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Novel Approaches in Genetic Toxicology of Pesticides Applying Fluorescent in Situ Hybridization Technique

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1. Introduction

The use of chemical substances in the pest control has been known since ancient times. Records have been found indicating that 2.500 years BC ancient Sumerians applied sulphur in mite control (Price, 1973). In ancient China, some 1.200 years BC inorganic mercury and arsenic compounds have been used in lice and bug treatment (Smith & Kennedy, 2002). First records of primitive fungicide and rodenticide use reach back to the time of Roman Empire where copper compounds were used in plant protection against moulds, and hellebore (lat. *Helleborus nigra*) containing poisonous baits against mice and rats (Smith & Secoy, 1975). Pyrethrium, which has remained in use as insecticide till nowadays, and is derived from *Chrysanthemum cinerariaefolium* flowers was brought to Europe from Persia by Crusaders (Wandahwa et al., 1996). However, the real revolution in use of chemicals in pest management started in early 1940-ties with the discovery of insecticidal properties of DDT by Paul Müller and the beginning of its massive production. Within next decade pesticidal properties of various synthetic chemical compounds had been discovered (hexachlorocyclohexane, 2,4-D, dithiocarbamates, chloradane, organophosphorous compounds, etc.) and they have been introduced in agricultural practice and household pest control (Ware & Whitacre, 2004). In the year 2009, more than 1.500 different active ingredients in more than 2.500 formulations were present in the world market (BCPC, 2009) with annual use exceeding 1.000.000 tones on global scale.

It was in 1962, after the publication of three parts serial in The New Yorker magazine and book entitled „Silent Spring" by Rachel Carson, when the general public became aware of possible adverse effects of unsustainable use of pesticides. The book has been based on records of the adverse effects of DDT on birds reproductive system and deaths of adults resulting from pesticide exposure. Furthermore, Carson pointed out the potential of pesticides to circulate within the ecosystems, accumulate within the organisms and affect all links in the food chain including humans (Carson, 2002). It has been ten years later when Environmental Protection Agency (EPA) estimated that evidences of adverse effect of DDT to the environment are sufficient to ban its use in the USA. Soon its prohibition was extended to European countries.

2. Carcinogenic risk of long-term pesticide exposure

Acute effects of pesticide poisoning are being recorded since their commercial use began (Green, 1949). However, more concern has been raised regarding the adverse effects that

long-term exposure to pesticides might have on human organism, specifically genomic material. The damage may be accumulating over the years without any noticeable health effects, but silently mediating cancer development. First studies indicating connection between exposure to arsenic insecticides in regular application and increased incidence of melanoma and bronchial cancer among European grape growing farmers appeared in late 1960-ties (Jungmann, 1966). However, since carcinogenic potential of arsenic had been documented already at the beginning of 20th century, these epidemiological studies did not raise much concern. In 1968, Klayman published first article suggesting association between chemically synthesized active ingredients and risk of carcinoma. The author reported several cases of larynx carcinoma in "never-smoking, never- or rarely-alcohol drinking" men with the record of more than 10 years of exposure to insecticides malathion or lindane working in either greenhouse or landscape (Klayman, 1968). Later, a case report indicating connection between lindane exposure and leukemia has been published (Hoshizaki et al., 1969). First more complex descriptive epidemiological studies aiming to evaluate carcinogenic risk arising due to occupational exposure to pesticides started in middle 1970-ties. In a study comprising the railroad workers applying herbicides amitrol or phenoxy acids, elevated occurrence of tumor deaths from stomach and lung cancer was observed (Axelson et al., 1974). With advances in knowledge on carcinogenesis it became evident that many life-style factors may elevate the risk of developing neoplasia by inducing genome damage. The profound effect on human cancer risk of smoking habits, alcohol consumption, diet, diagnostic procedures involving ionizing radiation or ultrasound, some medications, exposure to dyes and solvents may additionally pronounce or even prevail over the effect of pesticide exposure. Thus, beside simple recording of the type of pesticide exposure, all possible confounding factors should be considered in statistical analysis of data obtained by the study. Furthermore, the study should include population of individuals without any record of pesticide exposure that matches examinees by age, residence region, and life-style factors (Becher, 2005). In descriptive epidemiological studies there are three major epidemiological measures of cancer risk that are calculated. 1) Risk ratio or relative risk (RR) is the ratio of percentages of individuals developing cancer among the examinees and controls. If RR is above 1 then exposure increases the risk. 2) Odds ratio (OR) representing the ratio of the odd of cancer for the examinees and control. 3) Confidence interval (CI) represents sampling error inherent thus, statistical uncertainty of extrapolating the risk observed at the level of study group to the true population. These epidemiological measures should be adjusted for potential confounders by using Mantel-Haenszel estimation, regression methods (Cox, Poisson, multiple), or fractional polynomials. To overcome the problem of reduced statistical power in studies with small sample sizes meta-analysis is performed. It combines the results of several studies that address a set of related research hypotheses by using a form of meta-regression models. Additionally, to deduce the exact contribution of pesticides to the observed incidence of malignant disease multivariate approach in comparison between exposed and control group should be applied taking into consideration all confounding factors (e.g. MANOVA with post hoc comparison). Multiple regression analysis will identify the effect of each of confounding factors on the observed effect and canonical correlations analysis could be helpful (Ahrens et al., 2005). However, most of the individuals comprised by epidemiological studies are subjected to multiple pesticide exposures making impossible to separate the contribution of each individual pesticide to observed adverse health effect. Nevertheless, based on results of epidemiological studies, higher risk from developing neoplasia has been suspected for long-

term exposure to EPTC, pendimethalin, aldicarb, alachlor, chlorpyrifos, cyanazine, carbofuran, glyphosate and others (Dich et al., 1997). Short overview of indicated associations between pesticide exposures and increased risk of developing neoplasia published within last 3 years is presented in Table 1.

Exposure	Population	Cancer type/site	OR/RR (95% CI)	Reference
EPTC	Farmers	Colon	RR 2.09 (1.26–3.47)	van Bemmel et al., 2008
Trifluralin	Farmers	Colon	RR 1.76 (1.05–2.95)	Kang et al., 2008
Phenoxy herbicides	Plant workers	Myeloid leukemia	OR 6.99 (1.96–24.90)	van Maele-Fabry et al., 2008
EPTC	Farmers	Pancreas	OR 1.8 (1.0–3.3)	Andreotti et al., 2009
Pendimethalin			OR 1.7 (0.8–3.3)	
Metribuzin	Farmers	NHL	RR 2.42 (0.82-7.19)	DeLancey et al., 2009
Butylate	Farmers	Prostate	RR 2.09 (1.27–3.44)	Lynch et al., 2009
		NHL	RR 3.44 (1.29–9.21)	
Triazole fungicides	Farmers	HL	OR 8.4 (2.2–32.4)	Orsi et al., 2009
Urea herbicides			OR 10.8 (2.4–48.1)	
Organochlorines		Hairy cell leukemia	OR 4.9 (1.1–21.2)	
Phenoxy herbicides			OR 4.1 (1.1–15.5)	
Organophospha-tes	Residentials	Acute lymphoblastic leukemia	OR 2.5 (0.4-14.8)	Rull et al., 2009
Triazines			OR 4.1 (1.5-11.1)	
Permethrin	Farmers	Multiple myeloma	RR 5.72 (2.76–11.87)	Rusiecki et al., 2009
Terbufos	Farmers	Leukemia	RR 2.38 (1.35-4.21)	Bonner et al., 2010
		NHL	RR 1.94 (1.16-3.22)	
		Lung	RR 1.45 (0.95-2.22)	
Phenoxy herbicides	Plant workers	Urinary cancers	RR 4.20 (0.99-17.89)	Boers et al., 2010
		Genital cancers	RR 2.93 (0.61-14.15)	

Table 1. Most recent studies indicating association between pesticide exposure and cancer.

