รายงานวิจัยฉบับสมบูรณ์

โครงการการศึกษาการแตกหักของดีเอ็นเอในผู้ป่วยโรคปอดอุดตันเรื้อรังในจังหวัดเชียงใหม่ในช่วงที่มีมลพิษทางอากาศสูงและต่ำ

โดย ผศ.ดร.กลิ่นเทียน วรรณภักตร์ และคณะ

1 ตุลาคม 2561

สัญญาเลขที่ MRG5980190

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การศึกษาการแตกหักของดีเอ็นเอในผู้ป่วยโรคปอดอุดตันเรื้อรังในจังหวัดเชียงใหม่ในช่วงที่มีมลพิษทางอากาศสูงและต่ำ

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บทคัดย่อ

รหัสโครงการ: MRG5980190

ชื่อโครงการ: การศึกษาการแตกหักของดีเอ็นเอในผู้ป่วยโรคปอดอุดตันเรื้อรังในจังหวัดเชียงใหม่ในช่วงที่มีมลพิษทางอากาศสูงและต่ำ

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ระยะเวลาโครงการ: 2 ปี

บทคัดย่อ:

การได้รับสัมผัส PM10 ที่เกิดจากการเผาชีวมวลอาจทำให้การทำงานของปอดลดลงและเหนี่ยวนำให้เกิดการแตกหักของดีเอ็นเอเพิ่มขึ้น โดยเฉพาะอย่างยิ่งในผู้ป่วยโรคปอดอุดกั้นเรื้องรัง การศึกษานี้ทำการตรวจสอบการแตกหักของดีเอ็นเอ และการตายของเซลล์ชนิดต่างๆ ในเซลล์เยื่อบุกระพุ้งแก้ม โดยใช้เทคนิค buccal micronucleus cytome และทำการศึกษาความสัมพันธ์ระหว่างประสิทธิภาพการทำงานของปอด และความถี่ของการเกิดการแตกหักของดีเอ็นเอ โดยเปรียบเทียบการเปลี่ยนแปลงของการแตกหักของดีเอ็นเอในช่วงที่มีมลพิษทางอากาศสูง (PM_{10} > 50 $\mu g/m^3$) กับช่วงที่มีมลพิษทางอากาศต่ำ (PM_{10} < 50 $\mu g/m^3$) เพื่อประเมินผลของการได้รับสัมผัส PM10 และความเป็นพิษต่อยีนในผู้ป่วยโรคปอดอุดกั้นเรื้องรังจำนวน 58 คน และอาสาสมัครสุขภาพดีจำนวน 26 คน ที่อาศัยในอำเภอเชียงดาว จังหวัดเชียงใหม่ ผลการศึกษาพบว่าจำนวนเซลล์เยื่อบุกระพุ้งแก้มที่มีไมโครนิวเคลียส (1.09±1.95 vs 0.29±0.64 ในกลุ่มผู้ป่วย) และจำนวนเซลล์ที่มีสองนิวเคลียส (11.43±18.68 vs 1.60±1.31 และ 7.77±12.76 vs 1.00±1.17 ในกลุ่มผู้ป่วย และกลุ่ม

อาสามัครสุขภาพดีตามลำดับ) ในช่วงที่ระดับ PM10 เกิน 50 μg/m^3 สูงกว่าช่วงที่ระดับ PM10 ต่ำกว่า 50 μg/m^3 อย่างมีนัยสำคัญทางสถิติ ทั้งในคนไข้และกลุ่มควบคุม และพบจำนวนไมโครนิวเคลียสในคนไข้ที่มีอาการของโรคไม่รุนแรงสูงกว่าคนไข้ที่มีอาการของโรครุนแรง นอกจากนี้การได้รับสัมผัส PM10 ในระดับสูงสามารถเพิ่มอัตราการเกิดดีเอ็นเอเสียหายได้ถึง 295.23 เท่า ในคนไข้โรคปอดอุดกั้นเรื่องรัง

คำหลัก : Chronic obstructive pulmonary disease, buccal micronucleus cytome, micronuclei, PM_{10}, DNA damage, biomass burning, binucleated cells

Abstract

Project Code: MRG5980190

Project Title: Investigation of DNA damage among chronic obstructive pulmonary disease (COPD) patients during high and low air pollutants in Chiang Mai province

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Project Period: 2 years

Abstract:

Exposure to PM_{10} generated by biomass burning may reduce lung functions and induction of DNA damage especially in chronic obstructive pulmonary disease patients. This study investigated the frequency of DNA damaged cells and different types of cell death using buccal micronucleus cytome assay as well as determined the correlations between DNA damage cells and lung function. The changes in DNA damage and cell death during high pollutants (PM_{10} > 50 $\mu g/m^3$) and low pollutants (PM_{10} <50 $\mu g/m^3$) were evaluated to explore whether PM_{10} exposure increases genotoxic damages in COPD patients during high pollutant period (the daily average of PM_{10} concentration reached above 50 $\mu g/m^3$). Fifty-eight COPD patients and 26 healthy subjects living in Chiang Dao district, Chiang Mai, Thailand were recruited in this study. The results revealed that buccal cells with micronuclei (BCMN, 1.09±1.95 vs 0.29±0.64 in COPD patients) and binucleated cells (BN, 11.43±18.68 vs 1.60±1.31 and 7.77±12.76 vs 1.00±1.17 in COPD and healthy subjects, respectively) observed in high pollutant period were higher than those found in low pollutant period in both study groups. A weak negative correlation between

micronuclei and COPD severity were demonstrated showing more DNA damage cells in patients with mild conditions. Moreover, excessive exposure of PM_{10} increased the risk of DNA damage in COPD patients for 295.23-fold.

