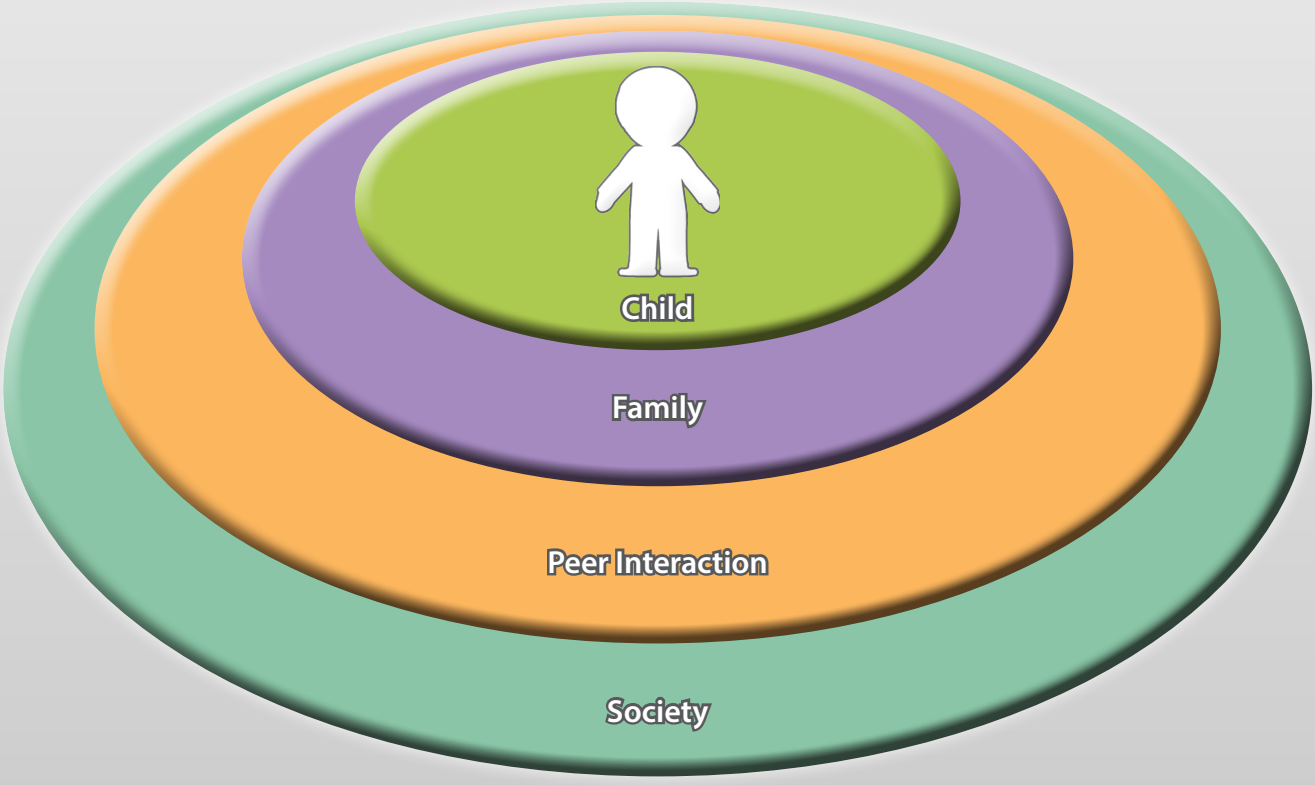

Tolerant environment and stimulating non-aggressive peer interaction is a favorable milieu for the growing child.

(See paper by Egloff and Djordjevic)

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12. Cerebrovascular embryology, in: power point; 2000. http://brainavm.oci.utoronto.ca/staff/Wallace/2000_curriculum/index.html

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Editorial

CHILDHOOD INTERVENTION AGAINST VIOLENCE AS AN EARLY PREVENTION TOOL

To raise the child in happy and healthy person is parental, community and society obligation and privilege. Children's behavior is a reflection of parental and social maturity. Tolerant environment and stimulating non-aggressive peer interaction is a favorable milieu for the growing child. We should offer and provide new models of childhood intervention with the important goal to prevent the violent behavior from kindergarten to adulthood. In the invited article Goetz Egloff and Dragana Djordjevic are sharing with us the German experiences of implementation of such an intervention, invented to cope with the global surge of violence. In a worldwide view, limitation of tolerance is the predominant notion underpinning today´s ideology.

Faustlos (meaning ´No Fists´) is a program, designed by Prof. Dr. Manfred Cierpka, who the authors worked with, as an emotional learning program to shape skills and competencies through an interactional framework. Upon his achievements and services rendered in early preventive care for children and their families, Professor Cierpka was recently bestowed the German Federal Cross of Merit, First Class. At the heart of Faustlos there are three issues to be transferred to children: (1) getting to know empathy and the training to be empathic, (2) learning to be capable of controlling one´s impulses, and (3) dealing with emotions of anger and rage.

"When dealing with people, remember you are not dealing with creatures of logic, but with creatures of emotion." - Dale Carnegie

To find more on this issue read the article of our invited authors.

Editor-in-Chief

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Invited Review Article

CHILDHOOD INTERVENTION AGAINST VIOLENCE

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Abstract. *Violence has its roots in many factors. Structured interventions for children can be useful when it comes to building emotional competencies. Self-regulation of negative emotions, impulse control, and empathy are important competencies to be achieved in order to prevent violence. The 'Faustlos' ('No Fists') program, which was designed to learn how to deal with emotions as well as to develop empathic dealings with one another, offers a wide variety of techniques and strategies for children. Furthermore, children are given a secure realm of learning and transfer in which no-one is excluded and stigmatization is avoided. With the additional involvement of parents, new modes of intra- and interpersonal conflict solutions in families can be developed. Its special relational approach makes the program convincing as a tool for childhood intervention. The development of pro-social emotions should be a base for the prevention of bullying and violence. Implementation in Serbia might be useful*.

Key words: *childhood, intervention, empathy, impulse control, coping*.

Introduction

Children with aggressive and violent behavior inflict injuries on others, either physically, psychically, or both. As to a multi-factorial concept of the generating of aggressive and violent behavior, it is mostly important to intervene early in the socialization process of children. Not only does the personal organization of emotions take place in childhood [1] but interactional processes of recognition, of boundaries and of intersubjective experiencing allow the creation of subjectivity. Therefore, especially the family and institutional surroundings of childhood play a significant role in supporting personal individuation, which allows for a communicative mode of non-violent dealings. Expansion and initiative in children are not to be eliminated but aggressive impulses of destructive color directed toward pro-social application instead. Destructive aggression has to undergo a subtle transformation into behavioral modes that are socially acceptable.

Violence

Although the expression of violence in everyday life is easily imaginable, it should be said first that quite different phenomena have to be subsumed under the term violence. In his influential writings, Žižek [2] reasonably differentiates subjective from objective violence. Subjective violence, which is the common notion of violence, is performed by a clearly identifiable agent. Then there is objective violence in two forms: symbolic violence is embodied in language and its forms, and systemic violence is the phenomenon of consequences of the smooth functioning of economic and political systems [3]. Subjective violence is experienced as such against the background of a non-violent zero-level. Objective violence is invisible since it sustains the very zero-level standard against which we perceive something as subjectively violent. Irrational explosions of subjective violence can better be understood via its counterpart, which is the objective one [4]. Systemic violence as inherent in western capitalism affects the formation of subjectivity, i.e. psychic development of people. It is not visible, yet it has effect on people via the more subtle forms of coercion that sustain relations of domination and exploitation [5]. Additionally, in a worldwide view, limitation of tolerance is the predominant notion underpinning today's ideology [6,7]. It might be that a broad range of personal freedom, such as in today's liberal societies, will entail its opposite, which is restriction. From this perspective the question arises whether liberalism itself leads to an incline of fundamentalism automatically [8], encompassing violence in all its different forms.

In the following we are dealing with subjective violence, which is commonly referred to as aggressive and violent behavior in people. As social scientists like Hurrelmann [9] and Heitmeyer [10] have broadly shown, such behavior is a “social disease”, generated by intrapsychic, interpersonal and societal conflicts. Children may express threats, or destroy objects. Such actions appear in contexts such as family, kindergarten,

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and school. Yet, the location of conflicts is often not identical with the location of expressed aggression or violence. Conflicts at home may be enacted in school or kindergarten, and experiences of victimization and conflict from school and kindergarten may be brought back home, leading to aggressive behavior, e.g. in siblings or in family interaction. At any rate, aggressive behavior is mostly used as a personal "solution" of interpersonal conflicts. Escalating situations that seem to leave no other option of communication often lead to violent acting [11].

There is a consensus that the expression of emotions in the course of human development is learned to some extent, as has internationally been discussed in conferences such as those on social-emotional learning [12]. This holds true for aggressive and violent behavior. Aggressive and violent children often have a dysfunctional family background [13] in which parents are not capable of taking enough care of their children, either physically or psychically. Sometimes there is a lack of attachment in the mother-infant-relation existing from birth on, or there are disorders of early attachment that have developed in children's first year of age [14]. Different sorts of dysfunctional modes in parents' behavior can affect the infant's emotional development since parent-infant interactional processes are prone to dysfunction. Intuitive parental competencies fundamentally influence the infant's emotional development and are vulnerable [15]. The loss of societal structure may disturb families in developing consistent educational modes [16]. Even social status and the overall status of societal development may compromise these competencies [17,18]. Dysfunctional and non-coherent educational practices in some families can puzzle and disturb children and direct their development toward dysfunctional modes of behavior. Moreover, aggressive children have often been victims of violence themselves [19]. Additionally, TV programs, video games and other media of violent contents mediate violence as a means of conflict solution. Children consuming many of these programs tend to use violence in dealings with their peers more often than those who do not [20]. As one can see, in order to prevent violence many subjects have to be dealt with.

To sum it up, children have partially learned to react in violent ways. Aside from an intrapsychic processing perspective, there is a social learning perspective that is still useful for the development of new modes of dealing with one another. It can be seen that in the last hundred years there have been great improvements in dealings with children [21–23], and when we look at publications on childhood development [24], it can be stated that scientific knowledge has hugely grown. Developing of subjectivity remains a multi-factorial process [25,26] which today is even more threatened by commodification, as health services are by economization of policies [27].

What Children Don't Know About

Early childhood is the most sensitive period of behavioral learning. Pro-social behavior can be learned to some extent. It is often impaired both in clinically conspicuous and in "normal" children. Modes of pro-social dealings with one another are by no means common in many families, nor do such come out of the blue. This is why, quite often, children [28]:

- don't know about appropriate behavior in certain situations since they don't have an inner working model of alternative conflict solution
- they do know about appropriate behavior but haven't been supported enough in doing so
- they show emotional reactions like anger, fear or anxiety that keep them from developing appropriate behavior
- they are not able to assess aggressive behavior appropriately
- they may have physiologically-based developmental deviation or disabilities stemming from genetic or parental influence.

Faustlos and Second Step

Faustlos (meaning 'No Fists') is an adaptation of Second Step [29], designed as an emotional learning program to shape skills and competencies through an interactional framework. At the heart of Faustlos there are three issues to be transferred to children: (1) getting to know empathy and the training to be empathic, (2) learning to be capable of controlling one's impulses, and (3) dealing with emotions of anger and rage. These issues are playfully dealt with in the kindergarten curriculum by way of 28 continuous lessons. Each lesson contains a story that is told by the educator and is illustrated by an accompanying picture. Each lesson is structured the same way: at first, the topic of the lesson is outlined by playfully fantasying what the lesson will bring. Moreover, in theater atmosphere, hand puppets (a toy dog and a toy snail) open up getting in contact with each other, further illustrating the issue of the lesson to come. This is followed by the actual lesson in which the story is told, shown in the picture, and discussed with the group. Role-playing, or alternative exercises at the end of the lesson will make sure the transfer to everyday life of children is initiated. The children are encouraged to try new approaches in kindergarten and at home too, therefore the parents are separately educated, too. Additionally, the educator is advised to return to the contents of the lesson during the following week. The program is conducted by a constant relational person, i.e. childcare worker as educator, in a closed group cycle of one year. Ideally, one lesson per week is conducted.

Lessons of the Kindergarten Curriculum

The lessons follow a consecutive order that will become more complex the further the topics develop. At first, fundamental emotions are dealt with, and the focus is on empathy. After that, coping strategies for dealing with negative emotions in interaction are focused at.

I Empathy:
1. What is Faustlos?
2. Emotions (joy, grief, anger)
3. Emotions (surprise, fear, disgust)
4. Same or Other
5. Emotions Change
6. If… Then…
7. Not Now – Maybe Later
8. Mishaps
9. What is Just?
10. I am Feeling…
11. Active Listening
12. I Care

II Impulse Control:
1. Calming Down
2. What is the Problem?
3. What can I do?
4. Choosing
5. Will it Work?
6. Sharing
7. Taking Turns
8. Negotiating
9. Listening
10. Interrupting Politely

III Dealing with Anger and Rage:
1. Am I Angry?
2. Calming Yourself Down
3. Dealing with Violations
4. Dealing with Name-Callings
5. Dealing with Getting Something Taken Away
6. Dealing with Not Getting What You Want

Parental involvement is part of the curriculum, too. By way of continuous parental meetings, parents grow accustomed to dealing with their children, and with one another, in rather empathic and non-violent terms. Ideally, new modes of intra- and interpersonal conflict solutions are developed with the parents. Specific accompanying courses and additional literature are offered to the parents, too [30].

Self-regulation, especially, has been proven to be difficult in traumatized and insecurely attached children, even sometimes in anxious children [31]. Faustlos offers a wide variety of techniques and strategies for children to learn how to cope with inner impulses by broadening the range of possible reactions in stressful and conflict situations. A special accent is given to change of perspective through stories viewed from different personal viewpoints in teaching lessons; something which has regularly been experienced revelatory in groups and audiences. Perspective changing and role-taking is highly important for psychic development: it was Sigmund Freud who observed early in his carrier that parts of what one observes can be fiction, i.e. something may not be what it seems to be [32].

Also from a differently conceptualized perspective of hysteria and borderline dynamics [33], children will respond to a well-structured offer of interactional experiencing, even when it cannot supply solutions to each and every shortcoming of personal and social issues. An anthropological constant of psychic pathology might have to be conceded [34], as well as the fact that there are culturally biased views of pathology, e.g. in psychic trauma theory [35]. Ratings highly depend on subjective construction [36].

Evaluation and Implementation

International evaluations of both Faustlos and Second Step proved these anti-violence programs to be effective [37]. Faustlos has been implemented at many educational institutions in Germany since 2001, such as in kindergarten and elementary schools, later on also in secondary schools.

The German Faustlos kindergarten curriculum was developed and evaluated between 2001 and 2004 at the University of Heidelberg. A process evaluation [38] was followed by a pre/post randomized control trial (RCT) study which compared the behavior of children in kindergartens which did and kindergartens which did not conduct the program. The study proved the program to be effective especially as to a decrease of verbal aggression in children [39,40]. Identifying emotions turned out to be easier for children who took part in the program than for those who did not; the same was shown for pro-social dealings with conflicts. Generally, Faustlos has been proven to have a specific anxiety-reducing effect supporting the transfer of competencies to everyday life [41] which is highly important since effects on the level of personal emotion entail even more appropriate interpersonal, pro-social behavior [30]. Further development and evaluation of the program in secondary education institutions followed from 2005 to 2007, so that evaluation of the program was conducted both in elementary and in secondary education [42–45]. Having been well-implemented at many educational institutions in Germany [46], the 'No Fists' program has even found its way into educational science text books [47]. The overall response of acceptance which is also due to the special relational approach tells of the program to be convincing.

Concerning violence prevention in Serbia, there is a program titled 'School without Violence – Towards a Safe and Enabling Environment for Children' whose implementation started in 2005 through UNICEF, the Ministry of Education and Science, the Ministry of Labor and Social Policy, the Ministry of Health, the Council for Child Rights, and the Office for Educational Development and Improvement [48]. The project involves everyone at school in a process of training and education during which they learn how to recognize, prevent and properly react to any instances of violent behavior. An

educational component of this program contains education for teaching staff about skills of constructive communication, open dialogue between children and adults in schools, and conflict management [49,50]. The term violence in this project does not only imply physical violence but all other forms of insults and verbal harassment. Six years later, in 2011, it was reported that sensitivity to violence increased significantly, popularity of violent pupils dropped, while both children and adults in schools that took part in the project increased their capacity to recognize violence and respond to it better, whereas cooperation with the local community and with parents was rated the least successful component of the program. Only about 20% of elementary schools have been involved [48].

Although there have been great efforts, violence is present worldwide. In order to prevent violence, it is highly important:

(1) to take prevention steps before violent behavior actually happens;
(2) to build an emotional foundation for non-violent dealings with one another in each child, the earlier in life the better;
(3) to make pro-social behavior second nature. The scientific findings show that this is possible via early interventions;
(4) to involve parents and make them familiar with principles of non-violent and respectful dealings with one another. Parental involvement is an important component of the Faustlos program;
(5) to approach all children, not excluding any child, not differentiating them into violent and non-violent and, especially important, not stigmatizing them after having been violent. This is also an advantage of Faustlos;
(6) to use well-evaluated programs. Faustlos is a scientifically evaluated program.

Therefore, Faustlos might be appropriate for implementation in Serbia, too.

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Review Article

THE CELL-FREE DNA DETECTION AND ANALYSIS AS THE NEW NON-INVASIVE PRENATAL DIAGNOSTICS OPTION

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Abstract. *Screening procedures for chromosomal abnormalities in fetuses are a standard of care for pregnant women. Ultrasound and maternal serum analysis are traditional prenatal screening methods with detection rates between 75%-95%, and considerable false-negative and false-positive results. Also, both require follow up by invasive diagnostic tests in screen-positive cases, mostly amniocentesis and chorionic villi sampling, which are associated with notable risk of pregnancy loss. One of the innovative non-invasive prenatal testing (NIPT) options is the analysis of cell-free DNA (cfDNA) in plasma, which is detected in maternal circulation in a relatively high concentration. Commercial tests for cfDNA in maternal blood have recently become available. Cell-free DNA detection tests do not separate fetal from maternal DNA but use full cfDNA complement and analyze difference in total amount of sequenced DNA fragments, with the help of sophisticated data analysis software. It seems that cfDNA technology testing is highly accurate and has a very high sensitivity, so the difference compared to routine serum sample screening shows its significant superiority. However, cfDNA positive results still need confirmation by the invasive testing. The cell-free DNA analysis aims to become the first choice NIPT option due to its safety and high accuracy rate. The final goal is to develop the reliable method that could eventually replace invasive prenatal testing procedures*.

Key words: *cfDNA, aneuploidy, prenatal diagnosis, amniocentesis, chorionic villi sampling, maternal-fetal exchange*.

Introduction

A standard care for pregnant women includes the use of prenatal diagnostic tests. Screening procedures are done early in pregnancy in order to identify the presence of chromosomal abnormalities, especially aneuploidies. These anomalies are the most common factor causing the failure of an embryo's growth and normal development of fetus. The most frequent chromosome abnormalities in miscarriages include trisomy or monosomy for chromosomes 13 (Patau syndrome, T13), 15, 16, 18 (Edwards syndrome, T18), 21 (Down syndrome, T21), or 22, as well as triploidy and abnormalities of sex chromosomes. Most fetuses with aneuploidies succumb to an early miscarriage, and only few percent survive to the newborn period, but may suffer significant morbidity and mortality [1,2].

The prenatal diagnostic techniques comprise non-invasive diagnostics - ultrasonography (nuchal translucency) and maternal serum screening (alpha-fetoprotein, estriol, beta-hCG) and invasive diagnostic methods - amniocentesis (AC) and chorionic villus sampling test (CVS). Ultrasound and maternal serum analysis are considered to be screening procedures that both require follow up by CVS or AC in screen-positive cases for a definitive diagnosis of a chromosome abnormality in the fetus. Invasive procedures are associated with notable risk of pregnancy loss, thus most female do not undergo the procedures unless there is a high risk indication. Invasive prenatal diagnosis is not a feasible option for all low-risk mothers and would eventually cause more miscarriages than detection of aneuploidy [3–5].

In order to improve the efficiency of non-invasive prenatal diagnostics and reduce the risk of currently available invasive procedures, the creation of novel, more sophisticated methods is of primary importance. Considerable effort has been devoted to developing a more accurate, reliable and safe non-invasive prenatal testing (NIPT), that would have a high detection rate (~95%) and low false-positive rate (~1%) [2].

In 2012 the American Congress of Obstetricians and Gynecologists (ACOG) approved the use of noninvasive testing of cell-free fetal DNA (cfDNA) in maternal circulation for women at high risk [6]. Since then, numerous reports on the use of cfDNA for NIPT have been published, and a number of commercial products were created [7].

