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Original Article

Effect of Occupational Exposure to Herbicides on Oxidative Stress in Sprayers

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ABSTRACT

Background: Herbicides such as glyphosate, paraquat, and 2,4-dichlorophenoxyacetic acid have been reported to cause adverse side effects through production of reactive oxygen species. However, there were no data representing the adverse effects of a mixture herbicide usage in farmers, especially the changes in oxidative marker and antioxidant defense. This study aimed to determine the urinary malondialdehyde (MDA) and glutathione (GSH) level in farmers using mixed herbicides.
Methods: Ninety-three farmers were recruited, and two spot urine samples (before and after work) were collected. The urinary MDA level was evaluated by thiobarbituric acid reactive substance assay, and the urinary GSH level was determined using the enzymatic recycling method.
Results: Sixty-two percent of the participants were men, and 59% of the participants worked in a farm for 20–40 years. The common combinations of herbicide usage were glyphosate with 2,4-dichlorophenoxyacetic acid (36.5%). There was no significant difference between pre- and post-work urinary MDA and GSH levels among the 3 groups of herbicides. However, the urinary MDA levels in farmers using the combination of glyphosate and paraquat were significantly higher than those found in farmers using glyphosate alone. The associated factors with changes in MDA levels found that the exposure intensity index ($B = 0.154$), the cumulative exposure intensity index ($B = 0.023$), and wearing gloves while working ($B = -2.347$) were found to be significantly associated with MDA level.
Conclusion: The results suggest that the combined use of glyphosate and paraquat caused a significant increase in urinary MDA levels. Moreover, intensity of exposure to herbicide and wearing gloves were associated with the level of MDA.

1. Introduction

Herbicides, namely, glyphosate, paraquat, and 2,4-dichlorophenoxyacetic acid (2,4-D), are commonly used in Thai agriculture to enhance productivity [1]. However, several studies demonstrated numerous side effects of exposure to these herbicides [2–4]. These herbicides can directly enter into the body through inhalation, skin absorption, and oral ingestion routes [1], which can cause short-term and long-term adverse health effects [5]. The main toxic mechanism of these substances is initially by reactive oxygen species (ROS) generation in the cell, contributing to imbalance between free radicals and antioxidant defenses [6–8]. It has been well established that high levels of ROS can cause direct damage to lipids [9]. Lipid peroxidation produces a wide variety of oxidation products including malondialdehyde (MDA), propanal, hexanal, 4-hydroxynonenal, and F_2-isoprostanes [10]. Among these toxic by-products, MDA has been recognized as the most mutagenic product of lipid peroxidation, whereas 4-hydroxynonenal is the most toxic [11]. To scavenge ROS toxicity, antioxidants play an important role in detoxification of ROS. One of the important antioxidant defenses is γ-L-glutamyl-L-cysteinyl-glycine known as glutathione (GSH) [12]. The nonprotein thiol of GSH acted as the catalyst to reduce H_2O_2 (most toxic substance) to H_2O (nontoxic substance) [13].

Herbicide-induced oxidative stress has been reported in many studies. It was assumed that a single exposure to herbicide could

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induce oxidative stress. The significant increase in ROS levels and lipid peroxidation was found in mice injected intraperitoneally with paraquat at a sublethal dose [14]. Glyphosate-induced ROS accumulation has been reported to be associated with the oxidation and antioxidation system imbalance as well as lipid peroxidation [15]. Moreover, the increase in ROS levels and lipid peroxidation was observed in herbicide-exposed farmers who used glyphosate, 2,4-D, paraquat, and atrazine [16–18]. In fact, most Thai farmers usually repeatedly used the single product or combinations of herbicide products to gain the highest weed control effectiveness. The use of the combination of herbicides with different modes of action on weeds exhibited broad-spectrum activity; however, the combination of these substances also represents a different toxicological profile compared with toxicity of the single herbicide [19]. This behavior probably increased the risk of herbicide intoxication. A previous study found that the combined use of two pesticides, chlorpyrifos and carbofuran, showed synergistic effect on cytotoxicity and neurotoxicity through oxidative stress induction in *vitro* [20,21].