Keywords: Chronic obstructive pulmonary disease, buccal micronucleus cytome, micronuclei, PM_{10}, DNA damage, biomass burning, binucleated cells

Executive summary

Introduction

The incidence of chronic obstructive pulmonary disease (COPD) patients has become more concerned by Ministry of Public Health, Thailand over the decade [1]. Cigarette smoking is the major risk factors for COPD, however, air pollution has now become more recognized as one of the risk factors as well [2-5]. The pathogenesis of COPD and its severities are complicated due to the interactions of several mechanisms including genetic susceptibility, inflammation, oxidative stress, chromatin modifications, DNA damage, apoptosis and defective DNA repair [2, 3, 6]. A number of reports have revealed an increase in numbers of patients with respiratory problems and hospital admissions or emergency room visits after exposure to excessive level of air pollution [2-4, 7-10].

Air pollutants have been implicated as causes of serious health effects especially during high polluted season which known as open burning season starting from December to April every year in Chiang Mai, Thailand (latitude 18°N and longitude 98°E) [4, 11, 12]. The significant sources of air pollution in this area are biomass or agricultural debris burning for land clearing, wildfires, as well as vehicle emissions [7, 9, 11, 13]. This haze has been concerned as a major health risk in Northern Thailand as well as neighbor countries [9, 11, 14]. According to the Pollution Control Department of Thailand (PCD), 89% of the pollutants found in Chiang Mai originate from forest fires, 5.4% from solid waste burning, and 2.3% from burning of agricultural waste [15]. Diesel combustion and industry sources contributed only less than 4% [13, 15, 16]. The dust and ash material from the burning materials, or airborne particulate matter (PM) with diameter less than 10 (PM_{10}) have become the significant threats to local population [16]. The PM_{10} measured in

Chiang Mai ambient air contained several inorganic and organic species, including nitrogen oxides (NO_x), sulfur oxides (SO_x), PM_{10}-bounded ions and polycyclic aromatic hydrocarbons (PAHs) [7, 9, 13, 14, 17, 18]. These components, especially PAHs, are carcinogens, which can induce DNA damage and respiratory distress [7, 19-21].

The pattern of PM_{10} concentrations in Chiang Mai, Thailand are similar every year since 2010. The annual pattern of monthlyPM_{10} concentrations were started to high levels at the beginning of December, reached their peaks in March, and subsequently decreased in May till November [14, 22]. Chiang Dao, Mae Rim and Mae Chaem districts are the dominant areas where rice and maize were produced [12, 23]. Even though Mae Chaem district was reported to be the area that had high rate of agricultural residual burning during the harvest season, biomass burning from Chiang Dao district seem to emit more chemicals ions into the air [12].

This study hypothesized that weather DNA damages and cell deaths of buccal cells of COPD patients and heathy subjects would be high during burning season due to excessive pollutant exposure. In addition, an increase in DNA damage-micronuclei and cell deaths may correlate with the severity of COPD. Therefore, we aimed to evaluate an increase in DNA damage using the Buccal Micronucleus Cytome (BMCyt) assay as an indicator in different stage of COPD during biomass burning season or high polluted period (daily average PM_{10} >50 $\mu g/m^3$) in comparison with low pollution period (daily average PM_{10} <50 $\mu g/m^3$) as per WHO Air quality guidelines for particulate matter [24]. Moreover, the relationship between abnormal buccal cell types and severity of COPD status were determined.

Materials and methods

Subjects and location

This study was conducted in 58 patients with mild to very severe COPD stages and 29 healthy control subjects lived in Chiang Dao, Chiang Mai Thailand (Fig.1).

