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Genotoxicity following Organophosphate Pesticides Exposure among Orang Asli Children Living in an Agricultural Island in Kuala Langat, Selangor, Malaysia

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Abstract

Background: Agriculture is an important sector for the Malaysian economy. The use of pesticides in agriculture is crucial due to its function in keeping the crops from harmful insects. Children living near agricultural fields are at risk of pesticide poisoning.

Objective: To evaluate the genotoxic risk among children who exposed to pesticides and measure DNA damage due to pesticides exposure.

Methods: In a cross-sectional study 180 Orang Asli Mah Meri children aged between 7 and 12 years were studied. They were all living in an agricultural island in Kuala Langat, Selangor, Malaysia. The data for this study were collected via modified validated questionnaire and food frequency questionnaire, which consisted of 131 food items. 6 urinary organophosphate metabolites were used as biomarkers for pesticides exposure. For genotoxic risk or genetic damage assessment, the level of DNA damage from exfoliated buccal mucosa cells was measured using the comet assay electrophoresis method.

Results: Out of 180 respondents, 84 (46.7%) showed positive traces of organophosphate metabolites in their urine. Children with detectable urinary pesticide had a longer tail length (median 43.5; IQR 30.9 to 68.1 µm) than those with undetectable urinary pesticides (median 24.7; IQR 9.5 to 48.1 µm). There was a significant association between the extent of DNA damage and the children's age, length of residence in the area, pesticides detection, and frequency of apple consumption.

Conclusion: The organophosphate genotoxicity among children is associated with the amount of exposure (detectability of urinary pesticide) and length of residence in (exposure) the study area.

Keywords: Organophosphates; Pesticides; Organophosphate poisoning; Mutagenicity tests; Comet assay, Child; Malaysia

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Introduction

Agriculture plays an important role in Malaysia's economy as it contributed 7.3% of the gross domestic product (GDP) and provided 11.5% of the number of total employment in 2011.[1] The pesticides industry has thus become one of the most important supporting industries in agriculture. This is because the use of pesticides in agriculture is crucial due to its function in keeping the crops from harmful insects and pest, which can cause destructions to the crops' quality and their yields.

Inherently, unethical agricultural activities such as excess use of pesticides and inappropriate method of waste disposal have become a major source of environmental pollution. In the meantime, a study conducted by Leong in 2007, which measured the contamination levels of selected organochlorine and organophosphate pesticides in the Selangor river between 2002 and 2003, found that the existence of residual pesticides in Selangor river can be attributed to extreme agricultural and urban activities.[2]

The increase use of pesticides can cause permanent damage to the environment as well as harm especially to infants and children. Exposure to pesticides at any point in a person's life can cause short- and long-term health problems. This is evident through a recent study by Hu in 2015 in China, which found that short-term health effects of pesticide exposure included changes in one's complete blood count and disruption in renal as well as hepatic functions.[3] Long-term effects of pesticides exposure include abnormality in the nerve system. The longer and more often a person is exposed to pesticide, the more dangerous it is and the greater the risk for him/her to develop health problems. In the meantime, reported cases of human organophosphate poisoning showed that the fatality rate was higher in children than adults.

Most studies conducted in Malaysia focused on occupational exposure of pesticides among agricultural workers. Limited studies were conducted on marginal groups such as children and women, and none was carried out on the aboriginal (Orang Asli) community. Most of the previous studies suggested that persistent exposure to pesticides could be one of the causes of several diseases—*eg*, cancer, neurological and mental illnesses, as well as reproductive problems—as pesticides can accumulate within the food chain.[4,5] Fetuses and children might be more susceptible to pesticides' potential neurotoxic effects as their brains are still immature and developing rapidly. Costa and Saxena provided evidence that fetuses can be exposed to pesticides.[6,7] Traces of pesticides can be found in the amniotic fluid because many pesticides can cross the placenta.

