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Micronucleus Analysis in Buccal Swabs of Paddy Farmers from the East Coast of Malaysia

(Analisis Mikronukleus Calitan Pipi Pesawah Padi dari Pantai Timur Malaysia)

ASMAH HAMID, KHOLILUL SYAFIQ BAHARUDDIN, NOR FADILAH RAJAB, SYARIF HUSIN LUBIS, ISMARULYUSDA ISHAK, HIDAYATUL FATHI OTHMAN, AHMAD ROHI GHAZALI, ZARIYANTEY ABDUL HAMID, NUR ZAKIAH MOHD SAAT, NIHAYAH MOHAMMAD, MOHD JAMIL MOHD RAFAAI, MOHD NAZIR NASROM & MOHAMAD ROFF MOHD. NOOR

ABSTRACT

Pesticide exposure may cause genotoxic effects by inducing the formation of micronucleus (Mn). Mn are fragments of chromosomes that remains after cells division. The increase in Mn may increase the risk of cancer formation. Our study aimed to determine the effects of lifestyle and pesticide exposure on the formation of Mn in epithelial cells from buccal swabs among paddy farmers in Malaysia. About 40 farmers who were exposed to pesticides were chosen as subjects and 30 personnels whose not directly exposed to pesticides, were chosen as the control group. Demographic and anthropometric data were obtained from questionnaires developed. Analysis of Mn formation was done using Giemsa staining (10% v/v) and the frequency of Mn formation was scored from 1000 cells per sample. Kruskal-Wallis test done between Mn frequency with age group showed a significant increase ($p < 0.05$) in Mn frequency in farmers as compared to the control in the age group of 30-39 , 40-49 years, and 50-59 years. Significant increased ($p < 0.05$) were observed between Mn frequency groups of normal BMI, pre-obese, and grade 1 obese as compared to control. Significant increase of Mn frequency ($p < 0.01$) was also seen among smokers and farmer's group (15.39 ± 3.34) as compared to controls (4.76 ± 1.26). The maximum numbers of Mn found in farmers are 7 Mn per cell whereas for control group is only 3 Mn. However, most farmers had only 1 Mn ($81.75 \pm 6.42\%$) and 2 Mn ($15.28 \pm 5.14\%$). Mn frequency with the duration of exposure to pesticides in a month and the use of PPE revealed no significant difference ($p = 0.27$). In conclusion, the increased frequency of Mn was influenced by age, gender, BMI and smoking status of farmers besides commonly repeated duration of exposures and the use of PPE. Further studies are needed to analyze the causes of an increased in Mn among farmers.

Keywords: Genotoxic; pesticides; micronucleus; farmers; buccal cells

ABSTRAK

Pendedahan kepada pestisida boleh menyebabkan kesan genotoksik melalui aruhan pembentukan mikronuklues (Mn) iaitu serpihan kromosom yang tertinggal selepas proses pembahagian sel. Peningkatan pembentukan Mn boleh meningkatkan risiko kanser. Kajian ini bertujuan untuk mengenalpasti kesan pendedahan peptisida kepada pembentukan Mn dalam sel epitelium calitan pipi dalam pesawah di Malaysia. Seramai 40 orang petani terdedah kepada peptisida dan 30 orang kumpulan kawalan yang langsung tidak terdedah kepada peptisida telah dikaji. Data demografi dan antropometri diperoleh daripada soal selidik. Analisa pembentukan Mn dilakukan dengan pewarnaan Giemsa (10% v/v). Analisa kekerapan pembentukan Mn dilakukan daripada 1000 sel bagi setiap sampel. Hasil ujian Kruskal-Wallis antara kekerapan Mn dengan kelas umur terdapat peningkatan signifikan ($p < 0.05$) antara kekerapan Mn kumpulan petani berbanding kawalan pada kelas umur 30-39 tahun, 40-49 tahun, dan 50-59 tahun. Peningkatan yang signifikan ($p < 0.05$) diperhatikan di antara kumpulan kekerapan Mn BMI biasa, pra-obes, dan obes gred 1 berbanding dengan kawalan. Peningkatan ketara kekerapan Mn ($p < 0.01$) juga dilihat di kalangan perokok dan kumpulan petani (15.39 ± 3.34) berbanding kawalan (4.76 ± 1.26). Jumlah maksimum Mn yang ditemui pada petani adalah 7 Mn per sel sedangkan bagi kumpulan kawalan hanya 3 Mn. Walau bagaimanapun, kebanyakan petani hanya mempunyai 1 Mn ($81.75 \pm 6.42\%$) dan 2 Mn ($15.28 \pm 5.14\%$). Kekerapan Mn dengan tempoh pendedahan kepada racun perosak dalam sebulan dan penggunaan PPE tidak menunjukkan perbezaan yang signifikan ($p = 0.27$). Kesimpulannya, peningkatan frekuensi Mn dipengaruhi oleh umur, jantina, BMI petani dan merokok selain tempoh pendedahan yang berulang dan penggunaan PPE perlu untuk menganalisis penyebab peningkatan Mn di kalangan petani.