Prenatal tests using cfDNA analysis are especially suitable for detection of chromosomal aneuploidies, trisomy or monosomy. Sex chromosomes aneuploidies (45, X0 - Turner syndrome, XXY – Klinefelter syndrome, and triple X syndrome) can also be detected with this method [2].

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The objective of this review is to present current knowledge and describe newly developed methods for application of cfDNA detection and analysis as the new NIPT option.

Traditional Prenatal Screening Methods

Non-invasive prenatal screening, recommended by the ACOG and the American College of Medical Genetics, includes a combination of first trimester risk assessment (FTRA) [11-14 weeks), maternal serum analyte (quad) screening (15-20 weeks), and a sonographic fetal structural survey (18-22 weeks). These tests are safe for the pregnancy but their primary target is detection of T21. The first trimester risk assessment and quad screening generally provide an adjusted risk for the presence of fetal aneuploidy. A sonographic fetal survey may suggest fetal aneuploidy by identifying so-called soft markers, with greater accuracy if several of these markers are present [3,5,8].

Detection rates of traditional prenatal screening methods are between 75% and 96% with considerable false-negative rates between 12-23% and false-positive rates ranging from 5% to 10%. If the results are positive, further checkup and confirmation is needed by direct genetic testing [3,5,8–11].

A definitive prenatal diagnosis of fetal aneuploidy requires direct fetal cells karyotyping and genetic analysis, for which two invasive procedures are routinely applied: CVS and AC. For CVS, a biopsy of placental cells is employed. The procedure can be performed early in pregnancy (10-13 weeks) and the results are issued within the following 10 days. Besides its invasiveness, the problem with CVS lies in the cell type which is sampled for the analysis, as these cells originate from the trophoectoderm and may contain placental mosaicism [2,12]. For AC, the primarily derived fetal cells are gained by aspiration of amniotic fluid and further subjected to analysis. Given the high pregnancy loss rates in early AC, it is usually offered after 15 weeks of gestation. Also, a low number of harvested cells require longer culture times and thus results can be issued within 8-14 days [2,13].

The diagnostic accuracy of karyotyping for specified invasive tests was found to be 97.5% to 99.8% [14,15]. Estimation of procedure-related miscarriage risk is 1% for CVS, while AC is regarded as a safer procedure with 1/300 to 1/600 risk [4,5,16-18]. In 2015, Akolekar et al. [19] reported in a systematic meta-analysis that the procedure-related risks of miscarriage for these invasive procedures are 0.81% and 2.18% for AC and CVS, respectively.

Cytogenetic analyses, ensuing invasive procedures, comprise fluorescence in situ hybridization (FISH) method and chromosomal microarray analysis (CMA). With FISH method, the results are available within 24 to 48 hours. The sensitivity and specificity of FISH is estimated between 99.6% and 99.98%. Disadvantage of the method is that it identifies only the most frequent cytogenetic abnormalities, thus the FISH should always be followed by routine chromosome analysis [20]. Besides detection of chromosome number abnormalities, CMA testing provides results for chromosomal imbalances (copy number variants) such as microdeletions and microduplications and unbalanced rearrangements of chromosome segments [21,22].

Fetal Cells and Cell-free DNA in Prenatal Diagnostics

At first, analysis of fetal cells in maternal blood was considered as a promising new candidate for non-invasive prenatal diagnostics. The possibility of their isolation and study for early chromosomal abnormalities in the first and second trimester provided exciting new opportunities for NIPT [2].

During pregnancy, fetal and maternal cells are exchanged across placenta [23]. It is suggested that this process has a physiological role in the development of maternal tolerance to the fetus. The precise mechanism by which this occurs is still unclear. Some of the proposed mechanisms include micro-traumatic rupture of the placental blood channels or leakage of placenta-uterine barrier, adhesion and transmigration across high endothelial venulae, and other [24,25].

There are several types of fetal cells detected in maternal blood during pregnancy. Cells that are most frequently found and examined are nucleated red blood cells or erythroblasts, CD34+ hematopoietic progenitors, trophoblasts, lymphocytes, and granulocytes. A fetomaternal microchimerism created in this way may persist for the lifetime [26,27].

However, there are specific disadvantages in management of all these cell types [26]. The major problem for successful usage of the cells is their scarcity in maternal blood (one fetal cell on every 10^5-10^9 maternal cells after the first trimester). This requires special techniques for their enrichment in maternal blood sample before any further analysis. Many different cell isolation methods have been developed in order to obtain the successful quantity of fetal cells (flow cytometry, density gradient centrifugation, micromanipulation), but all the methods require high technical approaches and still provide low yield [2, 28].

However, it was shown in 1997 that fetal cell-free DNA could be detected in maternal circulation, with relatively high mean concentration in total plasma DNA (3.4%-6.2%). This is 20 to 25 times greater level of fetal DNA in plasma compared to the DNA extracted from fetal cellular fraction [29,30].

The fetal cfDNA originates from the fetoplacental unit cells in the circulation or from various fetal organ systems. The main mechanism of fetal cfDNA release is supposed to be related to apoptosis of trophoblasts, as a result of normal aging, although an accidental breakage or necrosis may be the reasons as well [30]. This process is present continuously during pregnancy as early as from 5-7 weeks. The fetal fraction of cfDNA is lower in the earlier gestational age. Certain physiological

systems remove free DNA from the circulation within a few hours [16,31,32]. Additionally, very rapid clearance of fetal DNA occurs following delivery, which confirms the presumption that the most of fetal cfDNA in maternal circulation is derived from placenta [33].

Commercial tests for cfDNA in maternal blood have recently become available. Currently, a number of companies are trying to develop an optimal commercial product that could use cfDNA for fetal chromosome aneuploidies analysis. The absolute amount of fetal cfDNA is very small (less than 1mg/20ml of whole blood), which makes the separation of fetal cfDNA from maternal cfDNA technically challenging. That is why methods for their separation are dismissed and investigations are turned to the approaches that would use full cfDNA complement. Full cfDNA sequencing and sophisticated data analysis would detect abnormal amounts of chromosome specific DNA loci in the presence of fetal aneuploidy. The method of fetal cfDNA analysis was proved to be less demanding compared to isolation of fetal cells [7,11,31].

Methods of cfDNA Detection and Analysis

The first methods which have been employed for gathering and analysis of cfDNA were massively parallel shotgun sequencing (MPSS) and targeted sequencing. Both approaches use next generation sequencing technique, with high levels of sensitivity and accuracy for reliable analysis of the small cfDNA amounts [2].

The MPSS is a quantitative test, which relies on detecting difference in the total amount of plasma DNA fragments, while not distinguishing maternal from fetal DNA. The MPSS technique is based on the sequencing of large numbers of small DNA sequences (25-36 bp in length) from the entire genome. In the setting of NIPT, it would mean the sequencing of the whole amount of cfDNA from maternal plasma, or tens of millions of short-sequences in a single run [1,2]. After the sequencing, the chromosomal origin of each DNA fragment is determined by comparison with a reference copy of the human genome. Fragments (or reads) are categorized by chromosome, as well as their number per normal reference chromosome, which is referred to as counting. When the amount of a sequence fragment exceeds the threshold for a normal chromosome it is considered the positive result for trisomy. The increase in the quantity of genetic material occurs due to the 50% excess of genetic material originating from trisomic fetus extra chromosome [1,2,34].

This potential difference due to aneuploidy would be very small as the fetal DNA represents 10% at most of the cfDNA fraction, and the presence of extra DNA material (in T21) would change total cfDNA sample for only 0.075%. Because of this small DNA amount change, a large number of reads must be made in order to achieve satisfying degree of confidence, making the whole process robust. Also, it is estimated that in order to return sufficient data from the clinically significant chromosomes (13, 18, 21, X and Y; representing only around 14% of the genome) approximately 25 million raw sequencing reads are required per sample [1,2, 34,35].

The main limitation of MPSS is caused by the influence of GC chromosomal content on PCR amplification, leading to variability of the accuracy rate. The detection of T13 and T18 is especially challenging due to high GC content on these chromosomes. This issue has been notably reduced using novel bioinformatics algorithms [34–37].

The Targeted Sequencing test selectively amplifies specific genomic regions (loci of interest) which are read and counted. This significantly reduces the total number of reads, and all amplified sequences are utilized compared to MPSS method. The focus on clinically important chromosomes (13, 18, 21, X and Y) should provide higher sensitivity and specificity for the method. Still, as with quantitative read, the detection rates vary, depending on the chromosome tested, and are highest for T21 [7,38]. Similar to the MPSS method, post-hoc data analysis requires appropriate bioinformatics platform, such as z-score with GC correction and an internal control [2].

Starting from these two first approaches of NIPT, different companies made the effort to develop more accurate, sensitive and cost effective next generation test. The clinical implications for all newly developed tests are the same - they are screening tests that use a sample of maternal cfDNA and positive results still must be confirmed by invasive testing (CVS and AC).

One of the advanced techniques is called Digital Analysis of Selected Regions (DANSR). This is a targeted sequencing approach that initially amplifies specific chromosome loci of interest and then uses counting similar to MPSS analysis. It is coupled with post-hoc bioinformatics algorithm (Fetal Fraction Optimized Risk of Trisomy Evaluation - FORTE) that accounts for age-related risks and fetal fraction. This approach has greater efficiency than MPSS alone [38].

Another approach - Parental Support (PS) combines a targeted amplification, measuring single nucleotide polymorphisms (SNPs), and sophisticated statistical analysis. In this method, the number and identity of alleles of preferred chromosomal loci are determined, after which a model set of hypotheses is calculated using Bayesian statistics. A probability to each hypothesis is estimated and considering major individual variables and fetal cfDNA fraction, individual risk score is provided [39]. In this way, the problem of chromosome amplification variability is omitted and similar accuracy of fetal copy number at chromosomes is achieved. Using PS approach it is easier to detect sex chromosome aneuploidies, which is especially important as these abnormalities represent nearly half of all chromosomal defects at-birth [40].

The results of fetal aneuploidy risk assessment using these methods can be provided within the first 2 weeks, in early pregnancy. Also, cfDNA technology testing appears to be highly accurate and to have very high sensitivity, particularly chromosome 21 aneuploidy (99%), and the difference compared to routine serum sample screening (2-4% for T21) shows its significant superiority

[2,6,11]. Out of aneuploidies, Down syndrome has the highest incidence and it is the single most common cause of mental retardation accompanied by serious health disorders. Accuracy of its detection varies depending on a technical approach of a company. The sensitivity and specificity range from 98.6-100% and 99.8-100% for T21, 97.2-100% and 99.7-100% for T18, 78.6-100% and 99.0-100% for T13 [2,36,39].

Proposed indications for NIPTs and cfDNA analysis for aneuploidy are: maternal age above 35 years, fetal ultrasound findings indicating an increased risk, maternal serum screening test showing an increased risk, previous pregnancy with birth defects in child, family history of aneuploidy, positive test result for aneuploidy, parental balanced Robertsonian translocation. Testing can be performed as early as 9 weeks' gestation. However, positive results should be followed up with CVS or AC. What remains to be investigated is the accuracy of cfDNA method as NIPTs for females who are in the low risk population [6, 28].

Additionally, economic evaluation of newer NIPT methods is important in order to be accepted for wider clinical use. In the study by Song et al. [11], the cost-effectiveness model of NIPT for high-risk women in US population was assessed. The results showed better T21 detection and reduced unnecessary invasive procedures that lower the rate of euploid fetal losses, bringing to the lower total healthcare expenditures.

The new generation non-invasive prenatal screening for microdeletion syndromes test (Panorama^{TM}) incorporates maternal genotypic information, thus differentiating fetal genotypes in the plasma. It targets and analyses 19.488 SNPs from chromosomes 21, 18, 13, X, and Y. Additionally, the targeted screening was expanded for five microdeletion syndromes, by including SNPs within the microdeletion regions-of-interest [41]. The Panorama Extended Panel offers a risk evaluation for the 22q11.2 deletion (DiGeorge), 1p36 deletion, Cri-du-chat, Prader-Willi, and Angelman deletions. The sensitivity of this test was assessed to be greater than 93% and specificity greater than 99%. The importance of this test is emphasized by fact that these syndromes have very low detection rates by traditional screening tests, the risk for microdeletions is independent of maternal age, and their incidence is often underestimated. The evaluated combined incidence of these syndromes using Panorama test was calculated to be about 1/1000, showing that the incidence of DiGeorge syndrome is higher than for cystic fibrosis. All these circumstances assert the need for inclusion of microdeletion syndromes in prenatal screening options [41].

Besides the determination of the fetal aneuploidy risk, levels of circulating cfDNA are recognized as a marker of several pregnancy related complications. cfDNA levels were increased in preeclampsia, intrauterine growth restriction, preterm labor, placenta previa and hyperemesis gravidarum. Increased leakage of the cells is reported in the cases of fetal aneuploidy and preeclampsia. Because cfDNA levels decrease during diseases progression, it is suggested that they can be a predictive marker for early detection of these disorders [30, 42].

Conclusion

Cell-free fetal DNA in maternal blood is a valuable source of genetic information which has become increasingly available due to the progress in DNA sequencing and bioinformatics' techniques. The non-invasive prenatal testing with cfDNA represents the new generation of prenatal diagnostic screening, which strives to become the first choice testing option due to its safety and high accuracy rate. The final goal is to develop a feasible and reliable method that could eventually replace invasive prenatal testing.

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Review Article

DRUG-INDUCED LIVER DISEASE

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Abstract. *Liver impairment can be caused by a significant number of foreign compounds (xenobiotics); prescribed drugs, 'over the counter' (OTC) drugs, herbal and alternative medicines. Hepatotoxicity caused by drugs used for therapeutic, recreational or nutritional purposes as well as drugs of abuse is a drug-induced liver disease (DILD). Over 300 agents in use have been connected with causing DILD. Factors associated with increased susceptibility to DILD are: age, gender, genetic predisposition, dose, other drug reactions, concomitant use of drugs, excessive use of alcohol, nutritional status, liver disease and other diseases. Drugs may cause liver injury in a predictable, dose-dependant manner (intrinsic DILD), or in an unpredictable, non-dose-dependant manner (idiosyncratic DILD). Xenobiotics that cause liver impairment provide a wide range of lesions resembling many other liver diseases. Acute hepatocellular damage can be cytotoxic (hepatocellular necrosis), cholestatic (associated with the interrupted flow of bile), or mixed. Clinical expressions of DILD range from nonspecific abnormalities of liver tests, to cholestasis, acute hepatitis and acute liver failure. Nodular hyperplasias, chronic hepatitis, autoimmune hepatitis, fibrosis, NASH, cirrhosis, benign and malignant liver tumours have been reported. Diagnosis of DILD is based on history, blood tests, imaging examination of hepatobiliary tract and, if applicable, liver biopsy. Clinical and laboratory findings in DILDs are not always in line with liver pathology. Histologic changes can be minor compared to biochemical findings. Liver enzymes are not synonym of liver damage.*

Key words: *liver, injury, drugs.*

Introduction

Significant increase of scientific studies investigating drug-induced liver disease (DILD) in the last few years is making DILD an emerging safety issue that requires attention by medical professionals in clinical practice, regulatory authorities, pharmaceutical companies and academic institutions [1]. Liver impairment can be caused by a significant number of foreign compounds (xenobiotics); prescribed drugs, 'over the counter' (OTC) drugs, herbal and alternative medicines. Chemical agents widely used in households, drugs of abuse, pesticides, herbicides may have toxic and/or carcinogenic properties. Hepatotoxicity caused by drugs used for therapeutic, recreational or nutritional purposes as well as drugs of abuse is a drug-induced liver disease (DILD). About 14-19 per 100 000 inhabitants in general population is the reported frequency of DILD. Health care system records the incidence of about 30-32 per 100 000 persons [2,3]. According to available data, 462 medicinal products were withdrawn from the market between 1953 and 2013. Hepatotoxicity was the most reported adverse drug reaction causing post marketing drug withdrawal (81 cases; 18%). Withdrawals were significantly less common in Africa than in Asia, Europe, and America [4].

In the largest number of reports, DILD is unpredictable because of its idiosyncratic nature. Accurate underlying mechanisms (mitochondrial injury, reactive metabolites, biliary transport inhibition, and immune responses) have been identified rarely. DILD can occur in case of accidental or intentional overdose or during the use of a drug for therapeutic purposes in certain clinical circumstances, as in the case of paracetamol in patients who regularly consume alcohol [5]. Paracetamol is the leading cause of acute liver failure, whereas chlorpromazine, halothane, sulpiride and amoxicillin-clavulanate were found as most common drugs leading to hepatotoxicity in all prospective studies [6]. The list of top 10 drugs implicated in DILD consists of antibiotics, statins, antitumor necrosis factor antagonists (infliximab as leading); herbal and dietary supplements (most frequent causes of serious hepatotoxicity are weight loss and bodybuilding products) [7].

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Risk Factors for Incidence and Severity of DILD

Factors associated with increased susceptibility to DILD are: age, gender, genetic predisposition, dose, other drug reactions, concomitant use of drugs, excessive use of alcohol, nutritional status, liver disease and other diseases. Age more than 60 increases the frequency and severity of DILD caused by isoniazid and halothane. Children are more commonly affected by salicylates. Women are at an increased risk of developing hepatotoxicity from halothane, nitrofurantoin and men from amoxicillin-clavulanate and azathioprine. Concomitant use of acetaminophen and isoniazid, zidovudine and phenytoin lower the hepatotoxic dose and increase severity of DILD. Obesity increases the risk of liver injury by halothane, methotrexate and tamoxifen, while malnutrition increases the risk of liver injury by acetaminophen. Genetic variation at human leukocyte antigen (HLA) class I & II loci has been shown to be associated with amoxicillin–clavulanate DILD. The strongest association thus far identified is at a single nucleotide polymorphism in the gene encoding the class II HLA-DRB1* 1501-DQB1* 0602 allele [8,9]. Variations of genes for mitochondrial DNA polymerase gamma are associated with valproate hepatotoxicity [10].

Mechanisms of Drug Injury

Drugs may cause liver injury in a predictable, dose-dependant manner (intrinsic DILD), or in an unpredictable, non-dose-dependant manner (idiosyncratic DILD).

In most cases of the drug induced liver injury, the same happens in an unpredictable manner and only in susceptible individuals (idiosyncrasy or hypersensitivity). Impairment may appear from toxic metabolites which affect cell proteins. Toxic metabolites cause necrosis (metabolic idiosyncrasy) or form antigen (drug hapten) complexes which stimulate T cells, inducing an immune reaction and causing hepatic impairment (hypersensitivity or drug allergy). Drug-induced hypersensitivity reactions are commonly merged with systemic reactions, such as fever, rash and eosinophilia. They have a fixed latent period and prompt response to a repeated provocation. This reflects an underlying immunological mechanism. Vice versa, atypical metabolism of a drug which leads to formation of toxic metabolites, generally does not cause systemic allergic manifestations and it has a long or variable latency period and frequently a late response to a repeated provocation [11,12,13]. The most common causes of idiosyncratic damage are amoxicillin-clavulanate, nitrofurantoin, co-trimoxazole, ciprofloxacin, isoniazid, tyrosine kinase inhibitors [14].

A very small number of currently used medicinal products cause liver injury as a result of intrinsic toxicity or toxicity of one or more of their metabolites (predictable or intrinsic hepatotoxicity) [15]. Paracetamol is hepatotoxic due to production of the toxic metabolite as a result of accidental or intentional overdose or when used in recommended doses in circumstances of chronic use or alcohol abuse or starvation. The actual cause of damage to hepatocytes or cell death is damage or destruction of cell membranes or covalent binding of toxic metabolites to liver macromolecules causing a disturbance in calcium homeostasis, mitochondrial dysfunction or decay of other cell systems.

Acetaminophen (paracetamol) accounts for 50% of all drug-induced acute liver damage. Its metabolite, N-acetyl-p-benzoquinone imine (NAPQI), is created in hepatocytes. This toxic metabolite is reduced by glutathione. Reduced capacity of glutathione leads to impairment of vital processes in the cells and to their death. Paracetamol induced liver disease is treated with n-acetyl cysteine, in the first 8 hours of introduction of the drug [16].

Nimesulide, diclofenac, ibuprofen are non-steroidal anti-inflammatory drugs (NSAIDs) widely used in therapy of most rheumatological disorders, as analgesics and antipyretics, as prescription drugs and over the counter drugs. Nearly all NSAIDs are associated with hepatotoxicity; several NSAIDs have been withdrawn from the market (amphenac, ibufenac, phenylbutazone, fluproquazone). The new more selective COX-2 inhibitors (e.g. celecoxib, rofecoxib, nimesulide) are also connected with hepatotoxicity. Pathogenic mechanisms include oxidative stress alone or in combination with mitochondrial injury [17].

Oral contraceptive steroids and 17-alkylated anabolic steroids are associated with cholestasis, vascular lesions and hepatic neoplasms.