Available studies in *vivo* suggested that the antioxidant enzyme, GSH, content was reduced by a synergistic effect of pesticide mixture in zebra fish and freshwater gastropods [22,23]. According to Mishra and Srivastava [24], the researchers described the effect of exposure to the combination of pesticides on antioxidant defenses. The GSH level and GSH: glutathione disulfide (GSSG) ratio were remarkably decreased in rats exposed to the combination of pesticides. Nevertheless, there were no data representing the adverse effects of combined herbicide use in farmers on ROS induction and decrease in antioxidant levels, resulting in adverse health effects. In the present study, we hypothesized that the farmers who used herbicide mixtures containing glyphosate, paraquat, and 2,4-D probably could induce oxidative stress, leading to the release of MDA and GSH into urine more than a single-product use. Hence, the main objective of this study was to determine the urinary MDA and GSH levels in farmers using combinations of glyphosate, paraquat, or 2,4-D in agricultural activity in Long District, Phrae province.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Ethics Committee of the Faculty of Medicine, Chiang Mai University, Thailand (study code: FOR-2562-06349). All participants were informed about the protocol of this study and signed the consent form as per the guidelines of the Faculty of Medicine Ethical Committee.

2.2. Location and population

This study was conducted in Long District, Phrae province, Thailand (Fig. 1). The inclusion criteria consisted of farmers (1) aged between 30 and 60 years, (2) who have been using glyphosate, paraquat, or 2,4-D or the combination of these during the study period, and (3) who have never been diagnosed with kidney disease, diabetes, and gout. Ninety-three participants were eligible for this study. All individuals were interviewed face-to-face using a questionnaire documenting their demographic data, personal and health history, work characteristics (period of herbicide use, type of work, volume and concentration, frequency), and personal protective equipment (PPE) use.

2.3. Urine sample collections

Spot urine samples in the morning before work (prework) and next morning after work (postwork) were collected from each participant. All samples were stored at −20°C before the MDA and GSH analysis [25].

2.4. Cumulative herbicide exposure intensity index

To investigate the cumulative intensity of herbicide exposure in each participant, it was calculated using a slightly modified formula of Dosemeci et al. [26]. Factors relating to the intensity of herbicide exposure including mixing status, repair status, application method (e.g., backpack, hand spray), use of PPE (e.g., gloves, respirators, face shields, boots), duration of exposure, and frequency were used for calculation of an estimate level of herbicide exposure [26] as follows:

$$\text{EII} = (\text{Mixing status} + \text{Application method} + \text{Repair status}) \times \text{Personal Protective Equipment}$$

where Mixing status refers to never mixing (score 0) and mixed (score 9); Application method refers to does not apply (score 0), aerial aircraft (score 1), distribute tablets (score 1), application in furrow (score 2), boom tractors (score 3), backpack (score 8), and hand spray (score 9); Repair status refers to does not repair (score 0) and repair (score 2); and Personal protective equipment refers to four groups of PPE categories that are identified considering combinations of PPE used (Table 1) and then the score for each category [PPE-0 (score 1.0), PPE-1 (score 0.8), PPE-2 (score 0.7), PPE-3 (score 0.6), PPE-1 and PPE-2 (score 0.5), PPE-1 and PPE-3 (score 0.4), PPE-2 and PPE-3 (score 0.3), PPE-1 and PPE-2 and PPE-3 (score 0.1)].