Kata kunci: Genotoksik; racun perosak; mikronukleus; petani; sel calitan pipi

INTRODUCTION

Genomic damage is probably the most important fundamental cause of developmental and degenerative disease. It is also well established that genomic damage is produced by environmental exposure to genotoxins, medical procedures (e.g. radiation and chemicals), micronutrient deficiency (e.g. folate), lifestyle factors (e.g. alcohol, smoking, drugs, and stress), and genetic factors such as inherited defects in DNA metabolism and/or repair

(Holland 2008; Fenech 2010; Celik 2010). It is essential to have reliable and relevant minimally invasive biomarkers to improve the implementation of biomonitoring, diagnostics, and treatment of diseases caused by, or associated with, genetic damage. The MN assay in exfoliated buccal cells is potentially an excellent candidate to serve as such a biomarker.

Micronucleus is similar to the nucleus, but it is smaller than the original nucleus. Micronucleus size is one third the size of the original nucleus, or less than that. Micronucleus originate from chromosome fragment or whole chromosome that lag behind at anaphase during nuclear division (Fenech et al. 2003). Damage to chromosome or the spindle fibers causing failure to move toward the poles during the mitotic process. This will result in chromosome fragments isolated from the main nucleus of the daughter cells after cell division (Fenech et al. 2003).

Micronucleus assay test is a less invasive method of measuring the level of DNA damage in humans. This test was introduced in 1983 and began to be popularly used as biological markers of genetic damage (Stich & Rosin 1983). Micronucleus formation may serve as early warning for potential development of long-term health problems. Micronucleus assay test has proved an effective method for the detection of instability or chromosomal aberrations (Tolbert et al. 1992; Kayal et al. 1993; Pastor et al. 2001).

EXPERIMENTAL METHOD

SUBJECTS

The study was carried out on 40 farmers from Bachok and Pasir Puteh, Kelantan (8 smokers and 32 non-smokers) working as farmers more than 2 years. The control group consisted of 30 personnels (8 smokers and 22 non-smokers) who were office workers from UKM campus in Kuala Lumpur, with no indication of any exposures to pesticide or other potential genotoxic substances. Participants were informed about the study and were asked to sign a consent form before completing a standardized questionnaires. These to obtain necessary datas on anthropometry, period of exposure to pesticide and demografic factors such as age, gender, education, smoking status and personal protective equipment (PPE).

BUCCAL CELLS COLLECTION AND PRESERVATION

Exfoliated buccal mucosa cells were collected using a wooden tongue-depressor. The samples were transferred into Carnoy's fixative (Bortoli et al. 2009) after washing with buccal buffer (Moore et al. 1993). Washing was thought to be advantageous to remove cells debris and bacteria (Rosin & German 1985. All samples were stored in 4°C prior for analyzing.

STAINING

The polypropylene tubes containing the samples were centrifuged with Carnoy solution at 2,000 rpm for 10 minutes. Supernatant was removed by using a Pasteur pipette and the samples were sprayed on the tube's walls for several times before it dripped on the slide that has been heated to a temperature of 35°C by using a plate warmer. 100 µl droplets of each slide were completed. Slides were left to dry for 2 to 3 minutes before staining with Giemsa's stain. Giemsa staining was conducted overnight before rinsing with distilled water.

EVALUATION AND SCORING OF MICRONUCLEUS

Slides were viewed under a light microscope and micronucleus were analyzed. Mn scoring was done by calculating the frequency of Mn found in 1000 cells per sample. The numbers of Mn for each cells were calculated as well.

STATISTICAL ANALYSIS

The level of significance was taken as $p \leq 0.05$ and all analyses were conducted using theStatistical Package for Social Sciences (SPSS) for Windows, version 20.0.

