

## **THE LEVEL OF MICRONUCLEI AND NUCLEAR ANOMALIES IN BUCCAL CELLS OF THE ORAL CAVITY OF WOMEN INDIRECTLY EXPOSED TO CHLORINE PESTICIDES**

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### **ABSTRACT**

*The article presents the study results of the level of micronuclei and other nuclear anomalies in exfoliative cells of the oral mucosa of 70 healthy parturient women from 2 agricultural regions of Armenia.*

*The women were farmer wives and were indirectly exposed to chlorine pesticides because the residues of DDE (metabolite of DDT) were found in their breast milk and blood. As a control we studied 35 healthy parturient women who were not exposed to pesticides and live in the capital of Armenia - Yerevan. No traces of pesticides were found in biological fluids of these women.*

*Exfoliated buccal cells were obtained from both cheeks, washed with physiological saline, then fixed with 80% methanol on slides, stained with Feulgen reaction and counterstained with Fast Green. From each study participant 2000 differentiated cells were analyzed. All nuclear anomalies and basal cells were analyzed under bright field and fluorescent microscope.*

*It has been shown that indirect exposure to chlorine pesticides with presence of their residues in breast milk does not change significantly the level of the nuclear anomalies in exfoliated buccal mucosa cells except nuclear buds which reflect gene amplification.*

*Although the differences compared to the control do not reach statistical significance (except nuclear buds), the results show that further investigations in this area are certainly warranted with increased number of participants and more detailed biochemical analyses of the study participants.*

**KEYWORDS:** *chlorine compounds, pesticides, micronuclei, chromosome-defective, micronucleus tests, mouth mucosa*

### **INTRODUCTION**

Chloro-organic pesticide dichlorodiphenyltrichloroethane (DDT) was widely used some decades for prevention of malaria and also in agriculture as a pesticide [Snedeker S.M. et al., 2001].

Although DDT is banned in most countries, the Stockholm Convention permits the use of the pesticide for indoor spraying for malaria [Snedeker S.M. et al., 2001, Gajski G. et al., 2012]. Once sprayed in the environment, DDT can remain in soil for more than 30 years. DDT and its break-

down product, DDE (dichlorodiphenyldichloroethylene), are highly lipophilic. They persist in the environment and bioaccumulate in humans and animal fat tissues (also in mammary glands) because of their long half-lives (more than 6 years). These chemicals can be excreted via breast milk [Snedeker S, 2001; Bazikyan G, 2006; Ennaceur S et al., 2008; Gajski G et al., 2012].

At present DDT and/or its metabolites can be found at low concentrations almost anywhere in the environment including human blood and breast milk [Ennaceur S et al., 2008; Galassi S et al., 2008; Glynn A et al., 2011; Gajski G et al., 2012]. DDE, the main metabolite of DDT, was found at concentrations of between 15 to 720 ng/g of fat in human milk [Galassi S et al., 2008; Glynn A et al.,

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2011]. In Tunisia the level was much higher - 2100 ng/g [Ennaceur S et al., 2008]. The level of DDE in some samples of breast milk exceeds what is allowed to be sold commercially in any other milk product [Crinnion W et al., 2009].

The possible genotoxic activity of DDT and DDE has been studied, both in *in vitro* and *in vivo* investigations, and the data are inconclusive. Nevertheless, it seems that DDT and related compounds are believed to have no genotoxic hazards at environmentally relevant concentrations [Ennaceur S et al., 2008].

But some recent data suggest that DDT and DDE may induce genotoxic effects in cultured human lymphocytes [Gajski G et al., 2012; Geric M et al., 2012] and hemocytes of zebra mussels [Binelli A et al, 2008] at environmentally relevant concentrations.

DDT was considered by the International Agency for Research on Cancer (IARC) as compound of group 2B (probably carcinogenic to humans), but from 2015 the compound belongs to the group 2A (possibly carcinogenic to humans) [Geric M et al, 2012].