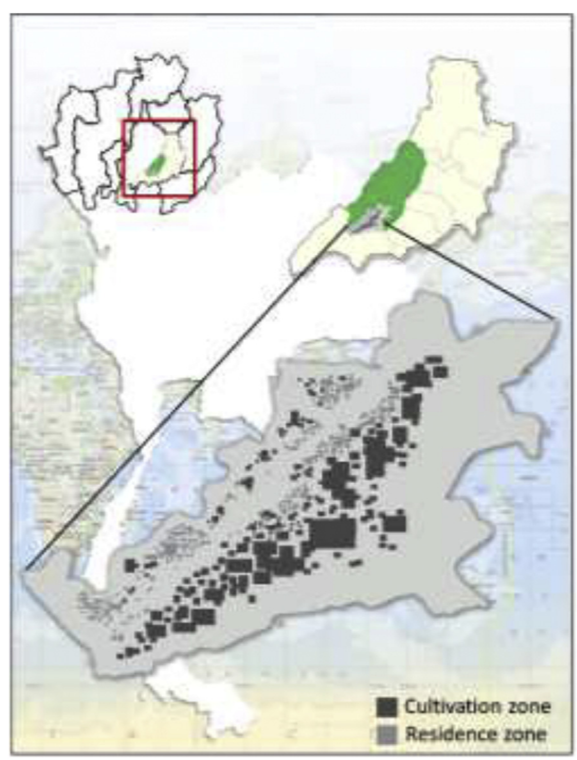

Fig. 1. The study location at Thung Lang subdistrict, Long District, Phrae province, northern Thailand. This area consisted of the cultivation zone (dark gray) and residence zone (gray).

Then, a cumulative herbicide exposure intensity index was subsequently calculated as follows:

$$\text{cumulative herbicide exposure intensity index} = \text{EII} \times \text{duration} \times \text{frequency}$$

where EII is the exposure intensity index, duration is the duration of exposure for the number of days applied, and frequency is the frequency of exposure in the number of hours of applications per day.

2.5. Quantification of urinary MDA levels

The urinary MDA levels in prework and postwork urine samples were determined using the thiobarbituric acid reactive substance method [27]. In brief, 200 μL of the urine sample was added into a mixture of 375 μL of 1% phosphoric acid (Merck, Darmstadt, Germany) and 125 μL of 0.6% thiobarbituric acid (Sigma-Aldrich, St. Louis, MO, USA). After that, the mixture was boiled at 90°C. After boiling for 30°minutes, the color reaction was then measured at 532 nm using a microplate reader (Synergy™ H4; BioTek Instruments, Inc., Winooski, VT, USA). The urinary MDA level was determined based on malonaldehyde bis(dimethyl acetal) (Sigma-Aldrich, St. Louis, MO, USA) standard curve, and the concentrations were expressed as micromolar [28].

2.6. Quantification of urinary GSH levels

The urinary GSH levels in prework and postwork urine samples were determined using the enzyme recycling system with 5,5′-dithiobis(2-nitrobenzoic acid) and glutathione reductase [12]. Sixty microliters of the sample was mixed with one-half volume of 0.6% sulfosalicylic acid (Sigma-Aldrich, St. Louis, MO, USA). The mixture was centrifuged at 8000 g for 10 minute at 4°C. After that, the supernatant was transferred into a new tube. In brief, 20 μL of the supernatant of the urine sample or standard was added into a 96-well plate. Freshly prepared 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, St. Louis, MO, USA) and glutathione reductase solutions (Sigma-Aldrich, St. Louis, MO, USA) were added. After incubation at room temperature for 30 s to facilitate the conversion of GSSG to GSH, 60 μL of β-NADPH (Sigma-Aldrich, St. Louis, MO, USA) was put into the well. The absorbance at a wavelength of 412 nm was immediately measured every 30 s for 2 minutes using a microplate reader (Synergy™ H4; BioTek Instruments, Inc., Winooski, VT, USA) [12]. The urinary GSH level was determined based on the reduced form of GSH (Sigma-Aldrich, St. Louis, MO, USA) standard curve, and the concentrations were expressed as micromolar.

2.7. Statistical analysis

Statistical analysis was conducted using SPSS for Windows, version 16.0 (SPSS Inc; 2007, Chicago, USA), and GraphPad Prism, version 8.3.0, for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). All demographic data (gender, year of work, smoking, alcohol consumption, type of herbicides, number of tanks, volume of pesticide use, working hours on the farm, and PPE) were described and analyzed by descriptive statistics. The levels of MDA and GSH in prework and postwork urine samples were compared using the Wilcoxon signed-rank test. Kruskal–Wallis analysis was used to compare the urinary MDA and GSH levels among the three groups of herbicide users. Linear regression was conducted to assess the association between the postwork levels of MDA and GSH with independent variables (age; smoking; alcohol consumption; types of herbicide use; herbicide exposure intensity index; cumulative herbicide exposure intensity index; use of gloves, boots, and facial masks while working). The regression model was weighted (weight cases) by the prework level of MDA to correct the overrepresentation or underrepresentation of certain characteristics in the sample. Cases with zero, negative, or missing values for the weighting variable are excluded from analysis.