DISCUSSION

Various factors may influence the formation of micronucleus (Mn) such as gender, age, smoking status, BMI, genetics, medications, exposure to X-ray and others. According to Bull et al. (2006) the formation of Mn as a genotoxic effect is a long-term effects. The formation is also influenced by various external and internal factors of a person (Albertini et al. 2000). According to a study by Suh et al. (2002), the age influence genotoxic effects of stress. Older people are at risk on getting genotoxic effects compare to younger people, this is because the rapid growth and metabolism process during younghood control the process of aberration by stimulating the necrosis or death of the cell.

Results showed that those aged 40 to 49 years gave the highest percentage of both study groups at 35% for the farmers and 50% for the control group. These results were similar to that obtained by Muller et al. (2004). However, there were significant differences between the frequency of Mn in the age groups between the farmers and the control group. Various studies had been conducted previously to study the effect of gender on genotoxic effects, but there were only two studies that would provide statistically significant results (Stich & Rosin 1984; Gonsebatt et al. 1997). There was no clear explanations about the cause of the differences between the sexes in the formation of Mn with their studies. However, Yesilada et al. (2006) reported that hormonal factor might influence the genotoxic effects. Hormones between men and women are different, this factor might cause significant differences in the study by Stich & Rosin (1984) and Gonsebatt et al. (1997).

Obesity is associated with the excessive production of free radicals than normal BMI class. Free radicals can induce the genotoxic effect and thus lead to the formation of Mn (Torres et al. 2009). Based on the results of the study, pre-obese was the highest group of personnels for both farmers and the control group ie with their respective percentages of 45% and 50%. However there was no significant difference between the frequency of formation of Mn by BMI class with the p value of 0.537. This was similar to the results obtained by Kažimírová et al. (2004) that examined the genomic stability between the different diet groups.

The results indicated that there was a significant increase of the frequency of Mn in the control group to a group of farmers in the different classes of BMI. However, there might be other factors that strongly influenced the formation of Mn such as PPE. It was found that most farmers from both underweight and normal group practice using PPE in the semi-complete category and most of them are smokers too. This could be the reason of having no significant relationship between the BMI and the grade earned for frequency of Mn.

In addition to the factors of BMI, there were many studies that examined the effects on individual smoking with cytogenetics. Most of the results claimed that smoking increases the formation of Mn (Lehucher et al. 1995; Bonassi et al. 2003; Konopacka et al. 2003; Gabriel et al. 2006). According to Surralles et al. (1997) there were various chemicals that cause genotoxic effects found in cigarette smoke. Genotoxic substances found in cigarette smoke were arsenic, benzene, ammonia, lead, and many more (Gorrod 1994).

Based on this findings, there was a statistically significant increase in the frequency of Mn with those who smoke. There were many studies support the results obtained (Lehucher et al. 1995; Bonassi et al. 2003; Konopacka et al. 2003; Gabriel et al. 2006). Smoking may be the strongest factor causing the increase in Mn formation in the epithelial cells analyzed in this study as compared to other factors such as exposure to pesticides, age, sex, and BMI. When smoking, genotoxic substances inside the cigarette smoke could act directly on the epithelial cells as compared to other factors that may act systemically to the body system before the formation of Mn induces the epithelial cells.

In addition, the frequency of Mn also can be caused by pesticides exposure. There are many substances shown to cause genotoxic effects of pesticides such as arsenic, paraquat, benzene, malathion, dithane, and more (Bolognesi et al. 2003). In a study conducted on farmers in Kelantan, pesticide exposure was measured by two methods, ie based on the use of PPE in dealing with pesticides and duration (hours) of exposure to pesticides. Previous studies such as by Vrhovac & Zeljezic (2002) and Kimura et al. (2005), reported significant correlation between the frequency of Mn with exposure to pesticides. While the results in this study indicated that the use of PPE when managing pesticides, were only partially completed (80%). There is an increased formation of Mn in those who wore incomplete PPE.

Types of active ingredients found in pesticides created the genotoxic effects on mankind. According to Shah et al. (1997) a different active ingredient causing a different genotoxic effects and this factor contributed to having no significant difference between the frequency of exposure to the formation of Mn. While the frequency of Mn between the farmers group and the control showed a significant difference. An increased frequency of Mn among farmers may be caused by various combinations of other factors that were not examined in this study. According to Albertini et al. (2000) another important factor to be taken into account in epidemiological studies is the formation of Mn medications, degenerative genetic disease that causes (mutations of BRCA1 and ATM), the factors genotype, diet, chewing betel, and exposure to carcinogenic substances such as ray-X and others.