Drugs of abuse like cocaine or 3,4 methyenediomatamphetamine (“ecstasy”) are related to hepatotoxicity. Cocaine toxicity is related to P450 catalysed N-demethylation to norcocaine, converted to N-hydroxynorcocaine. The latter redoxcycles to norcocaine nitroxide by receipt of an electron from NADPH, and transfers electrons to O2, generating oxidative stress [17].

The frequency of hepatic injury with antiretroviral drugs is at least 10%. Hepatic failure has been reported in patients taking zidovudine, but didanosine and stavudine have been most often involved in severe hepatotoxicity due to mitochondrial damage. Nevaripine has been implicated in causing severe hepatotoxicity. Ritonavir, Indinavir, Saquinavir, Nelfinavir have been reported for hepatotoxicity. Anti-retrovirals can induce direct toxicity in the liver, mitochondrial toxicity; hypersensitivity reactions have been reported relatively often with nevirapine and abacavir. Newer anti-HIV drugs like raltegravir, maraviroc and enfuvirtide have not been associated with significant hepatotoxicity [18].

The frequency of the DILD with recently introduced drugs will be known after larger studies. Nature of liver injury is presented in Table 1 [18].

Xenobiotics that cause liver impairment provide a wide range of lesions resembling many other liver diseases. Acute hepatocellular damage can be cytotoxic (hepatocellular necrosis), cholestatic (associated with the interrupted flow of bile), or mixed. In addition to

Table 1 Histologic pattern and clinical expressions of DILD

Drug	Liver injury
Alfuzosin	Hepatocellular od mixed hepatocellular-cholestatic injury
Beta interferon	Liver injury rare, autoimmune hepatitis
Bosentan, sitaxsentan	Acute hepatitis
Imatinib mesilate and other tyrosine kinase inhibitors	Acute hepatitis, massive or submissive hepatic necrosis(rare); acute liver failure with sunitinib
Leukotriene antagonists (zafurlukast, montelukast)	Massive or submissive hepatic necrosis (zafirlukast), acute hepatitis, cholestitis hepatitis (montelukast)
Infliximab and other tumor necrosis factor antagonists	Cholestasis, cholestatic hepatitis, hepatic granuloma, autoimmune hepatitis
Ximelagatran	Acute liver failure (not finally proven)

hepatocellular necrosis or cholestasis, other types of liver lesions could be induced by xenobiotics. Fatty change of the liver (steatosis) is common. Macrovesicular steatosis refers to large drops of fat and the core is replaced by a large intracytoplasmic lipid globule. Microvesicular steatosis is characterized by small drops of fat within the cytoplasm that do not suppress the core. Some drugs are associated with the formation of Mallory's bodies. Hepatic granulomas are a typical damage caused by certain drugs. Various forms of chronic liver impairment resembling chronic active hepatitis, chronic cholestasis and cirrhosis can be caused by xenobiotics.

Vascular disorders of the liver caused by medicinal products include a venous-occlusive disease, very similar to Budd-Chiari's syndrome. Peliosis hepatis is formation of blood cysts within the liver. Several drugs disrupt lipid metabolism in the hepatocytes by inhibiting phospholipase, which gives a foamy texture cytoplasm and characteristic ultrastructural liposomal appearance (phospholipidosis). Finally, certain drugs and chemicals are associated with hepatic neoplasia. Benign hepatic adenomas appear after the introduction of oral contraceptive steroids.

Clinical Expressions of DILD

Clinical expressions of DILD range from nonspecific abnormalities of liver tests, to cholestasis, acute hepatitis and acute liver failure. The most common form of presentation of DILD is an acute viral "hepatitis-like" syndrome, with jaundice, nausea, fatigue and abdominal discomfort or pain. DILI can virtually mimic any other liver disease such as chronic hepatitis, autoimmune hepatitis, fibrosis, NASH, cirrhosis, benign and even malignant liver tumours [19].

Clinicopathological classification of DILD is presented in Table 2.

Biochemical Classification

Biochemical classification of liver damage caused by drugs include hepatocellular, cholestatic and mixed pattern. R value is calculated to assist in diagnosis and management of DILD. In case there is evidence of drug or supplement use in previous 6 months, and elevated liver enzymes are detected, R value is calculated as follows:

$$R = (\text{ALT value} / \text{ALT ULN}) / (\text{ALP value} / \text{ALP ULN})$$

If $R < 2$ cholestatic damage is susceptible. Ultrasound of abdomen should be done and MRI/MRCP are to be considered. If R is between 2 and 5 mixed pattern and $R > 5$ hepatocelular liver damage is suggested. In these cases testing for hepatitis A, B, C and E should be done, as well as ultrasound imaging. In consideration are testing for EBV, HSV, autoimmune hepatitis etc.

Table 2 Clinicopathological classification of DILD

Damage type	Drug
▪ Acute hepatocellular injur	Isoniazid, aspirin, sulphonamide
▪ Autoimmune hepatitis	Nitrofurantoin, minocycline, ipilimumab
▪ Pure cholestasis	Anabolic steroid, oestrogens
▪ Macrovesicular steatosis	Tetracycline, steroids, gold, 5-fluorouracil, methotrexate, tamoxifen
▪ Microvesicular steatosis	Tetracycline, steroids, gold, 5-fluorouracil, methotrexate, tamoxifen,
▪ Cholestasis hepatitis	Phenytoin, AC, fluoroquinolones, macrolides, azithromycin
▪ Granulomatous hepatitis	Isoniazid, interferon, phenytoin, allopurinol
▪ Chronic hepatitis	Phenytoin, AC, fluoroquinolones, macrolides, azithromycin
▪ Non-Alcohol fatty liver	Tamoxifen, Amiodarone
▪ Fibrosis/cirrhosis	Metotrexate, amiodarone
▪ Liver Adenoma	Oral contraceptives

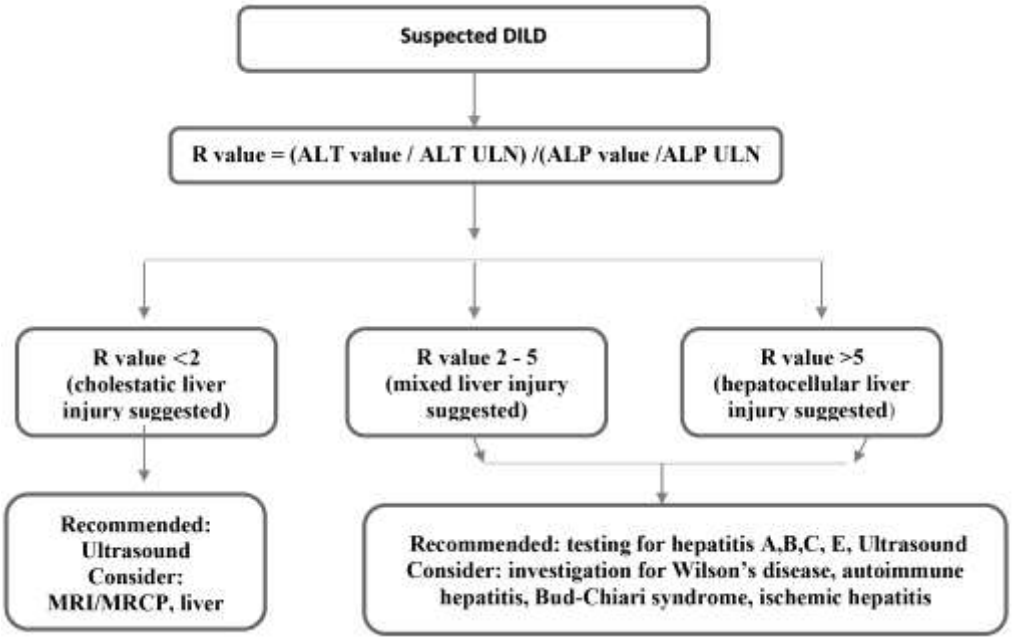

Fig 1 Calculation of R value and use in DILD management [15]

Diagnosis of DILD

Diagnosis of DILD is based on history, blood tests, imaging examination of hepatobiliary tract and, if applicable, liver biopsy. There are no specific laboratory tests, histological presentations, or clinical signs and symptoms enabling the diagnosis of DILD. Signs and symptoms vary with the drug, host, and severity of injury [20].

Some situations where the probability of the existence of DILD is likely are summarized in Table 3 [18].

The diagnostic evaluation of DILD usually includes evaluation of data summarized in Table 4

Liver biopsy is indicated in cases in which liver disease remains in doubt and this uncomfortable and risky procedure will make a difference in management of the injury. Liver biopsy is reasonable in case when continued use or re-challenge with a suspected drug is clinically necessary. For patients receiving methotrexate there are guidelines for biopsy [21,22]. Other situations where liver biopsy could be recommended are: exacerbation of liver function in spite of stopping drug exposure, unexpected decreases of ALT within 30-60 days in hepatocellular or ALP within 180 days in cholestatic DILDs despite termination of use of the suspected drug [13].

Table 3 Situations in which the existence of DILD is likely

- Introduction of a new therapy in the last 3-6 months;
- Evidence of extrahepatic manifestations like rash, eosinophilia, lymphadenopathy;
- Acute hepatitis not connected to hepatitis viruses, other infections, metabolic, immunologic disorders;
- Mixed hepatocellular and cholestatic injury;
- Hepatitis with microvesicular steatosis;
- Cholestasis with normal bile duct imaging;
- Chronic hepatitis without antibodies;
- Liver disease after years of taking steroids, immunosuppressive or other drugs, etc.

Table 4 The diagnostic evaluation

- history (use of drugs, herbal or dietary supplements; possibility of drug interaction; exposure time/latency, alcohol intake, chronic liver disease, concomitant diseases (diabetes, heart failure))
- signs and symptoms (weakness, fatigue, fever, yellow urine, nausea, vomiting, abdominal pain, abdominal bleeding, rash, pruritus, icterus, ascites)
- initial laboratory tests (compete blood count (eosinophilia), liver function testing (AST, ALT, GGT, R value)
- routine serological tests (Acute viral hepatitis A, B, C (Anti –HAV IgM, HbsAg, anti-HBc IgM, anti – HCV, HCV RNA, autoimmune hepatitis (ANA, IgG level))
- serological tests by patients history (hepatitis E (anti hepatitis E virus IgM), CMV, EBV, HSV)
- other investigations (for Wilson's disease, etc.)
- imaging studies (Ultrasound, CT, MRCT)

ALP (alkaline phosphatase); ALT (alanine aminotransferase); ANA (antinuclear antibody); CMV (cytomegalovirus), CT (computed tomography), EBV (Epstein Bar virus); GGT (gamma –glutamyl transferase, HAV (Hepatitis A virus); HBc (Hepatitis B core antigen); HBsAg (Hepatitis B surface antigen); HCV (Hepatitis C virus) ; HSV (hepatitis C virus); HSV (herpes simplex virus); IgG (immunoglobin G); IgM (immunoglobin M); MRCP (magnetic resonance imaging, RNA (ribonucleic acid); ULN (upper limit of normal range).

The Council for International Organizations of Medical Sciences has created a CIOMS/RUCAM questionnaire. Score count is based on timing of exposure and liver biochemistry washout, competing medications and diagnoses, re-challenge of data and risk factors for DILI. Additional methods have been developed. One of them is Naranjo Adverse Drug Reaction Probability Scale (NADRPS). The CIOMS/RUCAM is widely used and considered the best assessment method respecting sensitivity and predictive value. Likelihood levels are: 'highly probable' (> 8), 'probable' (6 – 8), 'possible' (3 – 5), 'unlikely' (1 – 2), and 'excluded' (score < = 0). RUCAM score system is separated into hepatocellular injuries; cholestatic or mixed injuries form [23].

Prevention

Liver function testing is recommended before starting the treatment along with safety monitoring during therapy with agents with known hepatotoxicity and in case treatment extends for longer than 2-4 weeks. However, with respect to the costs of such screening, it is difficult to define the threshold at which the drug should be discontinued especially in case of absence of symptoms.

Generally, it is recommended that the drug should be stopped if ALT level exceeds five times ULN. Abnormal bilirubin level, albumin concentration, prothrombin time and symptoms are clear indications to stop the therapy.

The monitoring of the liver tests is strongly recommended in case of treatment with the following agents: methotrexate, isoniazid, retinoids, ketoconazole, anticancer drugs, and minocycline in prolonged time [18,24].

Treatment of DILD

There are varied presentations and multiple possible drug causes. The treatment of all cases is withdrawal of the suspecting agent. If a DILD is caused by acetaminophen or in case of Amanita mushrooms intoxication, appropriate therapy should be administered. All patients can now be considered for NAC therapy, especially adults with early-stage of ALF (acute liver failure). Patients should be monitored for normalization of biochemical tests. In cases when it is recognized as lifesaving, Early liver transplantation is recommended in cases where it is recognized as a lifesaving procedure [25].

Prognosis

The prognosis is highly variable depending on the clinical presentation and degree of liver damage. In general, outcomes of idiosyncratic DILI are good, with about 10% reaching the ALF (coagulopathy and encephalopathy). The outcome of acute liver failure is determined by aetiology, the degree of hepatic encephalopathy, and complications such as infections. DILI developing to ALF carries a poor prognosis. Mortality rate of DILD is 9 to 12%. Only 20% to 25% of patients with acute idiosyncratic fulminant hepatic failure survive 3 weeks without liver transplantation. The causes of death include cerebral oedema, sepsis, multiple organs insufficiency, cardiac and respiratory failure [13,15]. In cases with existing liver disease, increased morbidity and mortality have been reported. Prognosis is worse the longer a patient is exposed to hepatotoxin.

Mixed type of liver damage often progresses into a chronic form with cirrhosis. Immune type of damage (eosinophils and granulomas on biopsy) has a better prognosis. Pure hepatocellular necrosis in biopsy has a worse prognosis [14].

Categorisation of the Probability of DILD

According to the Drug-Induced Liver Injury Network (DILIN) assessment causality of probability to induce liver injury, drugs are classified in five categories of probability to induce DILD. This assessment is based on published data and is more precise for widely prolonged use of medicines than nearly approved drugs or herbal products [26].

Table 5 Categorisation of the probability of DILD

Category A. Medicines from this category are well known, well described, well reported to cause either direct or idiosyncratic liver injury. The number of described cases is more than 50
Category B. Known or highly likely drugs reported to cause idiosyncratic liver injury. The number of described cases is between 12 and 50 cases including small case series.
Category C. Probably linked drugs to induce idiosyncratic liver injury, reported uncommonly. The number of identified cases is less than 12 without significant case series.
Category D. Possible hepatotoxic drugs that rarely cause liver injury. The number of identified cases is less than 3.
Category E. Drugs with no evidence that has caused liver injury. Mostly inconclusive single case reports have been published.
*Category E**. Agents with reported DILD in extensive clinical studies, but with insufficient supportive causality data. Hepatotoxicity is unproven, but suspected.
Category X. Medicines quite recently presented or seldom used in clinical practice with luck of data on risk for developing of DILD ("unknown" category).
A [HD], B [HD], C [HD] or D [HD] category. Medicines that induce liver damage in cases of overdose. Most common used agents from this category are aspirin, acetaminophen, naicin and vitamin A.

Liver disease associated with particular drugs

Amoxicillin-clavulanate

The incidence of DILD is 1.7 per 10,000 prescriptions. It is more common in the elderly with a concomitant therapy. It occurs more frequently in individuals who are

heterozygous for a mutant form of the gene for glutathione-S-transferase. The injury starts within 6 weeks of therapy with amoxicillin-clavulanate. Cholestatic type of damage is common and other forms are possible, as well.

Recovery period is 3-6 months. About 3% of injured finished with acute renal insufficiency or progression to cirrhosis [13].

Fluoroquinolones

The latency period is short (from 2 to 9 days). Common type of injury is immune damage. Prognosis is better than in case of DILD induced by amoxicillin-clavulanate. It is more common in people who are allergic to fluoroquinolones. Liver damage is a 'class effect' of fluoroquinolones [15].

Tyrosine kinase inhibitors

Liver damage occurs in the first 8 weeks after initiation of therapy. It can be manifested as a mild form - only an increase in transaminases, which passes spontaneously after discontinuation of medication. The severe form is presented as hepatocellular injury, and the incidence is 2-3% of total number of treated patients [13].

Direct-acting oral anticoagulants (DOACs)

Liver damage during administration of therapeutic doses of direct-acting oral anticoagulants has been reported in the past few years. Post marketed data reported rivaroxaban as the agent with the highest risk in the group. A pharmacological and chemical characteristic of direct-acting anticoagulants seems to be associated with drug-induced liver injury risk. Rivaroxaban, dabigatran and apixaban contain structural elements connected to metabolism and/or reactive metabolites connected to DILD occurrence in humans. Host factors seem to have influence on DILD occurrence. DILD induced by DOACs therapy of venous thromboembolism in surgical patients is reported more frequently then atrial fibrilation [27].

Herbal and Dietary supplement-induced liver injury

The increasing use of alternative medicines has led to many reports of toxicity. The spectrum of liver disease is wide. Herbal and Dietary supplements do not pass preclinical and clinical toxicology safety testing or clinical trials for safety. A dietary supplement consists of vitamins, minerals, amino acids, enzymes, tissues extracts, metabolites, etc. Herbal and Dietary Supplements (HDS) are widely consumed and in most cases without medical observation. Some of these products have been reported to induce liver injury. First of all, body-building products, which contain anabolic steroids are associated with an initial cholestatic hepatitis followed by prolonged jaundice [28]. Pyrrolizidine alkaloids can induce sinusoidal obstruction syndrome [29]. In some cases, flavocoxid, has been associated with severe liver injury [30]. The same diagnostic approach for DILI is applicable to suspected HDS hepatotoxicity. Patients should stop using HDS products and be monitored until hepatotoxicity has been resolved.

Individual susceptibility is important for herbal-induced drug injury. Kava, anxiolytic agent is connected to hepatotoxicity in Caucasians with low expression of CYP2D6. Some herbs initiate immunoallergic liver injury (jin bu huan). Rarely, herbal medicines may trigger latent liver disease (dai-saiko–to, black cohosh). Herbal hepatotoxicity could be presented as acute hepatitis, steatosis, fibrosis to submassive and massive hepatic necrosis (chaparral leaf). Some herbal agents and dietary supplements implicated as causing toxic liver injury are presented in Table 6 [13].

Discussion/Conclusion

Clinical and laboratory findings in DILDs are not always in line with liver pathology. There are significant differences between categories. Histologic changes can be minor compared to biochemical findings. Liver enzymes are not synonym of liver damage. Some drugs, like estrogens, are associated with high levels of AT and mild cholestasis on biopsy can be recorded.

Drugs like methotrexate, arsenic can cause cirrhosis with minimal changes in laboratory tests. Model of liver tests is nonspecific and often mixed. Various forms of injury can be seen: steatohepatitis, cholestatic hepatitis, chronic hepatitis, minor nonspecific liver injury.

Most cases of drug-induced dysfunction are reversible. In general, discontinuation of hepatotoxin results in rapid reversal of signs and symptoms if the

Table 6 Herbal agents and dietary supplements implicated as causing toxic liver injury

Herbal remedy	Indication	Pattern of liver injury
Atractylis gummifera	Purgative, diuretic	Acute liver failure
Black cohosh	Menopausal symptoms	Acute liver failure, could trigger autoimmune hepatitis
Chinese herbal medicines	Multiple use	Liver injury, Acute hepatitis
Germander tea and capsules	Weight reduction, health tonic	Acute and chronic hepatitis, acute liver failure, hepatic fibrosis
"Green juice"	Dietary supplement	Granulomatous hepatitis
Herbalife®	Health supplement	Acute hepatitis, Cholestasis
Kava	Anxiety disorder	Diffuse hepatocellular necrosis, Cholestatic hepatitis
Kombucha	Health tonic	Acute hepatitis
LipoKinetix®	Slimming aid	Acute hepatitis, acute liver failure
Shark cartilage	Food supplement	Abnormal liver tests

injury is mild to moderate. A 50% reduction of hepatic-associated enzymes can be expected within 1 week if the injury is hepatocellular, but this degree of improvement may take 6 months or longer if the injury is cholestatic.

In most cases, management of drug-induced liver dysfunction is limited to supportive care, as therapeutic treatment is applicable in only a small number of situations.

Liver function testing before starting of the treatment and safety monitoring during the therapy with agents with known hepatotoxicity and in case treatment will extend for longer than 2-4 week is recommended. Monitoring of the liver tests is strongly recommended in case of treatment with the following agents: methotrexate, isoniazid, retinoids, ketoconazole, anticancer drugs, and minocycline in prolonged time. Herbal and Dietary Supplements (HDS) are widely consumed and in most cases without medical observation. Some of these products have been reported to induce liver injury. Patients should stop using HDS products and they should be monitored until hepatotoxicity has been resolved.

