ENVIRONMENTAL SECURITY ASSESSMENT BASED ON THE CYTOGENETIC ESTIMATION OF MUTAGENICITY AND HUMAN HEALTH IN UKRAINE

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Abstract. The accumulation in different environmental objects of many toxicants defines the real risks for biota and human health. An especially serious risk for all living organisms is presented by environmental mutagenes that can affect the hereditary apparatus of somatic and sex cells, resulting in cancer increases and other ecologically dependent pathologies. Therefore, an investigation of mutagenesis in cells at genetic level is very urgent and real. Biological monitoring, which includes genetic and cytogenetic monitoring, provides a useful tool for estimating the genetic risks deriving from integrated exposure to a complex mixture of chemical, physical and biological environmental agents. A positive association between occupational exposure to complex pesticide mixtures, and the presence of different cytogenetic pathologies including micronuclei (MN), has been detected in the majority of studies. The application of cytogenetic testing, using the micronucleus assay (MN-test) on exfoliated buccal cells of children, substantiates the estimation of total mutagenicity in the studied territory in which they live. A positive modifying cytogenetic influence of natural adaptogens was established.

Keywords: Genotoxicity, cytogenetic monitoring, pesticides, exfoliated buccal cells, micronucleus, natural adaptogenes.

1. Introduction

One of the most significant global current problems is unquestionably the issue of ecology. An accumulation in the biosphere of harmful substances, together with an increase in radio-activity in the environment, threatens the state of

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ecosystems, genofonds and the population's health, and limits for the potential further development of civilization (WHO, 2011).

As a rule, the quality of different environmental objects is controlled in accordance with the system of, for example, maximum permissible concentrations of harmful substances, and the toxicological, organoleptic, sanitary and biological indices, through the use of numeral methods. However, mutagenic properties of the environment as the main risk from pollution, have remained undefined by such research. Therefore, a significant value is acquired by taking into consideration such problems as the control in the above mentioned processes of environmental contamination by mutagenes, as well as preventing the growth of mutagen contamination. It is also necessary to understand the nature of mutagenic actions and to search for methods of protecting living organisms from their negative influence. This can be achieved by the use of indicator biotests among which the cytogenetic tests are the most informative, as they are highly sensitive and sufficient for suitable estimations (Pilinska and Dybsky, 2004; Gorova and Klimkina, 2007; Fedak and Kim, 2008).

The advantages of biotesting at the cellular level are predefined by the theory about cellular structure and its similarity for all live organisms, as well as the universality of cell's chemical composition and its metabolism. It is considered that 60-70% of substances with an identified genetic activity have similar mutagenic and carcinogenic influences on plants, animals and human health (Dubinin, 1978). There are many scientific results confirming the mutagenic potential of pesticides (Bolognesi and al., 2010), heavy metals (Asakura and al., 2009; Kumar and al., 2010; Ivanova and al., 2008), radioactive nuclides (Mothersill and al., 2009; Dubrova and al., 2009), or the general influence of toxicants on plant and animal organisms (Selvaraju and al., 2011; Gil and Pla, 2001).

One of the specific features of mutagenic and carcinogenic substances is an ability to display biological activity even at weak concentrations. It complicates their analytical determination in biological tissues. Therefore, for the analysis of genotoxicity of environmental agents the different test-systems are worked out on the basis of use in the cells of bacteria and other microorganisms (Saratovskikh and al., 2007; Reifferscheid and Buchinger, 2010; Stepchenkova and al., 2006), plants (Christoffers and al., 2002), animals (Anitha and al., 2008; Zowail and al., 2010) including cells of human (Timoshevsky and Nazarenko, 2006).