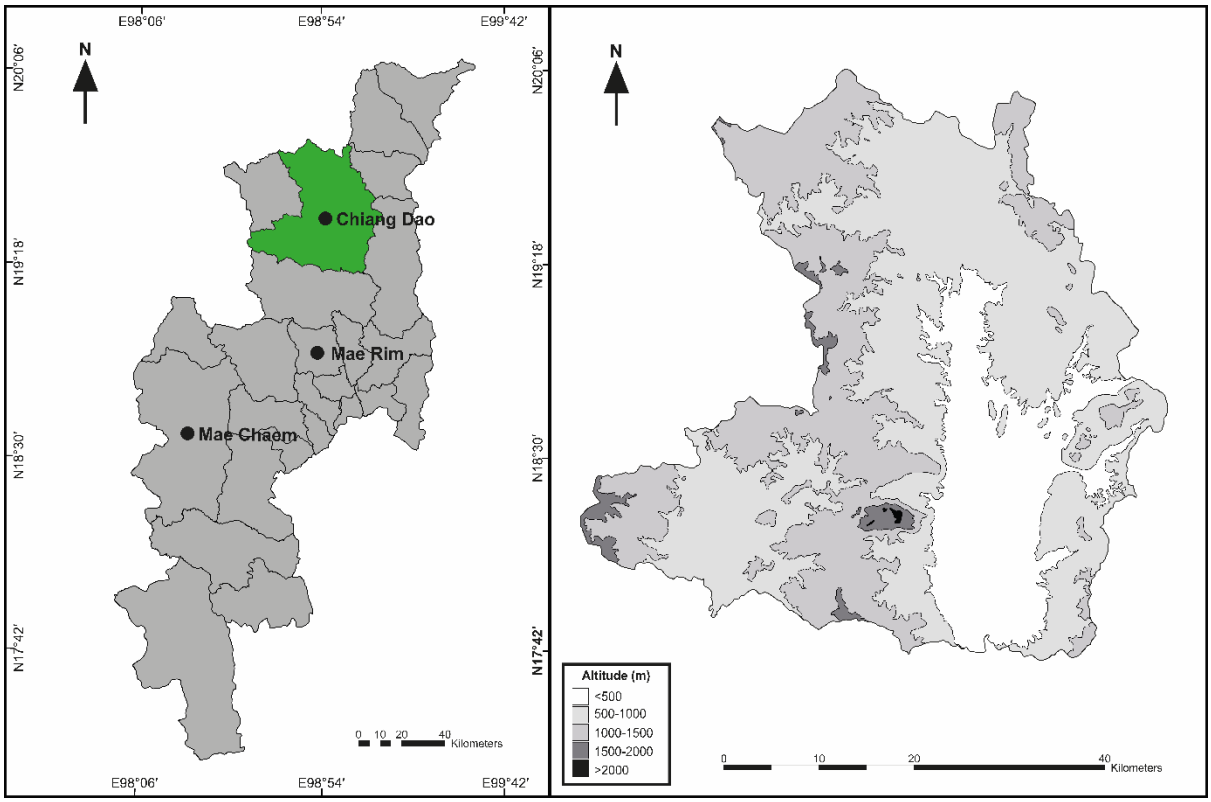

Fig. 1 Chiang Dao map

The COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease guideline (GOLD) [25]. The inclusion criteria as follow: 1) Subjects aged over 40 years old who have been living in Chiang Dao for more than 1 year. 2) The post bronchodilator FEV_1/FVC ratio is less than 0.7 for the COPD group and more than 0.7 for the healthy control group. 3) The chest radiograms reveal no other cardiopulmonary diseases related to their symptoms such as tuberculosis, bronchiectasis, lung abscess, interstitial lung diseases, lung mass, and left ventricular failure. The patients were grouped according to COPD stage as mild,

moderate, severe or very severe as per GOLD standard [26, 27]. This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethic Committee of Faculty of Medicine, Chiang Mai University, Thailand (Study code: FOR-2559-03852, FOR-2558-03434 and MED-2558-0303). The subjects were informed about the study and signed the consent form according to the guidelines of the Faculty of Medicine ethical committee. All individuals were interviewed face-to-face using demographic data questionnaire and COPD Assessment Test (CAT) to evaluate the health-related quality of life as well as a detailed personal questionnaire containing individual characteristics, potential cofounders such as smoking status, alcohol-drinking habits, diet, medication, and exercise habits were also obtained before sampling. High CAT score indicates COPD worsen severity of airflow limitation in stable COPD patients [28].

COPD GOLD stage

This study classified the **s**everity of COPD according to the GOLD 2017 Guidelines [29] as follows: stage 1 mild FEV_1 > or equal to 80% predicted; stage 2 moderate FEV_1 between 50 and 80% predicted; stage 3 severe FEV_1 between 30 and 50% predicted; stage 4 very severe FEV_1 < or equal to 30% predicted or FEV_1 < 50% predicted plus chronic respiratory failure.

Sample collection

The buccal cells from each subject were collected two times in March 2016 (burning season with PM_{10} >50 $\mu g/m^3$) [9, 14] and August 2016 (PM_{10} <50 $\mu g/m^3$). The stage and severity of COPD at the sampling time were verified in order to evaluate the relationships between DNA damage and COPD status. The buccal samples or oral mucosa were collected and processed in accordance with Thomas et al. [30]. Briefly, buccal cells were collected in a circular motion from inside of the left and right cheek wall using wood spatula. Then, the collected cells were placed

into the fixative containers containing Saccomanno's fixative solution. Afterward, the cell suspension was centrifuged at 4,000 rpm for 15 mins and the supernatant was removed. The cell suspension was washed twice using 500 μl of the fixative solution and centrifuged at 13,000 rpm for 10 min. Next, the supernatant was removed and replaced with 500 μl of the fixative solution. The final suspension was vortexed and then 100 μl of the cell suspension was placed directly onto a glass microscope slide. The slides were air-dried overnight and then placed in methanol: glacial acetic acid (3:1) for 10 mins before stained with Feulgen reaction solution before counterstaining with Fast-green reagent.

Buccal Micronucleus Cytome Assay

The slides were coded before scoring by two investigators (double-blinded scoring) and were examined at ×1,000 magnification using a good-quality bright field. The frequency of all the various cell types was scored in 2,000 buccal cells including the frequency of buccal cell with micronuclei (BCMN), micronuclei (MNi), bi-nucleated cells (BN), nuclear bud cells (NBUD), pyknotic cells (PY), condensed chromatin cells (CC), karyorrhectic cells (KR) and karyolytic cells (KL). Criteria for identifying and scoring cell types and nuclear abnormalities in the BMCyt assay was performed following Thomas et al. [30] and Bolognesi et al. [31].

PM_{10} data

The PM_{10} data used in this study was retrieved from the Thailand's air quality and situation reports of Pollution Control Department (PCD) Ministry of National Resources and Environment database (http://air4thai.pcd.go.th/webV2/) from January to December 2016 at the Chiang Mai City Hall station, Thailand. The PM_{10} concentrations were measure performed using beta-ray attenuation operated by the PCD. The data was reported as daily average concentrations of PM_{10}.

Statistical analysis

Results with numerical values were expressed as mean ± SD. Categorical data were demonstrated as absolute frequencies and percentages. The statistical analysis was performed on GraphPad Prism software Version 5.01 and IBM® SPSS® Statistics Version 22. The statistically significant difference was considered when $p \leq 0.05$. As the data was not normal distributed, the non-parametric test was employed. The Wilcoxon matched-pairs signed rank test was used to compare the means of variables of the same subjects between low and high pollution season. Chi-square test or Fisher's Exact test were used to compare the proportional categorical data. Statistical differences between the COPD and control group were tested using the non-parametric Mann Whitney U Test. The correlations between different variables were determined using the Spearman Rank Correlation Test. The Poisson log-linear model were applied to the data to estimate the association between BCMN, MNi and factors that involving micronucleus induction including PM_{10} levels, disease status, host factors, smoking habit, drinking habit, diet. Adjustments with dependent covariates were made for age, gender, FEV_1/FVC ratio, FEV_1, COPD severity, CAT score.