However, there are certain shortcomings of epidemiological studies that may lead to a possible bias in estimation of exposure risk and which results in studies reporting inconsistent results due to exposure to a specific pesticide (Eastmond & Balakrishnan, 2001). Due to poorly defined exposure levels, combinatorial exposure to other potentially carcinogenic agents, small study groups, or maladjustment of data for possible confounders, regulatory agencies and organizations authorized for carcinogenic classification of chemicals consider most of the reported associations of pesticide exposure with risk of cancer inconclusive. Because of these, in addition to epidemiological studies they turn to the results of long- and short-term animal and in vitro assays. Consequently, International Agency for Research on Cancer (IARC) and Environmental Protection Agency (EPA) have recognized

less than 10 active ingredients as proved human carcinogens. Yet, many substances have been assigned as likely to be carcinogenic to humans or with suggestive evidence for carcinogenic potential.

2.1 Relevance of epidemiological studies: pro and con

There is much controversy about the relevance of epidemiological studies in risk assessment of pesticide exposure. Since individuals occupationally or residentially exposed to pesticides are simultaneously affected by several substances it is not possible to determine contribution of the specific agrochemical to the observed health effect. As already mentioned this issue has been recognized by regulatory agencies. Their decisions mostly rely on surrogate short- and long-term testing performed under controlled laboratory conditions, and results of epidemiological studies may provide supportive information. There are potential endogenous confounding factors such as gender, age, genetic polymorphism, and exogenous such as smoking, alcohol intake, medications that may significantly influence observed adverse health effect and have to be considered in analysis and interpretation of the results (Anderson, 2000). Additional bias in estimation of risk may evolve due to poorly described exposure conditions, inconsistent level of exposure with time, lack of air quality measurements and data on personal protective equipment usage. Also, lot of skepticism has been raised about the value of short-term genotoxicity tests. As the results of acute testing had accumulated over the past years less and less correlation with results of chronic carcinogenicity tests on rodents has been observed (Casciano, 2000). Furthermore, though testing of active substances under strictly controlled conditions using different cell lines may provide valuable knowledge on adverse effect toward human genome, interpretation of obtained results is challenging. There is the likelihood of (a) false positive results mediated by rather high concentrations tested (which may also lead to increase in osmolality), pH value decline, or (b) false negative results mediated by neglecting the need for metabolic transformation or by testing highly pure active ingredients. The latest may also diminish the relevance of results obtained on animal models. Moreover, the results of carcinogenicity testing obtained on animals may not always univocally be extrapolated on humans since there are some distinctive differences in metabolic pathways between animals and humans. That may be crucial in activation of carcinogenic potential of substance of concern as it was reported for insecticide carbosulfan (Abass et al., 2009) and also raised controversy over saccharine risk assessment (Chappel, 1992). Major discrepancy between results obtained by using different short- and long-term experiments on both cell and animal models, and effects occurring in real-case exposures arises from the fact that conditions used in laboratory may differ significantly from those likely to be encountered in occupational and residential exposure to pesticides. By using active ingredients of high purity in experimental evaluations two important factors that occur in real exposure of humans to pesticide are completely omitted:

a. possible effect of impurities contained within pesticide formulations that are byproducts of active substance synthesis,
b. possible effect of “inert” ingredients (solvents, potentiators, surfactants, emulsifiers, stabilizers) as standard components of pesticide formulations.

There are several examples of contaminants with adverse effect on human genome exceeding those of active ingredients in whose synthesis they are by-produced. Low levels of 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) known as a potent human carcinogen, are

present in herbicide formulations containing chlorophenoxyacetic acids (Eastmond & Balakrishnan, 2001). Carbendazim may be found in sulphur pesticide formulations that are approved for application in organic agriculture (Balayiannis et al., 2009). It further contains 2,3 diaminophenazine (DAP) that is suspected to be responsible for carbendazim's carcinogenic effect in mice. Malaoxon impurities contained within malathion formulations are associated with its genotoxic effect (Blasiak et al., 1999) while pyrethroids contain contaminants that exhibit more sever toxic effects than active ingredient itself (Hadnagy et al., 1999). Inert ingredients are routinely added to pesticide formulation in order to facilitate the application and to secure rapid and efficient transport of the active ingredient to the target site within the pest organism. Organic solvents used as inert ingredients have been associated with elevated risk of non-Hodgkin's lymphoma (Blair & Zahm, 2008). It has been shown that toxic effects of herbicide formulations with atrazine, glyphosate, and fungicide vinclozoline may be increased by present inert ingredients (Cox & Surgan, 2006).

Consequently, as stated by Hill (2010), since detecting the effect of multiple chemical exposure epidemiological studies provide us with limited knowledge on the genotoxic mechanism of a single pesticide. However, their importance lies in the fact that they look directly at human risk in situ, and estimate impact of a specific exposure type on cytogenetic status of the population in question. Epidemiological studies of pesticide exposure and experimental evaluations mutually supplement each other, and both approaches represent inevitable segments of risk assessment mosaic of exposure to pesticide.

3. Genetic toxicology

Findings that cancer is triggered and promoted by occurrence and accumulation of genome damage induced by a series of physical and chemical agents from residential and occupational environment, genetic toxicology as a subfield of toxicology developed. Pioneer assays aiming to detect changes at the level of cell genome as the consequence of exposure to pesticides were conducted in 1970-ies. Though Kaszubiak (1968) was first to isolate Rhizobium mutants from the cultures treated with herbicides linuron, dinoseb, and dimethylurea, the first experiment intended to evaluate mutagenic effect was conducted in 1970 and it gave positive results for herbicide 3',4'-dichloropropionanilide (Prasad, 1970). The exponential growth of genotoxicity testing occurred in the middle of 1970-ties with the introduction of Ames test on mutant strains of bacterium *Salmonella typhimurium* and mouse lymphoma assay (MLA) on L5178Y cell line. Within a decade more than 100 genotoxicity tests for evaluation of genetic potential of chemicals emerged. Since most of them were flawed in providing results relevant for carcinogenic risk assessment they gradually disappeared (Casciano, 2000). The term "genetic toxicology" appeared already in 1975 in the title of the review paper of Legator & Zimmering (1975). This toxicological discipline aims to:

a. identify biomarkers of the exposure or effect at the level of the cell genome that would be affected by exposure to carcinogen in a dose-response manner and would highly correlate with risk of cancer development, and
b. interpret obtained results to deduce the mechanism of chemical-genome interaction, role of the observed effects in carcinogenesis induction, and their impact on human health.

Adverse effects that are subject of study of genetic toxicology arise at the exposure levels far beyond the concentrations that would induce observable toxic effects on cells, organs or

organism, which is somehow in disagreement with other fields of toxicology. These effects could even hardly be classified as toxic since they do not have any short-term impact on human health, and in most cases they are efficiently repaired. Nevertheless, under conditions of long-term exposure their occurrence may prevail over the repair or they may be misrepaired. Accumulation of these genomic lesions may induce cell transformation, immortalization and neoplastic growth.

Nowadays less than 15 assays are commonly applied in biomonitoring and even less are officially accepted for regulatory genotoxicity testing. Basically, assays applied in genetic toxicology may be classified in three major groups:

a. assays detecting mutagenic potential (Ames test, MLA, Drosophilla wing-spot test, mouse dominant lethal assay)
b. assays detecting genotoxic potential (micronucleus assay, structural chromosomal aberration analysis, sister chromatid exchange assay)
c. assays detecting nonspecific primary DNA lesions (alkaline comet assay).

Over the last decade, chromosomal aberration analysis, micronucleus, and comet assay have emerged as most reliable in evaluation of genotoxic effect of human exposure to pesticides. Among them, chromosomal aberrations and micronuclei as biomarkers of the effect were also proved to be good predictors in cancer risk assessment (Fenech, 2007; Rossi et al., 2009).

3.1 Chromosomal aberration analysis

Alterations in chromosome structure as the consequence of exposure to external agents has been known for more than 50 years. Their occasional application in health surveillance programs of individuals occupationally exposed to potential carcinogens started in 1960-ies. Soon structural chromosomal alterations in peripheral blood lymphocytes have been accepted as surrogate effect that reflects events triggered in the precursor cells for carcinogenesis under the exposure conditions of the issue (Hagmar et al., 2000).

