ORIGINAL ARTICLE

Glyphosate Suppresses the Ability of PON1 to Hydrolyse Oxidized-LDL in the Exposed Farm Workers in Pahang, Malaysia

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ABSTRACT

INTRODUCTION: Paraoxonase 1 (PON1) is a high-density lipoprotein (HDL)-associated enzyme which is known to hydrolyse most pesticides including organophosphates (OPs) and prevent atherosclerosis by inhibiting oxidative modification of low-density lipoprotein (LDL). Glyphosate is one of the common organophosphate pesticides used in agriculture in many developing countries including Malaysia. The aim of this study to is to assess the PON1 ability to hydrolyse oxidized LDL in glyphosate exposed farm workers. **MATERIALS AND METHODS:** In this cross-sectional comparative study, a total of 103 subjects (53 Glyphosate-exposed and 50 non-exposed) were recruited. Fasting serum samples were analysed for PON1 activities towards substrates paraoxon, phenylacetate, and diazoxon, as well as for lipid profiles and oxidized-LDL (ox-LDL). **RESULTS:** The results showed lower basal paraoxonase activity [156.96 (58.87) vs 177.06 (66.78)], arylesterase activity [90.06 (17.14) vs 96.92 (23.87)] and diazoxonase activity [850.93 (206.75) vs 990.48 (248.73)] in glyphosate-exposed compared to non-exposed, however, only diazoxonase activity was statistically significant ($p<0.05$). PON1 activity is not significantly different with different length of period of exposure except for arylesterase. There was also significantly higher ($p<0.05$) ox-LDL in the exposed group but no significant differences in lipid profiles ($p>0.05$) between the two groups. The PON1 to ox-LDL ratio which probably reflects the ability of PON1 to hydrolyse ox-LDL were also significantly lower ($p<0.05$) among the glyphosate-exposed group. **CONCLUSION**: The results suggested that the decreased PON1 activity in glyphosate-exposed individuals could predispose them to the development of atherosclerosis and coronary artery disease through decreased PON1 ability to hydrolyse ox-LDL.

Keywords
Glyphosate exposure, PON1 activity, lipid profile, ox-LDL, farm workers.

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INTRODUCTION

Pesticides are chemicals commonly used in agriculture to prevent, repel, or kill pests for better yield.[1] Several classes of compounds are still being used as pesticides despite the ban on some of them owing to their alarming toxic effects.[2] Glyphosate [N-(phosphonomethyl)-glycine] is one of the widely used organophosphorus with broad-spectrum non-selective herbicidal activity.[3,4] Glyphosate acts via the inhibition of S-enolpyruvylshikimate-3-phosphate synthetase (EPSPS), an enzyme required for the formation of aromatic amino acids.[5,6] Despite the relative safety of glyphosate, a number of adverse effects have been ascribed to it due to exposure in humans and animals.[7] It has been documented that pesticides including organophosphate produce reactive oxygen species (ROS) capable of inducing lipid peroxidation and impairment of natural antioxidants.[8] Due to progressive agricultural development in Malaysia, the use of pesticides has increased tremendously over the last decade.

Paraoxonase 1 (PON1) is a high-density lipoprotein (HDL) associated calcium-dependent esterase. The calcium atom in PON1 is required for its activity and stability. This differentiates PON1 from other esterases which require cobalt, manganese, or magnesium for their activity. It is a glycoprotein with molecular weight of 43 kDa and consists of 354 amino acid residues.[9] PON1 participates in detoxification of pesticides (including organophosphorus) and hydrolysing lipid peroxides.[10] It has been reported that PON1 activities are inversely proportional to oxidative stress in serum and macrophages.[11] Several factors/conditions have been reported to affect the activities of PON1. These include drugs such as antidepressants,[12] smoking,[13] renal failure,[14] diabetes,[15] and exposure to pesticides.[16]

Owing to the growing use of pesticides as a result of increase agricultural activities, the present study was undertaken to evaluate the levels of PON1 activities and lipid parameters in farm workers exposed to glyphosate pesticide and relate it to the possibility of being a risk to the development of atherosclerosis and coronary artery disease.