There was a significant correlation between the number of Mn between farmers and the control group. It can also be seen that most of the cells that have the number 1 Mn 2 Mn and 3 Mn. The more marked on the number of Mn, the more severe chromosomal aberrations would be (Bull et al. 2006). From this results, only those farmers having up to 7 Mn indicates to be more severe chromosomal aberrations.

RESULTS

Table 1 shows the frequency of Mn according to age group of the studied. There are significant differences in Mn frequency between groups of farmers and control at the age groups of 30-39, 40-49 and 50-59 ($p < 0.05$).

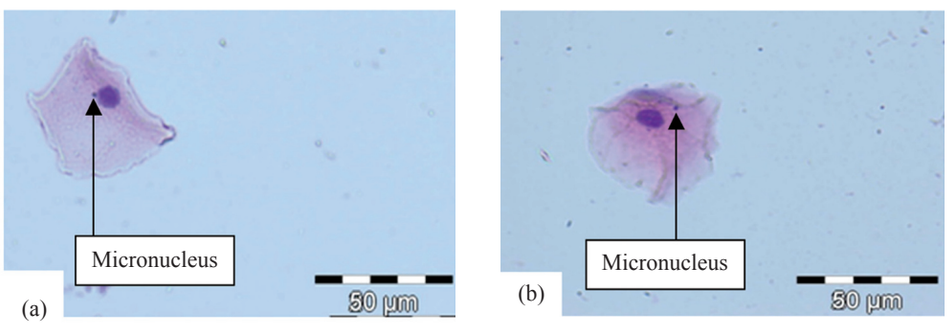

FIGURE 1. (a) and (b) showed the presence of the micronucleus in the epithelial of buccal cells using Micronucleus Assay with Giemsa staining under 400X magnification

TABLE 1. Frequency of Mn formation by age group

	Age Group	Farmers	Control
Frequency of Mn formation (%)	30-39	17.34 ± 0.91	5.08 ± 1.02
	40-49	15.85 ± 3.95	5.00 ± 1.32
	50-59	14.88 ± 3.31	3.82 ± 1.12
	> 60	14.75 ± 2.89	-

As shown in Table 2, there was no significant difference between gender and frequency of Mn formation ($p = 0.16$) within each groups. However, there was a significant difference in Mn frequency between groups of farmers and control among male and female ($p < 0.05$).

TABLE 2. Frequency of Mn formation by gender

	Gender	Farmers	Controls
Frequency formation of Mn (%)	Male	15.00 ± 3.52	5.15 ± 1.17
	Female	16.88 ± 2.10	3.79 ± 0.92

There was no significant difference between micronucleus frequency and gender ($p = 0.16$).

Table 3 shows the frequency of Mn by smoking status. Statistical analysis showed a significant difference between the frequency of Mn for smoking and non-smoking, within both farmers and the control group ($p < 0.05$). The analysis of smoking and non-smoking status between groups of farmers and controls was also showed an apparent significant different ($p < 0.05$).

TABLE 3. Frequency of Mn formation by smoking status

	Smoking status	Farmers	Controls
Frequency formation of Mn (%)	Yes	16.6 ± 3.39	5.51 ± 1.34
	No	14.35 ± 3.39	4.41 ± 1.08

Table 4 displays the frequency of Mn with the class of BMI for the study group. There are significant differences between the frequency of Mn for farmers and control groups at BMI class normal, preobese, and obese grade 1 ($p < 0.05$).

TABLE 4. Frequency of Mn formation by BMI class

	BMI Class	Farmers	Controls
Frequency formation of Mn (%)	Under weight	12.20 ± 4.43	-
	Normal	15.92 ± 3.76	4.50 ± 1.20
	Preobese	15.12 ± 2.79	4.76 ± 1.45
	Obese grade 1	16.16 ± 3.90	4.51 ± 1.07

Table 5 shows the frequency of Mn according to PPE category used by farmers, no significant difference between the frequency of micronucleus with category of PPE used by farmers ($p = 0.403$).

TABLE 5. Frequency of Mn formation by category of PPE used by farmers

Category of PPE	Frequency of Mn formation (%)
Complete	17.20 ± 0.47
Partially complete	15.11 ± 3.64
Not complete	15.52 ± 2.88

Table 6 shows the frequency of the Mn according to the number of Mn per cell for farmers and control groups. There are significant differences in number of total Mn between farmers and the control group for 1Mn and 2Mn ($p < 0.05$).