Formation of structural aberrations of chromosomes is a rather complex event, involving DNA replication process in S phase of the cell-cycle, and misrepair of induced DNA strand breaks in post-replication phases. Under physiological conditions the lymphocytes are mainly in "resting" G_0 phase. Most of the genomic lesions induced by chemical agents will be efficiently repaired, especially if they occurred in transcriptionally active regions of chromosomes. Nevertheless, unrepaired lesions will interfere with DNA replication process forming DNA strand breaks thus, chromatid breaks, but also chromosome breaks which may result from DNA breaks due to additional topoisomerase II impairment (Maynard et al., 2009). Post-replicate repair mechanisms, through specific error prone pathways may convert chromosome breaks that occurred within "rejoining distance" into more complex rearrangements in chromosome structure such as chromatid exchanges and dicentric chromosomes (Obe et al., 2002). Insect chemosterilants tepa and apholate were first pesticides proved to affect morphology of human chromosomes in 1968 (Chang & Klassen, 1968). They were followed by insecticides propane sultone, aldicarb, malathion, fungicides ziram, thiram, herbicides 2,4-D, symazine and others.

Due to their good correlation with the level of exposure to chemicals in dose-dependent manner, structural chromosomal aberrations have been accepted as valuable cytogenetic biomarker of effect in epidemiological studies and risk assessment. Additional support of their application in human biomonitoring lies in their predictive value in cancer epidemiology – elevated frequency of aberrations indicates a population in increased cancer risk (Rossner et al., 2005).

3.2 Micronucleus assay

Within the last 5 years micronucleus assay has been recognized as most reliable and efficient cytogenetic test in detection of potential carcinogens. It took over the primacy of being the most relevant biomarker of effect from chromosomal aberrations (Cavallo et al., 2009). Micronuclei as manifestations of adverse effect of physical and chemical agents on cell genome have been known for more than 40 years (Matter & Schmid, 1971). They are small chromatin structures visible in cytoplasm of interphase cells, with maximum of 1/3 of nuclear diameter in size.

Micronuclei originate from:

a. chromosomal fragments formed as the result of induced DNA strand breaks (as discussed in 3.1) that lagged in anaphase for not possessing the centromere to be attached to mitotic spindle and pulled to one of the mitotic poles, or
b. whole chromosomes that lagged in the anaphase due to spindle or kinetochore protein damage and that remained unsegregated (Fenech, 2007).

Following mitosis, one of the newly formed cells will be deficient in the genetic information within the lagged chromosome/fragment, while the other micronuclei containing cell will be in surplus. Micronucleus assay owes its preference over chromosomal aberration analysis to the ability to detect two different mechanisms of genotoxicity:

a. clastogenic mechanism meaning direct interaction of genotoxic agent with DNA molecule. It mostly results in micronuclei harboring chromosomal fragments
b. aneugenic mechanism meaning interaction of genotoxins with mitotic spindle proteins not DNA molecule itself, leading to genomic instability by loss and malsegregation of chromosomes. It results in micronuclei harboring whole chromosomes (Muller et al., 2008).

Additional efficiency of micronucleus assay in detecting genotoxic chemical has been gained by implementation of scoring criteria proposed by HUman MicroNucleus (HUMN) project group (Fenech et al., 2003). Beside micronuclei (MN), other aberrant chromatin structures such as nuclear buds (NB) and nucleoplasmic bridges (NPB) are considered in evaluating genotoxic potential. NBs are chromatin structures observed as nuclei extensions. They are formed in the process of elimination of (a) genomic regions harboring the genes related to metabolism of or resistance to exogenous chemical substance that have been amplified due to chronic exposure, or (b) DNA-repair complexes. NPBs, as chromatin structures connecting newly formed nuclei in telophase, are mostly manifestations of dicentric chromosomes, telomeric fusion of chromosomes, or union of sister chromatids (Fenech, 2007). In 1974, metepa was one of the first pesticides reported to induce micronuclei formation (Richardson, 1974). Soon, potential for micronuclei induction was reported for herbicidal phenylalkylureas, fungicide thiram, insecticide malathion etc.

Hence, micronuclei formation has been accepted as valuable cytogenetic biomarker of exposure to chemicals and there are ever more scientific evidences of its possible applicativity in epidemiological studies estimating the cancer risk of exposure to chemicals (Fenech, 2007; El-Zein et al., 2008).

3.3 Fluorescent in situ hybridization

In 1969 for the first time hybridization of small radioactively labeled RNA fragments has been successfully applied in microscopic localization of specific genes (Buongiorno-Nardelli & Amaldi, 1969). Some 20 years later, RNA molecules have been replaced with DNA probes, and radioactive labeling with antigen labeling and immunocytochemical detection of

sequences. Finally, when in early 1990-ties a fluorochrome labeled DNA probe has been produced, fluorescent in situ hybridization (FISH) as a powerful cytogenetic technique with high potential of discovering new biomarkers of effect in epidemiological studies of exposure to carcinogens was introduced. Basically, FISH technique enables visualization of specific genes, chromosome regions (centromeres, telomeres) or whole chromosomes within the cell genome in both, interphase nuclei and metaphase chromosomes. Thus, it provides us with the information regarding

a. the copy number of a specific gene or chromosome in detection of aneuploidy that may arise as the result of chromosome malsegregation or loss of broken fragments in exposure to genotoxic chemical,
b. the chromosome regions, or a specific chromosome harbored by micronuclei,
c. the occurrence of translocations as stable chromosomal rearrangements (Raap, 1998).

First attempts to categorize content of micronuclei occurred in 1990 (Becker et al., 1990). Several years later began its occasional application in human biomonitoring (Titenko-Holland et al., 1994), but it has not been before 2003 that micronuclei content has been indicated as a valuable parameter in characterization of the effect of exposure to chemicals on human genome (Norppa & Falck, 2003). The presence of whole chromosomes in micronuclei may be detected by anti-kinetochore antibodies that will target kinetochore proteins in centromeric region. However, it has been deduced that many micronucleated chromosomes may possess disrupted kinetochore or it may be detached thus, exhibiting no signal following immunocytochemical detection. Therefore detection of pan-centromeric regions within the micronuclei by hybridization of fluorescent DNA probe will render more relevant results in determining the micronuclei content. A year after the first application of pan-centromeric FISH to analyze the content of micronucleus in evaluation of the effect of exposure to pesticides was reported, and three years later according to the HUMN recommendations other aberrant chromatin structures in relation to pesticide exposure have been categorized regarding the centromere content.

Translocations are persistent alteration in chromosome morphology that significantly affect genome integrity. They are of similar ethiology as dicentric chromosomes, originating from double strand DNA breaks (DSB) that are misrepaired mostly by non-homologous end joining (NHEJ) and ectopic homologous repair. Translocations are considered as most valuable biomarker in cancer risk assessment (Obe et al., 2002). High translocation frequencies have been observed in all tumor cells, some type of them being highly associated with a specific type of cancer thus, being etiologic for the neoplasia in question (Mitelman et al., 1997). Due to their high correlation with risk of developing cancer, translocations are considered as a valuable cytogenetic biomarker of effect in evaluating human exposure to genotoxic agents. Their application in biomonitoring began in early 1990-ties, but it has been restricted to studies of exposure to ionizing radiation (Tucker et al., 1993). Several years later, Steenland et al. (1997) introduced application of translocations as biomarkers of effect in occupational exposure to pesticides by evaluating the effect of ethylenebis(dithiocarbamate) (EBDC) fungicides. However, until 2009 no further use of chromosome painting by FISH in evaluation of pesticide genotoxicity has been reported. The application of FISH technique in translocation analysis and in revealing the content of aberrant chromatin structures provides us with more precise knowledge regarding the extent of genome affected by pesticide-induced damage and its relevance to carcinogenicity.