Previous studies implicated a link between pesticides exposure and impaired neurodevelopment as well as autism, hearing loss, intellectual impairment and attention deficit hyperactivity disorder (ADHD) among children. A study conducted in 2010 on children in the USA found that children with high urinary concentrations of organophosphate metabolites had symptoms of ADHD.[8]

In the current study we used organophosphate pesticides urinary metabolites as biomarkers for pesticide exposure. Genotoxic assessment was done to provide information on the genotoxic risk among children following exposure to organophosphate pesticides. The alkaline comet assay method was used to measure the level of DNA damage. The objective of this study was to evaluate the genotoxic risk of children living in an agricultural area and were exposed to pesticides use.

For more information on green tobacco sickness among Thai traditional tobacco farmers see http://www.theijoem.com/ijoem/index.php/ijoem/article/view/540

Materials and Methods

This cross-sectional study was conducted on 180 Orang Asli children from the Mah Meri tribe aged between 7 and 12 years. These children lived in an agricultural island in Kuala Langat, Selangor, Malaysia. This is a 160-km^2 island where more than three quarters (117 km^2) of its area is planted with oil palm trees. The land, which was previously a mangrove area, was converted into a fully agricultural land.

Data Collection

Data were collected using a set of modified validated questionnaire and a food frequency questionnaire. It was a modified validated questionnaire translated into Malay language to document information on the demographic data, medical history, information on parents cigarette smoking, parents working exposure, children dietary information, lifestyle activities, and symptoms experienced by the respondents in relation to health effects caused by the agriculture activities. Pre-testing of the questionnaire was conducted prior to the actual research. Sixty Mah Meri respondents from Pulau Indah and Klang area were involved; they were excluded from the actual research. This was to validate and revise the format and presentation of the questionnaire. To get an accurate result and minimize bias, a guided questionnaire was used. The researcher clarified any queries raised by the subjects, thus, limiting the probability of an error. The interviewer was trained and had the ability to communicate well with study participants.

Six organophosphate dialkylphosphate (DAP) metabolites were measured: they were dimethyphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), diethylthiophosphate (DETP), dimethylthiophosphate (DMDTP), and diethyldithiphosphate (DEDTP). These organophosphate pesticides were detected using gas chromatography-mass spectrometry (GC-MS, model GC-CP-3800 chromatograph [Varian], Model MS-1200L Quadrupole MS/MS [Varian]), in urine samples obtained from the children. Exfoliated buccal mucosa cells were collected for genotoxicity assessment.

Interview

Interview with the parents or the guardians of the child was carried out at the same time the urine sample was collected. The interview was conducted in Malay language and was assisted by Tok Batin (head of village), where general information namely children's socio-demographic characteristics, parent's income, education level, occupation, length of residence in the current home, health and lifestyle status, pesticides use at home, and children's fruits and vegetables consumption were obtained. Children's dietary consumption was measured using a 24-hour dietary recall where the parents or the guardians were asked regarding their children's food intake frequencies and the serving size of their meals.

The food frequency questionnaire consisted of 131 food items in which the frequency of food consumption was classified using a 5-point Likert scale—'5' = daily, '4' = 2–3 times a week, '3' = once a week, '2' = once a month, and '1' = never, based on

TAKE-HOME MESSAGE

- Exposure to organophosphate pesticides would cause DNA damage in children. Longer exposure is associated with a higher damage.
- The main sources of exposure of children to organophosphate pesticides include their parents who are working in agricultural sector and spraying pesticides, and consumption of unwashed fruits and vegetables.

Chee, *et al.*[9] Then, this scale was divided into two categories: "frequent intakes" (score >3) and "less frequent intakes." (score 1–3).