MATERIALS AND METHOD

Materials

All chemicals used in this study were of analytical grade. The substrates paraoxon (o-o-diethyl-p-nitrophenyl phosphate), phenylacetate, and diazinon were purchased from Sigma Chemicals, USA. $CaCl_2$, Glycine buffer and methanol, NaCl, and lipid profile reagents were from Merck KGaA, Germany. Tris-HCl was purchased from Bio-Rad laboratories Australia. Oxidized- low density lipoprotein (Ox-LDL) ELISA assay kit was purchased from Mercodia, Sweden.

Study population and selection

This cross-sectional comparative study involved 53 glyphosates exposed and 50 non-exposed farm workers at 4 farms in Kuantan, Pahang namely: Ladang Penor Legenda, Penor Idaman, Sri Resak and Pulau Manis. The study was approved by the Kulliyyah Ethical Committee of International Islamic University Malaysia, Kuantan Campus. All pesticide sprayers above 18 years old were recruited whilst clinically diagnosed obese, hypertensive and those on regular medication were excluded. The comparative non-exposed group were selected through the matching process of the same age, sex, race, smoking status, BMI, and income bracket. Consent of the screened respondents were obtained, and pre-validated questionnaire was filled for them under guidance.

Specimen collection

The respondents were told to fast overnight before the day of sample collection. On the day of sample collection, blood pressure, weight and height were taken, and body mass index was calculated. Ten (10) millilitre of fasting blood was collected from each of the respondents by venepuncture and transferred into plain tubes. The serum was separated by centrifugation at 3000 rpm for 10 minutes, divided into aliquots and immediately stored at -70^{0}C until further analysis. The serum was analysed for PON1 activities and lipid parameters.

Methods

Determination of PON1 activities

Paraoxonase, arylesterase and diazoxonase activities were determined using their respective substrates hydrolysis. The 3 substrates analysis were done due the substrate specific differences in catalytic efficiency of PON1 genotypes/phenotypes which gave inter-individual variation.

Paraoxonase activity

The paraoxonase activity was determined according to modified Eckerson et al. (1983) method.[17] The basal paraoxonase activity was determined by mixing (i) 200µl of 5mM CaCl2 (ii) 250µl of 0.2M glycine buffer pH 10.5 (iii) 250 µl of distilled water (iv) 50µl of diluted serum (equivalent to 5µl of the serum) and (v) 250 µl of 4mM paraoxon was added finally. The salt-stimulated paraoxonase activity was determined using similar protocol but with addition of 200µl of 5M NaCl and 50µl

of distilled water in the assay. The absorbance was taken immediately at 412 nm wavelength at room temperature using spectrophotometer. The increase in absorbance was recorded at one minute interval for 4 minutes. The non-enzymatic hydrolysis (spontaneous) of paraoxon was corrected by using reagents without substrate as blank and the absorbance was read when the substrate was added (Ap). The enzyme activity was determined by taking absorbance when serum and substrate were added to the reagents (A), using the reagents without serum and substrate as blank. The true absorbance was calculated by subtracting the spontaneous hydrolysis (Ap) from the enzymatic hydrolysis (A) i.e. A-Ap. The average absorbance per minute (ΔA/min) was calculated, and paraoxonase activity was calculated using the molar extinction coefficient, 18290 M^{-1} cm^{-1}. One unit of paraoxonase activity (U) is defined as one nmol of p-nitrophenol formed per minute and activity was expressed as units per ml of serum (U/ml).

Arylesterase activity

The arylesterase activity was determined according to modified method of Gan et al. (1990).[18] The determination was done by mixing 600 µl of 5mM $CaCl_2$, 2ml of 30 mM TrisHCL buffer pH 8.0, 50 µl of distilled water, 50 µl of diluted serum (5 µl of the serum), and 300 µl of 10mM phenyl acetate was added finally. The mixture was vortex-mixed and transferred into quartz cuvette and absorbance reading was taken immediately. The absorbance was read at 270nm wavelength at room temperature using spectrophotometer The increase in absorbance was recorded at one minute interval for 4 minutes.