TABLE 6. Number of Mn per cell for farmers and control groups

No of Mn	Total Mn per cell	
	Farmers	Controls
1 Mn	81.75± 6.42	88.07±4.67*
2 Mn	15.28± 5.14	11.28±4.59*
3 Mn	2.75± 2.08	0.65±0.23
4 Mn	0.13± 0.03	-
5 Mn	-	-
6 Mn	0.06± 0.03	-
7 Mn	0.02± 0.01	-

* There are significant differences ($p < 0.05$) in number of total Mn between farmers and the control group for 1Mn and 2Mn.

Figure 2 shows the average frequency of Mn for the farmer is 15.39 ± 3.34 % while for the control is 4.76 ± 1.26%. There is a significant difference for the average frequency of Mn between farmers and control ($p < 0.05$).

Spearman correlation tests performed between the period of exposure to pesticides with micronucleus frequencies found that there was no strong correlation between them ($p = 0.27$) as shown in Figure 3.

CONCLUSION

The increase of Mn formations among farmers not only caused by pesticides exposure, chemical fertilizers, and any demographic factors but might also be caused by other factors which were not being investigated in our study.

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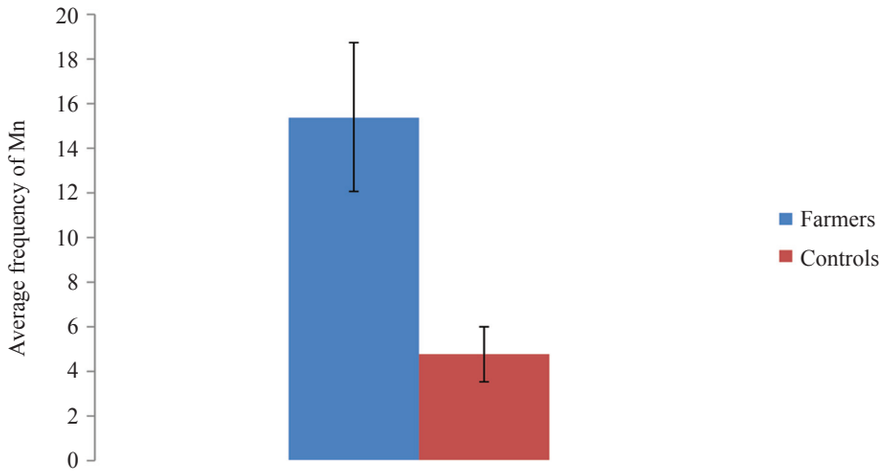

FIGURE 2. Average frequency of Mn by group

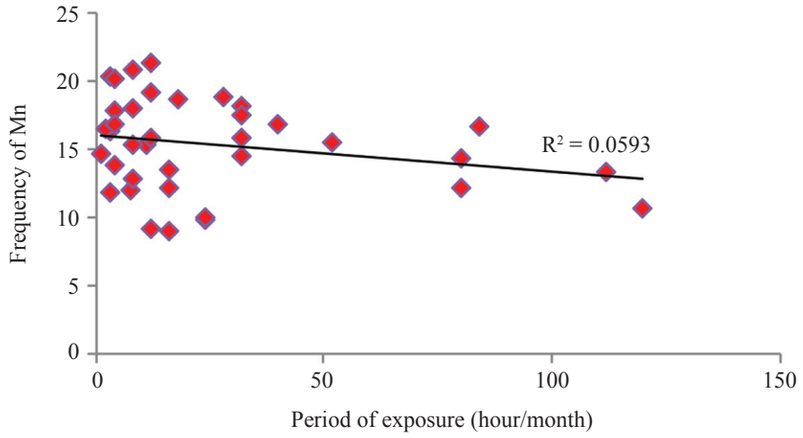

FIGURE 3. Correlation between periods of exposure to pesticides and the frequency of micronucleus

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Asmah Hamid
Kholilul Syafiq Baharuddin
Nor Fadilah Rajab
Syarif Husin Lubis
Ismarulyusda Ishak
Nur Zakiah Mohd Saat
Nihayah Mohammad
Hidayatul Fathi Othman
Ahmad Rohi Ghazali
Zariyantey Abdul Hamid
Mohd Jamil Mohd Rafaai
Mohd Nazir Nasrom
Department of Biomedical Sciences
Faculty of Health Sciences
Universiti Kebangsaan Malaysia (UKM)
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur.

Mohamad Roff Mohd. Noor
Horticulture Research Centre, MARDI

Corresponding author: Asmah Hamid
Email: asmah0901@ukm.edu.my
Tel: 03-92897644
Fax: 03-26929032

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