Measurement of Organophosphate Pesticides Urine Metabolites

Spot urine samples were collected in 60-mL urine specimen containers during the time of interview and were stored at 4 °C for a maximum of 24 hours until it reached UKMMC environmental health laboratory where they were frozen at -20 °C. Within one month of collection, urine samples were transported to a private laboratory (Chromelab) where they were analyzed. Urinary organophosphate metabolites were determined using GC-MS according to Hemakanthi de Alwis, *et al.*[10] This method provided a detection limit for the assessment of low-level exposures such as the non-occupational exposure of organophosphate pesticides. The limit of detection (LOD) for the six metabolites mentioned earlier ranged from 0.10 to 0.15 ng/mL—0.1 ng/mL for DMP, 0.1 ng/mL for DEP, 0.15 ng/mL for DMTP, 0.1 ng/mL for DMDTP, 0.1 ng/mL for DETP, and 0.1 ng/mL for DEDTP.

Genotoxicity Assessment using Comet Assay Method

Examination of the exfoliated buccal mucosal cells is a non-invasive method to assess DNA damage. The cells were collected from the children by scraping the inner part of their cheeks using a sterile cytology brush for three times. The cells were then washed with pre-prepared phosphate buffer solution in 1.5 mL Eppendorf tube and stored at room temperature.

Meanwhile, the comet assay was performed according to the method described in the comet assay kit (Trevigen, USA). In this procedure, lysis solution was first prepared and cooled at 4 °C for at least 20 min before use. The low melting-point agarose was then melted in a beaker of boiling water for 5 min and placed at 37 °C in a water bath for at least 20 min to allow cooling.

The cells collected were first centrifuged at 200 *g* for 5 min and subsequently pipetted and combined with melted low melting-point agarose (1:10 v/v) at 37 °C. As much as 50 µL of the sample was then immediately pipetted onto the comet slide. The slides were then placed in dark at 4 °C for 10 min. Then, a 0.5-mm clear ring appeared and the slides were immersed in the lysis solution at 4 °C for at least 30 min. The excess buffer was then drained and immersed in freshly prepared alkaline unwinding solution (pH>13) for 20 min at room temperature in dark.

Electrophoresis was then carried out for 30 min using 21 volts applied for 40 min. A total of 850 mL alkaline electrophoresis solution was added into the comet assay tank, where the slides were placed in electrophoresis slide tray and covered with slide tray overlay. In the meantime, the power supply was set at 21 volts and applied for 40 min. The excess electrophoresis solution was drained and gently immersed twice in distilled water for 5 min each and once in 70% ethanol for 5 min. The samples were then dried at room temperature for 15 min, and the slides were stained with 50 µL of diluted SYBR gold. Finally, the slide was allowed to dry completely at 37 °C and viewed through epi-fluorescence microscopy.

Data Analysis

The data were analyzed using SPSS® for Windows® ver 20. Urine sample readings below the LOD were regarded as '0.' The DNA cell images captured on epi-fluorescence microscopy were analyzed using Tri-Tek Comet Score software ver 1.5. The tail length, which is the distance of DNA migration from the body of nuclear, was used as an indicator of DNA damage level. A $p < 0.05$ was considered statistically signifi-

Table 1: Socio-demographic characteristics of the study population (n=180)

Variable	n (%), (n=180)
Gender	
Male	98 (54.4)
Female	82 (45.6)
Age (yrs)	
7–9	104 (57.8)
10–12	76 (42.2)
Mother's education	
Primary and below	157 (87.2)
Secondary and above	23 (12.8)
Mother's occupation	
Housewife	145 (80.6)
Fisherman	23 (12.8)
Office/Administration	12 (6.6)
Father's education	
Primary and below	128 (71.1)
Secondary and above	52 (28.9)
Father's occupation	
Agriculture	47 (26.1)
Fisherman	45 (25.0)
Office	33 (18.3)
Traditional craft/others	55 (30.6)
Use of pesticides at home	
Yes	93 (51.7)
No	87 (48.3)
Parents smoking status	
Yes	101 (56.1)
No	79 (43.9)
Length of residence (yrs)	
<10	126 (70.0)
≥10	54 (30.0)

cant.