The rate of non-enzymatic (spontaneous) hydrolysis of phenylacetate was corrected by using reagents without substrate as blank and absorbance was read when the substrate phenylacetate was added (Aph). The arylesterase activity was determined by reading the absorbance when serum and substrate were added to the reagents (A), taking reagents without serum and substrate as blank. The true absorbance was calculated by subtracting the spontaneous hydrolysis (Aph) from the enzymatic hydrolysis (A) i.e. A-Aph. The average absorbance per minute (ΔA/min) was calculated and arylesterase activity was calculated using the molar extinction coefficient, 1310 M^{-1} cm^{-1}. One unit (U) of arylesterase activity is defined as one µmol of phenol formed per minute and activity was expressed as units per ml of the serum (U/ml).

Diazoxonase activity

This was determined according to the method described by Davies et al., 1996.[19] A mixture of 200 µl of 10mM $CaCl_2$, 200 µl 0.5M TrisHCL buffer pH 8.5, 400 µl 5M NaCl, 50 µl distilled water and 50 µl of diluted serum (equivalent to 5 µl of the serum) was made; and 100 µl of 5mM diazoxon was added finally. This was vortex-mixed and transferred into quartz cuvette and absorbance reading was taken immediately. The absorbance was read at 270nm wavelength at room temperature using spectrophotometer The increase in absorbance was recorded at one minute interval for 4 minutes.

The rate of non-enzymatic (spontaneous) hydrolysis of diazoxon was corrected by using reagents without substrate as blank and absorbance was read when the substrate diazoxon was added (Ad). The diazoxonase activity was determined by reading the absorbance when serum and substrate were added to the reagents (A), taking reagents without serum and substrate as blank. The true absorbance was calculated by subtracting the spontaneous hydrolysis (Ad) from the enzymatic hydrolysis (A) i.e. A-Ad. The average absorbance per minute (ΔA/min) was calculated and diazoxonase activity was calculated using the molar extinction coefficient, 3000 $M^{-1}cm^{-1}$. One unit (U) of diazoxonase activity is defined as one nmol of diazoxon hydrolyzed per minute and activity was expressed as units per ml of the serum (U/ml)

Determination of lipid parameters

Lipid profile

Lipid profile analyses were performed in serum from blood collected after an overnight fast. Samples were analysed for total cholesterol,[20] HDL-cholesterol, and triglycerides[21] on ADVIA 1200 chemistry analyser. LDL-C was calculated using Friedewald formula.

Oxidised low-density lipoprotein (Ox-LDL)

Ox-LDL was determined by enzyme-linked immunosorbent assay (ELISA) method using ox-LDL ELISA kit purchased from Mercodia, Sweden according to the manufacturer's instructions. The optical density was measured using a microplate reader at 450 nm. The result was calculated from the standard curve equation.

Statistical analysis

The data from this study were analysed using SPSS IBM version 20. The value of $p < 0.05$ was taken as statistically significant at 95% confidence interval. The normally distributed data were presented as mean and standard deviation (SD) and not normally distributed as median and interquartile range (IQR). Categorical data were presented as frequency and percentage (%). Comparison of lipid parameters and paraoxonase activities between the glyphosate exposed group (sprayers) and the comparative non-exposed group (non-sprayers) was done using independent t-test (Gaussian) or Mann-Whitney U test (non-Gaussian). One way ANOVA was used to compare PON1 activities with respect to period of exposure category among the exposed group. Correlation of PON1 activities with TC, LDL-C, Ox-LDL, TC: HDL, and LDL: HDL was tested using Pearson correlation. Spearman's correlation was used for HDL-C and TG.

