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Genotoxicity test methods – a tool for DNA and chromosome damage biomonitoring

Тестови генотоксичности – алатке за биомониторинг оштећења днк и хромозоме

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SUMMARY
Nowadays is highly influenced by intense growth of various industries, high level of pollution, and other environmental factors with harmful effects on human health. Therefore, cytogenetic monitoring is essential for detection of changes in the structure of chromosomes, which occur because of the effects of various genotoxic agents. In this review we apprize the theoretical and experimental aspects of several tests for assessment of genotoxicity in humans such as Micronucleus assay (MA), Comet assay (CA), Chromosomal aberrations (CAs) assessment and Sister chromatid exchanges (SCEs) analysis. These methods are accepted by WHO (World Health Organization) as standard tests for genotoxicological screening in humans. The methods are sensitive and confirm the cellular genotoxic effects of various genotoxicants.
Keywords: genotoxicology, cytogenetic screening, biomonitoring, Micronucleus assay, Comet assay

САЖЕТАК
Данашњи живот је под великим утицајем интензивног ефекта различитих индустрија, високог нивоа загађења и других фактора животне средине са штетним утицајем на здравје људи. Због тих разлога, цитогенетски надзор је од суштинског значаја за детекцију или потврду различитих промена структуре хромозома, који настају због дејства различитих генотоксичних агенаса. У овом прегледу анализирамо теоретске и практичне или експерименталне аспекте, како и позитивни и негативне стране неколико тестова за процјену генотоксичности код људе, као што је микронуклеусни тест, комет тест, процена хромозомских аберација како и анализа размена сестринских хроматида. Нотиране генотоксичне тестове су прихваћене и одобрене са стране Светеске здравствене организације као стандардне тестове у процјени или скрининг генотоксичности на хуману популацију. Све те методе или тестове су осетљиве и потврђују челијске генотоксичне ефекте настале под утицајем различитих гентоксичних средстава.
Кључне речи: гентоксичност, цитогенетски скрининг, биомониторинг, микронуклеус тест, комет тест

INTRODUCTION

On a daily basis, humans are directly exposed to various genotoxic agents of physical or chemical nature, both professionally and incidentally, or they occur due to their different lifestyle, habits, or addictions. Genotoxic agents, which are present in our immediate surroundings, represent a potential health risk for each exposed individual, and increase the risk of various non-communicable diseases, especially risk for cancer diseases. The effect on each exposed individual depends on the degree of exposure to a given factor, the way of the genotoxicant is eliminated and the genetically determined differences among individuals [1-3]. Some of the human genotoxicants come as pollutants from technological processes or from uncontrolled manufacturing of certain chemical substances and their products [4-8]. Genotoxic substances that are present in various manufacturing processes mainly represent a direct hazard to the workers involved in the process, as well as to local population in these

industrial areas. Agents that are produced during manufacturing processes and that eventually enter the composition of those products, represent a potential health risk for a larger population, which include the consumers of these products. This group of potentially genotoxic agents includes acrylamide, organic solvents, organic compounds of metals, and heavy metals. The main features of genotoxic agents are essential for understanding their toxic effects, as well as the comprehension of the transport dynamics, distribution and excretion from the body [2,8]. Genotoxic agents may be broadly classified into factors of chemical nature (acrylamide, polychlorinated organic compounds, pesticides, organic solvents, and heavy metals) or factors of physical nature (short-wavelength electromagnetic energy such as ultraviolet (UV) radiation and ionizing radiation (IR)). IR is one of the main sources of genotoxicity [2, 6]. Some genotoxic agents are capable of damaging DNA due to their chemical and physical features. The harmful effects of genotoxic agents for mutations induction do not necessarily manifest in the organism that is exposed to them, sometimes not even in the first generation. Instead, these harmful effects can be manifested in the next generations. There is an evidence that long-term exposure to low doses of certain genotoxicants induces changes in the structure of chromosomes that would not be phenotypically visible, but can be passed on to the future generations. However, while high doses of these agents are lethal or toxic, small doses are cumulative, and mutagenic effects are activated or manifested in the next generation (s) [7, 8].

