

# Biological monitoring of genotoxicity to organophosphate pesticide exposure among rice farmers: Exposure-effect continuum study

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## Abstract

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**Background:** This study has used biomarker of exposure-effect continuum to examine the biological characteristics of organophosphate (OP) toxicity and its genotoxic effect among rice farmers.

**Materials and Methods:** A cross-sectional study was conducted among 160 pesticide exposed rice farmers and 160 adults from the fishing village as the unexposed group. They share the common socio-economical background for inter-individual comparison in human toxicology assessment. In this study depression of blood cholinesterase is used as a biomarker of exposure to OP toxicity. Two genotoxic assays (micronuclei and comet assay) were conducted as a biomarker of genotoxic effect among the adult population. In this context, micronuclei assay is used to indicate the chromosome breakage and comet assay to estimate the possible DNA damage.

**Results:** The study showed a significant difference of blood cholinesterase level ( $p=0.001$ ) between the exposed-unexposed groups. Besides, the results showed that farmers had at least 2-2.5 folds of significant increase ( $p=0.001$ ) in MN frequency (in 1000 cells) and comet tail length ( $\mu\text{m}$ ) compared to the unexposed group. In addition, regression analysis among farmers showed that blood cholinesterase level decreased with the genotoxic effects. A small variation ( $R^2=0.148$ ) of MN frequency could be explained by the depression of blood cholinesterase level; however, a significant reduction ( $p=0.001$ ), with strong changes ( $R^2=0.712$ ) in comet tail length was attributed to the depression of blood cholinesterase levels. Risk factors like age, body mass index, smoking status and years of working showed the different strength of the relationship with these genotoxic effects.

**Conclusions:** This study suggests that chronic exposure to OP shows an inhibition to blood cholinesterase level, which is associated with the potential DNA breakage as indicated by comet assay. Age, smoking and years of working are the contributing factors influencing the biomarker of effects.

**Keywords:** Organophosphate, Cholinesterase, Depression, Chromosomal Breakage, DNA Damage.

## Introduction

Over the past 60 years, various organophosphates (OP) insecticides have been introduced as the replacement for legally banned organochlorine (OC) used in the markets. In a global rice production area in Southeastern Asia, at least 40% of the total cost was spent to subsidize OP insecticides used by rice farmers [1]. Unfortunately, by

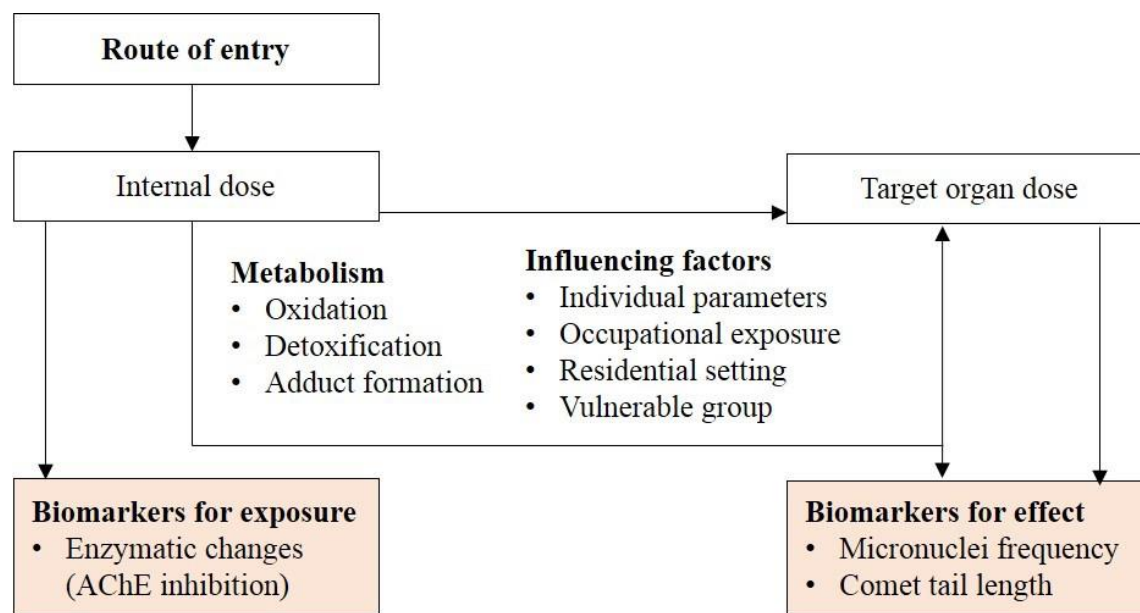
the year 2005, a report from Pesticide Action Network Asia and the Pacific (PANAP) highlighted that at least 39.5% of

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the insects resistant to OP, with approximately 542 species showed resistance to the total of 316 different insecticide compounds [2].