Recently the environmental monitoring was carried out in some regions of Armenia where chloro-organic chemicals were used for long time. It was found that DDT and DDE were found in water, soil, and eatable plants such as apples and potatoes [Tadevosyan N et al, 2012]. Also DDE was found in breast milk of parturient women in agricultural regions of Armenia at concentrations of 129 ng/g fat. In the frame of the same study, micronucleus assay in buccal mucosa cells of the women living in agricultural regions of Armenia was studied but no increase in micronucleus frequencies was found [Parsadanyan G. et al, 2013].

Recently the buccal mucosa micronucleus cytome assay was validated and suggested for application in biomonitoring studies [Thomas P et al, 2009]. This method gives possibility to evaluate not only cytogenetic aberrations but also to register cytotoxic action [Bolognesi C et al, 2013].

Present study aimed to evaluate possibly cytogenetic and cytotoxic action of indirect exposure to DDT of women living in agricultural regions of Armenia.

## MATERIALS AND METHODS

The work was carried out on the basis of the Laboratory of Environmental Hygiene and Toxicology by Scientific Research Center of State Medical University of Yerevan, Armenia. In total, 105 women were under investigation, 35 in each group of the different regions of Armenia (piedmont, flatland). The agricultural regions are Aragatsotn and Ararat, as well as the city of Yerevan. All subjects were born in mentioned regions and live there permanently. Written consent was received from all the participants of the study. None of the women abused harmful excesses (smoking before and during pregnancy, drinking alcohol). The women were wives of farmers and they were sometimes involved in the work with pesticides but mostly dealt with husbands' clothes contaminated with DDT and possibly other pesticides/herbicides. In breast milk samples of all these women the presence of DDE was observed at mean concentration of  $5.4 \pm 1.3 \mu\text{g/L}$  milk [Tadevosyan N et al, 2012]. As a control, 35 parturient women living in Yerevan were recruited who never were exposed to pesticides. In no one breast milk sample of these women DDE was observed.

Exfoliated cells (Figure 1a) were collected from both cheeks of all parturient women involved in this study with wooden spatula. Cells were washed twice in plastic tubes containing 10 ml of buffer solution (0.1 M EDTA, 0.01 M Tris-HCl and 0.02 M NaCl, pH=7.0) and fixed in 80% cold methanol overnight.

The cells were centrifuged at 1000 rpm/min, supernatant (methanol) was discarded, and 50  $\mu\text{l}$  of the cell suspensions were dropped onto wet cold glass slides and dried overnight in the dark at room temperature. For Feulgen staining, the slides were placed in beakers with 5.0 M HCL at room temperature for 15 min, rinsed with distilled water (15 min) and subsequently stained with Schiff's reagent for 90 min. The analysis of 2.000 differentiated cells was carried out according to established protocols

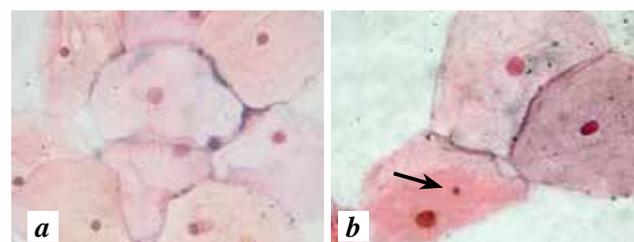


FIGURE 1. Exfoliated cells: a) normal and b) with micronucleus

[Thomas P et al., 2009] both under bright light and fluorescence with the microscope (Micors, Austria).