Results

Out of 189 Mah Meri Orang Asli children who participated in the study, only 180 children fulfilled the inclusion criteria; 9 had incomplete information and were thus excluded from the study.

Participants had a mean weight of 25.9 (SD 9.6) kg and height of 124.6 (12.3) cm. Table 1 presents the socio-demographic characteristics of the participants. The majority (54.4%) of the respondents were boys; 57.8% aged between 7 and 9 years. Most of the parents (87.2% of mothers and 71.1% of fathers) had primary school or lower education. Mothers were predominantly housewives (80.5%) More than a quarter (26.1%) of fathers worked at the agricultural area. It was assumed that the parents who worked in the agricultural area were highly exposed to pesticides compared to those who worked in other sectors. The median household income for the families was US$ 114 (IQR 68 to 205) per month. Almost half (56.1%) of the parents were smokers and 51.7 % had history of pesticides usage at home; 30.0% of the children lived in the island for 10 years or more.

Of 180 respondents, 84 (46.7%) showed positive results for urine organophosphate DAP metabolites. The organophosphate pesticides urinary metabolites identified in the study were DMP (46.7%), DEP (16.7%), and DMDTP (3.3%). From the 84 (46.7%) children who were tested positive for urinary DAP, 48 (57.1%) had only DMP exposure, 30 (35.7%) had DMP and DEP exposures, and 6 (7.1%) had DMP and DMDTP exposures.

Table 2 presents the socio-demographic characteristics of the 180 studied children and their association with pesticides detection. Fathers of 69.0% of the children with detectable levels of pesticides were working in the agricultural area; 64.0% of these fathers were highly exposed to pesticides during their work. Furthermore, 54% of parents of the children with detectable level of pesticides had a history of pesticides usage at home.

Table 3 shows the extent of DNA damage and its association with socio-demographic factors and fruits and vegetables intakes among the studied children. The median comet tail length of all respondents was 37.1 (IQR 17.5 to 54.5) µm. Children with detectable urinary pesticide had a longer tail length (median 43.5; IQR 30.9 to 68.1 µm) as compared to children with undetectable urinary pesticides (median 24.7; IQR 9.5 to 48.1 µm). There was a significant association between the extent of DNA damage and the children's age, length of residence in the area, pesticides detection, and frequency of apple consumption. Multiple linear regression anlysis revealed only two independent predictors for the length of comet tail. Those with detectable levels of pesticides ($p<0.001$) and children who had stayed for 10 years or more in the island ($p=0.015$) had a significantly higher comet tail length.

Discussion

In this study, urinary metabolites of organophosphate pesticides were used as biomarkers for pesticides detection among the studied children. Almost half of the respondents (46.7%) showed positive traces of organophosphate pesticides in their urines. Most (69.0%) of the fathers of children with positive urinary pesticides were working in agricultural areas; 54.0% of parents of these children had a history of using pesticides at home. These findings indicate that fathers who worked in agricultural areas posed a major risk for pesticides exposure in their children. This is consistent with other studies that showed children living in agricultural area might

Table 2: Socio-demographic characteristics of the studied children (n=180) and its association with detection of organophosphate pesticides in their urine samples

Variable	Pesticides Detected, n (%), (n=84)	Pesticides not detected, n (%), (n=96)
Gender		
Female	39 (48)	43 (52)
Male	45 (46)	53 (54)
Age (yrs)		
7–9	50 (48.1)	54 (51.9)
10–12	34 (44.7)	42 (55.3)
Mean (SD) weight (kg)	26.4 (10.1)	25.5 (9.2)
Mean (SD) height (cm)	125.5 (12.1)	123.7 (11.9)
Mother's education		
Primary and below	71 (45.2)	86 (54.8)
Secondary and above	13 (56.5)	10 (43.5)
Mother's occupation		
Housewife	61 (48.0)	66 (52.0)
Fisherman	0 (0)	23 (100)
Office/Administration	4 (100.0)	0
Father's education		
Primary and below	55 (43.0)	73 (57.0)
Secondary and above	29 (56)	23 (44)
Father's occupation		
Agriculture	49 (69)	22 (31)
Fisherman	6 (13)	39 (87)
Office	6 (67)	3 (33)
Traditional craft/others	23 (42)	32 (58)
Father's occupational exposure to pesticides		
Low	54 (40.6)	79 (59.4)
High	30 (63.8)	17 (36.2)

