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Evaluation of the mechanism of nucleoplasmic bridge formation due to premature telomere shortening in agricultural workers exposed to mixed pesticides: Indication for further studies

Davor Zeljezic [a], Marija Bjelis [b], Marin Mladinic [a,*]

[a] Division for Mutagenesis, Institute for Medical Research and Occupational Health, Ksaverska 2, 10000 Zagreb, Croatia
[b] Economic High School Velika Gorica, Ulica Kralja Stjepana Tomaševića 21, 10410 Velika Gorica, Croatia

HIGHLIGHTS

- Effects of pesticide exposure on relative telomere length was evaluated by Q-FISH.
- Theory of nucleoplasmic bridge formation due to telomere shortening was considered.
- No significant correlation between exposure and telomere shortening was found.
- Exposure significantly increased formation of nucleoplasmic bridges (NPB).
- Telomere-end fusion is not a way of NPB formation in studied pesticide exposure.

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ABSTRACT

Agricultural workers are often exposed to high levels of pesticides over prolonged periods of time. We attempted to determine whether exposure to multiple pesticides shortens relative telomere length (RTL) and causes nucleoplasmic bridge (NPB) formation via the mechanism of telomere-end fusion in the lymphocytes of agricultural workers.

For measuring RTL, we used quantitative fluorescent *in situ* hybridization, while NPB frequency was measured as part of the cytome assay.

Multivariate analysis of variances taking into account confounding factors (age, gender, years of exposure, smoking, and alcohol intake) did not show a decrease, but rather an increase of RTL in agricultural workers compared to control individuals. In the exposed population, NPB frequency was significantly higher compared to controls (6 times, $p < 0.05$). Multiple regression between NPB, RTL, and confounding factors was not significant. Using Spearman correlation, we did not find proof for our initial hypothesis.

Our hypothesis that telomere shortening is a mechanism of NPB origin was not proven, indicating that telomere-end fusion is not a mechanism of NPB formation under our experimental conditions for agricultural workers.

1. Introduction

The huge demand for food has led to an increase in pesticide use aimed at enhancing agricultural productivity. According to the European pesticide database, some 1300 active pesticide substances have been approved for agricultural use (EU). Everyday use of these substances may cause adverse effects for human health (Infante-Rivard and Weichenthal, 2007). In occupational settings, workers are often simultaneously exposed to a range of different chemicals and their cumulative effect on health and genome status should be determined to yield a relevant risk assessment (Bull et al., 2006). The most affected populations on a daily basis are employees in pesticide production and applicators, who are exposed to several times higher levels of pesticides over a prolonged period of time (Bhalli et al., 2006). There have been several studies assessing telomere length and occupational exposure (Zhang et al., 2013) but only one has evaluated the influence of pesticide exposure on telomeres. The aberrant maintenance of telomeres has been strongly associated with increased cancer risk (Artandi and DePinho, 2010). The telomere is a special functional complex located at the end of chromosomes that consists of

* Corresponding author. Tel.: +385 1 4682500; fax: +385 1 4673303.
E-mail address: mmladinic@imi.hr (M. Mladinic).