Table 1
The groups of PPE categories for EII calculation.

Categories	Description
PPE-0	Never used PPE
PPE-1	Face shields or goggles, fabric/leather gloves, other protective clothing
PPE-2	Cartridge respirators or gas masks, disposable outer clothing
PPE-3	Chemically resistant rubber gloves

EII, exposure intensity index; PPE, personal protective equipment.

3. Results

Ninety-three participants were randomly selected from five villages in Thung Lang subdistrict, Long District, Phrae province. The characteristics of the study participants are shown in Table 2. The majority of agricultural workers were men (62.4%). Most of the participants worked for at least 20–40 years (60.4%). Fifty-six percent of the participants used only glyphosate, and approximately 36.5% of the participants used glyphosate with 2,4-D, whereas only 7.5% used glyphosate and paraquat during their farm work. Most farmers (42.9%) sprayed 4–6 tanks of herbicide per day and usually prepared herbicide dilution in water at a ratio of 1:200.

Table 2
Demographic characteristics, types of herbicides, number of tanks, volume of pesticide use, working hours in farms, and use of personal protective equipment.

Characteristics	N (%)
Gender	
Male	58 (62.4%)
Female	35 (37.6%)
Years of work	
<19 years	13 (14.3%)
20–40 years	55 (60.4%)
>40 years	23 (25.3%)
Cigarette smoking	
Current smoker	22 (23.7%)
Nonsmoker	71 (76.3%)
Alcohol consumption	
Alcohol drinker	52 (56.0%)
Nondrinker	41 (44.0%)
Types of herbicide use while working	
Glyphosate	52 (56.0%)
Glyphosate and paraquat	7 (7.5%)
Glyphosate and 2,4-D	34 (36.5%)
Number of tanks	
1–3 tanks	34 (37.3%)
4–6 tanks	39 (42.9%)
More than 6 tanks	13 (19.8%)
Volume of pesticide use	
1–3 L/day	22 (23.6%)
4–6 L/day	32 (34.4%)
More than 6 L/day	39 (42.0%)
Working hours in farms	
1–5 h/day	49 (63.0%)
6–12 h/day	29 (37.0%)
Use of personal protective equipment	
Masks	90 (96.7%)
Gloves	81 (87.1%)
Boots	92 (99.0%)

2,4-D, 2,4-dichlorophenoxyacetic acid.

Approximately 34.4% of farmers used 4–6 L of herbicides per day in their work. Sixty-three percent of farmers worked in a farm for 1–5 h per day. The interval time to collect urine sample before and after work was 21.84 ± 3.66 h. Regarding the PPE usage, the results showed that almost every participant wore masks, gloves, and boots while working.

For the determination of the urinary MDA level, the participants were divided into three groups based on the herbicide use (glyphosate, combined glyphosate and paraquat, combined glyphosate and 2,4-D). The average levels of urinary MDA in the prework sample of workers who used glyphosate, glyphosate with paraquat, and glyphosate with 2,4-D were 1.78 ± 0.28, 4.03 ± 1.73, and 3.86 ± 0.48 μM, respectively. The prework urinary MDA levels in participants using glyphosate and 2,4-D were significantly higher than those found in participants using only glyphosate. The average levels of urinary MDA in the postwork sample of workers who used glyphosate, glyphosate with paraquat, and glyphosate with 2,4-D were 1.92 ± 0.32, 4.47 ± 1.02, and 2.24 ± 0.41 μM, respectively. The postwork urinary MDA levels in participants using a combination of glyphosate and paraquat were significantly higher than those who work with only glyphosate. However, there were no significant differences of the urinary MDA levels between the prework and postwork sample among the three groups, as shown in Fig. 2.