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Review Article

FORMULA FOR THE WELL-BEING OF EXPERIMENTAL ANIMALS: 3R + 1R

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Abstract. *Animals were first used for research purposes at the beginning of the development of both biology and medicine. However, the expansion in the use of animals for laboratory purposes began in the 19th century. During an experiment, animals may experience fear, deprivation, disease, and various degrees of pain. Animal Protection activists oppose to animal experiments and it is, therefore, necessary to harmonize the worldwide regulations on the use of animals for scientific purposes. More than 50 years ago, Russell and Burch were the first to define the 3R rule. It consists of the following three principles: Replacement, Reduction and Refinement. Over time, one more R was added to stand for Responsibility, meaning a responsible behavior of those who implement the 3R rule. Replacement means that, if possible, each experimental animal model should be replaced by an in vitro method or be reduced to a smaller number of animals used. Reduction is defined as a reduced number of animals used to obtain certain experimental information, while Refinement is a reduction in the frequency or severity of inhumane procedures applied to animals that have yet to be used. The 3R (+1R) rule has its drawbacks, but it is a very important aspect of animal use regulation, which is essential. These rules are used to direct animal users towards an adequate experimental model, but also to be a reminder of the appropriate use of experimental animals at a given time.*

Key words: *experimental animals, ethics in biomedicine, 3R + 1R rule*.

Experimental Animals

The use of animals for research purposes began at the dawn of science (more specifically of biology and medicine). However, in the 19th century, together with the development of a new scientific discipline called physiology, the true expansion of animal use for laboratory purposes began [1]. During the 18th and 19th century, anesthetics were not administered to animals during experiments, although diethyl ether (the first anesthetic) and its potential action had been known to science since the 16th century [1]. A series of experiments on non-anesthetized animals led to the establishment of the Royal Committee in the UK. This committee adopted the first law to regulate the issue, the Cruelty to Animals Act in 1876 in order to limit/control the use of animals in experiments [2].

The list of experimental animals available for use in scientific experiments today contains a large number of lower vertebrates and invertebrates (Table 1), as well as non-human primates (Table 2) [3].

Among these animals (Table 1 and 2), mice are by far the ones used most frequently due to a high degree of their biological similarity to humans, easy handling and a short reproductive cycle. Nowadays, in the era of genetic manipulations, mice with specific genetic codes, that mimic some of the disorders found in humans, are an excellent experimental model [4]. A large number of researchers base their research, which eventually leads to the discovery of new drugs and/or efficacy of pharmaceutical products and vaccines, on *in vivo* experimental animal models. Likewise, many researchers cannot even imagine a clinical trial without the initial toxicity testing performed on animals (mouse, rat, etc.) [5]. During the development of science, some of the breakthroughs would not have occurred had it not been for animal experiments. A certain_number of scientific discoveries were incorrect and/or were "slowed down" due to previous studies on experimental animals [6,7]. The use of experimental animals led to the revolutionary breakthroughs in the 17th century and these included the discovery of the circulatory system, antibodies, effects of hormones and vitamins, mechanisms of nerve impulses, a large number of genes associated with hereditary diseases, numerous medications and their effects, organ transplantation, etc. [6]. However, there were cases where despite the positive outcomes from animal experiments, results could not be transferred to humans. For instance, in the case of thalidomide, for instance, the drug had passed all the stages of animal testing and no side effects had been reported, however, in infants born to the mothers who used thalidomide the agent caused aplasia of the extremities. On the other hand, various other

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Table 1 Some of the lower vertebrates and invertebrates most frequently used in experiments

Name	Latin name	Name	Latin name
Mouse	*Mus musculus*	Rabbit	*Oryctolagus cuniculus*
Rat	*Rattus norvegicus*	Dog	*Canis familiaris*
Guinea pig	*Cavia porcellus*	Cat	*Felis catus*
Syrian hamster	*Mesocricetus auratus*	Frog	*Xenopus* (*laevis*, *tropicalis*) and *Rana* (*temporaria*, *pipiens*)
Chinese hamster	*Cricetulus griseus*	Zebrafish	*Danio rerio*
Mongolian gerbil	*Meriones unguiculatus*		

breakthroughs have not resulted from the use of experimental animals and these include the inventions of stethoscope, penicillin, artificial respiration, computed tomography, HIV virus, etc. [7].

Table 2 Some of the *non-human primates* most frequently used in experiments

Name	Latin name
Marmoset	*Callithrix jacchus*
Macaque monkey	*Macaca fascicularis*
Rhesus monkey	*Macaca mulatta*
Baboon	*Papio* sp.

During an experiment, animals may experience fear, deprivation, disease and pain of various degrees. These may occur both separately and in combination, under a variety of experimental conditions. However, they may be absent altogether. Thus, for example, mice that are tied up experience temporary fear, while those injected with a pathogen besides experiencing temporary fear and mild pain from inoculation, also develop a disease during the period of observation [8]. A number of different systems (scales) have been proposed to help in the assessment of the degree of invasiveness and severity of experimental procedures. One of these, proposed by the British Laboratory Animal Association, classifies the seriousness of some procedures as minimum, intermediate and maximum [9]. In order to obtain the corresponding score, this scaling takes into account the degree of animal alertness, the method of injection of a drug/tested sample, sampling of different tissues from live animals and certain surgical procedures on animals.

Do animals feel pain? This is a big dilemma among the scientists and a large number of them agree that animals do feel pain. Great philosophers, such as Bentham, advocated the view that even though animals cannot discern or speak, they, nevertheless, can feel pain [1]. The International Association for Pain defines pain as "*unpleasant sensory and emotional experience associated with real or potential tissue damage, or caused by such a damage or injury*" [10]. Therefore, if we know that there is a difference between pain and nociception, that pain can occur without nociception, and that emotions are one of the mental functions that characterize a conscious person, the question of whether animals feel pain is raised again.

Ethical Problems and Regulation of Animal Use in Experiments

Despite the progress of biomedical research and the benefits for humanity achieved by experimental research, the concerns related to animal experiments have always existed. It has a long history, starting with the letters of protest and peaceful gatherings, to the more aggressive outcries in recent times, primarily in Europe and America [6]. According to the animal rights activists, each experiment involving the use of animals potentially breaks six widely accepted moral norms: respect for the animals, good scientific practice, being a good citizen, responsibility to future generations, environmental responsibility, respect for the lifestyle and religion of other people.

Bringing up animals in cages, special diets, treatment of animals in experiments and their sacrifice in the end, grossly endangers a specific way of life of a biological species. Causing pain, suffering, anxiety, and in recent times the manipulation with animal genome constitute disrespect of life itself. Toxicity testing on animals is the most controversial one due to the belief that it is unnecessary to test so many compounds. Although not all animals suffer during these tests (e.g. control groups or experimental groups which receive a low dose of the tested substance), a large number of animals is subjected to suffering due to inherent characteristics of toxicity testing to cause undesirable effects in animals [6,11].

There is a growing tendency of resolving the above ethical problems related to the use of animals in scientific research in a satisfactory way. There are many national and international bodies commissioned to care and responsibility for the welfare of animals in experiments. This concept is called "a responsible experiment". Among the first institutions that should be mentioned here is the International Committee for Laboratory Animal Science (ICLAS), involving more than 100 countries, with the headquarters in the United States. This Committee has set the international guidelines for experimental procedures and trainings for researchers [12]. In Canada, animal experiments are regulated by the Canadian Council on Animal Care (CCAC), a national organization founded in 1968 in Ottawa. Its goal is simple: "*work to improve the care of animals in Canada*" [13]. In India, several institutions are concerned with the welfare of experimental animals, in both state and private sectors. The National Center for Laboratory Animal Sciences (NCLAS) in Hyderabad and the Central Drug Research Institute in Lucknow, regulate this segment of science, both primarily through legislation. The guidelines

published by NCLAS in 1992 and amended in 2000 should be addressed as well. They regulate the way of animal handling during the experiments [6]. The South African Medical Research Council (SAMRC) recognizes the moral dilemma involved in the use of animals in experiments, in teaching and testing, and the Council is committed to support only the projects that promise progress in science and knowledge and bring about certain benefits to the mankind, animals, and to the environment [8].

On November 24th 1986, the European Parliament and the Council of the European Union (EU) adopted the first Directive 86/609/EEC in order to eliminate the differences across the laws and regulations of the EU countries concerning the use of animals for experimental purposes [3]. The European Parliament and the EU Council adopted the directive on the protection of animals used for scientific purposes in 2010 [3]. In this extensive and comprehensive document, the EU refers to the reduction and replacement of animals in experiments; the origin, method of conservation, nutrition and care; ways of handling animals by laboratory staff and researchers and, most importantly, the requirement for the evaluation and pre-approval of projects and experiments in which animal use for research purposes is planned.

Our country (the Republic of Serbia) adopted the first Animal Welfare Act as late as 2009. This Act regulates a number of important issues for the preservation and improvement of animal welfare in various situations, including the use of animals in experiments [14]. This law stipulates the conditions (in terms of the purpose, area and ways of performing the experiments, authorized persons to carry out experiments, as well as the animals themselves) that must be met for conducting experiments on animals that would be considered legitimate. This law subsumes all living vertebrates and invertebrates under the concept of experimental animals, as well as their developing forms to be used in experiments [15].

The Veterinary Practice Act regulates that scientific experiments on animals can only be carried out by experts in veterinary, medical, pharmaceutical and other research institutions, and that the animals should not be subjected to any ill-treatment or suffering during the experiments [16]. Animal experiments can be performed only by the physical and legal entities registered in the Animal Experiments Registry kept by the competent Ministry. The persons and/or institutions entered in the Registry can perform such experiments only if they possess the certificate of approval to perform experiments on animals. This certificate is issued by the Minister, based on the expert opinion of an Ethics Committee in order to safeguard and upkeep the welfare of experimental animals, in accordance and in response to the previously submitted request of such individuals/ institutions. The law prescribes the content of such a submission or request [17].

3R Rule

More than 50 years ago, Russell and Burch [18] were the first to define the 3R rule. It consists of three principles - Replacement, Reduction and Refinement. This rule has been modified (perfected) over the years to gain its current form. It still consists mainly of the 3Rs, but another R has been added as well – Responsibility. This concept, responsibility, stipulates that those who implement the 3R rule should be held liable for their actions and behavior in the experiments they are performing [19].

Replacement

Each experimental model that can be replaced and/or leads to the reduction of the number of animals represents an alternative method of animal testing. Nowadays, there is a tendency to get as many details as possible from an animal (or group of animals) in order to avoid the experiment repetition, which can be achieved with a good experiment prediction [20]. This can also be achieved by a cost-benefit analysis of the experiment [21]. The difference in the replacement of experimental animal models exists for different types of research (applied, fundamental and innovation), due to the requirement to have a validated alternative method when engaging in applied research. The methods of replacement can be as follows: absolute (where animals are completely excluded from the experiment) and relative (the use of lower vertebrates and invertebrates), direct (using isolated human material or the one from dead animals) or indirect (the use of other means of carrying out the same experiment), as well as total (human models and *in vitro* methods) or partial (non-animal models) [6].

When contemplating on a replacement, a question should be answered whether this is an adequate replacement. If so, we are encountering difficulties with the validation of the replacement method. This problem is particularly evident in the attempts to replace animals in toxicological studies, but recent findings suggest a potential new, alternative *in vitro* model [5]. This refers particularly to the models that evaluate the carcinogenic potential of a compound [22]. We are presenting here only some of the suggestions for the application of replacement principle:

1) Animal and plant tissues culture – in *in vitro* conditions, different cell populations mimic *in vivo* conditions (cultured kidney cells, liver cells, lymphocytes etc.),
2) Isolated organ methods - the contraction of smooth muscles of the gastrointestinal tract, the hippocampus function testing in the brain tissue sections,
3) *In vitro* methods (reactions) – various chemical/ biochemical reactions which mimic isolated reaction processes in an organism (enzyme inhibition/ activation),
4) Computerized simulation – the use of computer programs for biomolecule interaction with certain receptors simulation; (Q)SAR experiments [23].

Reduction

Reduction is defined as: "*a reduction in the number of animals used in order to obtain certain information*". Similar to other "Rs", reduction serves to reduce the number of animals, where possible, and eliminate the suffering and inhuman treatment of animals [24]. *The Principles* do not define the reduction as minimization of the number of animals used to obtain certain information. The reduction is defined simply as a decrease, which is not synonymous with minimization. Russell and Burch do not explain why reduction is not defined as a minimization of the animal number, since their goal is to minimize animal pain. However, if we could define reduction as minimization, then simply the reduced number of experimental animals in a given experiment or the type of research would not be reduction, unless absolutely the smallest number of animals is used to obtain the desired results. Russell and Burch emphasize that it is often impossible to know the minimal number of animals needed before the experiment is carried out. Speaking of the importance of using statistical methods in the reduction, they state that: "*For the purpose of reducing, as we have noted, statistical methods play a key role – they give the minimum number of animals needed for the experiment*".

In addition to a simple reduction of the number of animals used, the reduction can be achieved in many other ways as well. Good experimental design and statistical analysis are to ensure that researchers are using the optimal number of animals. If kept in a clean environment, the animals suffer less from disease or secondary infections that can interfere with the study, thus reducing the number of animals more readily attained. New scanning techniques mean that tumors can be traced in a non-invasive manner, with more data collected from the same animal [25].

At first glance, it seems that the reduction is an easily measured target – it comes down to count. The data available to us, however, show that there is no progress in reducing the total number of animals used for experimental purposes, despite the researchers' efforts. On the contrary, this number has been steadily rising since the 1990s [26]. The explanation for this failure is often simple, and its reason lies in the implementation of more biomedical research. Today, many rodents and fish are counted in the total number of laboratory animal experiments, even if they are only used as breeding species to produce better animal models for the testing of serious diseases such as heart disease, cancer or Alzheimer's disease. These animals can also be used as a substitute for other animals, such as monkeys and dogs. The advantage of reduction is certainly the reduction of the number of animals exposed to manipulation, discomfort and suffering, but we must not forget to mention its deficiencies. In the first place, insufficient numbers of experimental animals may produce unreliable and inaccurate results. This disadvantage could be nullified by detailed study planning [24].

Refinement

Russell and Burch define refinement as "*any reduction in the frequency or severity of inhumane procedures applied to animals that have yet to be exploited*". This involves stress reduction to an absolute minimum. Any simple improvement in the animal housing/keeping conditions and animal care during the study means a great deal. This improvement can often be achieved through "environmental enrichment", meaning that the animals live a better, less stressful life. Additionally, this improvement increases the reliability of research results. There are numerous specific improvement techniques and they can be applied in almost all aspects of animal life. For example, a food reward can be used to train a monkey to sit on the measurement scales and thus completely eliminate the stress that the animal is experiencing. Blood pressure, heart rate and activity levels can be measured via an implant, so that animals do not have to be restrained on several occasions. Rodents can be placed in a special red plastic "house", so that they are under the impression of being in a dark place (they cannot see through red materials) and one can observe and study them. Animals should be routinely kept in groups and in stimulating environments as well. Animal welfare is not only an ethical concept, but it also represents good science. It is also against the law for any researcher to cause undue suffering to any animal.

Nowadays, when applying for an experiment to the Ethics Committee (or other relevant bodies) the principle of improving the living conditions of laboratory animals is taken into account. The study of Hagelin and associates has shown that in Sweden, as much as 18% of the applications to the Ethics Committee are refused and/or a modification of the study protocol is asked for. These modifications are commonly referred to as "improvements". The most common requirements include the design of the study, euthanasia and animal housing [27]. Moreover, there has been an increase in the requested amendments related to animal anesthesia and the presence of a licensed supervisor during the experiment [27].

4R - Responsibility

Another, newer, concept in the 3R rule is the fourth R (4R). This R refers to the responsibility imposed as necessary to comply with the 3R rule [19]. Researchers and people in general who use experimental animals and those who grow them and care about them are considered responsible for the proper care and animal use. Also, responsibility is directly connected to the level of training (to work with/handle animals) of that specific individual. The expertise of the persons carrying out the experiment should also be taken into account, meaning that it is necessary that they thoroughly know proper animal handling techniques. On the other hand, it is essential that the performed experiments are adequately substantiated in the relevant literature, i.e. that the experiments result in sufficiently relevant and significant scientific information [8]. This

rule also applies to the rehabilitation of animals which survived the experiment, i.e. their further destiny. They can be reused in another experiment if the treatment (or control group) and the experiment did not cause any permanent damage [8].

Even Russell and Burch gave an example of how it is possible to implement the 3R rule adequately. They exploited as an example the use of animals in virological analyses [28]. One animal is sufficient for obtaining large amounts of tissue for *in vitro* experiments - the replacement. Moreover, by the use of just one animal it is possible to acquire sufficient amount of information and a large number of animals is then not required. This concept fits in the reduction principle. Finally, the animals used in the experiment could be painlessly killed and would not have to experience the symptoms of disease arising from virus inoculation - refinement. We can now add the 4^{th} R, which involves a degree of responsibility of the researcher who conducts experiments on animals.

Conclusion

In the end, a remark should be made that 3R (+1R) principle has its shortcomings, e.g. these rules do not allow the use of certain animals (e.g. chimpanzees) in situations where it is acceptable and the usefulness of that is clearly visible. In addition, a situation may arise when two R rules cannot be applied at the same time because they nullify one another, e.g. in an attempt to decrease the use of animals through the possibility of animal re-use on one side, with an effort to decrease the experienced stress/pain occurring during the experiment on the other. Putting aside any personal attitudes towards the 3R (+1R) rules, one can not diminish their significant impact on the regulation of animal use that is most certainly needed. These rules attempt to guide a researcher towards an adequate experimental model and to remind us how to use experimental animals at a given moment.

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Review Article

THE ASSOCIATION OF CELIAC DISEASE WITH OTHER AUTOIMMUNE DISEASES IN CHILDREN

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Abstract. *Celiac disease (CD) is an autoimmune disease with estimated prevalence of 1% in European and North American population. All four components of CD autoimmunity are well known, such as: genetics, autoantigen, autoantibodies and the environmental triggers. Besides gastrointestinal manifestations, celiac disease has a wide array of extraintestinal symptoms and clinical signs. This article briefly summarizes celiac disease pathophysiology and association of celiac disease with other autoimmune diseases in children. It puts emphasis on possible protective role of gluten-free diet for the development of other autoimmune diseases in patients with celiac disease.*

Key words: *celiac disease, autoimmune diseases, diabetes mellitus.*

Introduction

Over the last few decades increased prevalence of many autoimmune diseases in the human population has been documented. Celiac disease (CD) is one of the most common autoimmune diseases in human population and the only disease with well-known environmental triggers of autoimmunity. Patients with one autoimmune disease are more susceptible to other autoimmune diseases. Coexistence of two or more autoimmune diseases in the same individual (autoimmune polyendocrine syndrome) has been published in literature [1].

At the beginning of the twentieth century, with the advent of the highly sensitive and highly specific serological screening tests for CD, large screening studies have been put into practice with the aim of identifying CD prevalence in general population and patients suffering from other autoimmune diseases.

Celiac Disease

CD is common, lifelong disease characterized by autoimmune damage of the small bowel mucosa. Recent epidemiological studies indicate that CD affects about 1% of European and North American population [2,3]. Nowadays, all 4 components of CD autoimmunity are well known, such as: genetics, autoantigen, autoantibodies and the environmental triggers. Genetic predisposition is needed for CD occurrence. More than 95% of patients are carriers of HLA-DQ2 haplotype (encoded by alleles DQA1 * 05 and DQB1 * 02). The rest of the patients are carriers of HLA-DQ8 (encoded by alleles DQA1 * 03 and * DQB1- 0302). The role of the genes out of the HLA system was examined by GWARS (genome-wide association studies) [4,5]. More than 115 of these genes have been involved in the regulation of the innate and adaptive immune responses. However, the importance of each individual gene outside the HLA system for the development of CD is very small.

The enzyme tissue transglutaminase (TG2) is located in lamina propria of small bowel mucosa. TG2 leads to deamination of glutamine residue of gliadin [6]. This reaction provides glutamine residue a negative charge and ability to bind to HLA DQ2 / DQ8 molecule on the antigen-presenting cells. TG2 participates in many physiological processes, such as extracellular matrix building, tissue reparation, signal mechanisms of receptors, cell proliferation, cell motility and endocytosis [7]. In addition to the small intestine, TG2 can also be found in the liver, muscles, and lymph nodes.

Dietrich et al. (1997) were the first who discovered IgA antibodies against TG2 (Anti-TG2) in the sera of CD patients [8]. Deployment of tests based on Anti-TG2 detection substantially improved the diagnostic accuracy of CD serologic testing.

Development of CD requires food intake that contains gliadin fraction of gluten from wheat or similar prolamins from rye (secalins) and barley (hordeins). There are gliadin peptides which demonstrate two different effects on small bowel mucosa: toxic and immunostimulatory [9]. Alpha-gliadin peptides (31-43, 44-55, 56-75) with toxic activity are responsible for the damage of the small bowel mucosa and initiation of the pathophysiological processes in CD. Peptides with

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immunostimulatory effects initiate T helper 1 (Th1) response and secretion of interferon-γ. It is assumed that there are more than 50 gliadin peptides with this kind of effect.