Genetic toxicology (or genotoxicology) studies the impact of genotoxic agents on the process of transmission of inherited traits, with particular emphasis on the possible health effects. It also studies the mechanisms of genetic damage, the substances that cause it, and improves the methods and experimental models that can determine such changes [7, 8, 9]. Potential changes in the chromosomes include: a) chromosome breakage and b) rearrangement of the fragments as a result of destruction of chromosomal structure [9. 10,

11]. Genotoxic effects of various agents can be detected with several tests for genotoxicity assessment. These tests have ability to reveal damage in the DNA molecule, as well as the changes in the structure and the number of chromosomes, which are extremely important for the transmission of hereditary traits and are involved in induction of carcinogenesis [9–17]. In genotoxicology there are several available tests applicable on human cells. In this paper we apprize the theoretical and experimental aspects of commonly used tests for assessment of genotoxicity in humans such as Micronucleus assay (MA), Comet assay (CA), Chromosomal aberrations (CAs) assessment and Sister chromatid exchanges (SCEs) analysis. Our aim is to give a brief review of these genotoxicity testing methods, as a tool for DNA and chromosome damage biomonitoring.

Micronucleus assay (Procedure and Principles)

Micronucleus assay (MA) is widely used assay for measurement of DNA damage and genotoxicity in the cells. Upon exposure to genotoxic agent/s, the cell may be damaged and divided, and after that it will form small micronucleus in addition to the main nucleus. To perform Micronucleus assay (MA), venous blood sample is being collected in heparinized blood collection tubes. In our laboratory, we follow the blood culture protocol according to Fenech [13]. After 44 h of incubation of the cells, Cytochalasin B (CytoB) is added to each culture to block cell cytokinesis, and cultures are re-incubated at 37 °C for further 28 h. CytoB blocks the cytokinesis because it inhibits actin assembly and thus prevents separation of daughter cells after mitosis, leading to formation of binucleated cells. Treatment with CytoB ensures that only the cells, which were divided, are scored. After 72 h of cultivation cells are harvested, fixated and fixed lymphocytes are stained by Giemsa and at last examined with light microscopy (×40 and ×100). Micronuclei are independent chromatin structures,

completely separated from the core. They are created as a result of condensation of acentric chromosome fragments or whole chromosomes that failed to incorporate into the core of the newly formed nuclei. Average size of micronuclei may vary from 1/3 to 1/16 of the nucleus size. The appearance, as well as the number of micronuclei is an important quantitative biomarker for DNA damage, resulting from various genotoxic agents *in vitro* or *in vivo* [13, 16-21]. During the examination of the microscopic slides must take into account, not to score binucleated cells with irregular shapes or with two nuclei different greatly in size, neither should binucleated cells be confused with poorly spread multi-nucleated cells [22].
The micronuclei are scored as positive if they are distinguishable from the two main nuclei, in case if they are less than one-third the size of the main nuclei, and if they have similar staining intensities to the main nuclei. Cells with irregularly shaped nuclei, more than two nuclei, and those with nuclei of different sizes in a single cell, should not be scored [13, 18].

The number of micronuclei per binucleated cells provides a measure of chromosome breakage. Micronuclei are generated due to exposure to genotoxic agents, especially IR [6,23]. Increased formation of small and large micronuclei, as an indicator of chromosomal instability, has been found in medical workers who are professionally exposed to IR [24].

Comet assay (Procedure and Principles)

The Comet assay (CA) is rapid and sensitive procedure for quantification of damage and repair of DNA molecules at the level of individual cells [12, 25, 26, 27]. This method is sensitive in detecting low levels of DNA damage (measuring DNA strand breaks) in the cells with absence of mitotic activity. The method does not require a large number of cells per sample, it is inexpensive, relatively easy to apply, and is widely used as a test for genotoxicity. However, it is considered optimal for of genotoxic effects detection on various