The urinary GSH content in urine samples is shown in Fig. 3. No significant difference was found in prework and postwork GSH content in each group of herbicide usage. The average levels of GSH in the prework urine sample of workers who used glyphosate, glyphosate with paraquat, and glyphosate with 2,4-D were 0.16 ± 0.02, 0.09 ± 0.01, and 0.19 ± 0.04 μM, respectively. Likewise, the average levels of GSH in the postwork urine sample of workers who used glyphosate, glyphosate with paraquat, and glyphosate with 2,4-D were 0.12 ± 0.01, 0.07 ± 0.02, and 0.14 ± 0.02 μM, respectively. However, the GSH content in urine of workers who used a combination of glyphosate and paraquat tends to decrease compared with the level in workers who used glyphosate alone and glyphosate with 2,4-D. In addition, the GSH content was compared between the prework and postwork urine samples. The result showed no significant difference in urinary GSH levels between the prework and postwork samples among the three groups of herbicide use. However, the GSH level in the postwork sample likely declined compared with the level in the prework sample of farmers using the combination of glyphosate and paraquat.

Linear regression was used to study the association between independent variables (age; smoking; alcohol consumption; types of herbicide use; herbicide exposure intensity index; cumulative herbicide exposure intensity index; the use of gloves, boots, and facial masks while working) and the postwork urinary MDA levels (Table 3). The results showed that the MDA level in the postwork urine sample was significantly positively associated with the herbicide exposure intensity index (β = 0.207) and cumulative herbicide exposure intensity index (β = 0.259). Moreover, our findings found that wearing gloves during work can reduce the MDA level (β = −0.180). This result also indicated that an increase in one unit of herbicide exposure, namely, the herbicide exposure intensity index and cumulative herbicide exposure intensity index, is associated with the increase in the MDA level in the postwork urine sample by 0.154 and 0.023 units, respectively. In addition, the decrease in wearing gloves is associated with the decrease in the MDA level by 2.347 units. However, the other factors including age, smoking, alcohol consumption, types of herbicide uses, wearing boots, and wearing facial masks did not influence the postwork urinary MDA levels. In linear regression analysis of the GSH level and independent variables, the result showed that the urinary GSH levels in the postwork sample were not associated with all independent variables (data not shown).

4. Discussion

Many studies have demonstrated the toxic effects of herbicide *in vitro* and *in vivo*. However, the observation of toxicity among workers who were occupationally exposed to the combined application of herbicides has been less reported because oxidative stress induction has been proposed to play a pathological role in herbicide poisoning especially glyphosate and paraquat. The oxidative stress biomarker of lipid peroxidation, MDA levels, and antioxidant defenses, GSH, were determined in two spot urine samples of farmers. Our result found the significant difference of the urinary MDA level between farmers using glyphosate and those using combined glyphosate and 2,4-D in the prework sample. It was probable that other factors such as smoking and history of pesticide use including the use of other pesticides before research

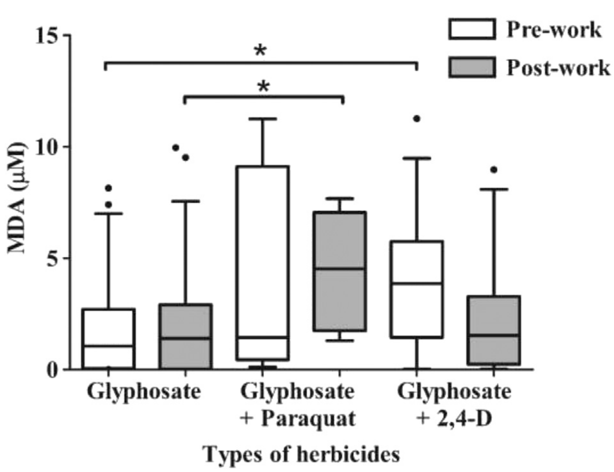

Fig. 2. The urinary MDA level in herbicide-exposed workers using glyphosate, glyphosate with paraquat, and glyphosate with 2,4-D in the prework (open bar) and postwork (solid bar) urine sample. The data are represented as mean ± standard error of mean (SEM). *The MDA level was significantly different between the groups ($P < 0.05$). 2,4-D, 2,4-dichlorophenoxyacetic acid; MDA, malondialdehyde.