4. FISH in genetic toxicology of pesticides

4.1 Background epidemiological studies

Concept of using structural chromosomal aberrations in peripheral blood lymphocytes in cytogenetic biomonitoring of subjects occupationally or residentially exposed to potential carcinogens has been based on the finding that level of genetic damage in lymphocytes reflects the effects occurring in precancer cells of target tissue. Until the late 1990-ties it has been generally accepted that occupational exposure to chemicals may influence chromosome structure mostly by inducing chromatid-type of aberrations such as gaps and chromatid breaks. It has been assumed that chromosome breaks may also occur but at much lower frequency than chromatid breaks, while more complex rearrangements as dicentric and ring chromosomes have been considered as cytogenetic biomarkers of exposure to ionizing radiation or a small group of radiomimetic chemicals (e.g. antineoplastic drug irinotecan) which interact with DNA in pathways that resembles the one of ionizing radiation (IAEA, 2001). Accordingly, epidemiological studies evaluating cytogenetic effects of occupational exposure to pesticides have reported increased level of alterations in chromosome morphology, but chromosome breaks being the most serious reported lesion. As discussed in section 2.1, all such studies published within last 2 decades comprised groups of examinees in multiple pesticide exposure. Significant effect on induction of chromatid and chromosome breaks in farmers has been published by Hoyos et al. (1996) due to exposure to mixture of dithiocarbamates, carbamates and organophosphates, by Garry et al. (1996) in applicators of broad spectrum of insecticides and herbicides, by Antonucci & de Syllos Cólus (2000) in applicators of organophosphates, carbamates, and some herbicides (Mann-Whitney *U*-test, $P<0.05$). Although Hoyos et al. (1996) presented detailed information regarding exposure conditions and adjusted their statistical analysis for confounders, none of that has been done by Garry et al. (1996) and Antonucci & de Syllos Cólus (2000). In the later study on chlorophenoxy herbicide applicators and exposed foresters Garry et al. (2001) showed significant increase in chromatid breaks but only in lymphocytes of applicators using more than 3,785 liters of herbicides per season (Wilcoxon rank-sum test, $P=0.017$). Again, no adjustment for confounders has been done. Conversely, Lander et al., (2000) analyzed results using a multiple log-linear Poisson regression model for smoking, age, and coffein intake as possible confounders. The authors monitored chromosomal aberrations in greenhouse workers exposed to residues of 10 different insecticides, 6 herbicides, and 3 growth regulators prior to and after the spraying season. Number of cells harboring structural chromosomal aberrations significantly increased after the spraying season ($P=0.05$). However, among all recorded structural alterations the effect has been significant only for chromatid gaps ($P=0.001$), and most prominent increase has been observed for non-glove using smokers ($P=0.04$). In spite of excellent study design, aberration nomenclature used by the authors does not follow the IAEA (2001) recommendations nor has been elaborated, which bias comparison of results with those reported in previously discussed studies.

Studies reporting complex rearrangements are summarized in Table 2. Contrary to previously cited papers, Kourakis et al. (1996) were first to report the presence of dicentric and even ring chromosomes in the absence of significant increase in number of chromatid-type aberrations, in group of both, outdoor and greenhouse spraying workers exposed to complex mixture of pesticides (organophosphoric and organochlorinic compounds, carbamates, dithiocarbamates). However, occurrence of complex alterations was not

statistically significant. Since the workers did not use any personal protection equipment the complex cytogenetic effects may be attributed to high level of exposure due to direct pesticide intake by inhalation, through the skin and eye contact. Non-significant presence of complex structural alterations accompanied by significantly increased chromatid breaks was observed in vineyard growers mostly exposed to insecticide diazinon and fungicide dithiocarbamate (Joksic et al., 1997). Unfortunately, though results were presented at the level of subgroups regarding smoking habits and gender, in both studies statistics has been done without considering life-style factors as possible confounders. Although Amr (1999) did report significant increase of dicentrics (Student's t-test, $P<0.001$) among pesticide handling workers exposed mostly to mix of DDT, chlorinated hydrocarbons, organophosphates, pyrethroids and carbamates, due to major lack of data regarding the group characteristics, and inclusion criteria, reported results are considered inconclusive. For instance, of 300 included examinees that were subjected to biochemical analyses and medical diagnostic procedures, only 30 of them were chosen for chromosomal aberration analysis without any explanation of criteria for selecting them. Furthermore, most of the examinees had medical history of suffering from various disorders. Again, no multivariate analysis of obtained data has been applied. Other study reporting elevated frequency of unstable chromosome rearrangements comprised pesticide plant workers primarily exposed to atrazine and cyanazine, with minor exposure to alachlor, 2,4-D, and malathion (Zeljezic & Garaj Vrhovac, 2001). Production of pesticides was organized seasonally and workers have been exposed to pesticides during 8 months of their production. Until next production season they were transferred to the working places out of the exposure zone. For the first time it has been reported that following exposure period, beside chromatid and chromosome breaks, dicentric chromosomes may also be significantly increased (MANOVA, $P_{ScheffePostHoc}<0.01$). Further, significant occurrence of chromatid exchanges in the form of quadriradials has been observed (MANOVA, $P_{ScheffePostHoc}<0.01$).

Exposure type	Chromosome rearrangements exposed vs. control			Reference
	Dicentric	Ring	Exchange	
Organophosphates, carbamates, dithiocarbamates, organochlorines	0.07±0.03 N/R	0.03±0.02 N/R	0.00±0.00 N/R	Kourakis et al., 1996
Ethofumesate, diazinon, vinclozolin, 2,4-D, dithiocarbamate, metalaxyl etc.	0.02±0.00 0.00±0.00	0.19±0.00* 0.02±0.00	N/R	Joksic et al., 1997
Mancozeb, methamidophos, captan, chlorpyrifos	0.08±0.30 0.05±0.09	0.02±0.05 0.03±0.07	N/R	Steenland et al., 1997
Atrazine, cyanazine, alachlor, 2,4-D, malathion	0.42±0.95** 0.00±0.00	0.00±0.00 0.00±0.00	0.10±0.40* 0.00±0.00	Zeljezic & Garaj Vrhovac, 2001
Captan, mancozeb, endosulfan, methiocarb, glyphosate, linuron etc.	Dicentric and ring chromosomes reported to be observed without providing quantitative data			Costa et al., 2006

Table 2. Studies reporting complex alterations in chromosome structures in pesticide exposed subjects. Results presented as average per 100 cells ± S.E., N/R data not reported by authors, *$P<0.05$, ** $P<0.01$.

At the end of the non-exposure period frequency of dicentric chromosomes significantly decreased, and no quadriradials were observed confirming the unstable nature of those chromosome-type aberrations (IAEA, 2001). Residual dicentrics were only detected in individuals with more than 18 years of employment in pesticide production. Conversely to previously cited studies, the authors applied multivariate analysis of variances considering smoking, gender, and age. None of them did significantly influence intergroup variations in aberration frequency. Furthermore, to avoid possible effect of x-ray diagnostics on induction of chromosome-type aberrations only workers without the record of being subjected to such procedures were allowed to participate in the study. Still, alcohol intake, nutrition, medications and other life-style factors have not been considered. Publishing of those results on pesticide genotoxicity coincided with the review article of Obe et al. (2002). The authors proposed the mechanism by which chemical genotoxins including pesticides may give rise to chromosome-type aberrations that had been previously considered as cytogenetic biomarkers of exposure to ionizing radiation. Accordingly, chemicals may induce DNA lesions such as single-strand breaks as the consequence of interaction with DNA that would result in hindered DNA replication, alkylations, bulky adducts formation, or oxidative DNA damage. During DNA replication or by error-prone DNA repair pathways, these lesions may be transduced into DSB. Further, improperly repaired DSB may give rise to complex alterations in chromosome structure such as dicentric chromosomes and exchanges. As stated by Obe et al. (2002) there are three major pathways of DSB repair: (a) ectopic homologous recombination repair (EHRR), (b) non-homologous end joining (NHEJ), and (c) single-strand annealing. Error-prone activity of two of them, NHEJ and EHRR are responsible for dicentric formation. Conversely to NHEJ that requires two initial DSB within "rejoining distance", for EHRR to form a dicentric a single DSB is needed. Furthermore, EHRR may involve homologous sequences of different chromosomes resulting in dicentric or translocation. However, it has to be stated that the repair of chemically induced DNA damage may be slower than that of ionizing radiation; thus, the probability of misrepair induced aberrations would be low (Preston, 2000) which could also be observed from the results summarized in Table 2.