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tandem repeats of (TTAGGG)n DNA sequences. It is vital for maintaining genome integrity and stability (Cong et al., 2002). Among the various environmental factors affecting telomere maintenance, chemical exposure has been detected but insufficiently studied. Many studies have shown that the attrition of telomeres leads to genomic instability associated with various age-related disorders including cancers (Murnane and Sabatier, 2004). Certain types of cancer proven to have short telomeres such as non-Hodgkin's lymphoma, leukemia, brain, breast, kidney, and prostate cancer have been linked with pesticide exposure (Bassil et al., 2007). In the process of DNA repair by non-homologous end-joining mechanism, such progressive telomere shortening may result in end-to-end chromosome fusions that generate chromosomal instability or, more precisely, dicentric chromosomes that result from the fusion of two chromosomes with prematurely shortened telomeres (Mladenov and Iliakis, 2011). Nucleoplasmic bridges (NPB), manifested in the telophase of cell division, are recognized as a sensitive measure of disturbances instigated by loss of telomere integrity and resulting chromosome end fusion (Fenech, 2007). Several studies have reported that cancer patients have increased numbers of NPBs in peripheral blood lymphocytes regardless of the cancer type (McHugh et al., 2013). In a model of human intestinal cancer *in vivo*, NPB formation was linked with critically short telomeres (Rudolph et al., 2001) and similar event was observed in an *in vitro* experiment with human mammary epithelial cells (Pampalona et al., 2010). In the present study, we aimed to test several hypotheses proposed by previously cited studies regarding the effect of long-term chemical exposure on premature telomere shortening and its possible role in NPB formation. We tested whether the exposure of agricultural workers to 17 pesticides affects relative telomere length (RTL) and/or NPB formation in an exposure-duration dependent manner. By testing the correlation strength of these two cytogenetic biomarkers, we aimed to evaluate the suggested theory of NPB formation via the mechanism of telomere-end fusion for conditions of prolonged pesticide exposure, since findings suggest that such exposure may lead to elevated number of NPBs.

2. Materials and methods

2.1. Study population

Approached subjects (75 individuals) were recruited from the Neretva river valley, which is known for extensive agriculture. They completed detailed questionnaires regarding their medical history (exposure to X-rays, vaccinations, medication), lifestyle (smoking, alcohol, diet), years of exposure. Among the agriculturists approached, only those with no record of any inflammatory and/or autoimmune disease, malignancies, psychological disorders, consuming less than 4 units of alcohol per day and/or smoking less than 10 cigarettes per day, leaving 30 individuals eligible to participate in the study. Selected population of professionals (27 males; 3 females) reported occupational use of 17 different pesticides: copper (II) oxide, refined mineral oil, mancozeb, fosetyl, lambda-cyhalothrin, thiamethoxam, tiacloprid, chlorpyrifos, propineb, captan, abamectin, acetamiprid, imidacloprid, dimethoate, methomyl, spinosad and glyphosate. In Table S1 (see Supporting information) pesticides are classified according to WHO and IARC classification of pesticides. The average age of the examinees was 42.5 ± 12.9 (range 25–62), with average seasonal exposure of 13.4 ± 13.5 years (range 1–40). All individuals considered as occupationally exposed individuals were those registered as agricultural workers at "family farms", directly involved in processes of pesticide formulation handling, both dilution and application. Agriculturists in our study used all of the pesticides listed. Seasonal exposure of agricultural workers lasted from February/March to October/November, depending on weather conditions in the investigated area. Detailed information gathered by the questionnaire is shown in Table 1. Examinees reported rare and irregular use of personal protective equipment such as gloves, masks and protective overalls. Control subjects were recruited from the same region as the exposed subjects. Only individuals with no record of occupational or household/vegetable/garden exposure to pesticides were considered for inclusion in the study as control volunteers. Household/vegetable/garden exposure has been taken into account in interviewing the subjects recruited for the control group. It refers to occasional use of biocidal products within the household, and use of plant protection products in gardening as hobby since control group does not comprise professional gardeners/applicators. The above described criteria produced a group of 30 controls who matched the exposed participants by sex, smoking habit, alcohol consumption, and approximate age (45.4 ± 13.6). Control subjects completed the same questionnaires as the other group of subjects. All of the participating subjects were informed regarding the planned procedures and analyses and aims of the study. The study was reviewed and approved by Institutional Review Board and written informed consent was obtained from all study participants.

2.2. Sample collection and lymphocyte cultures

For both the exposed and control study group, blood sampling was done simultaneously – one month following the end of spraying season. Peripheral blood samples were collected by venipuncture into heparinized vacutainers (Beckton Dickinson, UK). Samples were coded and kept on ice during transportation to the laboratory for further processing.