agents. Cells embedded in agarose gel [5, 12] on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Nucleoids are subjected to electrophoresis, resulting in formation of structures, which resemble comets. The former cell nucleus, which does not migrate, and the broken fragments stretched in the electric field, forms the "head" and the "tail" of the comet, respectively. The method itself was named after the characteristic appearance of the nucleoids. The intensity of the comet tail relative to the head reflects the number of DNA breaks. This is due to loops which contain a break and become free to extend towards the positive charged electrode (anode). Shorter DNA fragments travel faster through the gel, because of the difference in molecular weight. For better understanding of the results from the CA it should be taken into consideration: the tail of the comet is formed by DNA loops attached to the nuclear matrix while cut-off fragments leave the nucleus and could not be observed [12, 25, 26]. Also, Georgieva's results et al. [28] are proof of the principle that Comet assay may be used for studying the higher-order chromatin structures at a single-cell level. Most oftenly, the gel electrophoresis is followed by fluorescence microscopy in order to visualize the migration of damaged DNA. Fluorescent microscope connected to a computer, and a special computer software, are used to measure several important parameters of the comets, including their tail length and the percentage of DNA in the tail (tail intensity). The comet tail length reflects the size of the DNA fragments and the level of damage. The tail intensity indicates the portion of the genome that is affected by the damage [25, 26]. In the literature, the most commonly mentioned embodiment of the CA in alkaline conditions is the method of Singh et al. [29].

In the genotoxicological research, two versions of the CA are widely used: the neutral method for detection of DNA double-strand breaks, and the alkaline method, which detects DNA single-strand breaks and alkali-labile lesions [25]. In addition to the staining with fluorescent dyes (propidium iodide, ethidium bromide, SYBR Green and others), the comets

can be also stained with the silver staining method [30-32]. Therefore, the comets can be visible on conventional microscope, which is an important advantage for some laboratories. However, after the fluorescence staining, the agarose gels could be dried and re-stained with silver, for their documentation and future analyses. Nadin et al. [32] also described modifications of the silver staining method, which significantly increases the sensitivity/reproducibility of the CA and preserves the comets for long periods. In 2014, Osipov et al. [33] reported that DNA comets can also be visualized and analyzed using Giemsa staining. They explain the high sensitivity of Giemsa staining with the Romanowsky-Giemsa effect. They propose this staining as a result of the stain photo-stability and the higher resolution of bright-field imaging compared to fluorescence imaging.

Chromosomal aberrations

Human peripheral lymphocytes, particularly T-lymphocytes are commonly used cells in the studies of genotoxicity [34-36], since approximately 80% of lymphocytes recirculate throughout the body, which means that lymphocytes are able to leave the peripheral blood, pass through the spleen, lymph nodes and other tissues and re-enter the circulation. Therefore, lymphocytes with damaged DNA arising in any part of the body shall appear in the peripheral blood. Chromosomal aberrations (CAs) are considered biomarkers for assessment of exposure to occupational genotoxicants in human biomonitoring studies.

Scoring the specific unstable CAs, such as acentric fragments, dicentric chromosomes and ring chromosomes in peripheral blood lymphocytes in persons exposed to various genotoxic agents, is reliable method to detect and possibly measure the exposure [37-39]. High rates of CAs are observed in subjects occupationally exposed to low levels of IR [37]. In meta-analysis of cytogenetic studies performed in four Italian laboratories in the period

1965-1993, Bonassi et al. [38] reported significantly higher frequencies of CAs in medical workers exposed to low doses of IR. Garaj-Vrhovac et al. [37] also reported higher rates of CAs in workers exposed to IR, but the differences among those were not significant. Multiple studies have shown a significant correlation between induction of CAs and the risk of cancer [14, 15].

Sister chromatid exchanges

Sister chromatid exchanges (SCEs) are currently considered to be a biomarker of exposure on genotoxic agents such as carcinogens. Monitoring the exposure to them, as a DNA damaging agents, their frequency increases substantially so they have been commonly used as an indicator of genotoxic effects in cells. As an excellent tool for the quantitative and qualitative evaluation of DNA damage, detects the ability of genotoxic agents to enhance the exchange of DNA between two sister chromatids. SCEs are defined as a symmetrical exchange at one locus between sister chromatids, and appears to involve DNA breakage and repair mechanism during the S phase of the cell cycle, which does not alter the overall chromosomal morphology. Significant increases of SCEs frequencies have been reported in studies of the human population occupationally exposed to various biohazardous agents, including radiation, dust, fibers, fumes and organic and inorganic chemicals [39-42].