Continued

Table 2: Socio-demographic characteristics of the studied children (n=180) and its association with detection of organophosphate pesticides in their urine samples

Variable	Pesticides Detected, n (%), (n=84)	Pesticides not detected, n (%), (n=96)
Median (IQR) household income per month (US$)	137 (114 to 228)	114 (68 to 182)
Use of pesticides at home		
No	34 (39)	53 (61)
Yes	50 (54)	43 (46)
Parents smoking status		
No	38 (45)	41 (43)
Yes	46 (55)	55 (57)
Length of residence (yrs)		
<10	60 (47.6)	66 (52.4)
≥10	24 (44)	30 (56)

be exposed through contacts with pesticides drifts and residues during the application process and through traces brought home by their parents. Azaroff found that children who lived near agricultural areas experienced adverse health effects caused by pesticides exposure in the environment, even though they did not work in the field.[11] Their parents might bring in pesticides residues from the agricultural fields into their homes or pesticides may drift from the fields into the areas where the children play. Moreover, some children might lay and crawl on the floor and engaged in hand-to-mouth or object-to-mouth habits that could cause the ingestion of soils or house dusts that contained pesticides residues.

Fathers who work in the agricultural area and had history of using pesticides might expose their children to pesticides that causes short- and long-term health effects. Short- and long-term effects of pesticides exposure could result in learning disability and reduction in intelligence quotient. There was also evidence on how prolonged exposure to pesticides causes permanent brain damage in children. A meta-analysis conducted in 2013 showed that parental occupational exposure to pesticides is a risk for brain tumors in children.[12,13]

We used the comet assay to measure the extent of DNA damage in the studied children as a result of exposure to organophosphate pesticides. The alkaline comet assay was used because it is a rapid and reliable test that could detect DNA damage in different exposure situations.[14]

These results were consistent with the results of a study done by How in 2014 where children living in rural farm areas had an increased risk of genotoxicity in response to higher exposure to organophosphate pesticides.[15] Furthermore, a study on paddy farm workers in 2013 showed that farmers who were exposed to chronic organophosphates had significantly higher risk of DNA damage compared to adults living and working in fishing villages.[16] Another study conducted in India on farmers who were exposed to pesticides revealed that there was a significant higher risk of DNA damage among the farmers and that the percentage of DNA tail increased along with increased exposure duration.[17] A study conducted in Croatia showed that workers who had a high level of exposure to pesticides showed an increased comet tail length compared to workers in the control group.[18]

The findings from the present study also suggested that children who had resided for a longer period in the study area had a significantly higher comet tail length. This was also consistent with the findings of another study on paddy farm workers where residence of more than 30 years was significantly associated with a higher comet tail length.[19] Therefore, the longer the children stay in the area, the longer they are exposed to pesticides with consequent more damage to their DNA. When this happened, the DNA repair mechanisms would not be able to repair the damages; the cell would ultimately die or be mutated that could eventually transformed into a malignant cell.[20]

We found that children who frequently consumed apples had a significantly higher risk of DNA damage compared to those who consumed apples less frequently. That could be attributed to the residual pesticides left on the fruits that was in concordance with the findings from a study by Muñoz-Quezada in 2012 where consumption of fruits containing insecticides residues was the main factor contributing to pesticides exposure among school children in Chile, Latin America.[21] On the other hand, while it might be difficult to trace the origins of the fruits and vegetables consumed by the children, apples were usually bought from the nearby markets. Therefore, it can be concluded that exposure to pesticides in the study area might be originated from a source outside the region because apples are not grown locally in Malaysia. This suggests that there was probably a lack of knowledge among the studied community on good hygiene practices for preparation of food, especially on the needs for the proper washing of fruits and vegetables before they are consumed. This calls for a need to create awareness among the parents on the importance of avoiding consumption of fruits and vegetables which are not properly washed.