To determine relative telomere length, we used metaphase spreads of lymphocytes arrested in the second *in vitro* metaphase (IAEA, 2001) and NPB frequency on standard micronucleus assay preparations (Fenech, 2007). For both techniques, whole venous blood cultures were established by adding 0.5 mL of whole blood to 6 mL of RPMI 1640 cell culture medium (Gibco, UK) supplemented with 15% fetal calf serum (Gibco, UK), 1% antibiotics (penicillin and streptomycin; Gibco, UK), and 1% phytohaemagglutinin (Remel, UK). Lymphocytes were incubated for 72 h at 37 °C.

2.3. Relative telomere length measurements with TFL-Telo software

To determine relative telomere length, metaphase spreads were prepared. Therefore, three hours prior to harvesting, colchicine was added to whole blood cultures at the final concentration of 0.2 μg/mL. Cultures were centrifuged, resuspended in hypotonic solution (0.075 M KCl; Sigma–Aldrich), and fixed in acetic acid/methanol 1:3 (v/v) according to IAEA protocol (IAEA, 2001). FISH was performed on one month old metaphase spreads following the supplier's instructions provided with Telomere PNA FISH Kit/Cy3 (Dako, Denmark). Slides were counterstained with ProLong® Gold Antifade Reagent with DAPI (Life Technologies, USA). Images were acquired using Olympus AX70 epifluorescence microscope (Olympus, Japan).

We used the CytoVision Fluorescence *In-Situ* Hybridizer (Applied Imaging, Germany) software to capture images of hybridized metaphases analyzed for telomere length. By applying the CytoVision Karyotyper (Applied Imaging, Germany), we obtained negative DAPI images of metaphases and karyotyped chromosomes in order to classify chromosomes within the metaphase and determine relative telomere length for each of the 24 chromosome classes.

Telomere length measurement was done according to the protocol set by Poon and Lansdorp (2001). Original images of hybridized metaphases taken with the CytoVision software were exported and used to determine relative telomere length with Telomere

Table 1
Data on demographic and lifestyle factors of occupationally exposed subjects covered by questionnaire. Corresponding data for control subjects are provided in brackets.

Questions	Answers (*n*)				
1. Gender	Women			Men	
	3 (3)			27 (27)	
2. Age	Mean value		S.D.		Range
	42.5 (45.4)		12.9 (13.6)		25–62 (25–62)
3. Years of exposure	13.4 (0)		13.5 (0)		1–40 (0)
4. Medical diagnostics 12 months prior to blood sampling	None	1 Procedure	2 Procedures		3 Procedures
	17 (18)	11 (6)	2 (6)		0 (0)
5. Pharmaceutical intake[a]	No intake	1 year	1–2 years		2–5 years
Total	24 (27)	0 (0)	0 (0)		0 (0)
Analgesic	0 (0)	3 (0)	0 (0)		0 (0)
Sedative	0 (0)	0 (0)	0 (0)		1 (0)
Antihypertensive	0 (0)	1 (1)	0 (0)		0 (0)
Antihistaminic	0 (0)	1 (0)	0 (0)		0 (2)
6. Vitamins intake	No intake	1 year		1–2 years	2–5 years
	27 (28)	2 (0)		1 (1)	0 (1)
7. Diet	Canned food			Cooked meals	Deep fried
	1 (0)			29 (29)	0(1)
8. Meat consumption	Every day			1–3 times a week	None
	11 (15)			19 (14)	0 (1)
9. Roasted meat/week	1–3 times			More than 3 times	None
	14 (24)			16 (5)	0 (1)
10. Cured meat/week	Every day	3–5 times		Rarely	None
	0 (3)	22 (11)		8 (15)	0 (1)
11. Vegetables	Boiled			Fresh	
	8 (9)			22 (21)	
12. Vegetable consumption	Few times a day	Once per day		Once per 3–5 d	Once a week
	5 (3)	13 (12)		11 (15)	1 (0)
13. Fruit	Boiled			Fresh	
	0 (0)			30 (30)	
14. Fruit consumption	Few times a day	Once per day	Once per 3–5 d	Once a week	Rarely
	0 (0)	14 (12)	11 (18)	2 (0)	3 (0)
15. Tea consumption	Few times a day	Once per day	Once per 3–5 d	Rarely	Never
	0 (6)	2 (3)	3 (3)	25 (18)	0 (0)
16. Coffee consumption	Few times a day	Once per day	Once per 3–5 d	Rarely	Never
	14 (18)	9 (6)	0 (0)	7 (4)	0 (2)
17. Smoking habits	No smoking			Smoking	
	22 (24)			8 (6)	
18. Alcohol consumption	Daily	Few times a week	Few times a month	Rarely	Never
	5 (3)	8 (10)	4 (4)	8 (10)	5 (3)