RESULTS

Demographic data

The demographic data of the study respondent is as depicted in Table 1. Out of the 103 respondents, 53 were sprayers (glyphosate exposed group) and 50 non-sprayers (comparative non-exposed group). Out of the 53 glyphosate exposed subjects, 22 were from Ladang Penor Legenda, 8 each from Ladang Penor Idaman and Sri Resak, and 15 from Ladang Pulau Manis. All the participants in the study were males. The median (IQR) age for glyphosate exposed and non-exposed were 28(8) and 29(11) respectively. There was no significant difference in age (p=0.549) between the two groups. The race and smoking status were uniformly distributed between the two groups. Most of the respondents from the glyphosate exposed and non-exposed were Indonesian (58.5 % and 60% respectively) while the rest were Bangladeshi. Most of the respondents were smokers which made up 62.3% glyphosate exposed and 60% non-exposed.

Table 1. Race and smoking status in glyphosate exposed and comparative non-exposed groups

	Glyphosate exposed (*n=53*) N (%)	Comparative non-exposed (*n=50*) N (%)	χ^2	*df*	*p*-value
Race			0.007	1	0.841
Bangladeshi	22 (41.5 %)	20 (40.0 %)			
Indonesian	31 (58.5 %)	30 (60.0 %)			
Smoking status			0.179	2	0.892
Smoker	33 (62.3 %)	30 (60.0 %)			
Used to smoke	4 (7.5 %)	3 (6.0 %)			
Non-smoker	16 (30.2 %)	17 (34.0 %)			

Chi-square test, *p*-value of <0.05 was taken as significant at 95% confidence interval.

The range for period of exposure to glyphosate among the sprayers was 6 to 204 months (17 years) with median (IQR) of 7 (14). Eight of the exposed subjects (15.1%) admitted complete use of personal protective equipment, 43 (81.1%) used personal protective equipment partially and only 2 (3.8%) did not use any personal protective equipment. About 61% were exposed for more than one year. Each of the exposed worker spray about 150 litres of glyphosate, most of the time in combination with either all or some of other pesticides which include paraquat, metsulfuron methyl and triclopyr. They sprayed for about 4 days in a week. None of the sprayers admitted receiving official training for spraying pesticides.

PON1 activities

The basal paraoxonase (BPA), salt-stimulated paraoxonase (SSPA), arylesterase (ARYL) and diazoxonase (DIAZ) activities were all lower among the glyphosate exposed group. However, the difference was significant only in

Table 2. Blood pressure and body mass index (BMI) in glyphosate exposed and comparative non-exposed groups

	Exposed (*n=53*)	Comparative non-exposed (*n=50*)	*t-stat*	*p-value*
Blood pressure (mmHg)				
Systolic	120 (20)	117.0 (15.3)		0.184
Diastolic	78 (10)	70.0 (15)		0.005*
BMI (kg/m²)	21.53 (2.06)	21.98 (2.66)	0.973	0.333†

Mann-Whitney U test. Data presented as median (IQR) for blood pressure, Mean (*SD*) for body mass index (BMI), †independent t-test, *Significant difference at 95% confidence interval

diazoxonase activity (p=0.002) (Figure 1). When the glyphosate exposed group were categorized into 3 groups according to the period of exposure (Table 3), both arylesterase and diazoxonase were found to be lowest in those who were exposed for more than 36 months, but only arylesterase showed a significant difference (p=0.042).

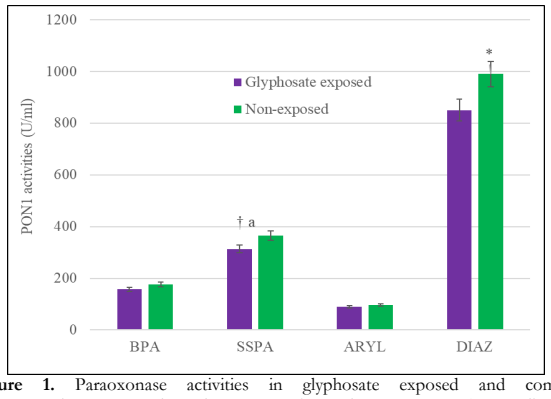

Figure 1. Paraoxonase activities in glyphosate exposed and comparative non-exposed group. Independent t-test, bars show mean SD), [a]Median (IQR), †Mann-Whitney U test, *significant difference p<0.05 at 95% confidence interval. BPA=basal paraoxonase activity, SSPA= salt stimulated paraoxonase activity, ARYL=aryesterase activity, DIAZ= diazoxonase activity.