The pathophysiology of autoimmune damage of small bowel mucosa in CD is not completely understood. It encompasses complex interactions between the innate and adaptive immune response, triggered by ingestion of gluten. At the beginning of this process paracellular permeability of small bowel mucosa is increased for gluten peptides due to peptide induced CXCR3 activated upregulation of zonulin, an intestinal peptide involved in tight junctions control [10].

Toxic gliadin peptides activate the innate immune system and increase production of interleukin 15 (IL-15) by epithelial and dendritic cells. IL-15 stimulates cytotoxic activity of intraepithelial lymphocytes through production of interferon γ (IFNγ) and stimulation of NKG2D cells. Activation of the adaptive immune response occurs after deamination of glutamine residues with TG2, their binding to dendritic cells and presentation to CD4 T lymphocytes followed by activation of the cellular (Th1) and humoral (Th2) immune response. Th1 response leads to the IFNγ induced cell death of enterocytes. Th2 response causes differentiation of B lymphocytes into plasma cells capable of producing anti-TG2 and antigliadin antibodies (AGA). It is not known whether anti-TG2 in a certain way influences the activity of the TG2. In addition to Anti-TG2 other types of auto-antibodies, such as antibodies against actin and reticulin were detected in CD [11].

CD may be manifested by typical (chronic diarrhea, loss of appetite, distended abdomen, malnutrition) and non-typical symptoms (stunted growth, refractory anemia, osteoporosis, unexplained hypertransaminasemia). The CD diagnosis is established on the basis of positive serology (anti-TG2 or anti-endomysial antibodies) and small bowel mucosa pathohistology.

Celiac Disease and Other Autoimmune Diseases

A group of autoimmune diseases is consisted of about 80 different diseases affecting 5-8% of the human population [12]. Patients with a single autoimmune disease are at 25% risk of development of other autoimmune diseases [13]. Many studies in children have reported an association between CD and various autoimmune diseases. Common genetic background is one of the predisposing factors for this association [14,15]. There is a possibility that persistent activation by proinflammatory cytokines leads to the unmasking of other autoantigens (besides TG2) and the initiation of a new autoimmune attack. Recent studies demonstrated that gut microflora plays an important role, not only in shaping the immune responses, but also in the development of autoimmunity [16].

Italian authors have found an increased frequency of organ-specific antibodies in CD patients [17]. Titer of antibodies significantly decreased following the commencement of the gluten-free diet (GFD). On the basis of this, they hypothesized that GFD may prevent the occurrence of other autoimmune diseases. However, subsequent studies have not confirmed the protective role of GFD [18,19]. Development of other autoimmune disease is more likely if the CD is diagnosed in young individuals with positive family history of CD [20].

Celiac Disease and Diabetes Mellitus Type 1

Diabetes mellitus type 1 (DMT1) is one of the most common chronic diseases in children with increasing annual prevalence from 3% to 4% [21]. Numerous screening studies conducted around the world showed the increased prevalence of CD (2.4% -16.4%) in patients with DMT1 [22]. The only screening study of this type in Serbia revealed the CD prevalence of 5.79% in children and adolescents with DMT1 [23].
DMT1 patients with CD can be asymptomatic (60%-70% of patients) or have classical CD symptoms. It should be kept in mind that positive titer of anti-TG2 in DMT1 patients does not always mean that they have CD. In a number of serologically positive DMT1 patients who do not have symptoms of the CD, anti-TG2 disappeared spontaneously, even though they continued to use gluten containing food. In these children anti-TG2 titer levels are usually slightly lower than in asymptomatic CD patients.

Some studies suggest that patients with newly diagnosed DMT1 and CD have frequent hypoglycemic episodes, higher average value of glycosylated hemoglobin, and reduced bone mineral density [24-26]. Conflicting evidence exist as to whether a GFD significantly improves glycemic control in DMT1 patients [24,27,28].

Children and adolescents with DMT1 and CD are prone to vascular complications later in life. CD is regarded as an independent risk factor for retinopathy and nephropathy in patients with DMT1 [29].

The International Society for Pediatric and Adolescent Diabetes (ISPAD) recommended serological screening for CD in all children with DMT1 at the time of DMT1 diagnosis and every 1-2 years thereafter [30] because positive titer of Anti-TG2 can be found many years after the beginning of DMT1.

Celiac Disease and Autoimmune Thyroid Disease

Many screening studies in children with autoimmune thyroid disease (Hashimoto's thyroiditis, Graves' disease) have found an increased prevalence of CD (2.3-7.9%) [31]. CD has been more commonly found in autoimmune thyroiditis than in Graves' disease. Serologic CD screening is recommended in children with autoimmune thyroid disease by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [32].

Investigations were carried out in the opposite direction as well, with the aim of determining the prevalence of autoimmune thyroid disease in children with CD. In one Italian study autoimmune thyroid disease was found in 26.2% of pediatric patients with CD [33]. Thyroid dysfunction in CD has a negative impact not only on growth, metabolism of proteins and cholesterol, but also on cognitive function and fertility.

Celiac Disease and Autoimmune Hepatitis

In case of elevated transaminases in children with CD, autoimmune hepatitis and non-specific reactive hepatitis (celiac hepatitis) should be considered. Autoimmune hepatitis (AIH) is a chronic disease in genetically predisposed individuals in whom the autoimmunity is directed toward liver antigens. Pediatric surveys have reported a wide prevalence of CD in AIH in a range from 3.6% to 12% [34]. Although there are still no recommendations for routine serological CD screening in patients with AIH, the association between these two diseases should be considered if one of them is diagnosed. Rare cases of CD associated with other autoimmune liver diseases such as primary sclerosing cholangitis (PSC) and "overlap" syndrome (AIH / PSC) were described in children [35,36].

Celiac hepatitis is found in 26-57% of children with CD [37,38]. It is manifested by the elevation of transaminases without any symptoms of liver disease. The increased intestinal permeability in CD may ease the entry of toxins, antigens, antibodies and cytokines to the portal system leading to hepatocytes function impairment [34]. In most cases (70-100%) transaminase levels are normalized within a year on GFD. All patients with unexplained hypertransaminasemia should be serologically tested for CD.

Celiac Disease and Dermatological Diseases

CD may be associated with various skin manifestations, such as dermatitis herpetiformis (DH), psoriasis, alopecia areata and vitiligo [39-41]. DH is most frequently found in adolescents, young and middle-aged adults. All patients with DH have some forms of enteropathy that can be found in CD. In 10-20% of DH patients, in addition to rash, there is a typical clinical picture of a CD while in the rest of 80-90% atypical or "silent" CD clinical forms are seen. GFD is standard treatment for DH with excellent clinical response. Since the reintroduction of gluten in a diet after clinical recovery on GFD triggers relapses of cutaneous and intestinal lesions, DH is regarded as skin manifestation of CD [39].

Celiac disease and rheumatological diseases

CD is found in in 1.5% -2.8% of children with juvenile rheumatoid arthritis [42,43]. The prevalence of CD is less studied in other rheumatological diseases in pediatric population. Studies examining positive effect of GFD on arthritis were not conducted in children, while in adults they are very rare. Sjogren's syndrome is considered the most common rheumatological disease associated with CD (in 12% -14.4% cases) in adults [44,45].

Conclusion

CD may be associated with many diseases of autoimmune origin. Currently, there are recommendations of international societies such as ISPAD and ESPGHAN for CD serological screening only in children with T1DM and autoimmune thyroid disease. Future studies with the involvement of large number of children, including a cost-benefit analysis, are going to show whether CD screening for children with other autoimmune diseases will be justified. Despite a very promising hypothesis, there is still no scientifically based evidence that GFD has prophylactic effect on the development of other autoimmune diseases in patients with CD.

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Original Article

THE EFFECT OF DEGREE AND TYPE OF LEFT VENTRICULAR HYPERTROPHY ON VENTRICULAR ARRHYTHMIAS IN HYPERTENSION

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Abstract. *Finding the arrythmogenic potential in patients with arterial hypertension as well as its correlation with left ventricular hypertrophy (LVH), and its type and degree. The research included 109 hypertensive patients (pts) (54 male and 55 female), 54.2 ± 7.9 years old without symptomatic coronary disease, myocardial infarction and systolic dysfunction. All the pts had a clinical examination, ECG, 24 h Holter monitoring with Lown classification of ventricular arrhythmias, an echocardiogram with left ventricular mass index (LVMI) and a specific type of LVH. QT interval dispersion (QTd) was calculated on 12 leads standard ECG. 75 pts had LVH (LVMI: 172.6 ± 42.95 g/m2) while 35 pts were without LVH (109.3 ± 15.9 g/m2). Non sustained ventricular tachycardia was registered in 13 pts (17.6%) with LVH and 1 female patient without LVH (2.9%). Patients with VT had a considerably higher ILVM (214.9 ± 6.8 vs. 151.9 ± 47.2 g/m2) than the average and higher QTd (73.7 ± 19.1 vs. 55.2 ± 20.2). VT was registered in 3/19 (15.8%) with eccentric nedilated type LVH, 6/38 (15.8%) with concentric LVH, 1/11 (9.1) (disproportional septal LVH) and 3/5 (60%) with dilated LVH. Univariate analysis showed a considerable correlation between the degree of arrhythmias and ILVM ($p<0.001$) and QTd ($p=0.012$). Ventricular arrhythmias in patients with arterial hypertension are considerably correlated to the degree of LVH expressed in ILVM and QTd.*

Key words: hypertension, left ventricular hypertrophy, ventricular arrhythmia, QT.

Introduction

The increased thickness of the left ventricular wall and enlargement of the left ventricular cavity that may accompany hypertension have a compensatory value in that the heart is better able to maintain stroke volume in the face of an increased afterload. As the structural changes progress, however, cardiac function diminishes and either concentric and eccentric left ventricular hypertrophy (LVH) develops. The change represents an additional risk factor for all types of cardiovascular disease in hypertensive patients [1].

Left ventricular hypertrophy on the electrocardiogram (ECG) has been identified as a major risk factor for cardiovascular disease for more than 35 years. The data from the Framingham Heart Study also showed that the detection of LVH by echocardiography provides persuasive prognostic information beyond that derived from traditional risk factors such as blood pressure, smoking, and lipid levels. In that study as well as in the investigation carried out at the New York Hospital-Cornell Medical Center, left ventricular mass and age were the strongest predictors of prognosis [2]. The importance of LVH is confirmed by the finding that, within 5 years of its appearance, one-third of men and one-fourth of women with LVH are dead [3].

A multivariate analysis demonstrated in men a relative risk of death from cardiovascular disease of 1.73 for each increment of 50 g/m^2 in left ventricular mass. The corresponding value for women was 2.12. The risk factor-adjusted relative risk of sudden death in men was for each increment of 50 g/m^2 in left ventricular mass. In a report restricted to patients with essential hypertension, the multivariate analysis showed that only age and echocardiographic left ventricular mass were independently associated with the cardiovascular death [4].

Analyzing patho-anatomic and electrophysiological characteristics of myocardium in LVH it can be shown that hypertensive myocardium has a tendency to show electric instability and arrhythmias. The hypothesis of hypertrophy per se leading to electrophysiological changes that may trigger lethal arrhythmias may be even more attractive.

It has been suggested that the increased incidence of sudden death in hypertensive patients, particularly those with LVH, may be casually related to the increased number and complexity of ventricular arrhythmias that have been demonstrated in these patients.

Electrophysiological studies suggest that QT prolongation is associated with increased regional

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homogeneity of repolarization, a factor that may contribute to ventricular arrhythmias. Recent clinical studies have suggested that the interload variability of the QT interval in the standard ECG, defined as QT dispersion, reflects regional differences in ventricular repolarization. Increased dispersion of recovery time is believed to increase the risk for serious ventricular arrhythmias. [5,6,7].

In view of this, the aim of our study is to prove whether there is an interrelation between markers of sudden death in hypertensive patients (left ventricular hypertrophy, its degree and type, ventricular arrhythmias and QTc dispersion).

Subjects and Methods

Subjects

One hundred and nine consecutive hypertensive patients (pts) (54 male and 55 female), 54.2 ± 7.9 years old, treated at the Health Center for Hypertension at the Institute "Niska Banja", were involved in the study. To be included, patients must have trough sitting diastolic blood pressure mean readings of 90 to 115 mmHg or sitting systolic blood pressure mean readings of 160 to 200 mmHg after 1 and 2 weeks without antihypertensive therapy. Patients were excluded if they had clinical or laboratory evidence of heart failure, renal failure, coronary artery disease, valvular defect, or secondary causes of hypertension.

Electrocardiography

Standard 12-lead ECGs were recorded with a paper speed of 25 or 50 mm/s. ECGs of technically good quality were recorded. QT interval analysis was done on 12-lead ECGs taken upon enrolment in the study [8]. A single observer measured QT interval in all leads, if possible, on a surface 12-lead ECG (25 mm/s speed). QT interval was taken from onset of the QRS to the end of the T wave (i.e., return to the T/P baseline). If U waves were present, the QT interval was measured to the nadir of the curve between the T and U waves. Three consecutive cycles were measured for each lead. QT intervals were corrected with Basset's formula (QTc=QT/RR sup 1/2). QTc dispersion, defined as the difference between maximum and minimum QTc, was calculated in ECGs in which at least 5 leads were measurable. Adjusted QTc dispersion was measured to correct for the known dependence of the index on the number of measurable leads [9].

Echocardiography

The echocardiographic studies were performed in the morning, with the subject in a supine left lateral decubitus position, after 30 minutes of rest. Only one physican was responsible for recording the echocardiogram. The measurements of left ventricular wall thickness and chamber diameter were made in diastole in accordance with methods outlined by the American Society of Echocardiography [10]. Left ventricular mass (LV mass) was estimated by the modified cubed formula using measurements obtained in accordance with the "Penn" convention [11]: LV mass (g) = 1.04 [(LVID + VST + PWT)^3 – (LVID)^3] – 13.6, where LVID is left ventricular internal dimension, VST is ventricular septal thickness, and PWT is posterior left ventricular wall thickness. LV mass indexed by body surface area (g/m^2). Cut-off values for LVH by Penn convention (g/m^2) criteria were ≥134 g/m^2 for men and ≥110 g/m^2 for women. Left ventricular wall thickness was defined as sum VST and PWT, relative wall thickness was defined as (left ventricular wall thickness/LVID)x100% and index LVID (LVID/ body surface area). On the basis of these parameters 4 types of LVH are defined: 1) concentric hypertrophy-C; 2) eccentric no dilated LVH - END, 3) eccentric dilated LVH - ED and 4) disproportional septal LVH - DS [12].

Ambulatory electrocardiographic monitoring

Twenty-four hour ambulatory ECGs were recorded with sistem Del Mar Avionics, model 465; Irvine, California during each subject's normal activities. Evaluation of premature ventricular complex followed the grading system of Lown and Wolf [13]. Grade 0, no premature ventricular complex; Grade 1,<30 premature ventricular complex/h; Grade 2, >30 premature ventricular complex/h; Grade 3, multiform prematureventricular complex; Grade 4A, couplets; Grade 4B, run of ventricular tachycardia (VT) (3 or more consecutive premature ventricular complex). Lown Grade ≥3 were considered as complex ventricular arrhythmias. Since the classification suggested by Lown and Wolf combines prevalence with morphology of ventricular ectopy, in a second analysis we compared hypertensive patients with complex ventricular ectopy with those without commplex ventricular ectopy.

Statistical analysis

The data from individual patients were summarized as mean ± SD. The statistical significance of differences in correlation coefficients and mean values was examined by ANOVA and by Student's *t* test for unpaired observations. A value of $P<0.05$ was taken as the minimal level of statistical significance. Statistical tests were carried using STATISTICA programs.

Results

From 109 hypertensive patients included in this study, 74 pts had echocardiographic evidence of left ventricular hypertrophy while 35 did not. There were no differences between the groups in terms of sex, body surface area and cholesterol degree. Systolic blood pressure, left ventricular mass index, the frequency of ventricular tachycardia and QTc dispersion were statistically significantly higher ($p<0.05$) in the patients with echocardiographic LV hypertrophy (Table 1).

Table 1 Clinical and hemodynamic characteristics of hypertensive patients with and without left ventricular hypertrophy established by echocardiographically.

	Echo LVH (n=74)	Echo no LVH (n=35)
Age (yr)	55.3 ± 7.8*	51.7 ± 7.5
Body surface area (m^2)	1.91 ± 0.2	1.87 ± 0.2
Sex (male:female ratio)	38/36	16/19
Systolic pressure (mm Hg)	169.4 ± 16**	158.1 ± 12.7
Diastolic pressure (mm Hg)	106.6 ± 15	104.6 ± 7
Heart rate (beats/min)	75.1 ± 9	72.9 ± 8.2
Left ventricular mass index g/m^2	172.6 ± 43**	109.3 ± 16
IVb degree Lown (n)	13 (18%)*	1(3%)
QTc ms	434.6 ± 28.5	421 ± 31
QTc dispersion	58.8 ± 20*	48.3 ± 20
Cholesterol	6.2 ± 1.1	6.1 ± 21.3

Values are expressed as means ± SEM , * p<0.05, ** p<0.01

Table 2 Clinical and hemodynamic characteristics of hypertensive patients with different types of LVH

	C LVH (n=38)	END LVH (n=19)	ED LVH (n=6)	DS LVH (n=11)
Age (yr)	54.6 ±7.3	56.9 ± 8.6	61.2 ± 4.8	51.4 ± 8.3
Body surface area (m^2)	1.87 ± 0.15	1.9 ± 0.2	1.96 ± 0.1	1.92 ± 0.1
Sex (male:female ratio)	16/22	11/8	6/0	6/5
Systolic pressure (mm Hg)	172 ± 16	169.5 ± 18.4	162 ± 15	160.5 ± 12
Diastolic pressure (mm Hg)	109.6 ± 10	105.3 ± 11	103 ± 4.5	106.8 ± 6.4
Heart rate (beats/min)	75 ± 8.6	73.1 ± 10	82.5 ± 13	77.2 ± 8
Left ventricular mass index g/m^2	166.8 ± 40.3	172.4 ± 23	260 ± 62.5*	152.7 ± 21.5
IVb degree Lown (n)	6 (16%)	3 (16%)	3 (50%)	1 (9%)
QTc ms	433.3 ± 25.2	430.8 ± 35.1	461.7 ± 24	442 ± 28.1
QTc dispersion	54.3 ± 14.1	62 ± 20.1	92.5 ± 38.4	58.2 ± 11
Cholesterol	6.12 ± 1.1	6.7 ± 1	5.9 ± 1.6	6.15 ± 1.2

Values are expressed as means ± SEM , * p<0.05

Type left ventricular hypertrophy

Most hypertensive pts had LVH of a concentric type, while a small but significant group of pts had a dilated LVH. This group was significantly different from the pts with other LVH types since they were older, they had lower systolic blood pressure, a high degree LVH (LV mass index: 260 ± 62.5 g/m^2), proportionally greater frequency of ventricular tachycardia and bigger QTc dispersion (Table 2). The examined parameters did not show any other significant differences in patients with other LVH types except for the fact that the patients with disproportional septal LVH were younger, had lower systolic blood pressure and lower LV mass index.

Relationship between left ventricular mass index and examined parameters

Systolic blood pressure (r = 0.27, p = 0.0047) and heart rate (r = 0.25, p = 0.016) are closely connected to the degree of left ventricular hypertrophy. The interconnection is even more prominent between left ventricular mass index and QTc dispersion (r = 0.34, p = 0.004). The frequency of ventricular arrhythmias graded according to Lown-Wolf is strictly correlated with seriousness of left ventricular hypertrophy (r = 0.4, p = 0.00002) (Table 3).

Table 3 Correlation coefficients relating clinical and haemodynamic data to left ventricular mass index

	ILVM
Age (yr)	0.17
Body surface area (m^2)	0.13
Systolic pressure (mm Hg)	0.27**
Diastolic pressure (mm Hg)	0.07
Heart rate (beats/min)	0.25*
Lown-Wolf degree	0.4***
QTc ms	0.14
QTc dispersion	0.34**
Cholesterol	0.03

*p<0.05, **p<0.005, ***p<0.001

Fig 1 and Table 4 show significant correlation between two examined markers of sudden death in our study. More serious degree of ventricular arrhythmias was accompanied by longer QTc, bigger QT dispersion and higher LV mass index.