In view of this, the frequency and dosage of insecticides used by farmers is expected to increase in order to cope with the insects' rapid evolution process of its genetic adaptation. In this study, the principle of human biological monitoring is used to

identify and quantify the level of exposure and health effect to the OP contaminants [3]. In order to characterize the risk of potential health effect, dose monitoring (=exposure biomonitoring) is complemented by studying together with biological effects (=effect biomonitoring). Pathway for biological measurements as suggested in this context is adapted as shown in Figure 1.



**Figure 1: Pathways for biological measurements of mixture of OP (Source: Adapted from [3])**

As shown in Figure 1, the primary mechanism of OP toxicity involves the inhibition of the acetyl cholinesterase (AChE) in the central and peripheral nervous system. This has been widely accepted by the occupational practitioner as an effective biomarker of OP exposure [4-6]. In fact, mixture of OP as the esters of phosphoric acid shares a common biochemical mechanism for additive chronic and/or acute health effects [7-8].

Over the years, monitoring of the OP induced chronic effect at the cellular level is of interest. Past studies suggested that the

reduction in activity of AChE demonstrates a significant association with the increase of oxidative stress and lipid per oxidation among farmers who were chronically exposed to OP [9-12]. In fact, the mechanisms of AChE inhibition from OP exposure to increase the potential oxidative stress have been studied by Banerjee [13]. Theoretically, increased formation of reactive oxygen species (ROS) during metabolism may cause oxidative damage to the cellular DNA [14-15]. The background of this principle further suggested that the elevated ROS level and reduction of ROS

scavenger and antioxidant enzymes are associated to cause damage to nucleic acids, proteins and lipids. This might partly exert their effect to induce chromosomal instability, mutations, loss of organelle functions, cause membrane damage and undergo a multistage of carcinogenesis process (16-18).

Currently, there is a growing concern on OP for its possible chronic effects to exert harmful genotoxic risk among farmers due to their long-term occupational exposure [19-22]. However, the potential association between the depressions of AChE to the potential genotoxic effect of chronic OP exposure has not been well investigated. Therefore, the question arises whether the evidence association between oxidative stress and depression of AChE level will also mark the AChE reduction and induce genotoxic risks [23]. The aim of this study is to fill the knowledge gap by biomonitoring the potential cholinesterase inhibition and genotoxic effects from OP exposure among the study population.

### Materials and Methods

The study was approved by the Ethics Committee of the University Research Involving Human Putra Malaysia (UPM/FPSK/100-9/2-JKEUPM). A total of 160 rice farmers ( $40.13 \pm 10.56$ ) who reported to have at least 2 years of farming experience were exposed to a mixture of OPs activity were recruited to participate in this study. They were considered as the rural farming community living in proximity to the paddy farmland. Therefore, they were presumed to have cumulative exposure to low level of pesticide through pesticide drift, deposition, sedimentation, leaching and drainage. An unexposed group consisting of 160 villagers ( $40.22 \pm 9.75$ ) from fishing village was

selected as an inter-individual comparison of toxicological effect.

### Biomarker of exposure

The primary mechanism of toxicity of OP pesticide is by phosphorylation of the acetyl cholinesterase enzyme (AChE) at the nerve endings [5-6, 24-25]. In this study, monitoring of blood cholinesterase level is a useful tool which was used as a biomarker of exposure to OP pesticide.

Invasive manner of collecting biological samples has been the obstacles in human health study, particularly among rural villagers. Therefore, blood cholinesterase test kit (Lovibond, AF267; Tintometer Ltd., UK) is used to determine the exposure level with only 10 $\mu$ l of capillary blood from the finger tips. This test kit which works based on the colorimetric principle is helpful to ease the laboratory analysis with an on-site blood cholinesterase level estimation.