Accordingly, it is necessary to implement a well-developed system of ecological and genetic monitoring that will allow adequate estimation of genetic risks to human health. Such a system must combine investigation results from the bioindication of mutagenes presented in the different environmental items,

the cytogenetic survey at the population level and direct genetic monitoring among the human population. Only on the basis of applying biological and medico-genetic research it is possible to estimate the real level of mutagenic load and the genetic risks for humans from the influence of harmful ecological factors (Gorova and Klimkina, 2007). It is necessary for substantiation of priority-oriented managerial decisions directed at a decrease in the technogenic loading, as well as improving of environmental conditions, biota and the nation's genofond (Gorova and Klimkina, 2002)

2. Genetic Monitoring

For estimating the possible influence of mutagenic environmental factors on human health it is necessary to have a system of genetic monitoring among the population living in regions with different ecological conditions. Implementation of the genetic monitoring should be based on the complex study of potential mutagenicity of total environmental contamination *in vivo* and *in vitro*, as well as epidemiological and cytogenetic changes in the human population.

Frequency in occurrences of spontaneous changeability of chromosomes in the somatic human cells can be used as a biological characteristic at the population level and at the same time can reflect the mutagenic environmental load on genetic human health. In addition, it is expedient to conduct a retrospective analysis of morbidity and death rates among the human population according to the following indices: frequency of spontaneous abortions and malformations; infantile death rate; level of cancer diseases; frequency of the inherited illnesses; and multifactorial pathology. Such no experiment-based prognosis of genetic risks from environmental contamination allows a plan to be defined for the realization of the next stages of the genetic monitoring.

Comparative analysis the frequency of chromosomal aberrations and level of sister chromatid exchanges in the cultivated leucocytes of peripheral blood from people living on the conditionally clean territories (control groups) and persons professionally contacting with some potentially dangerous mutagenic agents, and also comparing the control groups to the residents being under influence of factors of radiation or chemical nature, is presently adequate, sensitive and widespread method recognized for estimation of mutagenic effects both occupational and environmental factors. Moreover, existing methodology for effect analysis by comparing with the spontaneous level of mutations provides with sufficient certainty to the answer to the question about radiation (on frequency of two-hit types of chromosomal aberrations; Knudson, 2001) or chemicals (on frequency of one-hit types of chromosomal damages and level of

sister chromatid exchanges; Sarasin, 2003; Gadhia and al., 2005; Mrđanović and al., 2007) origin of mutagenic effect.

3. Pesticide Influence on Human Health

Acute poisoning caused by large doses of pesticides appears usually after accidents or fires on chemical enterprises. Chronic influence by measurable doses is connected, as a rule, with professional activity of workers involved in chemical production, or personnel directly engaged in the process of pesticide application.

Moreover, it is established that women living on land contaminated by pesticides suffer changes in their reproductive health (Aguilar-Garduño and al., 2012) including spontaneous abortions (Toichuev and al., 2012), a high frequency of obstetrical pathologies (Toichuev and al., 2012), and abnormalities in the rates and terms of physical and sexual development of girls (Lacasaña and al., 2012). A number of pesticides, including dibromochlorophane and 2,4-D, have also been associated with impaired fertility in males (Sheiner and al., 2003).

It is also shown that these are significant risk factors for mothers having professional contact with pesticides, using pesticides in housekeeping, and living within 0.4km of agricultural activities (Kumar and al., 2008).

Many studies have examined the effects of pesticide exposure on the risks of cancer (Ward and al., 2012). For example, associations have been found with leukemia and lymphoma, and cancers of the brain, kidneys, breasts, prostate, pancreas, liver, lungs and skin (Gilden and al., 2010). These increased risks occur with both residential and occupational exposures (Gilden and al., 2010), for example, increased rates of cancer have been found among farm workers who apply these chemicals (McCauley, 2006). Mothers' occupational exposure to pesticides during pregnancy is associated with an increase in their children's risk of leukemia, Wilms' tumor, and brain cancer (Gilden and al., 2010; Van Maele-Fabry and al., 2010).

Strong evidence links pesticide exposure to worsened endocrinic (Yang Jin-Hoon and al., 2012) and neuro-physiological outcomes (Toichuev, 2012; Finkelstein and al., 2012). Thus, the risk of developing Parkinson's disease is 70% greater in those exposed to even low levels of pesticides. Indeed, people with Parkinson's were 61% more likely to report having had direct pesticide application than were their healthy relatives. Both insecticides and herbicides significantly increased the risk of Parkinson's disease, and there are also concerns that long term exposure may increase the risk of dementia (Ascherio and al., 2006).