[a] Only analgesics, sedatives, anti-hypertensives and antihistaminics as pharmaceuticals that might exhibit genotoxic effect were considered as confounding factors in statistical analysis. The use of anti-rheumatics, anti-depressants, antilipemics, beta-blockers, anticoagulants was not reported by examinees.

Measurement software (TFL-Telo) (British Columbia Cancer Research Centre, Canada). To normalize variations in fluorescence intensities between metaphases caused by day-to-day variations due to the aging of the lamp, we used images of calibration slides with fluorescent beads that are captured prior to metaphase image capturing. Calibration slides with fluorescent beads were prepared by diluting 10 μL of FluoroSpheres® stock solution (0.2 μm carboxylate-modified microspheres; Invitrogen, USA) in 10 μL of PBS/fetal calf serum mixture 1:1 (v/v) (Gibco, Invitrogen, USA). On a periphery of 22 × 40 mm coverslip, 1.5 μL of microsphere suspension was deposited and a thin homogenous smear was produced on the surface of the coverslip using a microscope slide. On the day of telomere image capturing, 10 μL of ProLong® Gold Antifade Reagent (Life Technologies, USA) was dropped onto the microscope slide and covered with a microsphere-coated coverslip.

For each image capturing session, the fluorescence of 50 beads was measured by TFL-Telo software and the average fluorescence value was calculated. The average was used to calibrate the software and normalize fluorescence values of telomere images (relative telomere length) captured in the same session. Subsequently, 10 metaphase images per each individual were analyzed for relative telomere length (see Fig. S1 in Supporting information).

2.4. NPB frequency

We determined NPB on micronucleus preparations obtained according to standard procedure by Fenech (2006). Forty-four hours after the whole blood cultures for micronucleus assay were initiated, cytokinesis was arrested by adding cytochalasin B (Sigma, USA) at the final concentration of 6 μg/mL. Twenty-eight hours later, cultures were centrifuged, washed in saline solution (0.9% NaCl, Sigma) and fixed with 3:1 (v/v) methanol/acetic acid solution. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies, USA). For each subject, 1000 binucleated cells per each duplicate slide were scored to determine the total number of NPBs.

2.5. Statistical analysis

Normal distribution of data for RTL was checked using the Shapiro–Wilks test and logarithmic transformation was used in conforming them to normality. Afterwards, multivariate analysis of variances (MANOVA) with Bonferroni's *post hoc* comparison was used to test differences in RTL and NPBs between exposed and control groups considering confounding factors such as age, gender, years of exposure, smoking, alcohol consumption. Intra-group comparisons between male and female subjects were not possible due to the fact that there were only 3 female examinees.

