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Advanced Clinical Approaches for The Prevention of Dental Caries and Implicated Disease

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Micronucleus frequency of the buccal epithelial cells on pesticide-exposed female farmers in Dieng village, Central Java

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Abstract

Pesticide is a toxic material used to eradicate physical intruder of crop, including pests, diseases, or weeds. Long-term exposure of pesticide to human body may give genotoxic effects at cellular level, as its substances are chemically bond with the components of DNA triggering the damage of DNA and chromosomes. Micronucleus, additional nucleus on the cells, presents as a result of the termination of the chemical bonds of DNA. The aim of this study was to investigate the frequency of micronuclei of buccal epithelial cells on pesticide-exposed female farmers in Dieng Village, Central Java. This research was conducted on 36 female villagers of Dieng Plateau. The first group consisted of 18 subjects who had regularly and directly exposed by pesticides due to their job as farmers. The control group consisted of 18 subjects were unexposed to pesticides. Buccal epithelium cells of all subjects was collected by swabbing the right and left buccal mucosa using cytobrush, fixed and stained using Feulgen-Rossenbeck method. The micronuclei frequency was counted per 1000 epithelial cells. Data was analyzed using Independent Sample t-test. The statistical analysis showed a significant difference between exposed and unexposed groups (p< 0,01). It is concluded that there was an increase in the micronucleus frequency of buccal epithelial cells on pesticide-exposed female farmers in Dieng Village. Moreover, direct long-term exposure of pesticides may harm and damage the human body cells at the gene level.

Keywords: micronucleus, pesticide, genotoxic

Introduction

The use of pesticides and other chemicals on a large scale and continuously on the vegetable farms not only destroys the natural fertility of the soil, but also threatens the health of the farmers in the region of Dieng Plateau, Wonosobo, Central Java. A quick sampling conducted by Wonosobo Public Health (2009) on twenty farmers in Dieng village and surrounding area, showed that moderate to high level of pesticides was found in blood sample of all subjects [1]. This evidence shows that farmers are very susceptible to be exposed by these toxic chemicals. The toxicity effects of pesticides may reach deeper into the gene level. The genotoxic effects of pesticidemay cause metaphase and anaphase impairments, abnormality of chromosomes and micronucleus formation [2]. Micronucleus (micronuclei –pl.) is a small nucleus-like structure found in the cytoplasm of dividing cells that suffered from genotoxic stress, with the size of less than one fifth of nucleus. Micronucleus is formed from accentric fragments or lagging chromosomes during mitosis. Micronucleusstakes shape on the stage of telophase [3]. Evaluation of micronucleus from buccal epithelial cells smear is one of non-invasive DNA damage detection method that has been used in more than 40 laboratories from different
countries. The smear of buccal cells were stained with modified Feulgen-Rosssenbeck method[4]. The purpose of this study was to determine the frequency of micronucleus in buccal epithelial cells of pesticide-exposed female farmers in Dieng village.

Materials and Methods
Subjects / Materials
This research had been granted the ethical clearance sertificate and all subjects had understood and signed the informed consent form. Thirty-six female villagers of Dieng village, Wonosobo-Central Java, aged between 19-35 years old were involved in this research and divided into two groups. Exposed group consisted of eighteen subjects who had longerterm direct exposure to pesticides due to their job as vegetable farmers. Un-exposed group, as a negative control group, consisted of eighteen subjects who never had direct contacts with the pesticides. Main research material used in this research was modified-Feulgen-Rossenbeck dye [5].

Methods
Epithelial cells were collected by smearing buccal mucosa using cytobrush moistened in 0.09% NaCl. Swabbing was done by rotating the cytobrush at least 360° on the right buccal mucosa and continued on the left buccal mucosa. Cells attached to the cytobrush were rubbed on an object glass and fixed in fresh metanol-acetate (3:1) solution. The staining was based on the modified Feulgen-Rosssenbeck method [5]. The cells on object glass were immersed in 5M HCl solution at room temperature for 15 minutes, and then washed with distilled water for 10-15 minutes. Schiff's reagent, as the primary stain, and 1% Fast Green --as the counterstain, were dropped on the object glass for 90 and one minute, respectively. Micronucleus identification was done under light microscope (200x magnification) and observed using a computer monitor (100x magnification). Micronucleus was characterized as a spherical nucleus-like structure surrounded by a membrane, less than one-third size of the nucleus, with the similar texture compared to the nucleus. The color of both micronucleus and nucleus were dark green in Feulgen-Rosssenbeck staining. Micronucleus counted in this research was the nucleus that did not overlap with the nucleus, but the size, shape and color was clearly identified. Micronucleus frequency was calculated in units per 1000 cells.