Results

PM_{10} data

The monthly PM_{10} concentrations from January to December is shown in Fig. 2 indicating the PM_{10} levels were exceeded the WHO Air quality guidelines (50 $\mu g/m^3$) from January to April with the peak level of 187.33 $\mu g/m^3$ in March. Consistent with the previous work performed by Punsompong and Chantara [22] stating that the monthly PM_{10} concentrations from year 2010 to 2015 were similar showing the high levels of PM_{10} in dry season (January to April) and lower concentrations in wet season (May to October). The PM_{10} levels in 2016 showed the same pattern

as the previous observation which peak levels of PM_{10} in March resulting from active biomass burning [22]. Moreover, Chiang Mai is surrounded by mountains causing high accumulation of PM_{10} in dry season because of the low air-flow and inversion temperatures [9, 12].

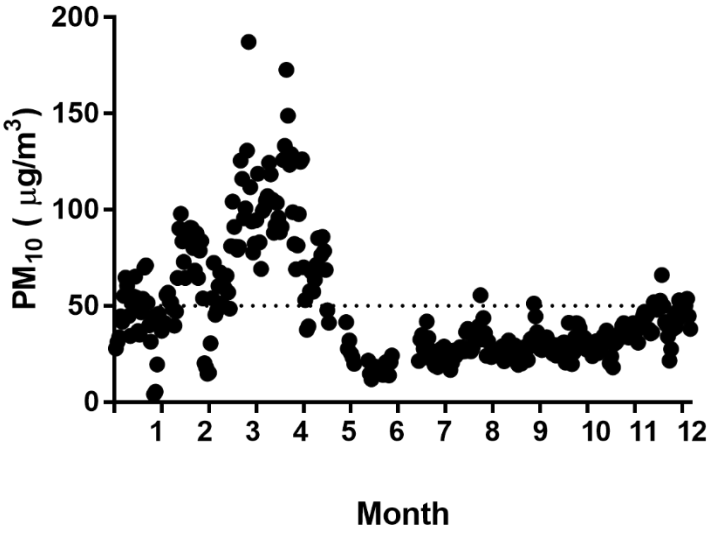

Fig. 2 The PM_{10} concentrations from January to December 2016.

Subjects

Fifty-eight COPD patients and 26 healthy subjects lived in agricultural area, Chiang Dao, Chiang Mai, Thailand were enrolled in this study. General characteristic of the study population (COPD patients and healthy control subjects) including gender, age, lung function, smoking and alcohol drinking status are shown in Table 1. The subjects from control group were 10 years younger than the COPD group. The control group had normal lung function according to GOLD standard. More than 50 percentages of the subjects were former smokers and most of the subject did not consumed alcohol. The post bronchodilator FEV_1/FVC ratio during polluted season was significantly lower than that measured during the low pollution in COPD patients as well as % predicted FEV_1 (Table 1). This finding confirms that PM_{10} levels involved in lung function status and disease progression as same as previous studies showing a strong correlation between PM_{10} exposure and exacerbations or COPD disease progression [4, 32, 33].

Table 1 Demographic data and general characteristic of the COPD and the control groups

Demographic data	COPD (n=58)	Control (n=26)
Male (%)	31 (53.45%)	13 (50.00%)
Age (years)	71.45± 7.93[a]	61.31±11.31[a]
FEV_1/FVC (%)		
PM_{10} >50 μg/m^3	57.08±10.26 [a]	82.03±7.32 [a]
PM_{10} <50 μg/m^3	58.58±11.92 [a]	80.78±6.83 [a]
FEV_1 (L)		
PM_{10} >50 μg/m^3	1.20±0.48 [a,b]	2.26±0.62 [a]
PM_{10} <50 μg/m^3	1.29±0.54 [a,b]	2.20±0.71 [a]
% predicted FEV_1		
PM_{10} >50 μg/m^3	64.78±24.46 [a,b]	103.80±20.46 [a]
PM_{10} <50 μg/m^3	70.80±24.65 [a,b]	102.16±21.47 [a]
FVC (L)		
PM_{10} >50 μg/m^3	2.09±0.68 [a,b]	2.74±0.66 [a]
PM_{10} <50 μg/m^3	2.20±0.69 [a,b]	2.70±0.79 [a]
CAT		
PM_{10} >50 μg/m^3	11.76±7.20 [a]	6.23±5.11 [a]
PM_{10} <50 μg/m^3	11.00±6.28 [a]	5.92±6.18 [a]
GOLD severity stage (n, %)		
Stage 1 Mild	14 (24.14%)	-
Stage 2 Moderate	17 (29.31%)	-
Stage 3 Severe	13 (22.41%)	-
Stage 4 Very severe	14 (24.14%)	-
Cigarette smoking (n, %)		
Never	8 (13.8%)	8 (30.8%)
Former	41 (70.7%)	14 (53.8%)
Current	9 (15.5%)	4 (15.4%)
Alcohol drinking (n, %)		
Never	32 (55.2%)	10 (38.5%)
Former	19 (32.8%)	7 (26.9%)
Current	7 (12.1%)	9 (34.6%)

Mean ± SD, FVC = Forced Vital Capacity, FEV_1= Forced Expiratory Volume in first second, [a] Mann Whitney U Test (COPD vs Control), [b] Wilcoxon matched-pairs signed rank test (within subjects, PM_{10} >50 μg/m^3 vs PM_{10} <50 μg/m^3), significant p value < 0.05.