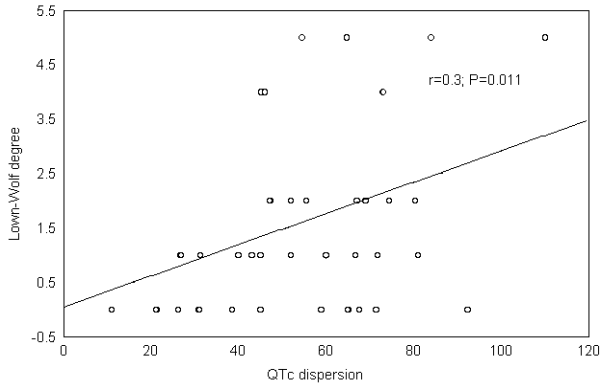

Fig. 1 Scatter plots illustrating the relationship of the premature ventricular complexes (Lown-Wolf) to the QT dispersion.

Table 4 Relationship between dispersion parameters and the grade of premature ventricular complexes.

	0–2*	3–4
LVM index	144 ± 35.3**	185.2 ± 58.5
QTc	425.7 ± 28.4**	443.5 ± 27.3
QTc dispersion	52.15 ± 18**	64 ± 21

Values are expressed as means ± SEM , *Lown-Wolf grades 0,1,2 vs. Lown-Wolf grades 3,4A,4B
** $p<0.05$

In 14 (13%) hypertensive patients non-sustained ventricular tachycardia was registered. Almost all patients were male but in other clinical parameters (age, body surface area, blood pressure and heart rate, cholesterol) they did not show any difference from other patients. However, the degree of their hypertrophy and QTc dispersion were significantly different compared to pts who did not have VT (Table 5).

Table 5 Clinical characteristics of patients with registered VT.

Age (yr)	57.4 ± 9
Body surface area (m^2)	1.9 ± 0.1
Sex (male:female ratio)	10/3
Systolic pressure (mm Hg)	168.6 ± 18
Diastolic pressure (mm Hg)	108.2 ± 12
Heart rate (beats/min)	76.9 ± 12.1
Left ventricular mass index g/m^2	214.9 ± 68.4
QTc ms	442.3 ± 28
QTc dispersion	73.7 ± 19
Cholesterol	6.1 ± 1.4

Values are expressed as means ± SEM,

Discussion

Our research shows that the increase of left ventricular mass is closely correlated with frequency of ventricular arrhythmias and QTc dispersion. Knowing the importance of ventricular arrhythmias and QTc dispersion as markers of sudden death, our results suggest that identification of left ventricular mass in hypertensive pts is a good marker for future prognosis.

The importance of LV hypertrophy in the morbidity of hypertensive disease has been underscored by the number of electrocardiographic and echocardiographic studies that have convincingly demonstrated its importance as an independent predictor of morbidity and mortality [14,15]. Not only does LV hypertrophy as a dichotomous variable predict adverse outcome, but the magnitude of LV mass a continuous variable is also associated with cardiovascular risk, even with values for LV mass within the "normal range".

Importantly, there is an association of LV hypertrophy with complex ventricular arrhythmia, a possible processor of sudden death in hypertensive patients, even in patients without coronary artery disease on angiography [16]. In patients with reversible myocardial perfusion defects on thallium scintigraphy, both LV hypertrophy and inducible ischemia are independently associated with ventricular arrhythmias [17]. Using programmed electrical stimulation, Coste et al. [18] demonstrated more intraventricular reentry and non-sustained ventricular tachycardia in hypertensive patients with LV hypertrophy compared to control group.

The pathologic alternant related to ectopic impulse generation in LVH are multifactorial [19] and include enlarged myocytes, the expansion of the collagen matrix, fibrosis, subendocardial ischemia, and medial hypertrophy of the coronary arteries impending homogeneous impulse propagation throughout the myocardium. The arrhythmogenic substrate of LV hypertrophy itself may explain reentry mechanisms, such as fibrillar stretching, anisotropy, and triggered activity due to after-potentials, depending on activation of slow calcium channels.

Concentric hypertrophy appears to be associated with a more severe overall prognosis compared with eccentric hypertrophy, whereas concentric remodeling seems to be of intermediate significance compared with normal geometry [20]. Eccentric LV hypertrophy was independently associated with increased risk of sudden cardiac arrest (SCA) in subjects with EF ≤40% [21]. In our study there was no (statistical) difference in arrhythmias between these two types of LV hypertrophy, however, significantly highest incidence of serious arrhythmia, despite the small number of patients, was registered in patients with ED type. These patients had the highest QT dispersion and the greatest left ventricular mass index.

QT dispersion has been associated with increased risk for ventricular arrhythmias and sudden death in patients with chronic heart failure [9], mitral valve prolapse [22], MI [23], familial long-QT syndrome [24], and with an increased risk for cardiac mortality in patients with peripheral arterial disease, MI and older men and women [25].

Clarkson et al. in their study of multivariate analyses demonstrated significant relationships between QT dispersion and office systolic blood pressure, and left ventricular mass index. Several studies reported that the most typical electrical abnormality associated with

cardiac hypertrophy is prolongation of the action potential. Left ventricular hypertrophy by severe hypertension induced by renal artery constriction produced a prolongation of action potential duration. An increased dispersion of ventricular refractoriness might represent another electrical consequence of the hypertrophic process. A prolonged duration of ventricular repolarization, in fact may be proarrhythmic, probably favoring the occurrence of after depolarizations and then triggering activity-related arrhythmias [26,27].

The results of our study have shown that QTc dispersion is significantly correlated with the increase of left ventricular mass index and that the highest values are found in pts with eccentric nondilated and eccentric dilated which proves the hypothesis that an important entity underlying QT dispersion is patchy myocardial fibrosis, resulting from ventricular dilatation and neurohormonal activation and myocardial ischemia.

The results of our study have shown a significant correlation between QTc dispersion and the degree of ventricular arrhythmias registered by Holter monitoring. It can be important for better understanding not only of the mechanisms of malignant arrhythmias in pts with LV hypertrophy (QTc dispersion shows differences in action potential duration) but also in estimating the risk that a hypertensive patient carries with himself.

Modern antihypertensive therapy means, among other things, a possibility of regression of left ventricular mass index. Recent studies prove that there is a chance of better prognosis in pts with regression of LV hypertrophy but there is not a study which shows what the arrhythmogenic potential of myocardium is after its regression. The observation of left ventricular mass, ventricular arrhythmias and QTc dispersion can probably answer the question to what degree and how regression of left ventricular hypertrophy are correlated with decrease of electric vulnerability of myocardium and with a risk of sudden death in this group of pts.

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Original Article

CYTOMORPHOLOGY OF THE BULBAR CONJUNCTIVAL CELLS IN PATIENTS WITH DRY EYE

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Abstract. *Dry eye is among the most common pathological conditions in ophthalmology. The aim of our study was to present possibilites of two different cytological methods for examination of cytomorphology of bulbar conjunctival cells-impression cytology (IC) and combined cytological method for scanning electron microscopy in the diagnosis of dry eye (ICSEM). A hundred and twenty-two patients of both sexes, in different age groups, were analyzed by clinical method (slit lamp, Schirmer I, TBUT, Rose Bengal) and two cytomorphological methods – IC and ICSEM. In patients with dry eye, squamous metaplasia, inflammation and severe loss of adhesiveness of the epithelium were present. ICSEM gives an advantage in early diagnosis of changes, before the lesion of superficial conjunctival epithelium in patients with dry eye. The phenomenon of metaplasia appears in the epithelium of the bulbar conjunctiva in the absence of manifest dry eye and represents the basis for understanding the increased incidence of this syndrome in older patients with dry eye.*

Key words: *dry eye, impression cytology, cytological method for scanning electron microscopy*.

Introduction

Dry eye is among the most common pathological conditions in ophthalmology [1].

According to the definition, it is a multifactorial disease of the tears and ocular surface that results in symptoms of discomforts, visual disabilities, unstable tear film and ocular surface damage potential [2]. Dry eye affects 2 - 14,4% of the tested population worldwide and it is more common among older people, especially among females. The dry eye is a result of various data obtained during several clinical and cytological diagnostic procedures [3-6].

Among the cytological methods of examination are conjunctival impression cytology (CIC) and a combined method of impression cytology and scanning electron microscopy (ICSEM). CIC is a noninvasive method of conjunctival biopsy which allows the examination of conjunctival changes at the cellular level, the etiology of various ocular surface disorders, documenting on age changes of ocular surface, monitoring the effects of the treatment of dry eye, staining of squamous metaplasia, and it contribute to the study of ocular surface diseases with immune histochemical and DNA analysis [7-10]. ICSEM shows the appearance of various types of conjunctival cells in three dimensions, as well as the appearance of apical cell surfaces and microvillus [11].

The aim of our study is to review the features of cytomorphology of bulbar conjunctival cell samples with CIC and ICSEM in patients with different dry eye etiology.

Material and Methods

After the approval of the local ethical committee, clinical and cytomorphological examination of 122 patients, out of which 100 by CIC and 22 by ICSEM, was conducted at the Ophthalmology Clinic and the Institute of Pathology, Clinical Centre Niš. The clinical methods of examination included slit lamp of anterior segment and tear layers, Schirmer test I, tear break-up time (TBUT), and Rose Bengal test. Cytomorphological examination of nasal and temporal parts of bulbar conjunctiva was done by CIC and ICSEM. All examined patients were divided into three categories, according to age: younger than 20 years, 21-40 years and older than 41 years.

Millipore membrane filter (Membrane-Filters, Whatman®, Schleicher&Schuell, OE 66 St., Germany) is biologically inert, with submicroscopic pores. It is cut into 4x4mm squares and it gently lies on the previously anesthetized ocular surface (on the bulbar conjunctiva for both methods, temporal/nasal location for CIC and temporal for ICSEM) for several seconds and it adheres

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to the fine layer of the superficial conjunctival epithelium. The obtained imprint is then fixed, dyed and visualized using scanning electron microscopy.

CIC imprint - after air drying for 15 minutes, the preparations were fixed in 95% alcohol and stained using a modified method of hematoxylin eosin (HE) and periodic acid-Schiff (PAS) for cytological specimens. Cytological analysis was performed to show the score impression cytology (IC-score) for each tested sample, which was used for statistical analysis (Pearson's $\chi 2$ tests) of the contribution of individual parameters relevant for the assessment of dry eye.

ICSEM imprint was immersed in 2% glutaraldehyde solution. Cells were first fixed in 2% solution of glutaraldehyde for 24 hours, washed by being kept in the Millonigbuffer for 30 min. Postfixation was performed in 1% OsO4, for 1.5 hours, and then the cells were washed two times for 30 min in Millonig buffer. The cells dehidratation was obtained by their imersion in the growing concentration of alcohol (50%, 75%, 95% and 100%), each concentration for 15 min and dried out at a critical point. The samples were mounted on pillars and covered with gold and thus prepared cells were observed in the scanning electron microscope.

Histological and morphological analysis of conjunctive cells samples, isolated by CIC and ICSEM, was performed using the methodology reported in literature [8-11] and presented in Figures.

We used SPSS statistical package, version 15.0 for statistical analysis. Categorical characteristics are given as absolute numbers, and in percentages (%).Pearsons χ^2 test or Fisher test were also used as statistical procedures.

Results

A comparative analysis of cytomorphological features of bulbar CIC samples from 100 patients, 50 males and 50 females, was performed. There are no statistically significant differences in gender representation in age groups (Table 1). Diagnosis of dry eye was established in 65 patients (Table 2) older than 40 years with rheumatoid arthritis (RA), hypertension (HTA), and diabetes mellitus (DM) by the following clinical criteria: Schirmer I $\leq$ 10 mm / 5 min; TBUT $\leq$ 10 sec; Rose Bengal positive, 3 of 4 per Bijsterveld. There is significant difference in health characteristics between genders ($p<0.001$) - Table 2. Also, in group patients for ICSEM there are statistically significant difference in gender representation in age groups (Table 3).

Table 1 Distribution of respondents by gender and age for CIC

Sex	Age		Total
	≤40 years	>40 years	
Male	18 (36.00%)	32 (64.00%)	50
Female	17 (34.00%)	33 (66.00%)	50
Total	35	65	100

χ^2 = 0,04, df=1, p=0,8348 (n.s.)

Table 2 Health characteristics of patients with dry eye for CIC

Sex	Health characteristics			Total
	RA	HTA	DM	
Male	2 (6.25%)	19 (59.38%)	11 (34.38%)	32 (100.00%)
Female	16 (48.48%)	8 (24.24%)	9 (27.27%)	33 (100.00%)
Total	18	27	20	65

χ^2=15.56, df=2, p<0.001

Table 3 Distribution by sex and age of patients for ICSEM

Sex	Age (years)		Total
	≤40 years	>40	
Male	6 (85.71%)	1 (14.29%)	7 (100.0%)
Female	5 (33.33%)	10 (66.67%)	15 (100.0%)
Total	11	11	22

χ^2=5,00, df=1, p=0,0253<0.05

There are no significant differences in health characteristics between gender of patients with dry eye for ICSEM (Table 4).

Table 4 Health characteristics of patients with dry eye for ICSEM

Sex	Health characteristics			Total
	RA	HTA	DM	
Male	1 (50.00%)	1 (50.00%)	0 (0.00%)	2 (100.00%)
Female	6 (46.15%)	6 (46.15%)	1 (7.70%)	13 (100.00%)
Total	7	7	1	15

χ^2 test, n.s.

Epithelial cells were presented as grouped and dense, intact in controls**,** or easily separated, weakly cohesive (Fig. 1 A), individual cells in the group with dry eye.

The degree of squamous metaplasia, estimated by nucleo/cytoplasmic relations, was unchanged if the ratio of the nucleus and cytoplasm was 1: 1 to 1:3, low grade with 1:4 to 1:6 ratio, expressed if the ratio was 1:6 to 1:10 and massive, if the ratio of these parameters was more than 1:10 in group with dry eye (Fig. 1 B). The degree of keratinization was low grade when there were few keratinocytes, or expressed and massive, if the sample exceeds the number of cytological non-keratinization cells in group with dry eye. Nuclear changes were evaluated based on the shape of sails, including the "serpentine sails" as a marked characteristic of squamous metaplasia in dry eye syndrome (Fig. 1A–C); a frequency change was graded according to the number of changed nuclei (sporadic, few and plenty of altered nuclei). Goblet cells were evaluated by number - normally present or easily reduced in group with dry eye, expressed a smaller

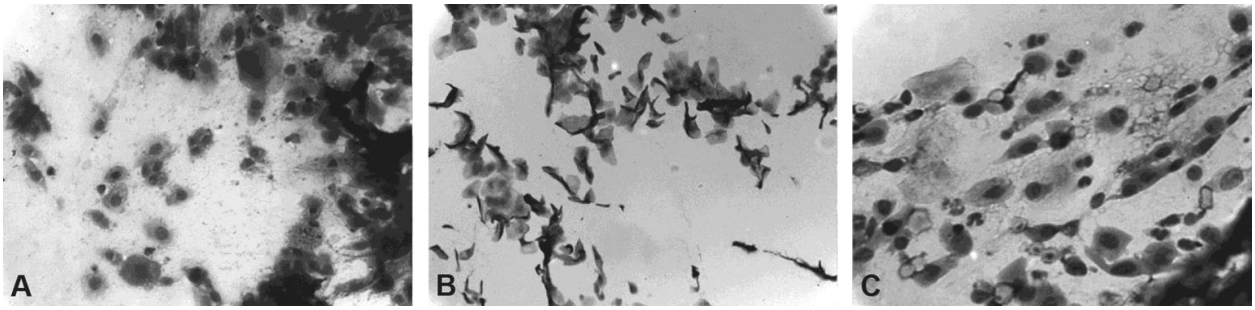

Fig. 1 A, B, C Cytomorphological feature of bulbar conjunctiva in CIC samples

number, sporadically or not found in the cytological sample. Morphological changes of goblet cells were evaluated for content as the normal mucous cells without mucus, a moderate reduction of the content and with abundant mucus. Mucus quality was assessed as normal if it is granular, seen with exudates, filaceous or in the aggregate. Inflammatory cells are described if they are present and the quantity of the sample.

The ten morphological parameters of IC samples were explored: the ratio of the cohesion of the epithelial cells, the degree of squamous metaplasia, degree of keratosis, frequency of nucleus changes, the type of nuclear changes, goblet cell density, morphology of goblet cells, the amount of mucus, mucus morphology and the presence of inflammatory cells.

The results of semi quantitative analysis of these parameters between controls and the group with dry eye, showed no significant differences in IC score on the nasal and temporal localization, except that the presence of inflammatory cells in both examined localizations is statistically more frequent in controls than in the study group (χ^2 = 13.61, p = 0.0035, p <0.01 in the nasal and χ^2 = 12.50, p = 0, 0059, p <0.01 in the temporal localization).

Characteristics of epithelial sheets were analyzed in controls and the group with dry eye on the basis of intactness, or the presence of reduced cohesion, severe levels of reduced cohesion or the findings of individual cells. There was no statistical significance between controls and the group with dry eye in the characteristics of epithelial sheets on the nasal IC scores (χ^2=1.66, p = 0.6445) and temporal localization (χ^2=0.46, p= 0.9270). Squamous metaplasia and keratinization were low grade, expressed or massive. There was no statistically significant difference between controls and group with dry eye in the nasal (χ^2 = 0.88, p = 0.8296) and temporal localization (χ^2 = 0.70, p = 0.8726) for presence of keratinization as well as squamous metaplasia (nasal χ 2 = 0.80, p = 0.8500, and temporal χ^2 = 1.38, p = 0, 7111). Nuclear changes were graded as sporadic, several or extensive. It is evident that the nuclear changes are presented in group with dry eye in both localizations (especially temporal), but no statistically significant differences were proven compared to the control group (χ^2= 1.26, p = 0.7394 the nasal localization and χ^2 = 2.40, p = 0.4929 in temporal localization). The type of nuclear changes was classified low grade, chromatin snake 1 + 2 + forms and nuclear fragmentation. It is evident that the nuclear changes represented in the group with dry eye in both localizations (especially temporal), but no statistically significant differences were found compared to controls (χ^2=1.66, p=0.6450, the nasal localization and χ^2 = 1.78, p = 0.6197 in temporal localization). Goblet cell density was normal or reduced. Even if the normal density of goblet cells was present in the control group, no significant differences in the density of goblet cells were found in relation to the group with dry eye in both localizations, nasal and temporal (χ^2 = 1.35, p = 0.7181, in the nasal localization and χ^2 = 2.67, p = 0.4459, in temporal localization) as well as the morphology of goblet cells in both groups (χ^2 = 0.09, p = 0, 7646 the nasal and temporal localization). The amount of mucus is normal or abnormal - heavy, medium and absent. There were no statistically significant differences in the amount of mucus between control group and group with dry eye in the nasal and temporal localization (χ^2=0.92, p= 0.8210, in the nasal and χ^2=0.75, p = 0.8614, in temporal localization).

Mucus quality was considered normal if it was granular, with exudate, filamentous or in aggregate. Statistically significant differences were not present in morphology of the mucus of the controls and group with dry eye in the nasal and temporal localization (χ^2=0.56, p = 0.7547 the nasal localization and χ^2 = 0.67, p = 0, 7135 the temporal localization).

Among the twenty two persons for ICSEM, 7 were male and 15 female (Table 3). The largest was the oldest category of subjects > 40 years, n = 11. The diagnosis of dry eye was established in 15 people according to the following clinical criteria: Schirmer I $\leq$ 10 mm / 5 min; TBUT $\leq$ 10 sec; Rose Bengal positive, 3 of 4 per Bijsterveld. This group included patients with rheumatoid arthritis (RA), hypertension (HTA), and diabetes mellitus (DM) (Table 4).

In physiological conditions, all epithelial types of the conjunctiva showed good cohesiveness, as well as exchange of information upon contact, tubular formations with high electron density which, like probes, penetrate adjacent cells. They are located along lateral cellular walls and are more expressed in younger population and in control groups (Fig.2A i C). These

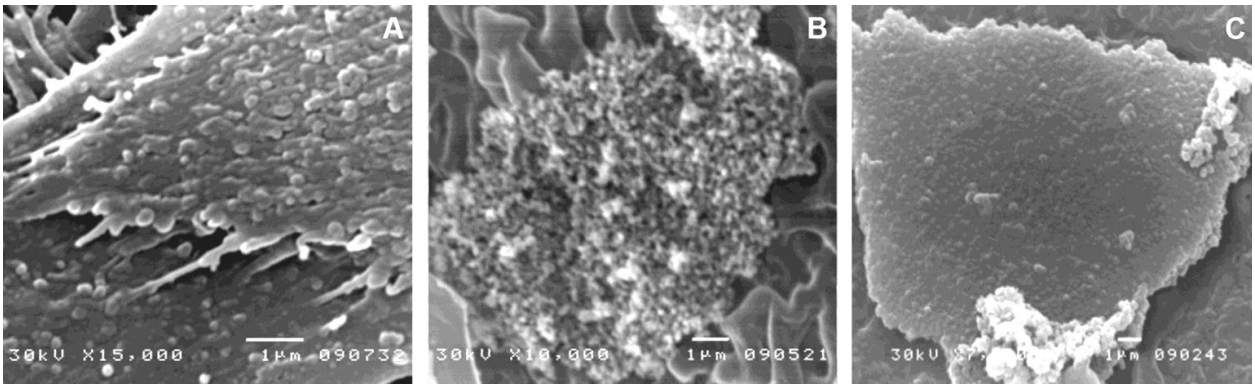

Fig. 2 A, B, C. Epithelial cells in physiological conditions (2 A et 2 C), tears (2 B)

structures are abundant in tear film after adverse factors. They are completely absent in pathological conditions.

