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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

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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.

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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

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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

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