Buccal Micronucleus Cytome Assay

For the Buccal Micronucleus Cytome Assay, the photomicrographs of scored abnormal cell types are shown in Fig. 3. The mean frequencies of abnormal cells (PY, CC, KR and KL) and different types of DNA damage cells (BCMN, MNi, BN, NBUD) are shown in Fig. 4. The BCMN and MNi cells were not statistically significant different between the COPD and control groups during both high and low PM_{10} levels. The MNi detected in the COPD and control groups during low pollution were used as a baseline MNi since spontaneous MNi can be found ranging from 0.05-11.5 MNi/1,000 cells even in healthy population [34].

A significant increase in DNA damage cells from their baseline were detected in both populations as presented in Fig.4. The mean frequency of BCMN observed in the COPD group during high-pollutionperiod was significantly higher than that found in low pollution period (Fig. 4 a), with the mean frequencies of 1.09±1.95 vs 0.29±0.64 (*p* value = 0.027), respectively. However, there was no different of BCMN frequencies between two periods (0.77±0.95 vs 0.65±0.61, *p* value = 0.449, respectively). The mean frequency of MNi (Fig. 4 b) during high-pollution period was slightly higher but not significant than that found during low pollution period (1.39±2.12 vs 0.88±1.05, *p* value = 0.681, 1.59±2.47 vs 1.05±2.95, *p* value = 0.162) in the control and COPD groups, respectively.

Moreover, the frequency of the BN cells (Fig. 4 d) was dramatically larger during high pollution than that detected during low pollution in both COPD (11.43±18.68 and 1.60±1.3, *p* value = 0.004) and control groups (7.77±12.76 and 1.00±1.17, *p* value = 0.012). The frequency of CC cells was higher during low pollution period in both COPD (132.40±104.30 and 230.20±87.35, *p* value = 0.006) and control groups (168.10±119.90 and 267.78±97.66, *p* value = 0.012) indicating the cells undergoing early stages of apoptosis when the pollution decreased.

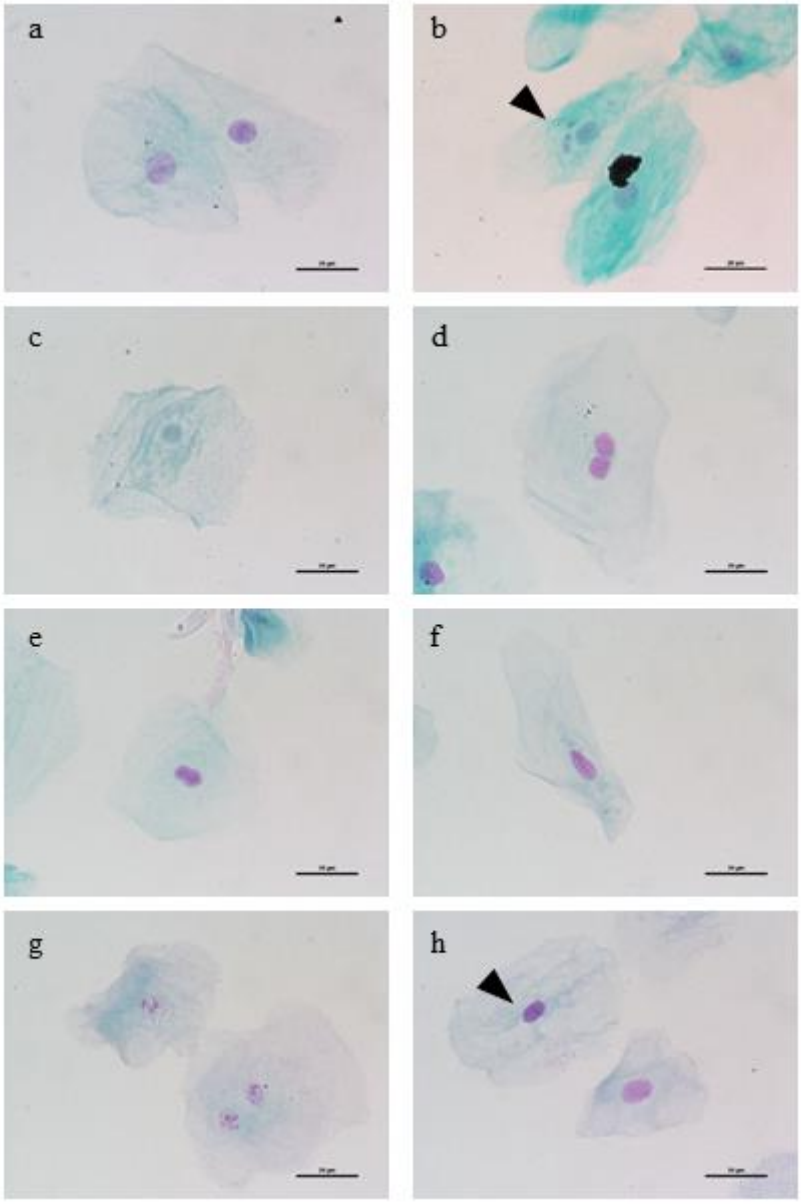

Fig. 3 Images of the different cell types stained using Feulgen and Light Green viewed by transmitted light microscope. a Normal differentiated cell, b Buccal cell with micronuclei, c Karyolitic cell, d Binucleated cell, e Nuclear bud cell, f Condensed chromatin cell, g Karyorrhectic cell, h Pyknotic cell. All images were taken at ×1,000 magnification, scale bar 20 μm.