The tear film presents as abundant granulated mass with dominant complex protein molecules or as filamentous structure, sometimes as lumpy protein mass containing crystal formations of cuboid sodium salts (Fig.2B).

In middle-aged and older patients (Fig. 3 A, B, C), apical surfaces lose the cytoplasmic and membranous ectropion, the cohesiveness is age-dependent and proportionate to exposure to the factors from external and internal environment – the severity of the damage of lateral conjunctiva gradually increases towards the nasal part. The number of cells with rough creases in the apical surface is proportionately higher and there are also keratin cells, which are typically small, robust, rough, individually distributed in filter paper imprint. The decrease in epithelial cohesiveness is manifested as widened intercellular connections and the findings of individual cells in the imprint.

The tear film is detected as a mass of rough protein and mineral deposits.

In patients with dry eye (Fig. 4 A, B, C), the number and distribution of cellular formations is contrary to the findings obtained from the control groups and young examinees. The dominant cells are those with rough morphology, robust squamous metaplastic epithelial cells, with smaller dimensions than normal conjunctival

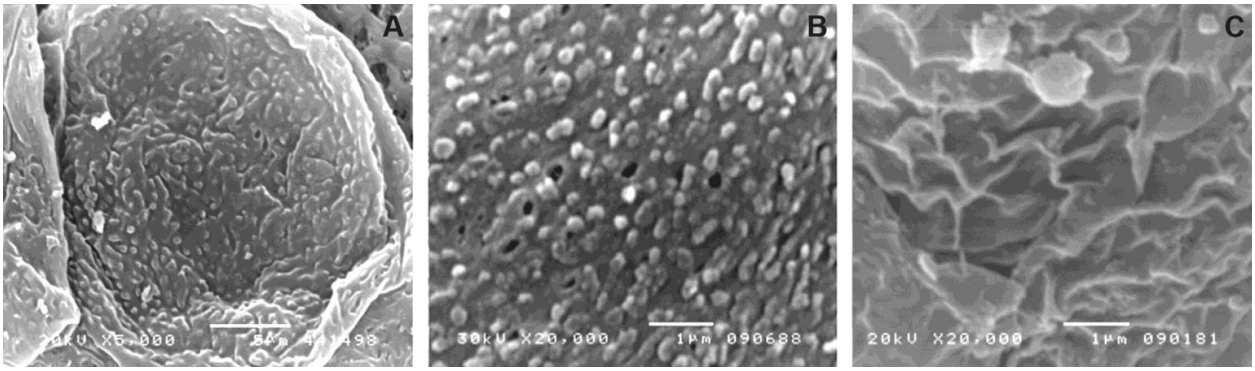

Fig. 3 A, B, C. Epithelial cells look like "cut stone" in older age patient group

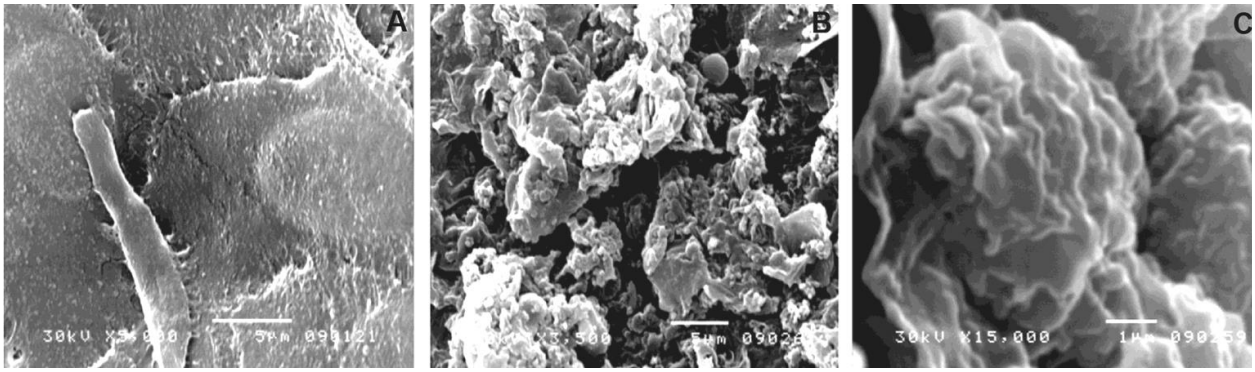

Fig. 4 A, B, C. Epithelial cells in patients with dry eye

cells. If present, villous cells are sparse and very often only rare, individual cells are obtained from the sample.

The fissured defect of the cell membrane after separation of the microvillus is a focal change, although diffuse defects have also been observed, which show a latticelike profile of the apical membrane. Under these circumstances, adverse environmental factors have a twofold effect – toxic and metaplasia induced by phenotype change in altered circumstances. Cells with rough and more expressed creases are found, which is the result of volume reduction or the first sign of evaporation caused by tear film defect.

Disscusion

Tear film and ocular surface is a complex and stable system whose equilibrium can be disrupted by many factors [1,9,13,14].

In dry eye syndrome the conjunctiva of our patients showed progressive stratification, hyperplasia with thinning and cell metaplasia. The appearance of binuclear cells or "incisions sails" in the cells can be seen in the early stages of this condition, while the form of pyknotic nuclei or loss of nuclei is a characteristic of the disease in advanced stage, which is similar to the results of other authors [15]. Separation of the epithelium is linear and follows the degree of severity of the syndrome. Reduced the number of goblet cells is a part of integrated morphological changes of the conjunctiva in relation to age changes, and that is not an isolated factor that contributes to the presence and / or highlighting the phenomenon of dry eye.

The ICSEM is an analysis of all available cells in cytological samples of the conjunctiva. This method of cytomorphological examination shows that except architectural changes, epithelial changes include a change in phenotype of cells of the conjunctiva. In the youngest category of subjects, the dominant epithelial types in the conjunctiva are gentle and rich villous structures without goblet cells, in the middle and older ages there were voluminous, cubic cells, with roughly wavy apical membrane surface, rounded and villous. The representation of both cell types was uneven and with variations in number, compared to age but not gender. In patients with dry eye and DM, HTA, RA these changes build on squamous metaplasia, inflammation and severe loss of adhesiveness of the epithelium. The appearance of papillary epithelial structures and focal irregular proliferation of cells with squamous metaplasia, and loss of adherence of the epithelium were observed in all analyzed groups of elderly patients.

The analysis of histological and cytological characteristics of the conjunctiva in relation to the age points to the main morphological features of the conjunctiva. It was noted that the number of epithelial sequences increases with age, while the number of goblet cells decreases. This adaptive phenomenon reflects the need for protection from external influences, or the response to proliferative stimuli from the environment. These findings agree with the findings from other authors[11, 12,15,16-22,24-26], but aging as a key factor for the appearance of dry eye syndrome was established in numerous studies [2, 24,25, 27,28].

Squamous metaplasia is a condition in which the non-keratinized epithelium is replaced with keratinized epithelium. It is a common pathological process in most epithelia, including the respiratory epithelium and the urothelium and it is a frequent occurrence in diseases with manifestations of dry eye, such as Sjögren's syndrome, Steven-Johnson syndrome, pemphigoid mucous membranes, and thermal eye injuries [15,18,29]. According to the findings, epithelial hyperplasia was observed, a process associated with aging and the continuous change of the epithelium which is seen in people with DM and RA.

Hyperplasia with squamous metaplasia of the epithelium is accompanied by a change in keratin expression and as shown in research, it comes exclusively from limbal stem cell pool, which involves transdifferentiation of terminally differentiated cells [30]. This study has shown that the phenomenon of metaplasia is present in the bulbar conjunctival epithelium in the absence of manifested dry eye, which forms the basis for understanding the increased incidence of this syndrome in the elderly. Transdifferentiation inhibitors can also be used as prophylaxis during life.

Conclusion

The ocular surface is a delicate and vulnerable structure. Investigation of the component parts of the ocular surface must necessarily be done without disruption (or at least minimal invasion) of physiological function.

The analysis of CIC parameters is a non-aggressive, reliable and representative method in the diagnosis of dry eye and can be used as a screening test for vulnerable age groups and persons at risk for developing the dry eye syndrome. Combined ICSEM methods show the evolution of conjunctival epithelial lesions. The advantage of this method is the identification of conjunctival epithelial damage which disrupted the tear film before the epithelial damage can be detected by optical microscopy.

A timely diagnosis of dry eye syndrome allows the application of adequate treatment and implementation of preventive therapy. Better knowledge of the pathophysiology and diagnosis of dry eye allows better and more effective treatment of dry eye.

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Case Reports

PROPTOSIS AND DIPLOPIA AS CONSEQUENCES IN TRAUMA OF CRANIOFACIAL JUNCTION

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Abstract. *In the trauma of craniofacial junction, frontal sinus wall fractures take up 5-15% of all facial bone fractures. The most common mechanism of their occurrence comes as a result of the action of high-energy impact force on the frontal area. Treatment of the injuries in frontal-orbital-ethmoidal regions largely depends on the responsible experts (otolaryngologist, maxillofacial surgeon or neurosurgeon) in all cases, because of the implementation of diverse surgical technics in order to achieve the best possible outcome for the patient. Bearing in mind the complex anatomical features of this region, it is clear that these procedures are often accompanied by series of possible complications, all of which are certainly neurosurgical. These can be expressed as early or late complications, and could be characterized by diverse clinical manifestations. Mucocele is formed, either due to partial obstruction of the sinus mucosa or due to the obstruction of the frontal sinus. The long term existence of mucocele and its progressive growth will result in strong pressure on the adjacent bones, and lead to their destruction followed by the process-propagation into surrounding tissues and spaces. In the further development if a bacterial contamination is detected, it will lead to the purulent inflammatory process and clinical picture of mucopyocele. In most clinical cases with complications proptosis and diplopia are dominant ophthalmic manifestations. In this paper we will present our experience in the treatment of proptosis and diplopia, as well as the ways of diagnostic evaluation in order to achieve timely diagnosis and assure swift healing of patients.*

Key words: *diplopia, proptosis, frontal sinus, craniofacial trauma*.

Introduction

Long persisting chronic pain syndrome can be an indicator of long-term complications, such as mucopyocele, osteomyelitis or mucocele that occur as a complication of trauma, mainly due to partial mucosal lining retention. Obstruction of sinus cavity can occur as a result of congenital anomalies, infection, trauma, allergies, and tumors or as a result of surgical procedures in the nasal cavity [1]. Continued secretion of mucus and its accumulation leads to formation of mucocele that grows over time causing atrophy or erosion of the bones of sinuses and surrounding bone structure, further extending by the path of least resistance [2,3]. Mucoceles are considered as tumors and destructive lesions [4]. The most common ways of spreading include propagation in the orbital cavity, surrounding sinus cavities, or nasal cavum with induration of surrounding soft tissue. In this manner, formed soft tissue cystic formation is covered with pseudostratified epithelium and its content is very often clear mucus, yellowish, sticky liquid, when we are talking about simple mucocele, or pus yellowish, thick and opalescent liquid, when we have secondary infections and then we are referring to an entity known as mucopyocele. Size, propagation time, duration and possible bacterial contamination of mucocele affect the formation of ophthalmic disorders [5]. Mucocele, in its development process, will grow in size and thus, provoke the thinning of the surrounding bone structure and eventually cause bone absorption. In the beginning, mucocele of the frontal sinus does not give any symptoms. Their growth and spreading through the bottom wall of the sinus results in protrusions, while a growth towards orbit causes exophthalmos (protrusion of the eyeball) and lateral position of the eyeball. Mobility of the eye balls is preserved. In mucocele of ethmoidal sinus tumefaction occurs in the inner corner of the eye and with long term dacryocystitis upward lateral positioning of ocular bulbus

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occurs. Also, in case of rare localization of mucocele in sphenoidal sinus we witness the appearance of the optic nerve atrophy, complications involving the pituitary gland function and exophthalmos. Furthermore, it may lead to destruction of the bulbus or to the suppression of peri-bulbar tissue, which is followed by occurrence of proptosis, diplopia or disturbance in the field of vision, or in worst case scenario could even lead to blindness. Long-persisting unilateral proptosis is a common consequence of injuries of craniofacial junction [6]. Craniofacial trauma is manifested as multisystem injuries in 20-50% of cases [7].

Risk factors that can allow the formation of posttraumatic complications largely depend on the method of treatment of these injuries in the primary act, the presence or absence of naso-frontal channel, and its level of obstruction [8,9]. Risk factors that are also mentioned as possible causes, and which have been mentioned in literature, refer to different technics in preforming plastic and reconstruction surgery of the walls of the sinuses and sinus cavity after trauma [10,11]. The importance of the presence or absence of naso-frontal channel and its condition in the trauma and after surgery is especially emphasized in prognostics. Rodriguez et al. [12] suggest a certain algorithm procedures in the treatment of the injury sinus, which is backed up by a comprehensive series of patients, and is based on the fact that the decision about treatment should derive from a variety of factors to consider: the type of fracture, the extent of damage to the back wall of sinus and naso-frontal channel, the neurological status of the patient and the presence of associated trauma and existence of nasal liquorrhea [13–16].

Case Reports

Case No.1

Patient (M.G.) is a white, 48 year-old male (Figure 1), with present proptosis, diplopia expressed in his right eye, experiencing persistent dull pain in orbit and head and the occasional outburst in the peripheral field of vision (tubular form) in the form of blurred and sometimes distorted vision of objects. The mobility of his right eye is limited and difficult, and when asked to look in all directions a distinct diplopia is noted. The anamnesis suggests that he had an injury in traffic 22 years ago as a driver (Figure 3 shows the presence of non-rehabilitated fracture in the frontal sinuses), and except for bleeding from both nostrils and bilateral infra orbital hematoma, he reports having no other problems. Throughout the past period of time he had occasional headaches that have reacted to painkillers and uncontrollable flow of tears over the edge of the lower eyelid. He stated that the symptoms intensified during last month thus he revisited his physician. After preforming diagnostics in the form of echo sonographic test of orbital cavity (cystic formation was detected in retrobulbar and posterior superior position), computerized tomography (CT) with 3D reconstruction (Figures 1, 2, 4) showed formations coming from the right frontal sinus and extending above the roof of the right orbit destroying on its way the upper bone dome, descending in retrobulbar direction, pushing out the contents of the orbit and communicating with muscular structures of the eye. Its mass suppressed the existing anatomical structures of the right orbit, leading to proptosis). On the basis of the above medical information, clinical, ultrasound and CT diagnostics, we can make a conclusion that it is a case of mucocele, which we verified later, both intraoperatively and by histopathology. The patient underwent surgery, where mucocele was identified. is the mucocele was removed in its entirety, followed by a reconstruction of the roof of orbit and the wall of the frontal sinus through designed bone graft from calvaria (skull cap). Figure 2 shows the removed mucocele, with horizontal diameter 38.4 x 32.5 mm and vertical diameter 37.5 x 46.7 mm. Postoperatively, after six months, there were no signs of proptosis and diplopia, while the patient reported absence of all symptoms.

Case No 2

Patient (R.M.) is a white 22. year-old female, with a history of a fall from a height which occurred five years previously, with reported trauma in the frontal sinus, with clinical picture of persistent headache for 6 months, stating that she had no previous surgery. Clinical examination shows mild proptosis of left upper eyelid with ocular bulbs movement slightly laterally and inferiorly. MRI showed tumor in frontal- ethmoid region that suggested the presence of mucocele (Fig. 5) in the left frontal sinus with significant penetration into orbit and much less present in the right side. Intraoperatively, we found mucocele in the left orbit along with destruction of part of a roof of orbit and compression to medial rectus muscle and ocular bulbus. Most of the surgery was done trough bicoronal approach arched section. Upon accessing and identifying changes, they were removed and sent to HP verification. Detected defects on the roof of the orbits were reconstructed with bone chip taken from external tabula, and then sealed with the tissue adhesive. Postoperative course was regular, clinical and local controls showed satisfactory finding. The aesthetic results were satisfactory and no complications related to the procedure developed, such as uncontrolled bleeding from sinus infections, damage to the posterior wall of the sinuses and brain.

Discussion

The anamnestic data gathered from the patient about gradually appearing unilateral proptosis presented us with clinical and diagnostic challenge, and lead us to doubt the persistence of mucocele [17–19], in addition to revising the possibility of the existence of diseases of organs of the eye, the retrobulbar orbital tumors, inflammatory pseudo tumor, sinus tumor and non-

metastatic lesions. Moreover, clinical reports commonly show patients presented with mucocele of frontal sinus, manifesting the eye motility disorders, lowered upper eyelid, decreased rima oculi, diplopia, unilateral proptosis, epiphora, dull periocular pain and sometimes the occasional outburst in the field of vision. In this case, pupils were equal and reactive. All patients had medical reports that confirm traumatic injuries in the frontal area with mild swelling of the area. Furthermore, if amansetic data report presence of an earlier trauma in craniofacial region we are more likely to conclude that posttraumatic mucocele is present. Progressive, unilateral painless proptosis, with gradual onset, should raise suspicion of a mucocele of sinus, mainly in frontal (Figure 1, Figure 2, Figure 4) or ethmoidal sinus, as these are the most common sites for this type of affliction [20–24]. Doubt is amplified even more if there are accompanying diplopia, present pain in periorbital area or at the forehead, and epiphora, as these are often accompanying symptoms of mucocele [5].

Size and expansion rate of mucocele are directly connected with multitude and gravity of accompanying symptoms that follow this disease.

The level of presence of the ptosis can vary in patients who experience cold or inflammation of the sinuses. The existence of mucocele at times can be associated with chronic sinus inflammation, nasal obstruction or traumatic consequences [25-27]. Patient can exhibit blurred or impaired vision or even a complete loss of vision, as a sign of evident proptosis. The reasons for the loss of vision can be diverse. It can be due to the direct compression of optical nerve in orbital area, and vascular or inflammatory process which pertains to the optical nerve, refractive error in the eye, keratopathy or secondary glaucoma. Ophthalmological manifestations which are described by patients are often evidence of persisting mucocele in craniofacial junction, deriving from frontal sinus. Usually, patients are noting the swelling formation under the eye, followed by occurrence of diplopia. Moreover, all of the above can also include mechanical pressure applied onto the optical nerve which will cause impairment of vision. Another well noted complication of mucocele is erosion of the frontal bone structure of the wall of sinus which leads to protrusion of the mass beneath the periostea of the frontal bone. Erosion of the back wall of the frontal sinus may result in epidural abscess, meningitis, subdural empyema and brain abscess. Rarely, the cranial nerve can be affected. RTG presentation of mucocele is manifested by bone dilution and expansion of the walls of sinus by the formation of mucocele, with the presence of bone erosion. RTG description of mucocele is characterized by homogenous hyperdense formation which fills up the lumen of frontal sinus. Standard RTG procedures will show the presence of the lesion, while CT will give us better insight in its size and its relation to the surrounding structures, such as bone and mucocele contact, and intracranial and orbital propagation which in final instance recommends the surgical extent of the treatment. In addition, CT scans will provide us with information about liquid presence inside mucocele and its density. It will also allow us the insight in the volume of the lesion and its propagation towards surrounding structures. MRI on the other hand can detect mucocele but may lead us to wrong conclusion by presenting condensed mucus in sinus as air filled cavity [16,22].

In patient No.1, computed tomography (CT) showed a picture of mucocele with its soft content, where its largest part occupies the front part of the zone of sphenoidal bone and frontal sinus on the right, with osteolytic process in the roof of orbital cavity and pushed eyeball and peri-bulbar tissues downwards and outwards, with all the signs of remodeled anatomy. We notice the old untreated fracture of frontal-nasal-ethmoidal complex.

In patient No. 2, the diagnosis was made by magnetic resonance imaging (MRI) with the idea to clarify that this is not a malignant process. The disadvantage of MRI is primarily in the inability of these procedures to assess the bone anatomy and relationship of the discovered entity with this structure. In the second case, we have discovered a benign process in the right and left frontal-ethmoidal aspect.

Due to the inability of MRI to differentiate bone walls separating the orbital cavity from adjacent sinuses, we were not able to assess whether mucocele propagate into orbit or not. However, with suggestive clinical data, which indicate that mucocele originates from the sinus cavities of the frontal sinuses and causes proptosis, CT of the orbit should be the first choice of diagnostic tool to use. MRI plays a role in diagnosis because it can provide information in the study of the orbital cavity and serve as a diagnostic procedure of choice in suspicion of other soft tissue tumors that affect the appearance of proptosis and are not caused by mucocele.