Finger pricked blood is pipetted to round test tube which contained of 0.5ml of indicator solution (Bromothymol blue solution) followed by 0.5ml of substrate solution (Acetylcholine Perchlorate). The test tube was then mixed thoroughly and transferred to 2.5mm cuvettes. Next, the 2.5mm of cuvettes was placed in the colour compartment to view the indicator colour through a prism. The result is read based on the acid-base blood cholinesterase level (%) obtained through the pH colour indicator. This colorimetric principle is based on the normal breakdown of acetylcholine as shown in Equation 1. The presence of acetic acid determines the normal workout between acetylcholine and acetyl cholinesterase; otherwise, it forms the bases due to the accumulation of acetylcholine.

Acetylcholine  $\rightarrow$  Acetic acid + choline (Equation 1)

**Biomarker of effect (Genotoxicity assessment)**

Biomarkers of effect are biological indicators of the body's response to exposure. The effect of OP exposure is determined by monitoring the genotoxicity consequences of sub-clinical changes. The genotoxic effect is measured through the micronuclei and comet assay. To ensure biological samples are collected in a convenient and less invasive manner, exfoliated buccal mucosa cells were collected as a sensitive biomarker of genotoxic damage in the target tissues [26 - 27].

**Genotoxicity test (Micronuclei Assay)**

Micronuclei (MN) assay is used as an internal dosimeter to monitor the presence of MN for chromosome [28]. This is to estimate the possibility of early cancer risk experienced by the study population. This assay was conducted based on the standard protocol from Thomas and Fenech [29].

Buccal mucosa cell specimens collected were first centrifuged at 1500rpm to wash the cells in the buffer solution (80% methanol, absolute ethanol). The supernatant is aspirated off and the buccal cells were smeared on the slide by using a pulling technique and keep air-drying. The cell was fixed with methanol: acetic acid (3:1) solution in a 0.1% phosphate buffer (pH 7.5) for 20 minutes. The slide was then stained by Feulgen reaction, followed by counterstaining of the slide with 0.1% of fast green for 30 seconds and rinsed well with deionized water (dH<sub>2</sub>O). The slide was then placed face-down to blot away any residual moisture and allowed drying for about 10-15 minutes before analyzing under light microscope with 100x magnification.

The end point is to measure the cells in the presence of MN (s), which is scored based on the cells presented with a main nucleus and

smaller nuclei called MN. The MN was usually round or oval in shape, and their diameter ranged between 1/3 to 1/10, the diameter of the main nucleus.

**Genotoxicity test (Comet Assay)**

The Comet Assay, also known as Single-Cell Gel Electrophoresis (SCGE) technique, is a fast and effective way to measure DNA damage by estimating the comet tail length ( $\mu\text{m}$ ). This assay was based on the standard procedure from Comet Assay Kit (Trevigen, USA).

Collected buccal mucosa cell specimens were first centrifuged for 1 minute at 2500 rpm. Next, Low Melting-point Agarose (LMA) melted in a beaker of boiling water, with the cap loose in 5 minutes and then kept cool at 37°C water bath for at least 20 minutes. This was followed by pipetted 75 $\mu\text{L}$  of 1:10 (v/v) aliquot onto comet slide. Lysis process initiated by placing the slide at 4°C in the dark for 10 minutes, then immersed in pre-chilled lysis solution for 60 minutes. Cells were further denatured by immersion in freshly prepared alkaline solution, pH>13 for 45 minutes at room temperature in the dark. The cells were now ready for electrophoresis system and set the power supply was set at 1volt/cm. After 1 hour, the slide was rinsed by dipping in deionized water (dH<sub>2</sub>O), and immersing slide in 70% ethanol for 5 minutes. The slides then were stained with 50  $\mu\text{L}$  of dilute SYBR green before viewing under fluorescent microscope (DM2500, LEICA) with magnification 100x and the images were captured. The cells were then analyzed by using the commercially available TriTek Comet Score (version 1.5) software (TriTek Corp., Sumerduck, VA, USA). The tail length was measured ( $\mu\text{m}$ ) to indicate the distance of DNA migration from the body of nuclear core and it was used to evaluate the extent of DNA damage.

**Results**

Table 1 summarizes the socio-demographic characteristic of the study population. The

320 participates were all male and Muslims, who did not drink alcohol. Both exposed and unexposed groups had similar socio-economical background.