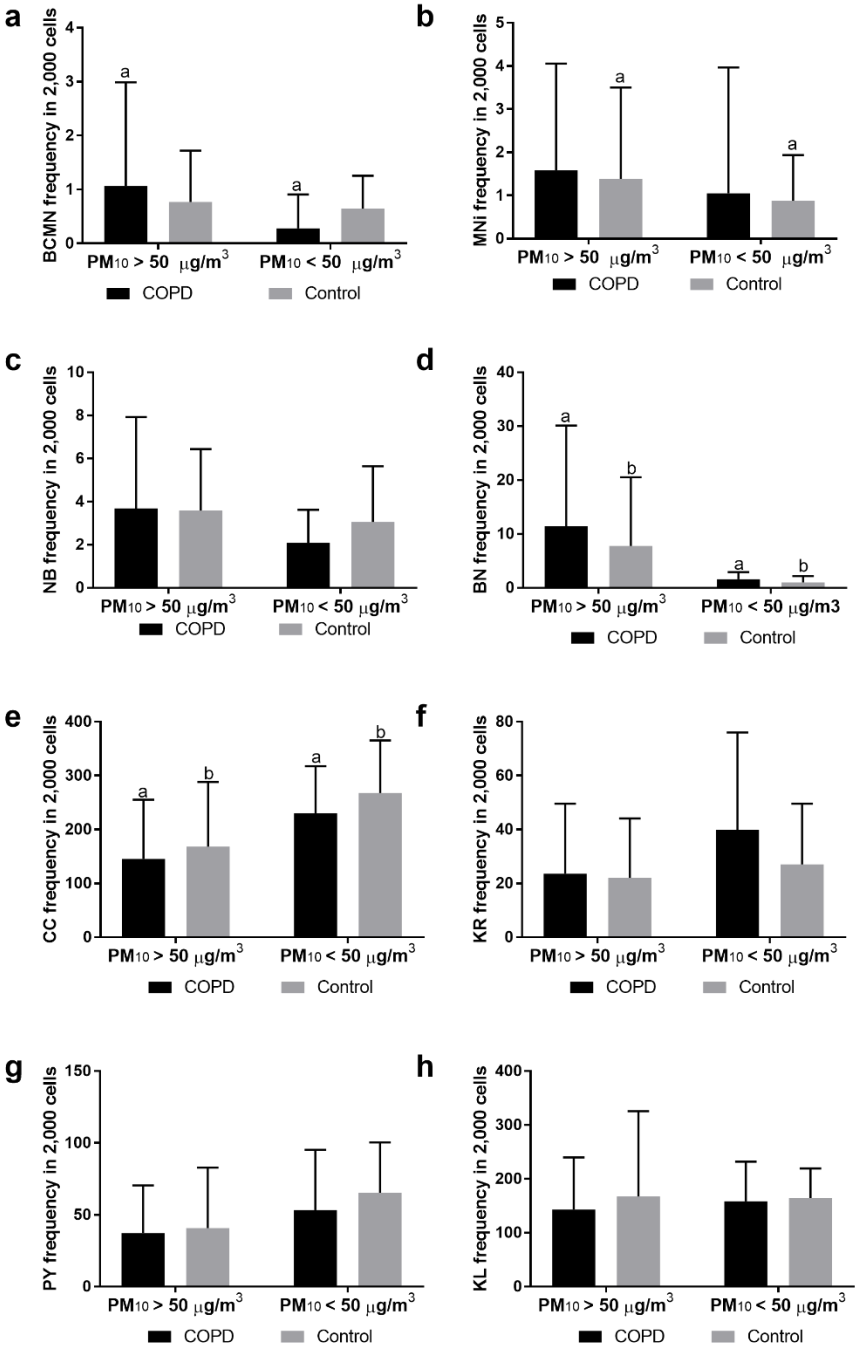

Fig. 4 DNA damage and cell death markers. The frequencies of: a Buccal cell with micronuclei, b Micronuclei, c Nuclear bud cell, d Binucleated cell, e Condensed chromatin cell, f Karyorrhectic cell, g Pyknotic cell, h Karyolitic cell. Groups not showing the same letter are significantly different from each other.

Only the BCMN, MNi and CC cells were significant different among COPD severity groups (Fig. 5). The frequencies of BCMN and MNi (Fig. 5 a and b) in patients with mild symptoms (stage I) were higher than the patients with moderate, severe and very severe stages. On the other hand, the frequency of apoptotic cells or CC cells was found more in patients with stage II to IV (Fig. 5 c).

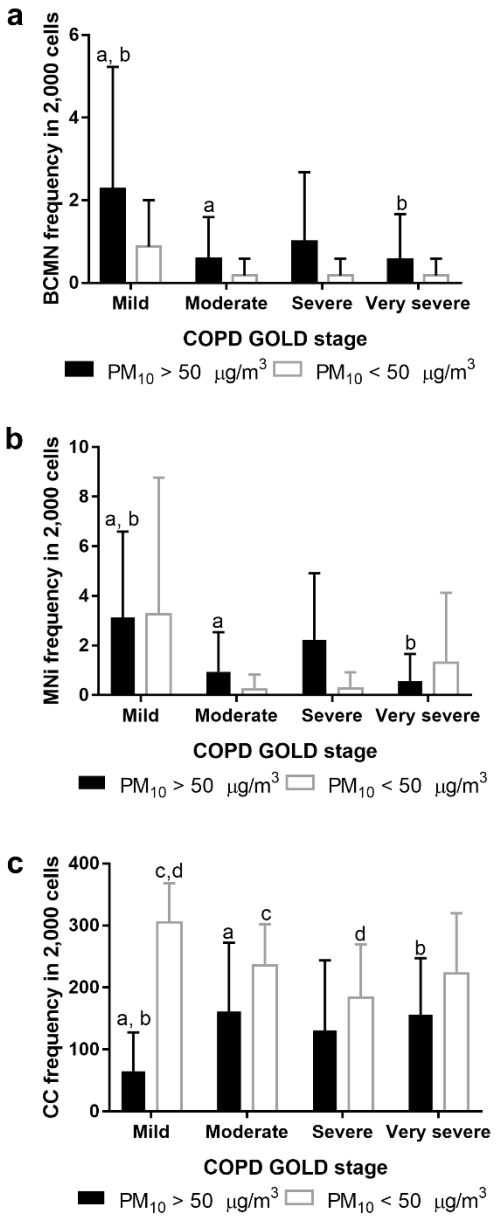

Fig. 5 Frequencies of a Buccal cell with micronuclei, b Micronuclei, c Condensed chromatin cell in COPD group according to COPD severity as defined by GOLD standard. Groups not showing the same letter are significantly different from each other.