More recent studies also reported appearance of dicentrics and quadriradials in lymphocytes of workers exposed to pesticides. Costa et al. (2006) detected complex structural alterations in lymphocytes of sprayers applying 33 different active ingredients without specifying the type. Ergene et al., (2007) detected dicentrics detected in residentially exposed subjects living in region contaminated mostly with organochlorines, organophosphates, carbamates, pyrethroids and benzoyl ureas. Both groups of authors did adjust statistical analysis for confounding factors. The incidence of chromosomal aberrations has been significantly affected (MANOVA, $P<0.01$) by pesticide exposure only in the study of Ergene et al., (2007) indicating that residential exposure to pesticides may also adversely affect genome integrity.

Nevertheless, findings of studies documenting the presence of dicentric chromosomes and other complex chromosome-type aberrations in lymphocytes showed necessity of using novel cytogenetic approaches that may provide more detailed knowledge regarding the potential of pesticides to affect genome integrity and induce complex genome rearrangements considered as a driving force for carcinogenicity.

4.2 FISH in analysis of pesticide induced aberrant chromatin structures

Generally, micronuclei represent the most known aberrant chromatin structure, and within the last 3 decades they have been used in evaluation of cytogenetic effect of exposure to

pesticides (Bull et al., 2006). Etiologically, they may originate either from chromosomal fragments as result of unrepaired chromosome breaks or whole chromosomes that lagged in anaphase due to damage of mitotic spindle or kinetochore. Identifying each of these two types of micronuclei provides us more detailed knowledge regarding the mechanism of genotoxicity and enables distinguishing between clastogenic pesticides that directly interact with genome and aneugenic that give rise to genomic instability by damaging protein structures. The latest leads to malsegregation of chromosomes and may result in their loss and aneuploidy. Application of fluorochrome labeled DNA probes that would hybridize in pan-centromeric region of each of 46 chromosomes makes it possible do recognize micronuclei harboring whole chromosome. When analyzing lymphocyte preparations under epifluorescence microscope, such micronuclei will contain one or more fluorescent signals. Conversely, micronuclei harboring chromosomal fragments remain without any signal.

Bolognesi et al. (2004) were among first to hybridize all-chromosome pan-centromeric probes to micronucleus slides in elucidating origin of micronuclei induced by exposure to mixture of pesticides. Study comprised floriculturists mostly exposed to organophosphates, carbamates, organochlorines, benzimidazoles, thiophtalimides, pyrethroides, and bupirimate, exposure duration ranging form 2 to 70 years. Although no statistically significant difference has been observed between pesticide users and control subjects, MN frequency increased with amount of pesticides applied, number of formulations individuals were exposed to, involvement in pesticide preparation, years of exposure, and non-use of personal protective equipment. While in the control group ratio of centromere containing micronuclei (61.9%) did not significantly differ from the reference values (±60%), in floriculturists the ratio of whole chromosome harboring micronuclei (C+MN) prevailed over the fragment harboring ones (C-MN). Though not associated with pesticide exposure duration, ratio of C+MN positively correlated with non-use of gloves in pesticide preparation. As expected, proportion of C+MN increased in both groups with age, which could be attributed to micronucleation of X and Y chromosomes associated with ageing. The effect of lagging sex chromosomes is more pronounced in females (Norppa & Falck, 2003). The most interesting conclusion made by Bolognesi et al. (2004) is higher ratio of C+MN in benzimidazolic fungicide appliers (66.52±16.11) compared to floriculturists applying other pesticide classes (63.78±14.02), which does not surprise knowing that benzimidazoles are proved as spindle microtubule poisoning agents. Single limitation of the study arises from the lack of multivariate statistical analysis of obtained data that would adjust them for age, wide range of exposure duration, and other possible confounders.

Though it renders deeper insight in mechanism of genotoxicity, revealing micronuclei content does not provide any information regarding the ability of pesticides to affect genome integrity by inducing complex rather unstable chromosome rearrangements as it has been indicated by application of chromosomal aberration analysis in section 4.1. To obtain that kind of knowledge, HUMN recommendations for considering other aberrant chromatin formations, especially nucleoplasmic bridges, had to be implemented in biomonitoring study coupled with all-chromosome pan-centromeric FISH analysis (Fenech et al., 2003).

Such an approach has been applied in evaluation of cytogenetic effect of occupational pesticide exposure in carbofuran production workers (Zeljezic et al., 2007). Though the use of carbofuran had been banned in the EU countries, in 2008 the application for its inclusion in Annex I of Council Directive 91/414/EEC concerning the placing of plant protection products on the market thus, reallowance of its use, has been resubmitted. In 2003 in some

EU countries carbofuran was still among top 5 active substances applied to vegetable crops. Epidemiological studies reported that individuals exposed to carbofuran may have increased risk for lung cancer (Usmani et al., 2004) and non-Hodgkins lymphoma (Zheng et al., 2001). In study of Zeljezic et al. (2007) lymphocytes of carbofuran production plant workers micronuclei, nuclear buds and nucleoplasmic bridges were analyzed for centromere content. Acethylcholinesteraze (AChE) activity in both, whole blood and plasma also has been recorded, as the biomarker of acute exposure to carbamate and organophosphorus insecticides. As shown in the Fig. 1. considering smoking, alcohol intake, gender, difference in age, medical procedures and exposure duration as possible covariates the incidences of MN, NBs, and NPBs have been significantly increased ($P_{DuncanPostHoc}$=0.0040; 0.0089; 0.046, respectively) in carbofuran exposed examinees. Although the ratio of centromere harboring MNs (69.6±12.5%) did not exceed referent values suggested by Norppa & Falck (2003), it has been increased compared to the unexposed group (54.3±10.3%). Statistical significance in the difference ($P_{DuncanPostHoc}$=0.0084) suggests that carbofuran, beside interacting with DNA to induce breaks in chromosome structure may also exhibit aneugenic effect by damaging mitotic spindle, and consequently resulting in chromosome loss and aneuploidy. Evident increase in the presence of heteromorphic sites of chromosomes 1, 9, 15, 16, and Y in C+MN of carbofuran handling workers ($P_{DuncanPostHoc}$=0.0036), suggests that micronucleation of whole chromosomes is not restricted to sex chromosomes as it may be expected in elderly subjects. Rather, it may indicate higher susceptibility of genomic regions rich in A-T base pairs toward aneugenic effect of carbofuran and/or its metabolites. Additional support for both clastogenic and aneugenic activity of carbofuran could be found in literature data. Stehrer-Schmid & Wolf (1995) reported adverse effect of the insecticide on formation of microtubuli and impairment of their function during chromosome segregation. Clastogenic effect of carbofuran observed as induction of C-MN may find its support in the paper of Zhang et al. (2005) who deduced carbofuran's ability to intercalate between base pairs and produce DNA-carbofuran adducts. Contrary to general perception that ACh-sensitive receptors are specific for cells of nervous tissue, they are also present on the membrane surface of most non-neuronal cells, including the periferal blood lymphocytes. Their specific physiological role has mostly remained unclear though they are confirmed to be involved in immune functions, control of gene expression, cytoskeletal organization, secretion, absorption etc. Consequently, as discussed by Rull et al. (2009) changes in AChE activity due to chronic exposure to anti-ChE compounds may induce amplification of AChE gene, and may affect cell proliferation and differentiation (Vidal, 2005). Both processes are closely associated with carcinogenesis, which may also provide a support for the results of previously cited epidemiological studies suggesting a correlation between carbamate exposure and increased risk of lymphoma. Possible amplification of AChE and butyrylcholinesterase (BuChE) genes under the chronic burden of exposure to carbamates may be the reason of elevated occurrence of NBs in lymphocytes of pesticide plant workers ($P_{DunvanPostHoc}$=0.0089). Amplification of AChE gene under chronic exposure conditions may be the reason of increased NB formation. Observed NBs might be also formed as the result of the elimination of DNA-repair complexes that have been overrepresented due to increased level of DNA damage induced (a) directly by genotoxic activity of pesticide or (b) indirectly by endogenous reactive oxygen species formed in the cells as the result of carbofuran-produced oxidative stress (Calviello et al., 2006). Although we did observe sporadic centromere signals in NBs of exposed subjects (5.8±2.21%) C+NB/C-NB ratio did not differ from the control subjects ($P_{DuncanPostHoc}$=0.72).