**Table 1: Demographic characteristics of study population (N=320)**

Demographic characteristics	Rice farmer (N=160)		Unexposed (N=160)	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Male gender	160 (100)	-	160 (100)	-
Age (year)	-	40.13 (10.56)	-	40.22( 9.75)
Body Mass Index (BMI)	-	24.05 (3.37)	-	23.06 (2.59)
Smoker	109 (68.1)	-	92 (57.5)	-
Farming experience (year)	-	17.63 (11.35)	-	-

The level of blood cholinesterase is to reflect the biomarker of OP exposure during the farm activity. Table 2 shows there is a significant difference ( $p=0.001$ ) of blood cholinesterase level between exposed and unexposed groups. When an acid-base (%) of

blood cholinesterase level is estimated, with the average farmers indicated an “over-exposed” (41.02%) level and unexposed group show an average of “normal” (75%) level.

**Table 2: Comparison biomarkers of exposure (blood cholinesterase level) among study population (N=320)**

Biomarker of exposure	Rice farmer (N=160)	Unexposed (N=160)	<i>t</i> -statistic <sup>a</sup>	<i>p</i> -value
	Mean (SD)	Mean (SD)		
Blood Cholinesterase level <sup>b</sup>	41.02 (24.57)	74.84 (15.42)	-14.751	<0.001**

\*\* *p*-value is significant at level 0.001

<sup>a</sup> Independent *t*-test

<sup>b</sup> Analysis of result based on blood cholinesterase test kit(Lovibond AF267, Tintometer Ltd., UK),

100.0-75.0 (%): Normal;

62.5-50.0 (%): Over-exposure;

37.5-25.0 (%): Serious over exposure;

0.0 (%): Very serious and dangerous over exposure

The study utilized two genotoxic assays, e.g. MN assay and comet assay to evaluate the genotoxic effect. Table 3 suggested that

farmers had at least an increase of 2-2.5 folds in genotoxic effects as compared to the unexposed group. The significant difference

of MN frequency (per 1000 cells) and comet tail length( $\mu\text{m}$ ) are crucial to highlight that farmers are at high risk of the genotoxic

effects due to the nature of their work as a pesticide applicator as compared to the unexposed group.

**Table 3: Comparison of biomarker of effect (genotoxic risk) among study population (N=320)**

Biomarker of effect	Paddy farmer (N=160)	Unexposed (N=160)	<i>t</i> - statistic <sup>a</sup>	<i>p</i> -value
	Mean (SD)	Mean (SD)		
Micronuclei (per 1000 cells)	14.48 (4.20)	5.46 (1.67)	25.2	<0.001**
Comet tail length ( $\mu\text{m}$ )	24.35 (8.20)	12.85 (3.10)	16.6	<0.001**

\*\* *P*-value is significant at level 0.001

<sup>a</sup>Independent *t*-test

The difference between the genotoxic effects examined by MN assay and comet assay is due to the variations in the type of DNA alterations in the test system. The MN assay is commonly used to detect fixed mutations which persist for at least one mitotic cycle; whereas, comet assay is a biomarker for the

repairable DNA lesions or single and double stranded DNA that breaks at a single cell. In other words, MN assay is widely used to indicate early genotoxic risk due to chromosomal breakage; in contrast, comet assay is used to estimate the potential of DNA strand breaks in a cell.

**Table 4: Relationship between blood cholinesterase level and genotoxic effects among rice farmers (N=160)**

Variables <sup>a</sup>	Micronuclei (per 1000 cells)		Comet tail length ( $\mu\text{m}$ )	
	$\beta$ -coefficient	<i>r</i> , correlation coefficient	$\beta$ -coefficient	<i>r</i> , correlation coefficient
<b>Block 1</b>				
Age	0.063	0.264	0.766**	0.830
BMI	0.180	0.209	0.049	0.165
Smoker <sup>b</sup>	-1.323*	-0.155	-1.745*	-0.102
Year of employment	0.162**	0.335	-0.126	0.697
Adjusted <i>R</i> <sup>2</sup>	0.136		0.699	
<i>F</i> -statistics	7.248**		93.113**	
<b>Block 2<sup>c</sup></b>				
Blood cholinesterase level	-0.023	-0.146	-0.042*	-0.166
Adjusted <i>R</i> <sup>2</sup>	0.148		0.712	
<i>F</i> -statistics	6.519**		79.662**	