The World Health Organization (2011) and the UN Environment Programme estimate that each year, 3 million workers in agriculture in the developing world experience severe poisoning from pesticides, about 18,000 of whom die. According to biomonitoring results of the Centers for Disease Control and Prevention's (CDC) National Health and Nutrition Examination Survey (2005-2006), as many as 25 million workers in developing countries may suffer mild pesticide poisoning yearly (Gilden and al., 2010). This report stated that detectable body levels of about 50 pesticides have been found in a representative blood sample of the U.S. population.

Thus, against the background of deterioration in basic medical and demographic indices that characterize the population's health (such as death rate, birth rate, an increase in allergic, infectious, reproductive, oncologic and other morbidity), pesticides promote a substantial risk factor for both the population's state of health and environmental quality.

The significance of the pointed factor is aggravated by the application of pesticides on land polluted by radionuclides and/or heavy metals, and also on land with deficit/excess of different microelements (such as iodine and fluorine). This is due to the combined influences which can cause an intensification of harmful action of the indicated factors on both human organisms and biota.

4. Genotoxicity of Pesticides on Biota

Mutagenic activity of pesticides is one of the most dangerous displays of negative influence on all living organisms including human health and its posterities. However, because of the enormous variety of pesticides used in agriculture, according to all available data the information about pesticide genotoxicity is not sufficient.

At the UN Conference on Environment and Development, 1992, pesticides (along with heavy metals) were attributed to the prevailing contaminants in the biosphere. Therefore, research of their toxicity and long-term effects is extremely important today. Some research show the genotoxicity of pesticides; for instance, in plants treated by Basagran much of the chromosomal damage at the different stages of merosis is detected (Reddy and Rao, 1982). Also, Herbicide Round Up induces the formation of anaphase bridges and other mitotical defects in the cells of root meristem of *Vicia faba* (El-Tabyev and Zaki, 2009) as well as reversion genes in prototrophic bacteria *Salmonella typhimurium* detected in the Ames-test (Saratovskikh and al., 2007). Senkor in concentrations of 0.01 and 0.05 % causes chromosomal aberrations in cells of *Crepis capillaries L*. The spectrum of chromosomal aberrations is presented by

chromatid and isochromatid deletions and micro-fragments (Voskanian and al., 1987).

Using the Ames-test on the strains of *Salmonella typhimurium* TA98 and TA100 a mutagenic activity based on a mixture of seven pesticides was studied: six herbicides (Round Up, Senkor, Basagran, Kusagard, Lontrel, and Setoxidim) and the fungicide Tachigaren. Also tested were complexes of herbicide of Lontrel (ML_2) with eight metals (Cu, Co, Zn, Ni, Fe, Mn, Mo, and Mg). It is established that the mutagenic indices of the investigated pesticides correlate with the value of complexing constants of these substances with DNA (Saratovskikh and al., 2007). Similar results are obtained from the study of mutagenic potential of mixture of the following pesticides: Aktar, Senkor, Mospilan, Penkozeb and Fastak, which are widely used for treatment of potatoes in Republic of Tatarstan (Karamova and al., 2007).

Studying the influence of «Decis» (deltametrine) insecticide and «Magnum» (metsulfuronmethyl) herbicide on morphological and the cytogenetic mutability of *Drosophila melanogaster*, authors concluded that specified concentrations cause damage to the stability of development and increase the frequency of puff-formation in polytene chromosomes of flies (Golosova and al., 2009).

A study aimed at clarifying the effects of environmental pollution, including from agriculture, on some genetic processes of *Tilapiane* fish showed an increase in the frequency of both chromosomal structural and numerical aberrations, such as deletion, gap, end-to-end association, fragmentation, polyploidy, stickiness and monosomy (Zowail and al., 2010).