Table 3. PON1 activities in different categories of period of exposure in glyphosate exposed group

	Period of exposure categories (Months)				
PON1 activities	**6-12 (*n*= 38)**	**13-36 (*n*= 11)**	**Above 36 (*n*= 3)**	***F*-test**	***p*-value**
Basal PON (U/ml)	145.72 (58.00)	192.75 (53.59)	164.21 (62.62)	2.90	0.065
Arylesterase (U/ml)	89.88 (16.21)	95.21 (16.53)	67.49 (18.27)	3.39	0.042*
Diazoxonase (U/ml)	879.64 (216.93)	812.08 (163.89)	671.67 (162.69)	1.71	0.191

One-way ANOVA, results presented as mean (SD), *significant at 95% confidence interval

Lipid parameters

The total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), triglycerides (TG) and high-density lipoprotein-cholesterol (HDL-C) were non-significantly lower in the exposed group (p>0.05) (Table 4). However, Ox-LDL was significantly higher in glyphosate exposed than that of comparative non-exposed group (P<0.001) (Table 4). When PON1 activities were expressed per unit of ox-LDL, the basal paraoxonase, arylesterase and diazoxonase to ox-LDL ratios were all significantly lower among the glyphosate exposed as compared to that of comparative non-exposed group (Figure 2).

Table 4. Lipid parameters in glyphosate exposed and the comparative non-exposed group

Test parameters	**Exposed (*n=53*)**	**Comparative (*n=50*)**	***t*-stat**	**df**	***p*-value**
TC (mmol/l)	5.10 (1.09)	5.36 (1.64)			0.196
TG (mmol/l)	1.15 (0.84)	1.17 (0.66)			0.505
HDL (mmol/l)	1.32 (0.44)	1.33 (0.39)			0.835
LDL (mmol/l)	3.09 (0.86)[a]	3.34 (0.96)[a]	-1.41	101	0.162†
Ox-LDL	4.89 (3.94) *	2.83 (1.63)			<0.001

Mann-Whitney U test, data presented as median (IQR), [a]Mean (*SD*), †independent t-test, *Significant difference at 95% confidence interval.

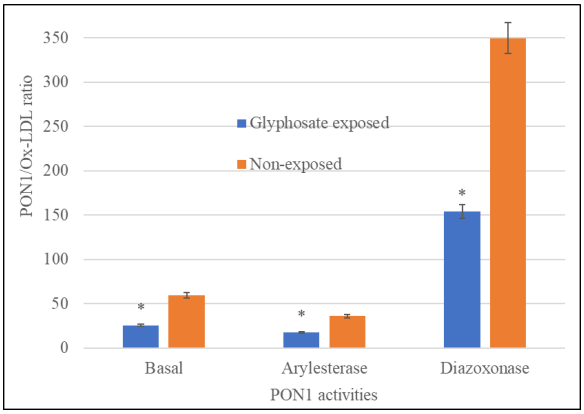

Figure 2. PON1 activities to Ox-LDL ratios. Mann-Whitney U test, data presented as median (IQR), *Significant difference at 95% confidence interval.

DISCUSSION

Use of pesticides such as glyphosate has negative effects to all organisms including humans especially those handling them. Several studies have examined the effects of these pesticides on a number of organisms. Therefore, it is imperative to examine their effects on human especially the sprayers of the pesticides. This cross-sectional comparative study involved 103 respondents, 53 sprayers (glyphosate exposed group) and 50 non-sprayers (comparative non-exposed group) were examined. All the participants in the study were males, because mostly males partake such jobs. Even though most of the respondents in the study were smokers, and smoking was reported to affect the activities of PON1,[22,23] for the fact that smoking status was uniform in the respondents, its effect on PON1 is presumed to be effaced.