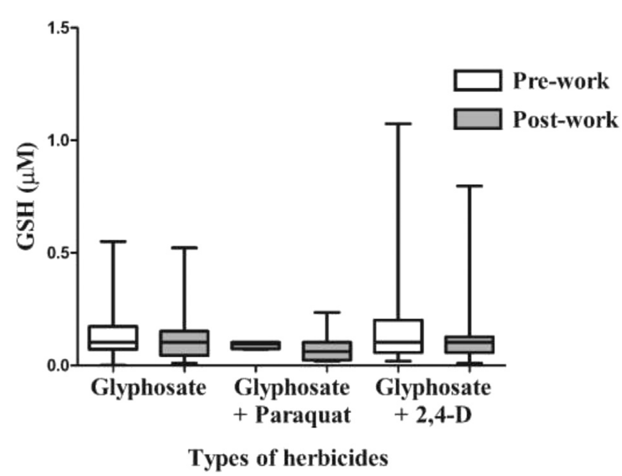

Fig. 3. The urinary GSH level in herbicide-exposed workers using glyphosate, glyphosate with paraquat, and glyphosate with 2,4-D in the prework (open bar) and postwork (solid bar) urine sample. The data are represented as mean ± standard error of mean (SEM). 2,4-D, 2,4-dichlorophenoxyacetic acid; GSH, glutathione.

Table 3
The factors associated with the change in the urinary MDA level in the postwork sample during herbicide application.

Independent variables	B ± SE (95% CI)	Beta (β)	sig
Herbicide exposure intensity index	0.154 ± 0.062	0.207	0.014
Herbicide cumulative exposure index	0.023 ± 0.008	0.259	0.003
Wearing gloves during work task	−2.347 ± 0.981	−0.180	0.018

B or unstandardized regression coefficient represents the amount of change in a dependent variable MDA due to a change of 1 unit of independent variables; SE represents the standard error of regression coefficient; Beta (β) represents the standardized regression coefficient ranging from 0 to 1 or 0 to −1, depending on the direction of the relationship (the closer the value is to 1 or −1, the stronger the relationship); sig represents the significant predictors at the significance level of 0.05.
95% CI, 95% confidence interval; MDA, malondialdehyde.

recruitment have influenced the MDA level. Wafa et al. [29] indicated the significant increase in the MDA level in farmers who had a working history with prolonged use of many pesticides. Furthermore, the synergistic effect between previous history of use of pesticide and current smoking behavior has been reported to affect the changes of the oxidative marker level by detection of a high level of MDA [30].

The urinary MDA level in the postwork sample of farmers who used both glyphosate and paraquat was significantly higher than the level in farmers who used glyphosate alone. It could be attributed to 2 reasons: (1) paraquat exerted the ability to induce MDA only, and (2) both herbicides glyphosate and paraquat exerted a joint effect to induce MDA. The second reason was previously supported by several publications. They found that these two herbicides also exhibited the adverse effects on oxidative stress. Shukla et al. [22] reported that exposure to pesticide mixture was more toxic than individual exposure and also resulted in the highest MDA level. In addition, the MDA level in the group exposed to combined glyphosate and paraquat was significantly increased. In the rat model, exposure to the combination of herbicides of diclofop methyl and difenoconazole showed increase in the MDA level compared with exposure to a single herbicide [31]. In addition, the previous study compared the effect of MDA between exposure to the mixture and single exposure to pesticide. The result showed that the combined exposure to pesticide represented the significant high level of MDA in sprayers [32]. Previous findings have proposed the combined mechanism of glyphosate and paraquat to induce toxicity. The paraquat-exporting protein known as multidrug and toxin extrusion 1 protein in renal cells was disturbed by glyphosate. Hence, the exportation of paraquat from the apical membrane of tubular cells into the renal lumen for excretion by urine was suppressed, resulting in higher accumulation of paraquat in renal cells [33,34]. Moreover, the disruption of ATP hydrolysis by glyphosate at the phosphate-binding site reduced the function of renal efflux protein, multidrug resistance protein 1, in the kidney against paraquat toxicity [34]. These events possibly supported the elevation of ROS generation in renal cells exposed to combined glyphosate and paraquat.