Unlike for C+MN, centromere signal in NBs would indicate budding of epicentric interstitial fragments or joining of chromosome harboring MNs with nucleus. Less likely scenario would be that whole chromosome may be expelled from the nucleus forming the NBs by triggering aneusomy rescue mechanism, which would be possible for highly aneugenic pesticides. Lagged chromosomes that have been incorporated within daughter nucleus instead of forming MN may cause distortion in chromosome territories and be eliminated in the budding process (Lindberg et al., 2007). However, this theory has not been proved yet nor did carbofuran exposure exhibit such prominent aneugenic potential considering observed C+MN/C-MN ratio. Finally, ratio of centromere signals has been significantly elevated ($P_{DuncanPostHoc}$=0.046) in NPBs of pesticide plant workers (20.3±12.3%) compared to the control subjects where none of detected bridges harbored the pancentromeric region. Elevated occurance of NPBs as biomarkers of dicentric and ring chromosomes, additionally supports results discussed in section 4.1 indicating that long-term exposure to pesticides is capable of inducing complex alterations in chromosome morphology. Since detected NPBs were mostly not accompanied by the presence of micronuclei (98.2±1.5%) it may be suggested that they are formed by misrepair of DSB by NHEJ or by telomere-end fusion (TEF). The latter occurs as a consequence of telomere shortening which has been commonly associated with aging, but also may be prematurely mediated by chronic influence of chemicals affecting cell proliferation and is observed in precancer cells (Fenech, 2006). Both NHEJ and TEF are characterized by the lack of acentric formation that would result in micronucleus formation. Further, increased frequency of NPBs has been recognized as a biomarker of elevated risk of lung cancer (Multivariate logistic regression analysis OR=29.05, CI=7.48-112.80, P<0.001; El-Zein, et al. 2006), for which an association has been indicated with occupational carbofuran exposure (Usmani et al., 2004).

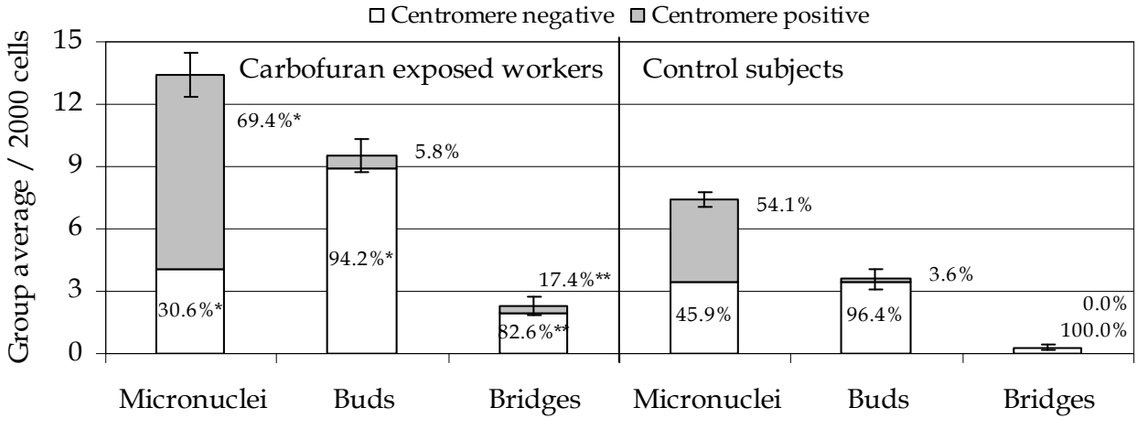

Fig. 1. Aberrant chromatin structures regarding to presence of FISH centromere signals in carbofuran production line workers and controls. *P<0.05, **P<0.01 compared to the control.

AChE activity, as a biomarker of exposure to carbamates, will not reflect possible cumulative effect of pesticides in evaluation of their adverse impact on human health. Consequently in presented study (Zeljezic et al., 2007) we did not find any correlation between plasma and whole blood AChE activity and years spent handling carbofuran formulations as assessed by multiple regression analysis (R=0.023). Average whole blood AChE activity was 94.5±1.33% (81-100%), and plasma activity 99.2±0.55% (86-100%).

Thus, to relevantly assess potential carcinogenic risk of long-term exposure to low-doses of carbamate and organophosphorous compounds, appropriate cytogenetic biomarker of exposure that will reflect cumulative effect should be determined. Interestingly, centromere containing NPBs (Fig. 2.) correlated with employment/exposure years (R=0.77, β=0.68), confirming previously reported finding that long-time occupational exposure to pesticides is capable of inducing complex chromosomal rearrangements. Additionally, multiple regression analysis considering age, gender, medical procedures and life-style factors as possible confounders revealed significant influence of carbofuran exposure duration on incidence of C+MN (R=0.83, β=0.76), NBs (R=0.86, β=0.79) suggesting that aberrant chromatin structures may be applied as nonspecific biomarkers in evaluation of cytogenetic effect of long-term carbamate exposure.

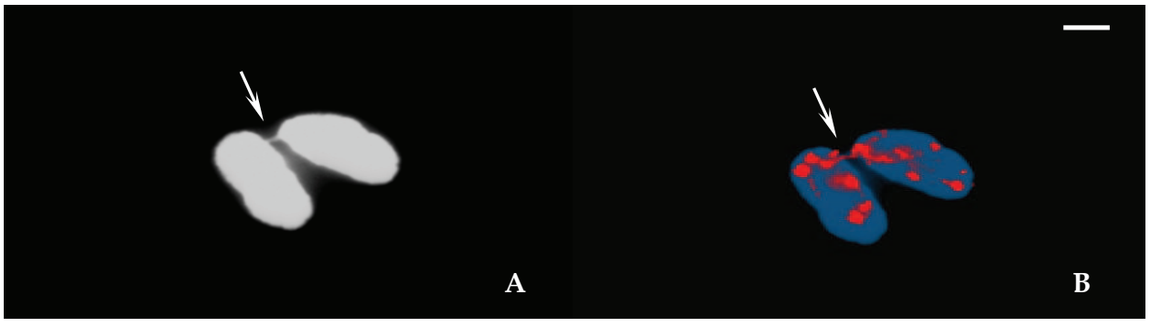

Fig. 2. Binucleated lymphocyte of a carbofuran plant worker with nucleoplasmic bridge indicated by arrow: A) DAPI staining, B) centromeric DNA dyed red (Texas Red-conjugated DNA probe), and nuclei blue (DAPI). Objective 100x, bar 1 μm.

Dynamics by which discussed cytogenetic biomarkers are being affected by pesticide exposure has been reported based on a case of acute carbofuran intoxication (Zeljezic et al., 2009). A single male worker on Furadan production line who also participated in previously discussed cytogenetic monitoring, has been transferred to medical facility with symptoms of acute anti-ChE poisoning after accidental inhalation of pesticide containing dust. The patient experienced cephalalgia, disorientation, suffocation, perspiration, weakness, fatigue, abdominal pain, and vomited. Measured whole blood AChE activity was 57% which has been significantly decreased (χ^2=16.10, P=0.0001) compared to the earlier records obtained for the same worker (83%). As shown in the Fig. 3., cytogenetic analysis revealed that 180 minutes upon intoxication frequencies of MN, C+MN, and NPBs, remained unaffected compared to the records obtained in the study prior to intoxication (P_{χ^2}=0.847; 0.683; 0.180, respectively). Only the number of C-NBs has been elevated significantly (χ^2=11.93, P=0.0006). It seems unlikely that observed NBs represent amplified AChE or BuChE genes that are being extruded from the cells. More reasonable explanation would be that these early NBs harbor expelled DNA repair complexes or which is more likely, broken chromatid/chromosome fragments as the result of genotoxicity. Latest theory might be supported by results of the alkaline comet assay. The level of primary DNA damage detected 3 hours following intoxication has been significantly increased compared to referent value for the same worker detected prior to the accident ($P_{Mann\text{-}Whitney}$=0.0019). Nevertheless, C-NBs may be indicated as possible early cytogenetic biomarkers of the effect of exposure to carbamate insecticides. In the next 72 hours, frequency of C-NB continued to increase insignificantly (χ^2=2.50, P=0.114), followed by significant occurrence of C+NBs

(χ^2=15.25, P=0.00009). Simultaneously, total MN, and proportion of MN originating from whole chromosomes were significantly increased (χ^2=10.40, P=0.0013; χ^2=10.30, P=0.0013) which categorizes them as late biomarkers of the effect.