Orbit ultrasonography is a noninvasive procedure that can be a very informative technique in the display of tissue as it helps to determine whether the lesion is cystic or solid.

Differential diagnosis of mucocele includes encephalocele, a cholesterol granuloma, epidermoid cyst, meningioma, chordoma, neurofibroma, para-ganglioma, nasal-angio-fibroma and malignant neoplasms. The diagnosis of mucocele is based on history, clinical examination and radiological findings.

The definitive treatment of mucocele is primarily surgical. The aim of the implementation of surgical treatment is largely dependent on the size, location and extent of mucocele. The main task is to empty the mucocele and allow ventilation of the sinuses and re-establish its adequate drainage, taking into account that there are no functional and cosmetic deformities, and respecting the principles of reconstruction of damaged bone structures that play a role in supporting vital structures. The lining of the cyst has to be removed completely and inspection should establish that the bone is left without impurities from mucosal cysts. We

approached these mucoceles by external open obliterative procedure, because it seemed to be more cosmetically acceptable and attractive procedure in order to lift the lid of frontal bone by osteoplastic technique [28−30], which is valid for adequate and comprehensive approach. The purpose of the surgery itself should enable us to achieve good surgical outcome.

Conclusion

The presence of frontal mucocele can occasionally be accompanied by ophthalmologic manifestations such as proptosis and diplopia. Since this is a benign, long developing disease, early recognition of symptoms of its presence, and it timely surgical removal is very important for the patients' swift recovery. Adequate evaluation of clinical symptoms and radiological evaluation of the appropriate method of examination are very important for timely establishment of the correct diagnosis, which will, then, result in a high percentage of therapeutic success.

It is up to the surgeon to decide on the choice of optimal treatment in a particular case, and depending on the size and volume of the mucocele, along with factors related to the patient (age, anatomy, spatial propagation, the size changes, etc.), the decision of best surgical technique in order to solve the existing problem will be made.

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Appendix

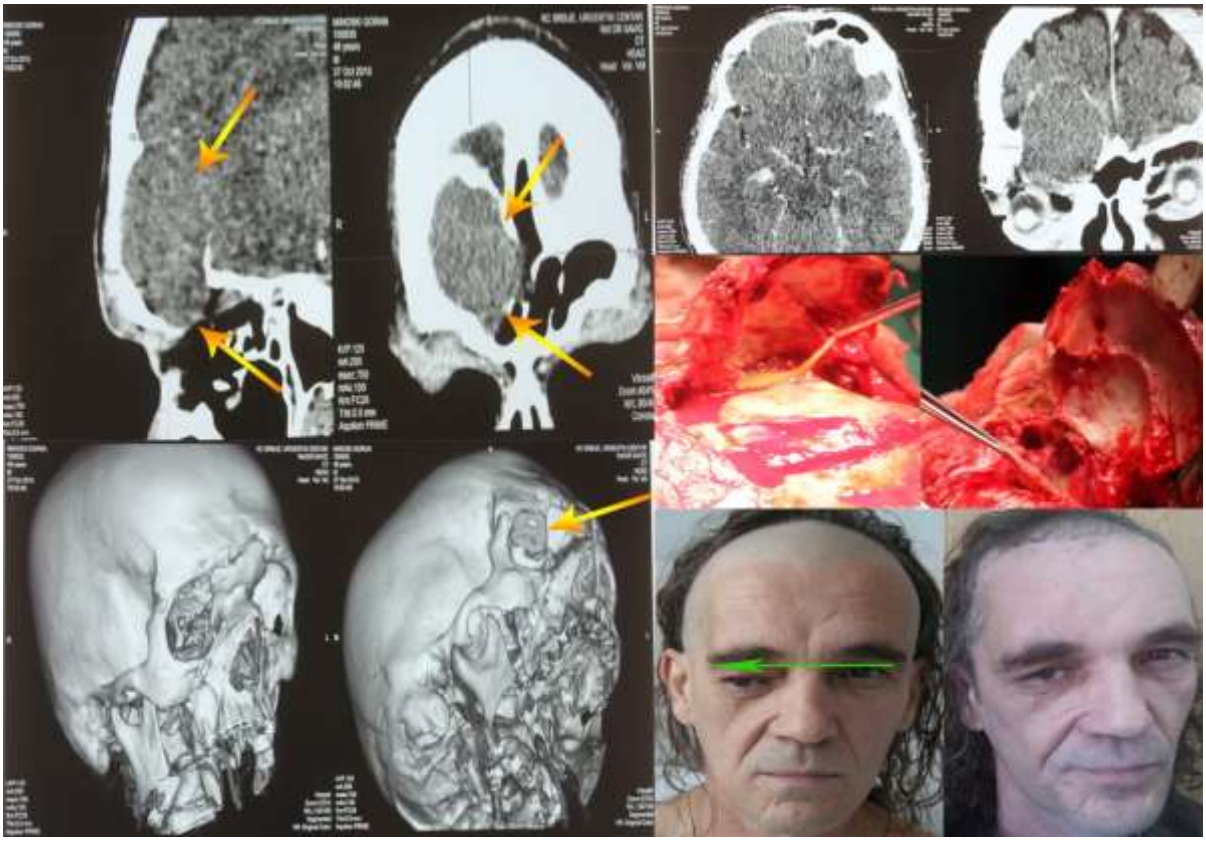
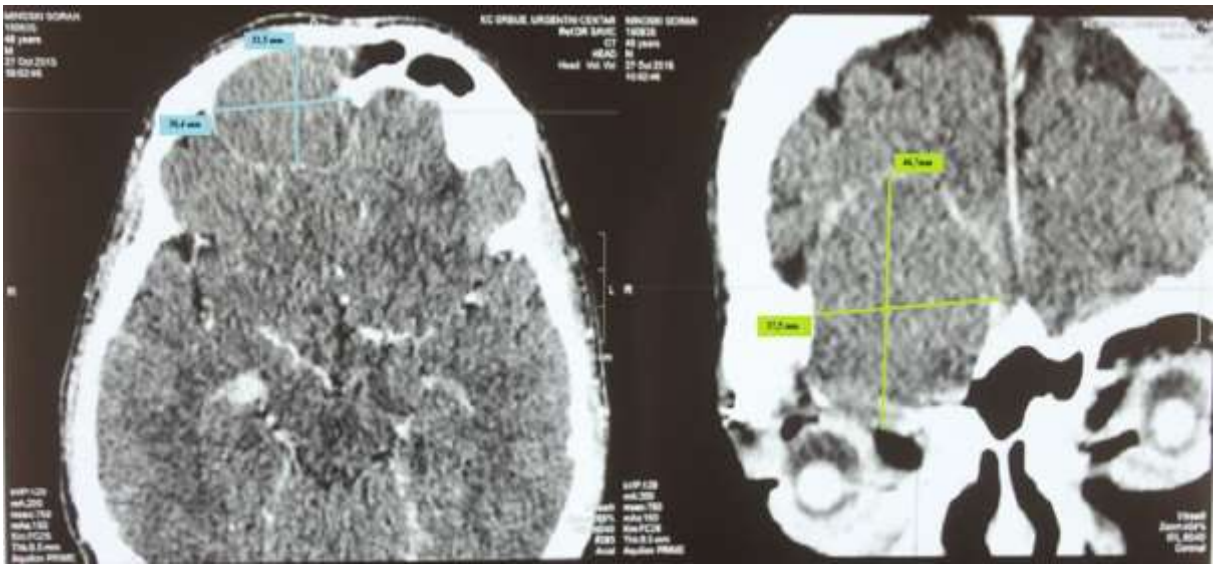


Fig.1 Preoperative evaluation MDCT with 3D reconstruction and operative findings
* Visible proptosis of the right eyelid, eye level below pupillary lines



**

Fig. 2 Diameter of persistent mucocele
Affected right frontal sinus mucocele (HP finding br.1248 / 15: Mucoccela [Prof. Dr. Boričić].
*** diplopia of the right eye. Horizontal diameter changes 38,4 x 32,5mm and vertical diameter of 37,5 x 46,7mm.
Mucocele with its propagation in orbit fits and suppresses its contents, manifesting proptosis and

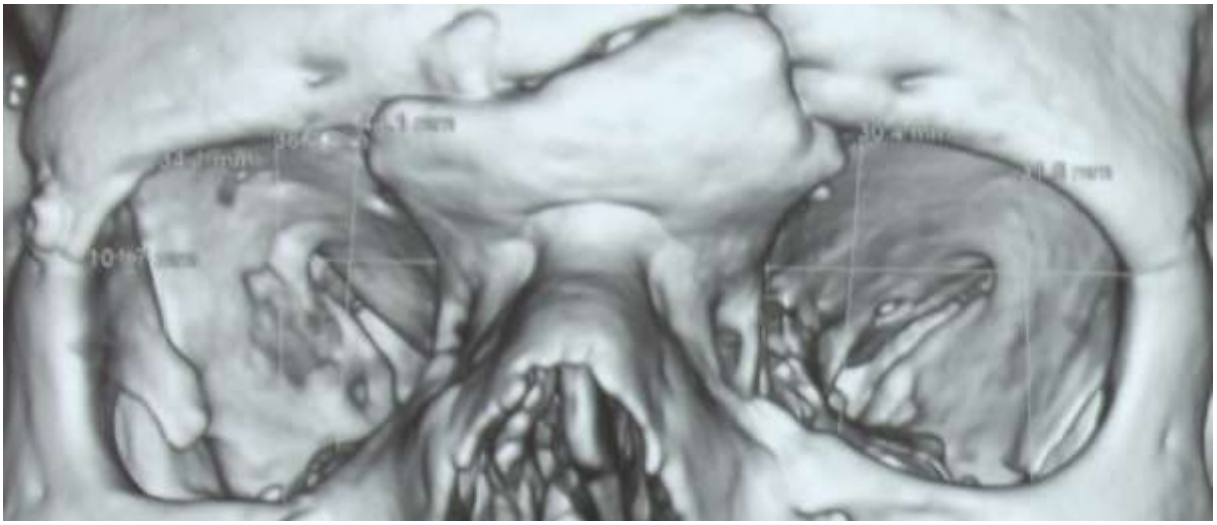


Fig. 3 An impaired diametrical relationship of orbit trauma
**** Unrepaired break in the area of the frontal sinus after traffic accidents

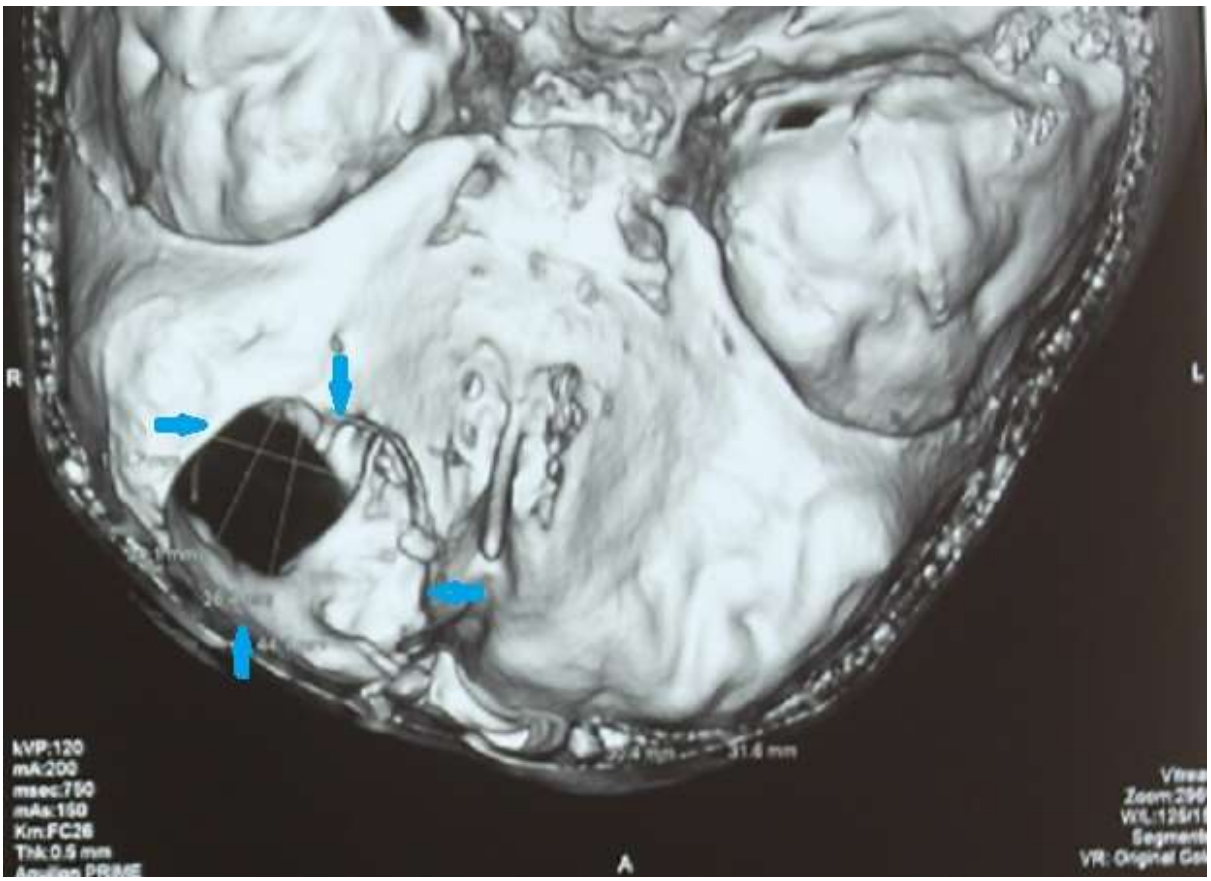


Fig. 4 View of the destruction and its scope on the roof of the right orbit
***** Consideration from the perspective 3D MDCT reconstruction

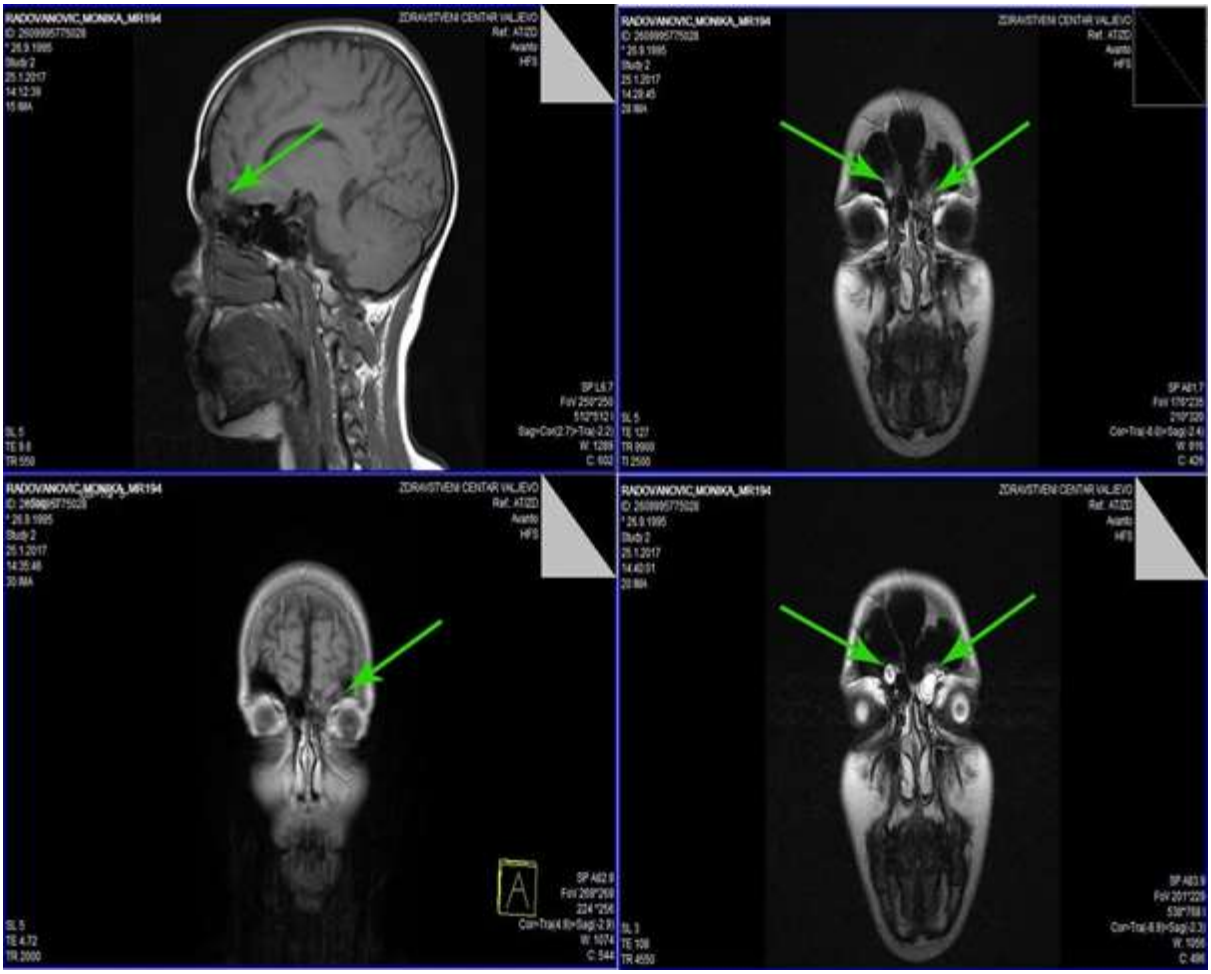


Fig. 5 MR diagnostic display of the patient with two entities of mucocele of the frontal sinuses, with its expansive growth penetrating into the left orbit

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Highlights

HIGHLIGHTS FROM THE 19th EUROPEAN CONGRESS OF ENDOCRINOLOGY (ECE) 2017, Lisbon, 20th-23rd May

The 19th ECE Congress has recently been held in Lisbon, the magnificent capital of Portugal. Endocrinologists could not imagine better place for congress venue. According to myth, the Greek hero Odysseus founded Lisbon on his journey home from Troy. The city is most famous for its history of maritime successes in particular for the voyages of Vasco da Gama who first navigated a sea route to India.

ECE in Lisbon hosted more than 3, 600 endocrinologists from all over the world. An interesting fact is that Serbian ESE members are very numerous and take a second place after Portugal members adjusted for the number of country population. Responsible for this popularity of ESE in Serbia is certainly our great Endocrinologist Professor Vera Popovic-Brkic, a former President of Executive ESE Committee, and its actual Vice President.

European Journal of Endocrinology Prize Lecture was awarded to Miguel Lopez (Spain) for amazing investigation presented in an extraordinary way about "Hypothalamic AMPK: a golden target against obesity?" John Wass (UK) presented "The fantastical world of hormones".

„The secret life of FGF21" , plenary lecture by David Mangelsdorf (USA) represents a paradigm of successful presentation where the scientific research meets the practice. Recently discovered FGF21 secreted by the liver is presented from physiologically relevant properties to practical directions for potential clinical use. Established as sensor for starvation, FGF21 also protects pancreas from protein overload and prevents pancreatitis by stimulating pancreatic secretion. Given intramuscularly in diabetics FGF21 decreases glycaemia and promotes insulin sensitivity, thus leading to weight loss. Among other numerous metabolic activities, it influences blood pressure rise and stimulates sympathetic system. Caloric restriction in mice leads to increased expression of FGF21 both in the liver and in the growth plate. It would be of practical importance to determine and explore the role of FGF21 in growth retardation of SGA neonates and in children with ISS (Idiopathic Short Stature).

The rising star of Erasmus University Tim Korevaar presented his scientific work of great practical impact: "Effects of maternal thyroid function on infant neurodevelopment". Neurogenesis period is of full capacity from the 5th week of embryonic life to 18-20th week of fetal development, when the full function of fetal thyroid function is achieved. He demonstrated that the low level of maternal fT4 leads inevitably to suboptimal IQ of the offspring, and it affects brain morphology. Low maternal fT4 is accused also of childhood autism, ADHD and schizophrenia in later life. Nevertheless, the attempt to improve IQ in offspring by increasing the substitution dose to 150 μg/daily in mild maternal hypothyroidism, failed.

Symposium on clinical updates in hypoparathyroidism was devoted to congenital and acquired forms of hypoparathyroidism, and offered new therapeutic approaches. Recombinant Parathyroid Hormone (rDNA), produced in E.coli using recombinant DNA technology is identical to the 84 amino acid sequence of endogenous human parathyroid hormone. It is indicated as adjunctive treatment of adult patients with chronic hypoparathyroidism who cannot be adequately controlled with standard therapy alone. It is a self-administered, once-daily subcutaneous injection using a pen device.

Besides prestigious prize and plenary lectures an exciting range of "Meet the Expert" sessions covered state of the art in endocrine practice. Participants enjoyed a highly interactive program providing the very best of endocrinology. We must be prepared for the next ECE in Barcelona, expecting at least the similar experience.

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