Results
Under various contrast of light microscope, a dark-green color of micronucleus could be easily distinguished from the nucleus, as well as debris or bacterial artefacts (Figure. 1)
The micronucleus frequency of pesticide-exposed control groups were expressed in the number of micronucleus-positive cells per 1000 epithelial cells. Shapiro-Wilk normality test showed that both groups had a normal distribution of data, therefore parametric statistical tests can be performed ($p > 0.05$). The mean and standard deviation of the frequency of micronucleus subjects exposed group and the control group was shown in Figure 2.

![Figure 2. Mean and standard deviation of micronucleus frequency of buccal epithelial cells](image)

Independent sample t-test was used to determine the significance of the mean difference between the two groups. Table 1 shows a summary of the independent sample t-test.
TABLE 1. SUMMARY OF RESULTS OF INDEPENDENT SAMPLE T-TEST BETWEEN TWO GROUPS

<table>
<thead>
<tr>
<th>Groups</th>
<th>Micronucleus Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t</td>
</tr>
<tr>
<td>Exposed</td>
<td>14.07</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
</tbody>
</table>

p<0.05 : significant difference

Independent sample t-test resulted a significant difference between the mean of micronucleus frequency of pesticide-exposed groups compared to a control group (p <0.05). These results suggested that pesticide exposure may increase micronucleus frequency of buccal epithelial cells.

Deeper analysis done on pesticide-exposed group showed that the increase of micronucleus frequency were in line with the duration of exposure. Based on quartile data, which were 3.5, the distribution of exposure were divided into 3 categories and then classified into three categories, <4 years, 4-6 years, and >6 years.

TABLE 2. SUMMARY OF ONE-WAY ANOVA BETWEEN THREE GROUPS OF EXPOSURE DURATION

<table>
<thead>
<tr>
<th>Duration of exposure</th>
<th>Mean ± SD</th>
<th>F</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;4 years</td>
<td>15.67±3.2</td>
<td>5.749</td>
<td>0.014</td>
</tr>
<tr>
<td>4-6 years</td>
<td>21.36±3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;6 years</td>
<td>24.75±3.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p<0.05 : significant difference

Analysis of One-Way ANOVA (Table 2) showed a significant difference of micronucleus frequency between groups (p = 0.014). The longer the duration of pesticide exposure, the higher the micronucleus frequency. Table 3 showed Post Hoc LSD test result of micronucleus frequency between groups of the exposure duration.

TABLE 3. SUMMARY OF POST HOC LSD TEST BETWEEN THREE GROUPS OF EXPOSURE DURATION

<table>
<thead>
<tr>
<th>Comparison between groups</th>
<th>Mean difference of micronucleus frequency</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 4 years to 4-6 years</td>
<td>5.7</td>
<td>0.025</td>
</tr>
<tr>
<td>&lt; 4 years to &gt;6 years</td>
<td>9.1</td>
<td>0.004</td>
</tr>
<tr>
<td>4-6 years to &gt;6 years</td>
<td>3.4</td>
<td>0.12</td>
</tr>
</tbody>
</table>

LSD Post Hoc Test results showed differences of micronucleus frequency between groups of pesticide exposure duration. Micronucleus frequency of <4 years group showed a significant difference (p <0.05) compared to the group of 4-6 years, as well as, between the group of <4 years and > 6 years (p > 0.05). Only between the group of 4-6 years and > 6 years showed no significant difference in micronucleus frequency. This results showed that after 4 years of pesticide exposure, micronucleus frequency performed a remarkable increase.
Discussion
Pesticides have long been used by farmers in Dieng village to boost agricultural production. The long term-use of pesticides increase the dosage time by time. According to the field observation and direct interview, wearing of body protective gears such as masks and gloves are less noticed by farmers in this area. This condition may increase the risk of pesticide exposures on the body. Oral cavity is the first barrier of the inhalation or ingestion of carcinogens and may play roles in metabolism of reactive substances [6]. Therefore, epithelium of oral mucosa acts as a susceptible target of genotoxic effects of carcinogenic substances through inhalation or ingestion.