Thus, for monitoring purposes bio-indicators are exposed to the environmental pollutants "*in situ*" or in laboratory tests "*in vivo*". Tests with eukaryotic cells or organisms might be more relevant for human and ecological risk assessment, but generally they are much more time-consuming. Several tests have been developed using the integrity of DNA as a non-specific endpoint of genotoxicity e.g. comet assay, alkaline DNA-elution assay, DNA alkaline unwinding assay and other. Most eukaryotic genotoxicity tests detect macro damage of chromosomes in the visible light microscope following appropriate staining (i.e. chromosomal aberration, micronucleus assay, and SCE assay). Plants, amphibians, fish and water mussels, as well as permanent mammalian cell lines such as V79, CHO or CHL, have been used as the test organisms. Newer technologies, such as transcriptomics, proteomics and metabolomics, provide the opportunity to gain insight into genotoxic mechanisms and also to provide new markers *in vitro* and *in vivo*. There is also an increasing number of animal models with relevance to genotoxicity testing. These types of models will undoubtedly have an impact on genotoxicity testing in the future (Masood, 2012).

5. Genotoxicity of Pesticides on Human Health

The relevance of research into the mutagenic properties of pesticides is defined by the fact that most mutagenic substances cause a carcinogenic effect and so represent a hazard for human health.

Investigation of the cytotoxicity and genotoxicity of Acephate on the peripheral blood of healthy humans under in vitro test conditions revealed that, after two hours of exposure to the pesticide, almost 100% cells became non-viable at a 70μM concentration. In addition, it was established that the percentage of chromosomal aberrations and cells with DNA damage studies by single cell gel electrophoresis technique increased with the increase in the concentrations of the pesticide. At further higher concentrations there was a predominance of necrotic cells (Das and al., 2008).

Cytogenetic investigations among greenhouse farmers exposed to pesticides showed a significant increase in sister-chromatid exchanges (SCEs) frequency in peripheral lymphocytes. The results of SCEs were expressed through two variables: (a) the mean number of SCEs per chromosome, and (b) the proportion of high frequency cells (i.e. cells with more than eight SCEs). A high correlation was found between these two variables that indicates a potential cytogenetic hazard for humans due to pesticide exposure (Shaham and al., 2001).

A study of structural and numerical chromosomal aberrations (CAs) in workers of a plantation of flowers located in Quito, Ecuador, South America, exposed to the Aldicarb and Fenamiphos pesticides concluded that workers exposed to these pesticides showed an increased frequency of CA compared with the control group. In addition, the authors determined the level of erythrocyte acetylcholinesterase which was below the optimal in 88% of exposed individuals. This clearly shows the negative effect of organophosphate pesticides on human health (Paz-y-Mino and al., 2002).

Several studies have shown a decline in human semen quality with respect to the effects of pesticide exposure *in vitro* and *in vivo* and increased risks of male sub-fertility. In epidemiology studies clear effects on male fertility have been demonstrated for some pesticides (e.g. dibromochloropropane and ethylene dibromide) (Bretveld and al., 2006).

As for realization of mass cytogenetic and other monitoring surveys it is difficult, even sometimes impossible, to use invasion methods, and so it is extremely important to use non-invasion methods for the prognosis of origins of genetic human abnormalities.

6. Use of the Micronucleus Test for Assessment of Genotoxic Influence

In recent years, in the conditions of the permanent worsening of ecological situation, it is important to assess the level of population health and the identification of potentially high-risk groups of people.

Damage to the genome is probably the most important and fundamental cause of the development of anomalies and degenerative diseases. It has been established that genomic damage is produced by exposure to (a) genotoxic substances, (b) medical procedures (such as from radiation and chemicals), (c) micronutrient deficiency (from folic acid), (d) life styles (relating to alcohol, smoking, drugs, and stress, for example), and (e) genetic factors (such as defects in metabolism and/or in the repair of DNA). Hence, it is essential to perform biomonitoring with minimally invasive markers. The micronucleus trial on exfoliated cells of the buccal mucosa is a potentially excellent biomarker candidate for monitoring studies (Holland et al., 2008). In addition, the micronucleus test (MN-test) in buccal mucosa cells is one of the less invasive methods to measure DNA damage in humans.