Strength of associations between the number of NPBs and RTL at the level of autosomes and allosomes were analyzed by Spearman correlation. Multiple regression analysis was used to test the strength of the impact of age, exposure duration, and recorded lifestyle factors on relative telomere length (at the level of genome, autosomes, each of allosomes) and frequency of NPBs. The low

number of female participants did not allow for an evaluation of the influence of demographic characteristics on RTL values and NPB frequency for female subgroups. Statistical analysis was performed using Statistica 12 (StatSoft, USA).

3. Results

RTL values in the lymphocytes of the examinees are presented in Fig. 1. Multivariate analysis of variances (MANOVA) considering demographic and exposure parameters as confounding factors (age, gender, smoking, alcohol intake) showed that there was no significant difference between exposed and control subjects in RTL for each chromosome pair (Table 2).

The number of NPBs in the lymphocytes of exposed subjects was significantly higher than in the controls ($F(1,54) = 11.60$, $p = 0.0024$) when confounding factors (age, gender, years of exposure, smoking, alcohol intake) are considered (Table 3). Bonferroni's *post hoc* analysis revealed additional significance ($p = 0.0007$).

Multiple regression analysis was done to evaluate the strength of influence of each recorded demographic factor on RTL values at the level of autosomes/allosome groups and on NPB frequency. None of the evaluated factors exhibited significant influence on RTL values of autosomes, either in agricultural workers or in the control group (see Table S2 in Supporting information). Among the workers, the highest influence on RTL values of autosomes was observed for age ($\beta = -0.450$) and exposure duration ($\beta = 0.513$). When the subgroup of exposed men was considered separately, the influence of age and exposure duration was even more prominent ($\beta = -1.00$ and $\beta = 0.824$, respectively). In the control group, autosomal RTL values were most influenced by alcohol consumption ($\beta = -0.570$) and smoking ($\beta = 0.253$). Similar results were obtained for the subgroup of control men ($\beta = -0.640$ and $\beta = 0.422$, respectively).

RTL values for *X* chromosomes in the exposed group were most affected by age ($\beta = -0.370$) and exposure duration ($\beta = 0.371$), while in the controls, the highest influence was observed for alcohol consumption ($\beta = -0.500$) and smoking habits ($\beta = 0.327$). A similar pattern of effect of evaluated factors on *X* chromosome RTL values was observed in both male subgroups (see Table S2 in Supporting information). Among male agriculturists, the *Y* chromosome RTL value was most influenced by age ($\beta = -0.930$) and exposure duration ($\beta = 0.727$), while among control men, the highest influence was observed for alcohol consumption ($\beta = -0.470$) and age ($\beta = -0.320$).

NPB frequency in the exposed group was most affected by smoking habits ($\beta = 0.336$), and age ($\beta = -0.260$); in control subjects by age ($\beta = 0.176$). The effect of gender ($\beta = 0.440$) could not be considered relevant due to the small number of female participants. For the duration of exposure to pesticides, multiple regression analysis indicated a negligible influence on NPB frequency (see Table S2 in Supporting information).

Spearman correlation analysis did not reveal a significant association between RTL values of either autosomes or allosomes and NPB frequency regardless of the study group (data not shown). In the exposed group, the highest but positive correlation was detected for NPB frequency and *Y* chromosome RTL values (Spearman's rho = 0.27, $p = 0.31$). Among the controls, the number of NPBs was most correlated with the RTL values of autosomes (Spearman's rho = −0.34, $p = 0.34$) and *X* chromosomes (Spearman's rho = −0.43, $p = 0.21$).

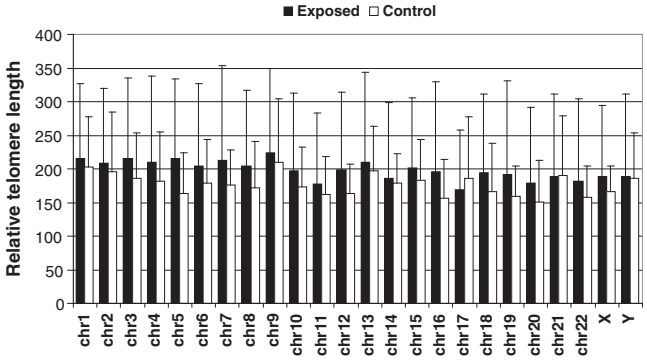

Fig. 1. Comparison of relative telomere lengths (RTL ± S.D.) expressed in arbitrary units for each chromosome pair in lymphocytes of exposed and control subjects.