Finally, the predictors of comet tail length among the studied children were a detectable level of urinary pesticides and their length of residence in the study area. Exposure to pesticides for a long period are associated with a higher risk of genotoxicity.

In conclusion, pesticides use should be

Table 3: Comet tail length (μm) and its association with socio-demographic factors and fruits and vegetables consumption in the studied children

Variable	n	Median (IQR)	p value
Socio-demographic factors			
Gender			
Female	82	37.4 (16.7 to 50.1)	0.790
Male	98	37.1 (17.5 to 56.5)	
Age (yrs)			
7–9	104	31.8 (13.4 to 48.8)	0.020
10–12	76	44.8 (26.0 to 57.2)	
Mother's education			
Primary and below	157	37.1 (16.7 to 55.1)	0.714
Secondary and above	23	37.1 (17.5 to 50.9)	
Father's education			
Primary and below	128	37.4 (17.9 to 55.7)	0.734
Secondary and above	52	35.8 (16.7 to 50.6)	
Father's occupation exposed to pesticides			
Low	133	36.6 (16.2 to 51.7)	0.188
High	47	40.8 (26.0 to 57.8)	
Use of pesticides at home			
No	87	30.7 (12.7 to 51.9)	0.083
Yes	93	39.2 (23.3 to 55.9)	
Parents smoking status			
No	79	36.6 (15.9 to 51.9)	0.754
Yes	101	38.2 (17.5 to 55.7)	
Length of residence (yrs)			
<10	126	32.9 (13.7 to 49.8)	0.001
≥10	54	45.3 (27.6 to 58.6)	
Pesticides detection			
No	96	24.7 (9.5 to 48.1)	0.001
Yes	84	43.5 (30.9 to 68.1)	

Continued

Table 3: Comet tail length (µm) and its association with socio-demographic factors and fruits and vegetables consumption in the studied children

Variable	n	Median (IQR)	p value
Fruits and vegetables intakes			
Cucumber (*Cucumis sativus)*			
Less frequent	156	37.1 (17.5 to 54.1)	0.684
Frequent	24	35.8 (11.9 to 56.5)	
Long bean *(Vigna unguiculata)*			
Less frequent	155	37.1 (17.5 to 54.1)	0.613
Frequent	25	37.1 (15.1 to 56.5)	
Pucukpaku (*Diplazium esculentum)*			
Less frequent	124	36.3 (13.4 to 51.8)	0.174
Frequent	56	39.5 (29.7 to 56.8)	
Potato sprouts *(Manihot esculenta)*			
Less frequent	121	36.0 (16.4 to 50.4)	0.073
Frequent	59	40.3 (23.9 to 58.8)	
Carrot (*Daucus carota*)			
Less frequent	164	36.8 (17.1 to 54.5)	0.574
Frequent	16	42.9 (22.0 to 55.0)	
Apple *(Malus pumila)*			
Less frequent	138	36.0 (13.1 to 50.1)	0.015
Frequent	42	45.1 (32.1 to 62.0)	
Banana (*Musa* species)			
Less frequent	170	37.1 (16.8 to 54.2)	0.287
Frequent	10	42.7 (34.1 to 56.6)	

limited in residential areas or areas where children play to minimize the exposure. Standard precautions to avoid contamination during and after the application of pesticides should be considered. Increasing awareness of at risk population through their education is also of paramount importance.

Conflicts of Interest: None declared.

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