Cells of buccal mucosa in the oral cavity are comfortable objects for estimating the physiology state of the organism (Holland and al., 2008; Kashyap and al., 2012) and the influence of environmental factors such as: pesticides (Bolognesi and al., 2002, 2004; Pastor and al., 2002, 2003; Costa C. et al., 2007); automobile exhaust-gases (Shastri and Pant, 2011; Çelik and al., 2003); oil products (Djambetova and al., 2009); chemotherapy (Burgaz and al., 2010; Torres-Bugarin and al., 2007); genotoxicity agents (Budak and al., 2010; Çelik A. et al., 2006; Chen and al., 2006; Martínez and al., 2005; Giri and al., 2012; Costa and al. 2007; Lucero and al., 2000; Godderis and al., 2006), as well as lifestyle habits (Reis and al., 2006; Ramirez and Saldanha, 2002; Proia and al., 2006).

As a criterion for estimation of negative influence of different environmental agents is presence and frequency of occurrence of cytogenetic abnormalities: cells with micronuclei (MN), binucleated cells, pycnosis, karyorechsis, karyolysis and other nucleus anomalies (Tolbert and al., 1991; Holland and al., 2008; Thomas and al., 2009).

High correlation is set between the increase of number of chromosomal aberrations, activity of mitosis process and induction of micronuclei (Thomas, 2009; Holland and al., 2009; Pastor and al., 2002). Micronuclei are formed from acentric fragments or whole chromosomes because of abnormalities of processes of cell division. The presence of micronuclei in cells is considered to be as the marker of genetic instability (Bolognesi and al., 2004).

Considering the above mentioned, we have used the MN-test for assessment of mutagenic influence on genome of children living in the industrial regions with a high technogenic load.

7. Research Subjects

The subjects of the research were the cytogenetic status of human organism and the general mutagenicity in the Dnepropetrovsk region which has cities of different types and technogenic load levels, namely Marganets, Zholtyye Vody (Yellow Waters), Nikopol and Dnepropetrovsk. These cities are characterized by high levels of development of such branches of industry as mining, metallurgical and chemical manufacturing, ore production and uranium ore dressing. In the capacity of a local "control" an area with a low technogenic load was chosen, namely the area of the medically-improving complex "Solyony Liman (Silted Estuary)" located in the Novomoskovsk part of the Dnepropetrovsk region.

8. Materials and Research Methods

In a group used in the cytogenetic survey the healthy and practically healthy children of 5-7-years-old were selected by a special questionnaire (Holland and al., 2008; Thomas and al., 2009).

Subjects were required to rinse their mouths with water before sampling. Exfoliated epithelial cells of buccal mucosa were obtained by scraping the middle part of the inner cheek with a wadded tampon on a spatula. The epithelial cells collected from buccal mucosa were smeared onto clean microscope glass slides which were then air-dried and fixed with a mixture of ethanol and glacial acetic acid (3:1) within one hour. Then the slides were stained with aceto-orcein.

A light microscope "Olympus" using 100-times magnification on coded slides was used for MN analysis. At least 1,000 cells per child were analyzed to determine MN frequency.

The scoring criteria used are mainly based on those originally described by Tolbert and al. (1992). Normal differentiated cells have a uniformly stained nucleus which is oval or round in shape. They are distinguished from basal cells by their larger size and by a smaller nucleus-to-cytoplasm ratio. No other DNA-containing structures apart from the nucleus are observed in these cells. These cells are considered to be terminally differentiated relative to basal cells because no mitotic cells are observed in this population group (Thomas and al., 2009).

The micronuclei are round or oval in shape and their diameter should range between 1/3 and 1/16 of the main nucleus. Also, they have the same staining intensity and texture as the main nucleus. Most cells with MNi will contain only one MN but it is possible to find cells with two or more MNi.

MN-index was calculated in accordance with frequency of cells with MNi. The received experimental data were used for the calculation of conditional indices of damage (CID) for biosystems. On this basis an estimation was made of the ecological situation on the mutagen background (Gorova and al., 2007, 2009). Using statistical analyses all the data were expressed as the mean ± standard error of the mean and results with $p < 0.05$ were considered significant.

9. Research Results

Analysis of results of cytogenetic survey of pre-school age children living in the technogenic loaded cities of the Dnepropetrovsk area (Table 1) showed an increase in frequency of micronuclei in buccal cells that is 3.5-4.8 times that found in those who live in the "conditionally clear" territory of the medically-improving complex "Solyony Liman".