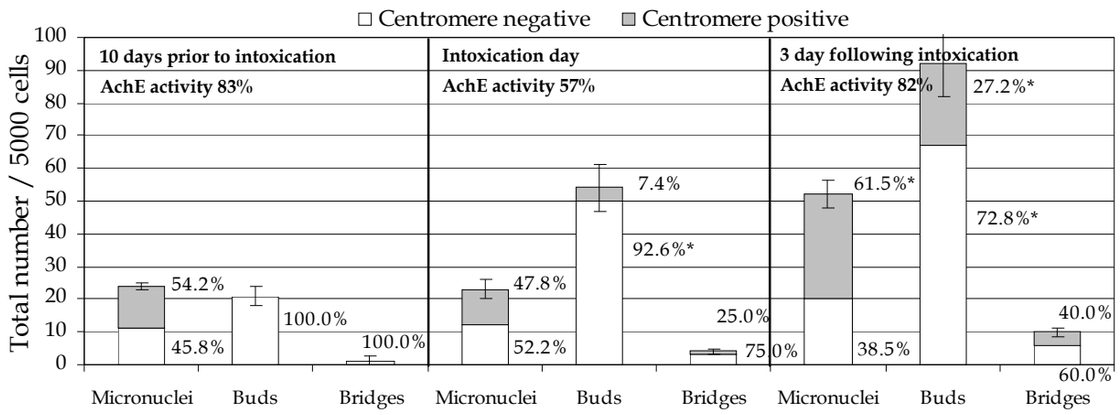

Fig. 3. Dynamics of formation of aberrant chromatin structures regarding to presence of FISH centromere signals in carbofuran intoxicated production plant worker. *P<0.05 compared to values prior to intoxication.

Conversely, even 3 days upon intoxication the level of NPBs was not significantly elevated (χ^2=2.58, P=0.1086). As already discussed, NPBs did significantly correlate with exposure to carbamate. However, NPBs are formed by dicentric or ring chromosomes whose formation is mediated through a cascade of misrepairs or significant telomere shortening. These cytogenetic events need longer period of time to occur and to form a critical pool of aberrant chromosomes that will be manifested as NPBs. Time consuming aspect of formation of chromosomes aberrant in their structure is even more pronounced in chemical than ionizing radiation genotoxicity. Some bridges will be lost due to their breakage when the daughter cells separate which also negatively reflects on their manifestation as NPBs. Thus, NPBs might be only considered as biomarkers of the effect in long-term exposure to pesticides. However, their presence in lymphocytes of individuals under pesticide exposure together with the reports indicating their presence in lymphoma patients stressed the need to look after the possible induction of translocations due to pesticide exposure. Their increased presence would indicate populations with elevated risk of tumor development.

4.3 Pesticide exposure and translocation yield assessed by FISH chromosome painting

Chromosomal translocations indicate severe impairment of genome integrity and are associated with the carcinogenic transformation of the cell, even more used as specific biomarkers in cancer diagnostics, classification, prognosis, deciding on the treatment and evaluating its efficacy. Thus, evaluation of translocation yield in population of concern is applied as a surrogate approach in epidemiological studies for estimation of cancer risk.

First attempts aiming to assess the level of translocations in peripheral blood lymphocytes of individuals exposed to pesticides started in early 1990-ties by applying G-banding technique. Method is based on treatment of metaphase spreads with trypsine and Giemsa staining that reveals a pattern of bands being specific for each of chromosome pairs. Any change in the band pattern indicates chromosomal rearrangement. Based on the

determination of patterns proceeding and following the rearrangement site chromosomes involved in the translocation may be identified. By using G-banding technique, Garry et al. (1996) detected increased (Wilcoxon rank sum test, P=0.003) frequency of rearrangements in phosphine mixing or applying subjects (1.7±0.5 per 100 metaphases) compared to the control group (0.5±0.1). However, 12 months upon some subjects ceased using the phosphine lymphocyte translocation frequency decreased to the control values. Conversely, it remained increased in individuals who continued with pesticide application. Translocations at 1p13, and 14q32 were observed in majority of applicators. Since these rearrangements are accepted to be related to non-Hodgkin's lymphoma (NHL) the authors assumed that long-term exposure to phosphine poses a risk of developing NHL. Similar results have been reported for mixed pesticide applicators (Garry et al., 1996). In applicators of multiple insecticides significantly elevated rearrangement frequency (Wilcoxon rank sum test, P=0.005), has been reported (1.4±0.3) compared to the control individuals (04±0.1). Though elevated (1.0±0.3) translocation yield in mixed herbicides applicators was not significantly affected (P=0.096). Again, 14q32 position has been involved in translocations common to most examinees indicating elevated risk of NHL. Unfortunately, both studies are characterized by rather poor pesticide exposure characterization and lack of multivariate statistical approach considering life-style factors as possible confounders; though exposed and control groups were matched by age and smoking habits. In their third study, Garry at el. (2001) applied G-banding in detection of translocations in forest and areal pesticide applicators with most prominent exposure to chlorophenoxy herbicides. Translocation, deletion and insertion yield (exposed 2.22±0.38 vs. control 0.65±0.30) has been significantly affected only in subjects applying more than 3.785 liters of herbicides per season (Wilcoxon rank sum test, P=0.003). Since Poisson regression analysis adjusted for smoking status revealed insignificant negative correlation between rearrangement frequency and measured 2,4-D urinary concentrations observed impact on induction of translocations could should be attributed to other than chlorophenoxy herbicides.

To obtain relevant results in assessing translocation frequency by analyzing G-band patterns on metaphase chromosomes requires well trained and experienced scorer, able to recognize any change in the banding sequence or width of bands that may indicate chromosomal rearrangement. Even then, due to significant variability in banding quality between metaphases of the same donor, and different donors, as well as due to terminal interchromosomal rearrangements that may not affect banding pattern, many translocations remain undetected which bias sensitivity and reliability of the technique. All these limitations classify G-banding technique laborious and inadequate for detection of cytogenetic effects resulting from low level pesticide exposures. Referred drawbacks may be circumvented by the use of FISH where single, several or all chromosomes are hybridized with DNA probes specific for each of them, and labeled with various combinations of flourochromes. As the result, each of the 22 of pairs of autosomes and 2 sex chromosomes are dyed in different color which makes easier to spot interchromosomal rearrangements and enhances the sensitivity of the method in detection of translocations. Table 3. provides an overview of studies applying FISH in translocation analysis in pesticide exposed subjects. More thorough evaluation of translocation frequency by applying chromosome painting FISH in regards to duration of pesticide exposure considering various confounders has been conducted on a group of pesticide plant workers exposed to mix of carbofuran, chlorpyrifos, metalaxyl and dodine (Zeljezic et al., 2009). In this study approach used in ionizing radiation biodosimetry has been applied, painting chromosomes 1, 2, and 4 by FISH in red,

Exposure type	FISH probe	Translocations per 100 cells vs. control	Significance	Correlation to exposure years	Reference
Mancozeb, methamidophos, captan, chlorpyrifos	Chr #1,2,4	1.21±0.97 0.92±0.82	P=0.05	N/A	Steenland et al., 1997
Carbofuran, chlorpyrifos, metalaxyl, dodine	Chr #1,2,4	1.63±0.78 0.51±0.23	$P=4x10^{-5}$	R^2=0.356, P=0.0003	Zeljezic et al., 2009
Dieldrin, toxaphene, lindane, atrazine	LSI IGH/BCL2 genes loci on Chr #14 & 18	N/A; OR($CI_{95\%}$) 2.1 (0.8-5.4) 1.0 (referent)	P=0.04	Yes; only descriptive	Chiu & Blair, 2009

Table 3. FISH in detection of translocations associated with long-term pesticide exposure.