Table 2
Results for multivariate analysis of variances for relative telomere length of each chromosome group in lymphocytes of exposed subjects.

Chromosome class	*F* value	*p* value
1	(1,54) = 0.03	0.85
2	(1,54) = 0.02	0.87
3	(1,54) = 0.58	0.45
4	(1,54) = 0.51	0.48
5	(1,54) = 1.62	0.21
6	(1,54) = 0.44	0.51
7	(1,54) = 0.76	0.39
8	(1,54) = 0.49	0.48
9	(1,54) = 0.02	0.86
10	(1,54) = 0.41	0.52
11	(1,54) = 0.13	0.71
12	(1,54) = 1.32	0.26
13	(1,54) = 0.11	0.74
14	(1,54) = 0.14	0.70
15	(1,54) = 0.29	0.59
16	(1,54) = 1.11	0.30
17	(1,54) = 0.23	0.63
18	(1,54) = 0.36	0.54
19	(1,54) = 0.72	0.40
20	(1,54) = 0.47	0.49
21	(1,54) = 0.01	0.96
22	(1,54) = 0.55	0.46
X	(1,54) = 0.65	0.42
Y	(1,55) = 0.01	0.92

Table 3
Number of nucleoplasmic bridges and micronuclei in lymphocytes of exposed and control subjects. Value represents the average calculated on the basis of 1000 binuclear lymphocytes analyzed on each of duplicate slides per subject.

Group	Gender	NPB ± S.D.
Exposed	Total ($n = 30$)	2.4 ± 1.6*
	Male ($n = 27$)	2.6 ± 1.6*
	Female ($n = 3$)	1.3 ± 0.6
Control	Total ($n = 30$)	0.4 ± 0.7
	Male ($n = 27$)	0.6 ± 0.8
	Female ($n = 3$)	0.0 ± 0.0

* $p < 0.05$.

4. Discussion

In the present study, we aimed to determine if long-term exposure to multiple pesticides affects premature telomere shortening and its possible role in NPB formation. Using the correlation of measured cytogenetic biomarkers, we attempted to evaluate whether the theory of NPB formation via the mechanism of telomere-end fusion is applicable to prolonged pesticide exposure, considering previous findings that such exposure may lead to elevated NPB frequency (Coskun et al., 2011). To our knowledge, only one previous study has focused exclusively on occupational pesticide exposure and telomere length. Currently, there are two