No significant difference in the BMI (p=0.333) was observed between the two groups. This is expected since the groups were matched during selection. In terms of the

blood pressure of the respondents, there was no significant difference in systolic blood pressure between the two groups. However, though within normal range, diastolic blood pressure was significantly higher among the exposed group (p=0.005). There was no family history of hypertension, diabetes mellitus or ischemic heart disease among the subjects except for one subject from exposed group who gave family history of hypertension. A report by Robb and Baker[24] showed that organophosphate exposure causes hypertension. This was suggested to be as a result of nicotinic receptors stimulation in the adrenal gland leading to hypertension, sweating, tachycardia, and leucocytosis. Recently, a study confirmed that organophosphates play role in the aetiology of blood pressure dysregulation.[25] These studies explained the observed significantly higher diastolic blood pressure in the glyphosate exposed group in this study.

Most of the subjects in this study were exposed to glyphosate for over 12 months and arylesterase was found to be significantly lowest (p=0.042) in those who were exposed for over 36 months. This could possibly be due to accumulation of the organophosphate and its reaction products such as dialkylphosphates over period. It has been demonstrated that the effect of organophosphate increases with the period of exposure even though the metabolites have short half-life in the body.[26] Though the use of PPE is expected to prevent direct contact with the pesticides, it has been established that absorption cannot be prevented[27] and hence the workers are still exposed upon repetitive regular long-term exposure. To some extent, our results indicated that there is variation in PON1 activities with respect to the period of exposure. Similar findings of varying changes in PON1 activity with respect to period of exposure were reported by Hofman et al. (2009)[28] and Hernandez et al., (2008).[29]

Data from this current study revealed that PON1 diazoxonase activity was significantly lower (p=0.002) in glyphosate exposed farm workers. Similar reduction in PON1 activity by glyphosate was reported in fish.[8] Kuang et al. (2006)[30] showed that organophosphate also reduced PON1 activities in humans. Here, they demonstrated that long term direct exposure to organophosphates significantly inhibits the activities of PON1, carboxylesterase, acetylcholinesterase, and butyrylcholinesterase enzymes in different levels. They also showed that the PON1 activity which presumably detoxifies organophosphate was affected by its polymorphic forms. MM polymorphic form was found to be predominant in the organophosphate exposed respondents implying that it was responsible for the lower PON activity in them compared to the control group.

With respect to lipid parameters, although previous study showed an increase in TC, HDL LDL-C and TG in animals following exposure to organophosphates,[31] glyphosate exposure in humans seems to have no effect on lipid profile as observed in the present study. The slightly lower LDL-C glyphosate exposed group is consistent with study by Ibrahim and El-Ghamal (2003)[32] following exposure to diazinon. This was suggested to be due to induction LDL receptors for the clearance of cholesterol from circulation.

The higher ox-LDL in glyphosate exposed subjects was expected since exposure to glyphosate lowered PON1 activities which by implication affected the ability of HDL to prevent LDL oxidation. The findings of higher ox-LDL and lower PON1: ox-LDL ratios among glyphosate exposed group indicated that glyphosate exposure causes a reduction in the ability of PON1 to hydrolyse ox-LDL which may subsequently decrease their protection against LDL oxidation, thus predisposes them to a higher risk of developing atherosclerosis and CAD. In a study similar to ours, it was demonstrated that PON1 activity could predict the severity of CAD in multiple vessel lesions, smokers and diabetic subjects.[33] Furthermore, the significant reduction of PON1 activity in severe CAD patients corroborate the significance of PON1 prevention of CAD.[34] To our knowledge, this is the first study in human reporting the level of ox-LDL among chronic glyphosate exposure in humans. Only Zheng et al. (2021) study which showed lipids elevation following exposure to glyphosate [35] was near to our study, however, their study was in tilapia fish and ox-LDL was not measured.

CONCLUSION

This study highlighted that glyphosate exposure reduces

the PON1 activities and suppress its ability to hydrolyse Ox-LDL. Thus, glyphosate exposure might predispose an individual to the risk of developing atherosclerosis and CAD through this mechanism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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