The mechanism of chromosome damage due to chemical pesticides is a process of trans-alkylation of DNA [7]. Electrophilic is the primary component of all types of pesticides that donates an electron pair to form a chemical bond called nucleophile to the nucleus. Nucleophile breaks $P = O$ and $C = O$ bonds of phosphorus and carbon of DNA through phosphorylation and alkylation processes. The chemical reaction of nucleophilic substitution causes lesions on cellular level called genotoxic effects [8].

Damage leading to the micronucleus formation occurs in the basal layer of epithelial tissue while undergoing cell-mitosis. Micronuclei are easily found in superficial epithelial cells instead of in the basal cells. This is likely due to the rapid turnover of epithelial tissues that brings the cells to the surface [9]. A constant turn-over process of superficial layer cells is followed by the division of basal stem cell. In the process of basal stem cells division, the damage chromosome persists to the segmented nucleus, and appears in the mitotic cytoplasm daughter cells as a micronucleus [6].

Results of statistical analysis using showed a significant increase in micronucleus frequency of buccal epithelial cells in pesticide-exposed subjects. The increase was due to the nature of the genotoxic chemical pesticides that can cause damage to the chromosomes in the nucleus of buccal epithelial cells. The chemicals ingredients of pesticides used by farmers in the village of Dieng are cypermethrin, beta-cyfluthrin, mancozeb, and isopropyl amine glyphosate. This chemicals not only leave residues on human organs, but also have some genotoxic effects [10]. Cypermethrin has the potential carcinogenic and genotoxic in mammals, including humans. Beta-cyfluthrin has a lower genotoxic potential and has been widely studied. Mancozeb pesticide active substances and isopropyl amine glyphosate in small doses cause irritation to the skin and eyes, while at high doses these chemicals accumulate in organs. In the long-term use, these substances are carcinogenic and genotoxic and may trigger tumor or cancer [11].

Chronic exposure of pesticides for more than two years increase the frequency of micronucleus significantly compared with the control group. Research conducted by Yadav et al. [12] showed an increase in DNA damage is directly proportional to the duration of exposure to pesticides. In this study, the duration of exposure to the pesticide-exposed subjects was two to eight years. Duration of exposure was categorized into 3 categories: <4 years, 4-6 years, and> 6 years. The significant raise of micronucleus frequency was inline with the duration of exposure ($p=0.014$). The exposure less than 4 years showed no significant increase in micronucleus frequency of subjects. Micronucleus frequency increased with the duration of exposure to pesticides farmers was over 4 years. Long-term use of pesticide may cause acute or chronic adverse health effects, including the occurrence of neoplasms [13]. Research conducted by Roulland et al. [14] showed an increased incidence of oral cancer in long-term users of pesticides.
In addition to the external chemical exposure, other factors such as age, sex and contraceptive use may affect buccal epithelial micronucleus frequency. All subject in this study were women aged between 19-35 and not the acceptor of hormonal contraceptives [15]. Hormonal contraceptives may increase the frequency of micronucleus, asestrogen and progestosterone may change intracellular biochemical processes [3]. Estrogen metabolism in the body induces the covalent bond between the DNA with carcinogenic chemical substances (free radicals) resulting in DNA and RNA damages [16].

Evaluation of micronucleus frequency in buccal epithelial cells may be used as a bio-monitor of genetic damage due to the exposure of carcinogenic agents. Epithelial cells are the first barrier to chemical agents, inculding carcinogenic agents, through inhalation and ingestion. Several studies have shown that genetic alterations caused by exposure to carcinogenic substances may occur in the bronchi, esophagus, mammary gland, uterus and cervix [5]. Evaluation of genetic damage using exfoliative cytology method has a relevant result to the evaluation of micronucleus in lymphocytes [17]. This procedure is easily done and non-invasive, so that it can be considered as a biomonitor of genetic damage in human populations exposed to genotoxic substances. This research can be used as a prospective early detection onpesticide-exposed oral diseases.

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