theories on the formation and origin of nucleoplasmic bridges (Fenech et al., 2011). The first proposed mechanism of NPB formation is that they originate from dicentric chromosomes formed from double strand breaks (DSB) via the error prone DNA repair pathway (Obe et al., 2002). Although the most efficient genotoxic agent in inducing dicentric chromosomes is ionizing radiation, such a result can also arise from long-term exposure to chemical substances (Costa et al., 2006). The second suggested mechanism, which is more relevant for occupational exposure to pesticides and which we aimed to test in the present study, is telomere-end fusion. This process can be triggered by premature telomere shortening (Fenech, 2007). In a model of human intestinal cancer *in vivo*, an association was proven between the observed number of NPBs and critically short telomeres (Rudolph et al., 2001). Pampalona et al. (2010) showed that cell cultures established from healthy human mammary primary epithelial cells following their immortalization exhibited high levels of telomere erosion and significantly increased the formation of abnormalities in chromatin organizations. The authors proposed that the elevated induction of NPBs was mediated by end-to-end fusion of chromosomes with critically short telomeres. For telomere-end fusion to occur, aberrant DNA replication in the telomere region, or unfinished telomere replication in one or two chromosomes, must take place. Telomere fusion is expected to occur more frequently in senescent cells or in the case of long-term exposure to chemical genotoxic agents (Thomas and Fenech, 2011). There are numerous studies linking pesticide exposure with an increase in the number of micronuclei, but very few considered analyzing cells for all chromatin instabilities suggested by micronucleus scoring criteria (Fenech, 2006). So far, there has been only one study showing the adverse effect of pesticide exposure on NPB formation in lymphocytes of open-field agricultural workers (Coskun et al., 2011). However, the observed increase was not statistically significant. Other studies indicate a significant association between an elevated number of NPBs and occupational exposures to different chemicals such as 1, 3-butadiene (Xiang et al., 2012), vanadium pentoxide (Ehrlich et al., 2008), organic bentonite particles (Huang et al., 2013), lead (Minozzo et al., 2010), and polycyclic aromatic hydrocarbons (PAH) (Duan et al., 2009). In our study NPB frequency was significantly higher among the agricultural workers, but multiple regression analysis did not reach significance between years of exposure and number of NPBs. According to general knowledge, increased level of telomere erosion is to be expected in long term exposure to genotoxic agents, but a gap that needs to be answered still exists, because the results of epidemiological studies are ambiguous. Besides, there have been no univocal outcomes of attempts to correlate telomere length with different lifestyle and demographic factors such as age, gender, exposure time, health conditions. A study by Shin et al. (2010) reported significant positive correlation between the serum concentration of persistent organic pollutants (POP) and telomere length of chromosomes of peripheral blood leukocytes, indicating that exposure to low levels of POPs (polychlorinated biphenyls, organochlorine pesticides, and polybrominated diphenylethers) positively affects telomere length. Moreover, Li et al. (2012) found telomere elongation in leukocytes of women exposed to arsenic through drinking water. Telomere length was inversely associated with age and body mass index (BMI). Likewise, Dioni et al. (2011) compared telomere lengths in chromosomes of leukocytes of steel workers at the first day of the working week and after 3 consecutive days of exposure. The authors found elongation of telomere length following exposure to inhalable particulate matter (PM). An increase in leukocyte telomere length has also been positively correlated with exposure to high levels of ambient PM in the blood of truck drivers compared to a group of office workers (Hou et al., 2012). For individuals occupationally exposed to benzene, Bassig et al. (2012) reported increased leukocyte telomere length compared to unexposed subjects. There were no data regarding any confounding factors influencing telomere length in this particular study.

Our results regarding the comparison of telomere length under conditions of seasonal exposure to multiple pesticides are in agreement with the above cited studies. There is still a knowledge gap in understanding the exact mechanism of telomere elongation caused by chemical exposure. Bassig et al. (2012) and Li et al. (2012) proposed that telomere elongation under the studied exposure conditions was mediated by increased telomerase activity, while Dioni et al. (2011) and Hou et al. (2013) assigned longer telomeres to the effect of sustained inflammatory mechanisms.