TABLE 1. Results of micronuclei testing in buccal cells of pre-school age children living in cities of Dnepropetrovsk region ($p < 0.05$)

City	MN-index, per cell; 1999 – 2004		MN-index, per cell; 2005 - 2009	
	Average	Min - Max	Average	Min - Max
Dnepropetrovsk	0.077 ± 0.006 (n = 127)	0.024 ÷ 0.100	0.080 ± 0.007 (n = 98)	0.040 ÷ 0.120
Zholtyye Vody	0.111 ± 0.008 (n = 85)	0.053 ÷ 0.160	0.092 ± 0.007 (n = 75)	0.080 ÷ 0.170
Nikopol	-	-	0.089 ± 0.003 (n = 52)	0.056 ÷ 0.142
Marganets	0.085 ± 0.010 (n = 30)	0.045 ÷ 0.130	-	-
Local control "Solony Liman"	0.023 ± 0.004 (n = 53)	0.001 ÷ 0.042	0.026 ± 0.002 (n = 35)	0.001 ÷ 0.050

It should be noted that, although during the period 2005-2009, there is a positive dynamic in the change of the studied index in the investigated cities, but these differences are not significant ($p > 0.05$). In addition, increases were observed in minimum and maximal values of MN-index ($p < 0.01$) during 2005-2009 in comparison with 1999-2004. This testifies the tension of

ecological conditions in the investigated cities as a result of presence and accumulation of environmental pollutants having mutagenic properties.

In accordance with the methodology used (Gorova and al., 2007, 2009), the calculated conditional indices of damageability (CID) of child organisms for cytogenetic parameters (considering the minimal (P comfortable) and maximal (P critical) values of the investigated parameter) testify that the total mutagen background in the Dnepropetrovsk region has to be considered "unsatisfactory" on the basis of a "threatening" condition for children's organism, and that the level of damage to their cells is "above average". Of the four investigated cities in the Dnepropetrovsk region the greatest index of micronuclei in epithelial cells of children is defined in the center of the uranium-extractive and uranium-processing mining industry, namely the city of Zholtyye Vody. In the control area there was defined a "low" level of genetic damages in epithelial cells and a "safe" condition of organism in respect of the cytogenetic status. It has allowed considering as "favorable" the ecological condition of the control territories of the medical-and-health improving complex „Solyony Liman".

In the investigated cities there were defined groups of children with an increased genetic risk having values of the MN-index equal to or exceeding 0.100. In Zholtyye Vody an elevated risk group included 48.4% of the examined children, while in Dnepropetrovsk it was 35.9%, in Marganets 40%, and in Nikopol 36.5%. As to the 'control' area, there are undetectable representatives with an elevated level of genetic disorders in somatic cells.

10. Rehabilitation/prevention Measures

The rehabilitation program held at the base of Pulmonary Sanatorium of Dnepropetrovsk included the combined oral administration of humic substances (humics), carotene oil (pro-vitamin A), enterosorbents (pectin), and probiotics (acidophilus). All were officially approved and previously tested for food supplements. A humic food additive in the form of a 0.05% solution of humic acid was used for 21 days according to instructions from the Pharmaceutical Committee of the Ukraine Ministry of Health.

Treatment was provided for 2 months to the 37 children suffering recurring bronchitis. All food supplement dosage levels were age-appropriate and reassessments were conducted at the end of the 2-month treatment period.

An anti-mutagenic effect was observed in 87.5% of cases. The number of cells with MN decreased from 0.071±0.008 to 0.037±006 (i.e. $p < 0.01$). In addition, experiments displayed normalization of the immune system condition of child organisms and a reduction in the level of respiratory diseases by 1.3 times within the next autumn and winter period.

11. Conclusions

Under the influence of harmful environmental factors in human organism it is observed that there is an increase in the frequency of occurrence of cells with micronuclei. However, the condition of one's organism can be improved on the basis of using special rehabilitation courses with natural adaptogenes. The obtained data present a theoretical basis for the formation of rehabilitation programmes for population health status in technogenically-loaded areas.

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