green, and yellow and analyzing their mutual rearrangements, and those with other chromosomes (IAEA, 2001). Pairs of chromosomes 1, 2, and 4 represent 22.34% of the DNA content of the female genome and 22.70% of the male translocations detected painting them in 3 colors will represent 39.4% of total translocations involving all other chromosomes. Due to different gene densities along these chromosomes efficiency of DNA repair varies between them classifying translocations involving Chr 1 among most persistent, and those involving Chr 4 among least stable. Formula derived by Lucas & Sachs (1993) is used to extrapolate observed translocations frequencies for painted chromosomes to total genomic translocation frequency. In lymphocytes of pesticide plant workers genomic translocation frequency was significantly higher than in matching controls ($P_{ScheffePostHoc}$=0.000004), being higher in females (0.0062±0.0027 per cell) than in males (0.0043±0.0016) regardless of adjustment for the age difference. Most of detected translocations were reciprocal (2 bicolor chromosomes in metaphase; Fig. 4.), though their frequency in examinees (67.8±1.34) has been lower than among the controls (80.1±1.83) indicating involvement of rather small chromosomal fragments into rearrangements that are beyond resolution of the technique. Furthermore, while among the controls no complex rearrangements have been detected, among pesticide handling workers 8.5±0.51% translocations involved 3 or more chromosomes. Multiple regression analysis adjusted for confounders showed significant correlation between translocation yield and age (R^2=0.274, P= 0.021) and pesticide exposure duration (R^2=0.356, P=0.0003) the effect of latest being more prominent (exposure β=0.623, age β=0.524). Although distribution of translocations between chromosomes was random, involvement of chromosome 4 in rearrangements positively correlated with years of employment in pesticide production (R^2=0.48, p=0.0008). Since no significance in dependence of translocations upon age has been detected among controls (R^2=0.10, P=0.088), and since age and years of exposure significantly correlated among examinees (R^2=0.50, P=0.0000), and finally due to higher impact of exposure it may be concluded than translocations detected in pesticide plant workers are a consequence of impaired genomic stability due to long-term exposure. No cytogenetic effect of dodine or metalaxyl was revealed thus, observed genotoxicity may be attributed mainly to mixed carbofuran and chlorpyrifos exposure. As discussed in section 4.1, chemically induced single-strand breaks, abasic sites, oxidative damage lesions and alkylated bases may be transferred into DSBs by

error-prone base excision repair (BER; Maynard et al., 2009). Both, strand breaks induced in the course of BER, and oxidized DNA bases due to their topoisomerase II poisoning activity may be converted into DSBs (Khan et al., 2009). By error-prone activity either of original or non-classic pathways of NHEJ in its standard or modified repair, translocations may be formed (Weinstock et al., 2006). Indications for such cascade of error-prone damage transformation may be indicated by results of multiple regression analysis revealing high correlation of translocations with chromatid-type aberrations (R^2=0.26, *P*=0.003) in pesticide manufacturing workers being highly correlated with years of employment (R^2=0.479, *P*=0.00001).

Steenland et al. (1997) also reported significant increase in translocations by FISH painting of chromosomes 1, 2 and 4 in EBDC fungicide applicators (age adjusted Poisson regression *P*=0.05). Analyses restricted to reciprocal translocations nonsignificant exposure effect (p = 0.24) which may be mediated by significantly lower resolution of earlier FISH applications. However, total, and reciprocal translocations significantly correlated (R=0.28, *P*=0.02; R=0.27, *P*=0.02, respectively) with incidence of sister-chromatid exchanges that are manifestations of DSB repair.

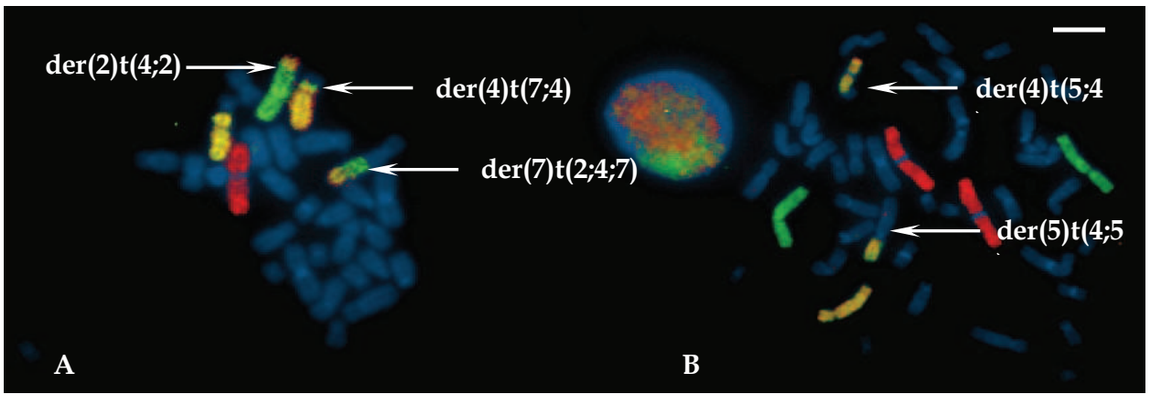

Fig. 4. Translocations in lymphocytes of the pesticide plant workers: A) complex translocations, B) reciprocal translocation t(4;5). Chr 1 – red; Chr 2 – green; Chr 4 – yellow; Objective 100x, bar 1 µm.

Similar but gene specific approach of FISH use in estimation of cancer risk due to pesticide exposure has been reported by Chiu & Blair (2009). Gene locus specific LSI IGH/BCL2 dual-fusion probe has been applied to evaluate association of t(14;18) rearrangement in NHL patients with their previous long-term pesticide exposure. Translocation t(14;18) represents the hallmark of follicular lymphoma as one of the most common adult NHLs. Exposure to crop and animal insecticides and herbicides have been associated with t(14;18) positive NHL (multivariate regression *P*=0.01). The risk of NHL for dieldrin applicators has been estimated to OR=2.4 (CI_{95}= 0.8–7.9), toxaphene OR=3.2 (CI_{95}=0.8–12.5), lindane OR=3.5 (CI=1.4–8.4), and atrazine OR=1.7 (CI_{95}=1.0–2.8) (Chiu & Brian, 2009).

5. Conclusion

Presented data indicate that within the last decade by applying the FISH technique in monitoring studies of subjects exposed to pesticides, a new set of cytogenetic biomarkers has been introduced providing us with more detailed insight in the effect of long-term

occupational pesticide exposure on genome integrity. Some biomarkers such as chromosomal rearrangements and translocation had been over a long period of time considered irrelevant in chemical carcinogenesis and cancer risk assessment due to pesticide exposure. Valuable knowledge regarding the error-prone effect of distinct DNA repair mechanisms helped us to understand how primary genome lesions induced by agrochemicals may be transformed into chromosomal rearrangements. The use of FISH in evaluation of the impact of occupational exposure to pesticides on human genome may provide us with more precise insight in the extent and type of genome damage, indicate possible specificity of pesticides of concern toward specific chromosomes or regions and help us in evaluation of pesticide exposure relevant for cancer risk.

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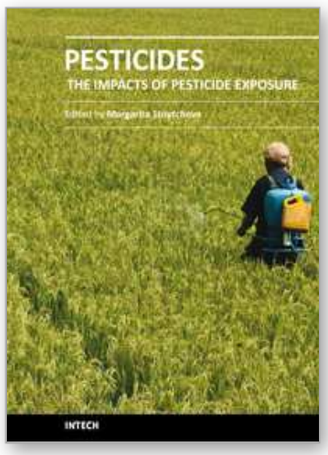

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Pesticides are supposed to complete their intended function without â€œany unreasonable risk to man or the environmentâ€. Pesticides approval and registration are performed â€œtaking into account the economic, social and environmental costs and benefits of the use of any pesticideâ€. The present book documents the various adverse impacts of pesticides usage: pollution, dietary intake and health effects such as birth defects, neurological disorders, cancer and hormone disruption. Risk assessment methods and the involvement of molecular modeling to the knowledge of pesticides are highlighted, too. The volume summarizes the expertise of leading specialists from all over the world.

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