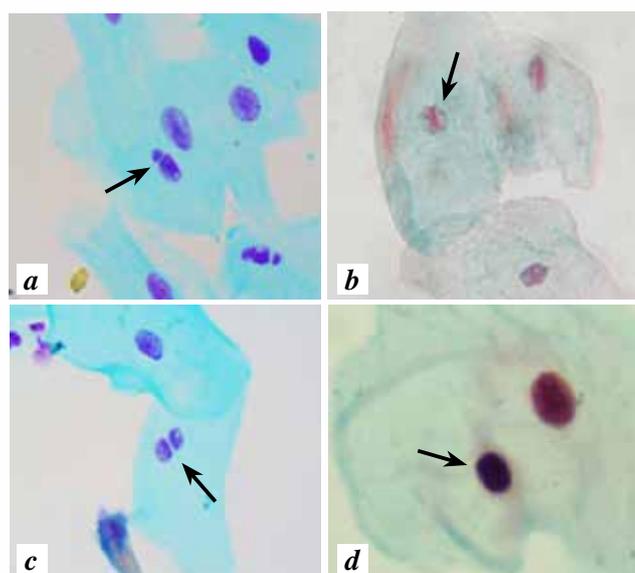
Cells with binucleates (2 nuclei), condensed chromatin, pyknosis (very small nucleus), broken egg (BE, MN attached to the main nucleus by the thread or stalk), karyorrhexis (nucleus broken to pieces) and karyolysis (cells with ghost nucleus) were registered along with cells with MN (Figure 1b, 2) [Thomas P et al., 2009; Bolognesi C et al., 2013].

All chemical were from Carl Roth, Germany. The statistical analysis was carried out by means of GraphPad Prism, version 3.02.

## RESULTS

Antropometric data of the study participants are presented in Table 1. As can be seen, no difference was between exposed and control women in regard of age and body mass index. The levels of DDE in breast milk in two groups of exposed women were almost the same. No traces of DDE were observed in the all samples of breast milk of women living in Yerevan.

As can be seen in Table 2, there was no significant difference between the groups in regard to the number of micronucleus cells. But both total number of micronuclei and the number of micronucleated cells were increased in exposed women although the difference did not reach statistical significance ( $p=0.8$  and  $p=0.59$ , respectively). The same regularity was found with all other parameters of the cytome assay. In the case of nuclear buds the difference was significant in



**FIGURE 2.** Other nuclear anomalies in exfoliative cells: a) broken egg, b) condensed chromatin, c) binucleate, d) pyknosis

**TABLE 1**

### Antropometric data of the study participants

Studied parameters	Investigated women groups (n=35)		
	Artashat	Ashtarak	Yerevan, control
Age, years	22.2±1.28	23.6±1.64	25.4±1.82
Body mass index	20.8±1.1	21.4±1.3	22.8±1.6
DDE content in breast milk, $\mu\text{g/L}$	5.9±1.7	4.9±1.5	ND

NOTES: The data are present as means  $\pm$  SE; ND – not detected

exposed women (increase by 76% and 93% in women living in Artashat and Ashtarak, respectively;  $p < 0.05$  in both cases). nuclear buds is considered to be connected with genetic toxicology events, such as exclusion of amplified DNA from the nucleus [Thomas P et al., 2009; Bolognesi C et al., 2013].

Some parameters reflecting cytotoxic effects, such as karyorrhexis, karyolysis and condensed chromatin were increased by up to 50%. Not significant increase of basal cells numbers (by ca. 50%) which indicates acceleration of cell proliferation was observed in exposed women.

## DISCUSSION

The increase of most parameters of the cytome assay (although not significant) indicates that there is genetic instability in organisms of exposed women. The only parameter increased in exposed women was nuclear buds which is considered to be connected with genetic toxicology events, such as exclusion of amplified DNA from the nucleus [Thomas P et al., 2009; Bolognesi C et al., 2013].

Engel L. and co-authors (2005) studied breast cancer frequencies in women, wives of farmers living in Iowa and North Carolina, either involved or not involved in pesticides use. They found increased levels of breast cancer compared with cohorts of women not exposed (directly or indirectly) to pesticides. A similar study was also conducted in Armenia in 2006. It was found that a high incidence of breast cancer was detected in the investigated two regions of Armenia, which is comparable to the level observed in the capital of Armenia. In other regions of Armenia the incidence was significantly lower. [Bazikyan G, 2006].