DISCUSSION

Advantages and disadvantages of genotoxicity test methods

Due to their numerous advantages, the genotoxicity test methods as tools are widely used for DNA and chromosome damage biomonitoring especially in human biomonitoring studies. However, they also have some limitations. MA is relatively inexpensive methods

which scoring of micronuclei can be performed on various cell types relevant for human biomonitoring such as lymphocytes, fibroblasts, exfoliated epithelial cells, and other cell types with mitotic activity. However, the main disadvantage of this assay is that it does not detect all structural CAs, but only the acentric fragments. Another disadvantage is the cytotoxicity of Cytochalasin B, which varies among cell types and sometimes even among the subtypes of the same cell type. Next, in order to perform the MA, a high number of cells should be taken (approximately 1000 binucleated lymphocytes per sample). With these improvements the MA will become more sensitive and specific, which will increase its applicability in large-scale screening studies.

One of the most important advantages of the CAs is that the DNA damage can be measured in any (nucleated) cell type [14], whereas the MA is limited to the cells having mitotic activity. The CA is rapid, inexpensive, relatively easy to perform and detects a broad variety of primary DNA lesions which cannot be identified by other tests. It is sensitive to very low levels of DNA damage, and requires a small amount of cells per sample. Common feature of MA and CA is that both micronuclei and comets appear because of the damage of nuclear DNA. However, FISH (*Fluorescent in situ hybridization*) analysis promotes better understanding of the mechanisms of their formation, and often complements the MA and CA [7].

Various cytogenetic end-points (including CAs, SCEs, and MN), have already been utilized as biomarkers of cancer susceptibility in non-carriers [43]. Epidemiological evidence supports the predictive value of elevated frequency of CAs in peripheral blood lymphocytes [44]. Indeed, in Nordic [44], Italian [45] and Czech cohort studies [46], the authors evaluated the association between the frequency of CAs, SCEs or MN in peripheral blood lymphocytes and the subsequent risk of cancer.

Luzhna et al. [47] confirm that hypomethylation of heterochromatin in the pericentromeric regions is related to chromatin decompensation, which leads to improper chromosome segregation and exclusion into MN. Global methylation has been found to be related to more relaxed chromatin, increased gene expression, elevated DNA damage, and chromosomal breaks, which form MN with acentric chromosome fragments. According to this, overall loss of DNA methylation has been proposed as a valid biomarker for cancer. Due to the alteration of DNA methylation patterns has been found to be related to many diseases, including cancer, this alteration has potential for clinical application as a prognostic biomarker. Recently, van Leeuwen et al. [48] developed a transcriptomic network analysis of MN-related genes based on the knowledge from literature and a case study on children and adults who were differentially exposed to air pollution. Using a pathway tool MetaCore, the authors retrieved 27 genes and gene complexes involved in MN formation. Such genes were mainly associated with cell cycle checkpoints, spindle assembly, and aneuploidy. In a biochemical approach, repair enzymes in the extract induce breaks at damage sites; and the breaks are measured with the comet assay. The nature of the substrate lesions defines the repair pathway to be studied [49].

The extent of DNA migration depends directly on the DNA damage present in the cells. It should be noted that DNA lesions consisting of strand breaks after treatment with alkali either alone or in combination with certain enzymes (e.g. endonucleases) increase DNA migration [14]. Other authors [50] suggest comet-FISH assay to be used for examination of the initial DNA damage and subsequent repair in some gene region. Horvathova's findings et al., [51] suggest that the patterns of migration of domain-specific signals may depend on the localisation of breaks within or around the probed region. Also, Zeller et al. [52] in their study of human exposure to formaldehyde, comparatively

investigated it in order to be able to identify a possible effect of the exposure schedule on changes in gene expression.

CONCLUSION

Considering the fact that today's living is highly influenced by intense growth of many industries and environmental pollution, genotoxicity test methods are extremely important for monitoring of the changes in the structure of chromosomes and DNA damage, which occur as a result of the influence of various genotoxic compounds. In this paper we focus on the effects of genotoxic agents on human cells, which can be analyzed with previously noted tests for assessment of genotoxicity. We define several basic genotoxicity test methods which can be applied as tools for DNA and chromosome damage biomonitoring in human population.

Conflict of interest: None declared.

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