<sup>a</sup> Simple linear Regression (SLR) by block

<sup>b</sup> For coding for categorical variables (1=smoker, 0=non-smoker)

<sup>c</sup> Included predictor: Blood cholinesterase level and covariate (Block 1)

\*\* *P*-value is significant at level 0.01

\* *P*-value is significant at level 0.05



Table 4 shows the relationship between blood cholinesterase and the genotoxic effects. Risk factors, such as, age, body mass index (BMI), smoking and year of employment (as farmer) are introduced as covariate which may influence the genotoxic risks in the study population. Both genotoxic effects indicated an inverse relationship with the level of blood cholinesterase. The result indicated that the blood cholinesterase significantly decreased with MN frequency ( $p=0.001$ ) and comet tail length ( $p=0.001$ ). Regression models for adult farmers suggested that 14.8% of MN frequency (per 1000 cells) were explained by the blood cholinesterase level; however, 71.2% of comet tail length ( $\mu\text{m}$ ) changes were attributed by the depression of blood cholinesterase levels among adult farmers. Overall, there is a significant increase in adjusted  $R^2$  from block 1(MN assay and covariate: $R^2=0.136$ ; Comet assay and in a way that covariate: $R^2=0.699$ ) to block 2 that the combination of the predictor and the covariates seems to contribute to the variance in the outcome genotoxic effect.

## Discussion

The present study used biomarker of exposure-effect continuum to examine the biological characteristics of OP toxicity and its genotoxic risk among rice farmers. The result is in agreement with other studies which showed a significant reduction in blood cholinesterase level among farmers [2-25, 30]. Besides, genotoxic effects shown in the current study are consistent with previous studies. Both the effect of chromosomal breakage (MNformation) and DNA strand breaks (comet tail length) showed a significant increase in farmers as compared with the unexposed group [20, 31-33].

As shown in Figure 1, there are factors which influence the biomarker effects' output, such as the role of lifestyle, individual parameters and occupational factors. In this study, we examine the changes in blood cholinesterase level with the genotoxic effects by evaluating the selected risk factors, such as, age, BMI, smoking and year of employment (as a farmer).

Among these, occupational exposure such as the year of working experience is of particular importance due to farmers' work nature as a pesticide applicator. This is considered as a crucial factor contributing to the genotoxic effects [34-35]. Besides, it is known that individual factors such as age, BMI, and smoking are also the predisposing factors contributing the cancer development [28]. This finding showed that age, smoking and obesity contributed to the bodyfunction impairments over time. In other words, dynamic interaction between individual predispositions and genotype were associated with the production of reactive oxygen species (ROS) under a sustained stress exogenous and endogenous environment, which characterize the increase in intracellular oxidative stress modulating the multistage carcinogenic process over an extended period of time [14,18,36].

The different response presented by MN assay and comet assay to cholinesterase inhibition in this study is in agreement with previous studies [22, 38]. These studies suggested that the spontaneous hydrolysis of OP from the active site (serine) of acetyl cholinesterase enzyme is very slow, and may cause irreversible impairment due to cumulative and long-term toxic effects among farmers. Under this continuous environmental stress, ROS are more inclined to accumulate and produce over a long period of time after chronic and low level of OP exposure [11, 22, 38-39].

Furthermore, the association between blood cholinesterase activities with ROS suggest a relevant gene-pesticide interaction which could further lead to genotoxic risk and carcinogenicity. Since the increases in stress of ROS and reducing of ROS scavengers and antioxidant enzymes may lead to a significant damage to cell structures [15, 18], a significant damage may occur to the cell structure and induce somatic mutation and neoplastic transformation over time. Indeed, cancer initiation and progression have been associated with oxidative stress through increased DNA mutations or induced DNA damage and genomic instability [36, 40].

### Conclusion

This study suggests that the chronic exposure to OP marks an inhibition to blood cholinesterase level, which is associated with the potential DNA breakage as indicated by comet assay. However, the biomarker for short-term acute exposure showed no chromosomal breakage from MN frequency. Nevertheless, further study is needed to further quantify the potential body burden perceived from OP exposure and estimate the adverse effect from chronic exposure at the cellular level of the organism by considering the weight of evidence.

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