Comparing the prework and postwork sample in each group, the urinary MDA level in prework and postwork urine samples was not significantly different among farmers who used glyphosate, combined glyphosate and paraquat, as well as combined glyphosate and 2,4-D. This might be due to oxidative stress repair after herbicide exposure. Wang et al. [35] suggested that the repairing and elimination process to clear ROS was effective immediately after exposure to pesticide. They found the level of 8-hydroxy-2'-deoxyguanosine (8-OHdG), oxidative stress marker, was decreased to baseline within 2 days after pesticide exposure [35]. A previous study in Thai male farmers showed no statistical difference in 8-OHdG and DNA damage before and after pesticide application seasons [36]. Owing to occupational exposure to herbicide, it is possible that the induction of the MDA level was not substantial enough to be detected because of low-dose exposure in the short period. The in *vitro* model was studied to show the effect of low concentration of glyphosate. After 24-hour incubation of glyphosate at the occupational exposure level, no significance different was observed in the ROS level between the control and treatment group [37]. Moreover, the use of PPE during working is an important factor to control herbicide exposure. Most participants in this study wore gloves, masks, and boots while working. It is likely that health protection by PPE might be an effective approach to avoid direct contact with herbicide. This reason was supported by previous findings. The workers involved in rice farming who wore PPE had a significantly lower level of MDA and were significantly associated with a lower level of 8-OHdG [38]. The health risk of herbicide exposure in humans was remarkably decreased approximately 32% when the workers wore effective PPE [35], and wearing PPE reduced the risk of exposure to other pesticides [36–38]. Hence, health surveillance by using good work practices and using PPE should be supported to reduce health effects.

Regarding comparison of the urinary GSH level in urine samples, there was no significant difference in the GSH level among the 3 groups of herbicide use. It might be the result of the protection rendered by wearing of PPE during working as mentioned previously. However, the GSH level in farmers who used a combination of glyphosate and paraquat tends to decrease compared with the level in farmers who used glyphosate alone and glyphosate with 2,4-D. Based on the mode of action of paraquat, it generates ROS and eventually leads to enhancement in lipid peroxidation [18] and decrease in the GSH level owing to antagonizing effect of oxidative action of paraquat. Thus, the GSH level represented a marker in the susceptibility of the human biological system to oxidant-induced tissue injury [39].

To study the associated factors in MDA and GSH levels, a linear regression using the weight cases method was used. It was found that the urinary MDA level of the postwork sample was significantly positively associated with the herbicide exposure intensity index and cumulative herbicide exposure intensity index, whereas it was negatively associated with wearing gloves. These algorithms are estimated based on the chance of farmers' exposure to herbicide during working, i.e., mixing, loading, application in farms, and cleaning equipment including time of exposure. All activities supported the risk of herbicide exposure in workers, leading to association with herbicide intoxication. Other findings represented that the accumulation and degree of herbicide exposure were based on the duration and frequency of agricultural activities [36,40].

In conclusion, it could be concluded from this study that there is increased MDA production with occupational exposure to combined herbicide of glyphosate and paraquat. There is no significant difference in the GSH level between use of single herbicide and combined use of herbicides. In addition, intensity of exposure to herbicide and wearing gloves while working were associated with the urinary MDA level after agricultural activities.

Conflicts of interest

The authors have no conflicts of interest.

Author contributions

K.W. and S.K.-a. conceived the present idea. K.W., S.K.-a., and R.S. were involved in planning and supervised the work. K.W., S.K.-a., U.I., and K.K. contributed to sample collection and management. U.I. and K.K. contributed to interviewing of the participants. U.I. and S.K.-a. carried out the experiments. U.I., S.K.-a., and K.W. contributed to the interpretation of the results and data analysis. U.I., S.K.-a., and K.W. contributed to original draft preparation. All authors have read and agreed to the published version of the manuscript.

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