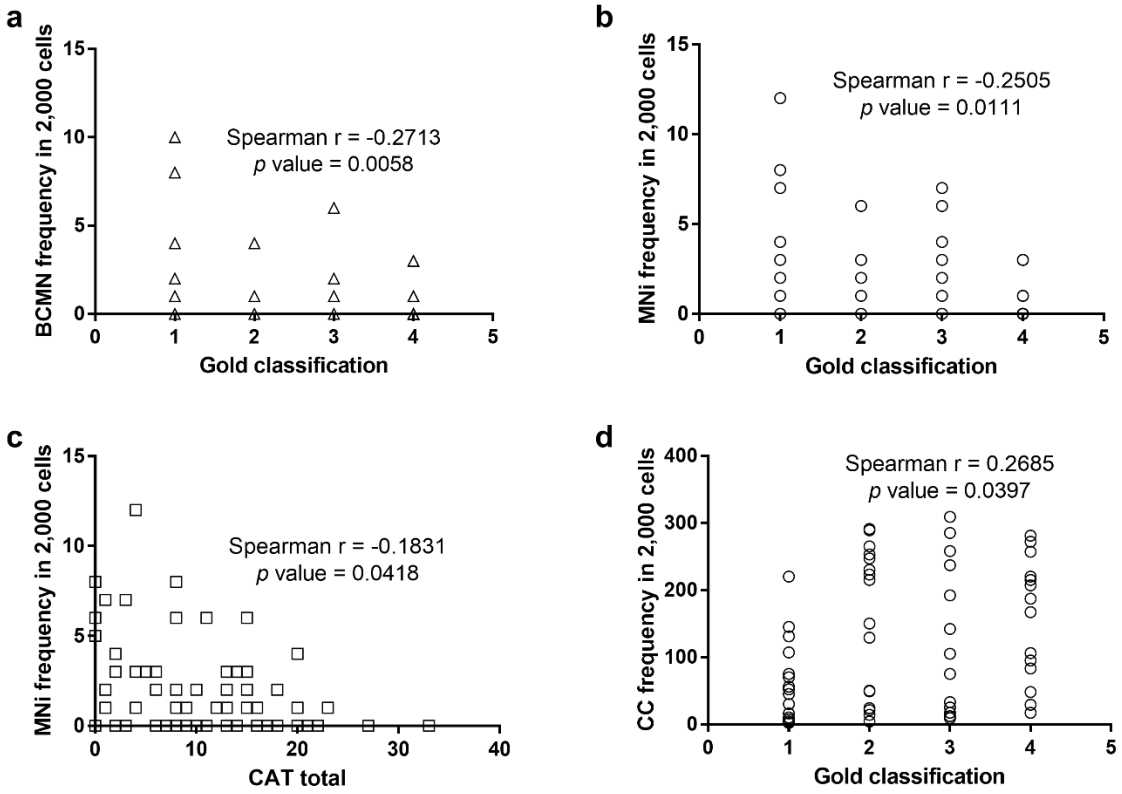

Fig. 6 Correlation between the DNA damage and COPD severity stages as defined by GOLD

1 = mild, 2 = moderate, 3 = severe, 4 = very severe

To evaluate the relationship between DNA damage cells, cell deaths and the COPD severities, Spearman's rank correlation was performed. pulmonary function indicators FEV_1 and FVC was not correlated with any DNA damage or cell death markers. The severity defined by GOLD classes and CAT score correlated negatively with the BCMN and MNi frequencies, however, only a weak correlation was observed with the Spearman r below 0.3 (Fig.6 a-c). This finding confirmed that DNA damage cells presented more in mild to moderate COPD patients. Severe COPD patients on the other hand had more CC cells indicating cells undergo apoptotic cell death program.

Association between DNA damage cells and confounding factors

The confounding factors that may involve in micronucleus induction were taken in to account to remove all bias from the analysis. After adjustment, COPD patients had a higher risk of PM_{10} induced BCMN and MNi formation with the frequency ratio of 295.23 and 64.51 when the PM_{10} levels were higher than 50 $\mu g/m^3$ as shown in Table 2. This indicates that COPD patients has more susceptibility to PM_{10} toxicity when compared to the corresponding control group. In addition, high pollutants enhance the genotoxic effect of PM_{10} in this finding as the frequency's ratio of DNA damages during PM_{10} levels more than 50 $\mu g/m^3$ was higher than that in the low pollution period.

Table 2 Risk of BCMN induction in COPD patients and control subjects compare between high and low PM_{10} levels

DNA damage cells	Predictors	Group	PM_{10} > 50 μg/m^3			PM_{10} < 50 μg/m^3		
			FR	95%CI	*p* -value	FR	95%CI	*p* -value
BCMN	Diseases	Control	1	-	-	1	-	-
		COPD	**295.23**	**4.59-18973.26**	**0.007**	**96.51**	**4.95-1881.65**	**0.003**
MNi	Diseases	Control	1	-	-	1	-	-
		COPD	**64.51**	**5.15-807.58**	**0.001**	**29.77**	**1.60-555.06**	**0.023**

[a] FR: Frequency ratio, significant results showed in bold.

The role of confounding factors was also important for micronucleus induction. The association between BCMN and life-style parameters in COPD group were presented in Table 3. The most associated factors links to BCMN cells were smoking and food consumption. The results indicated that former smokers had a possibility to have higher BCMN frequencies for 12.75 times than those who never smoked. Subjects who intake fruit and vegetable daily have less BCMN frequency (FR = 0.036) than those who do not consume with over 96.4% DNA damage reduction.