Conversely, findings published in several other papers have linked long-term chemical exposure with premature telomere shortening and only one study on pesticide exposure. Hou et al. (2013) reports a significant shortening of RTL in buccal cells of agricultural workers exposed to several pesticides. This work is not comparable with our findings because active substances to which individuals were exposed are banned for use in EU and as a consequence have completely different mechanism of action, levels and mechanism of geno/toxicity. Individuals included in our study use pesticides allowed for use in EU. Therefore, results of these two studies are understandably inconsistent. Further, Pavanello et al. (2010) observed a decrease in lymphocyte telomere length of coke-oven workers exposed to PAHs. The authors suggested that telomere shortening was mediated by the formation of PAH–DNA adducts. Additionally, telomere erosion was stimulated by oxidative damage to DNA induced by oxidative stress due to PAH activation. Shorter telomeres significantly correlated to years of employment but not age. The same adverse effect of exposure on telomere length was observed for individuals working in the rubber industry exposed to N-nitrosamines (Li et al., 2011) battery manufacturing plant workers exposed to lead (Wu et al., 2012) and traffic officers exposed to benzene and toluene (Hoxha et al., 2009). As suggested by the authors, observed premature telomere shortening was mediated by oxidative stress induced by inhaled substances. Contrary to the previously discussed results of Hou et al. (2012) this study found that longer exposure (up to 10 d) to PM in truck drivers shortened telomere length. In addition, McCracken et al. (2010) reported a decrease in leukocyte telomere length in the general population due to exposure to black carbon traffic-related particles. Their results on telomere length were inversely associated with age. Considering that the participants in the study were recruited from the general population, as was the case for our control subjects, we may emphasize the congruence of an inverse association of telomere length and age in both studies.

One of the aims of the present study was to assess the fitness of NPB formation theory by end-to-end fusion of chromosomes with eroded telomeres (Fenech et al., 2011) in conditions of seasonal occupational exposure to multiple pesticides. For this purpose, we tested the strength of dependence of 2 evaluated biomarkers: (a) NPB frequency and (b) RTL in lymphocytes of agricultural workers by Spearman correlation analysis. Even though we did observe a significantly increased level of NPBs in the exposed subjects, when compared to the controls, no significant difference in RTL values was found. Accordingly, no correlation between frequency of NPBs and autosomal or allosomal chromosomes, was found (data not shown). Furthermore, among the agricultural workers, RTL values for both chromosome groups were positively associated with NPB values, which was in contradiction with our initial hypothesis. Based on the results of the statistical analysis, we may suggest that the observed NPBs in pesticide-exposed subjects were not formed by the mechanism of end-to-end fusion due to premature telomere shortening. The NPBs that occurred did not necessarily have to be positioned in the telomeric regions of

chromosomes. Kisby et al. (2009) showed that agricultural workers exposed to pesticides had higher levels of oxidative stress than controls. Oxidative DNA lesions during replication, by acting as topoisomerase II poisons, may be converted to permanent DSB (Maynard et al., 2009). In turn, DSB via error-prone repair pathways such as NHEJ and ectopic homologous recombination may be converted into dicentric chromosomes that can be manifested as NPBs (Obe et al., 2002).

This study has several advantages and limitations. First of all, no similar study has focused exclusively whether occupational pesticide exposure shortens telomere length and causes chromatin instabilities such as NPB via the mechanism of telomere-end fusion. Second, a major advantage of our study is that agricultural workers had real multiple exposures of allowed pesticides reported through the questionnaire. As for limitations, we analyzed RTL in individuals within an appropriate age range (25–62), but the sample size ($n = 60$) was rather small. Therefore, a bigger sample size might be necessary in order to detect a significant correlation between RTL, age and/or other confounding factors and perhaps obtain statistical significance. Type of exposure as an outdoor application could not be entirely controlled and recorded. Real multiple pesticide exposure was evaluated only through the questionnaire because real field exposure was beyond controlled conditions. This issue was also present in majority of similar studies that investigated occupational exposures to multiple pesticides.

5. Conclusion

Agricultural workers seasonally exposed to multiple pesticides, in addition to higher levels of chromatin instabilities (i.e. nucleoplasmic bridges), exhibited slightly longer RTL in peripheral blood lymphocytes, indicating that nucleoplasmic bridges arise from dicentric chromosomes. These features were found in subjects intermittently exposed to pesticides, suggesting that they do not have higher cancer risk due to occupational exposure. However, future investigations are needed in order to confirm this observation and confirm whether other chemical exposures cause NPB formation by telomere-end fusion. Another method for determining telomere length such as qPCR of flow cytometry should be considered to further support or refute our findings with a broader follow-up study.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.05.085.

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