The International Agency for Research on Cancer classified DDT as “probably carcinogenic to humans (Group 2B)” (IARC 1991) because of high

TABLE 2

Micronuclei and other nuclear anomalies in exfoliated buccal cells of women from three regions of Armenia

Studied parameters (‰)	Investigated women groups (n=35)		
	Artashat	Ashtarak	Yerevan, control
Cells with micronucleus	0.96±0.51	1.06±0.44	0.80±0.51
Total number of micronucleus	1.24±0.89	1.18±0.90	0.90±0.76
Nuclear buds	3.56±1.09*	3.66±0.82*	1.85±0.45
Binucleates	21.65±3.73	20.58±3.18	18.56±4.12
Karyorrhexis	17.50±4.05	18.94±3.30	12.08±5.16
Karyolysis	43.46±4.14	42.53±2.64	30.52±5.56
Condensed chromatin	22.54±4.62	20.56±2.74	14.56±3.15
Pyknosis	0.67±0.60	0.48±0.42	0.60±0.68
Basal cells	12.00±1.93	13.06±1.71	8.16±1.83

NOTES: The data are presented as means ± SD; \* - p<0.05

rates of the induction of liver tumors in rats and mice. Epidemiological studies did not find an association between DDT exposure and cancer risk. The results of few studies suggested that DDT exposure might be associated with lung cancer and lymphomas. There is also some evidence that exposure to DDT and subsequently increased levels of DDE in women are associated with a higher risk of breast cancer. The data concerning genetic toxicology of DDT and DDE presented in the IARC monograph are inconclusive [Snedeker S, 2001].

Due to DDT use despite the ban from WHO and long half-life of this compound in the environment and in human body, interest to genotoxicity and carcinogenicity of DDT and its metabolites was again increased [Gajski G et al., 2012; Loomis D et al., 2015; Harada T et al., 2016].

Recently Canales-Aguirre A. and co-authors (2011) evaluated genotoxicity induced by inhalation of the pesticide DDT on lymphocytes and buccal exfoliated cells (micronucleus test) and mammary gland cells (the comet assay) obtained from adult female Wistar rats. In all tests, positive results were obtained which suggest that DDT is genotoxic agent. The number of micronuclei in buccal cells of exposed rats increased significantly up to 1400-fold compared with the control (28.0‰ vs. 0.02‰). This finding supports the possibility to found genotoxic effect in buccal cells after exposure to DDT.

Some authors studied micronucleus frequencies induced by DDT and DDE in human lymphocytes at various concentrations [Ennaceur S et al., 2008; Gajski G et al., 2012; Geric M et al., 2012]. Geno-

toxic concentration of DDT and DDE were comparable with environmental concentrations of these compounds in the first study. It is noteworthy that the rates of nucleoplasmic bridges (which reflect dicentric chromosomes) and nuclear buds (which reflect gene amplification) were also significantly increased. Furthermore, the compounds at the same concentrations induced DNA damage in human lymphocytes in the comet assay [Geric M et al., 2012]. In the third study, the concentrations which induced genotoxic effects, were much higher. It is noteworthy that we also found increased levels of nuclear buds in buccal cells which is an evidence of genetic instability.

Potential danger of DDT and DDE at environmentally relevant concentrations was also shown by Binelli A. and co-authors (2012) in mussels which support the results of Geric M. and co-authors (2012).

The IARC experts [Loomis D et al., 2015] and Harada T. and co-authors (2016) stated that DDT is not directly genotoxic agent and its genotoxicity is due to oxidative stress induced in target cells.

In conclusion, in this pilot study we found in epithelial cells of women indirectly exposed to DDT (possibly also to other chlorine pesticides) increased level of total number of micronucleus and the frequencies of most nuclear anomalies reflecting cytotoxicity. Although the differences compared to the control do not reach statistical significance, except the rates of nuclear buds, the results show that further investigations in this area are certainly warranted with increased number of participants and more detailed biochemical analyses of the study participants.

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