Table 3 Effects of smoking habit and food consumption on BCMN frequency

Predictors	Group	PM_{10} > 50 μg/m^3			PM_{10} < 50 μg/m^3		
		FR	95%CI	*p* -value	FR	95%CI	*p* -value
Smoking	Never	1	-	-	1	-	-
	Former	**12.75**	**2.74-59.85**	**0.001**	**25.33**	**3.21-199.82**	**0.002**
	Current	4.32	0.75-24.78	0.101	4.87	0.53-44.48	0.160
Fruit	Never	1	-	-	1	-	-
consumption	**Once a week**	**0.046**	**0.00-0.79**	**0.056**	217117.93	0.00-α	0.992
	Every day	**0.036**	**0.00-1.08**	**0.035**	136621.80	0.00-α	0.990
Vegetable	Never	1	-	-	1	-	-
consumption	Once a week	0.077	0.00-0.504	0.156	0.55	0.02-14.20	0.722
	Every day	**0.015**	**0.00-2.66**	**0.019**	0.37	0.01-10.62	0.565

[a] FR: Frequency ratio, significant results showed in bold.

Discussions

The BCMN as well as MNi frequencies are the markers of chromosome loss or fragmentation due to the genotoxic exposure [34]. An increase in DNA or chromosome damages during high pollution demonstrated in our findings may possibly link to an excessive exposure of PM_{10} from agricultural waste burning in dry season. Several studies indicated that air pollution especially from biomass burning cause severe genotoxic effects on COPD patients as much as cigarette smoking [2, 3, 5, 21, 35]. Furthermore, Ceretti et al. stated that an increase in 10 $\mu g/m^3$ unit of PM_{10} was significantly related to the elevation of MNi, CC cells and KR cells (Ceretti et al. 2014).

Micronucleus formation occur originally in the basal layer after systemic exposure of genotoxic substances during cell division. The cells with MNi differentiate into the stratum spinosum layer (the prickle cell layer) and the stratum corneum layer (the keratinized superficial layer) and then exfoliate into the buccal cavity. The time frame of cellular migration from basal layer to the keratinized superficial layer is between 7 to 10 days [30, 34]. Meanwhile, some of the cells containing DNA fragments may undergo program cell death and turn into cells with condensed chromatin, karyorrhectic cells, pyknotic nuclei or karyolitic cells.[34]. However, the epithelial tissues have a rapid turnover rate of about 7-21 days. Consequently, the genotoxic effects observed in our study could be a result of PM_{10} – induced DNA damage 1 to 3 weeks before buccal cell collection [34, 36].

Binucleated cells are an indicator of defective of cytokinesis due to aneuploidy [37]. Aneuploidy reflects gain or loss of whole chromosomes as well as non-balanced rearrangements of chromosomes, including deletions, amplifications or translocations of large regions of the genome [38]. Moreover, aneuploidy cells or chromosomal instability cells have an elevated rate of DNA mutations and chromosome missegregation which can lead to micronucleus formation

[39]. In addition, the elevation of binucleate:mononucleate cell ratio can be used to identify a cytokinesis failure caused by higher-than-normal rates of aneuploidy which is related with cancer risk [30, 40]. Therefore, the high frequency of BN observed in our study during open burning season, and subsequently return to baseline in wet season, could be a result of cytokinesis failure during cell division induced by biomass burning exposure. Our finding is in agreement with Mondal et al. stating that chronic exposure to biomass fuel not only causes chromosomal and DNA damage resulting MNi induction but also induced cytokinesis defect leading to higher frequency of binucleated cells [41]. The frequency of BN was also slightly associated with air pollution as reported by Ceretti et al. The studied stated that an increase in the PM_{10} concentration every 10 $\mu g/m^3$ unit resulting in the elevation of BN for 0.01 unit [42].

Furthermore in our study area, there are three main types of the biomass including rice straw, maize residue and leaf litter that emitted high concentration of PM_{10}-bound PAHs after burning [23]. The PAHs profiles from Wiriya et at. study revealed that high molecular weight PAHs, for example benzo[a]pyrene, benzo[k]fluoranthene, benzo[b]fluoranthene, chrysene, benzo[a]anthracene, pyrene, fluoranthene, phenanthrene were the most abundant pollutants [23]. These PAH species involve in DNA and oxidative damage [43], consequently, initiate and promote cancer [44]. The chromosome break or cytokinesis dysfunction demonstrated in our study could be an impact of PAH exposure. Nonetheless, the limitation of this study was the lack of individual pollutant exposure data as a real-time particulate matter measurement for each subject have not been performed.

An increase in the CC cells may indicate repair and elimination process of DNA damage in buccal cells (Fig.4 e) [30]. In addition, a lower frequency of CC cells found in our present work may suggest the reduction of regenerative capacity of epithelial tissue during high pollution [45].

Similar results were reported in the study in open-cast coal mine workers by Rohr et al. A frequency of CC cells in the exposed group was lower than in non-exposed group. They concluded that an increase of this cell type in non-exposure group occurred randomly [46].

Rohr et al. determined the relationship between genetic damages and cell deaths in open-cast coal mine workers using the buccal micronucleus cytome assay. The results showed that subjects with high MNi frequency had a lower frequency of CC cells **[46]**. In our study, the cells with genetic damages occurring during PM_{10} exposure may be eliminated via the apoptosis resulting in the high frequency of CC cells and less DNA damage cells in the severe COPD groups [34].

Ceretti et al. studied the association between the PM_{10} exposure and MNi frequency in children. Linear regression was performed to analyze using air pollutant mean levels at time 0 (buccal cell sampling day), 1, 2 and 3 weeks before exfoliated cell collection. The results showed modest association between MNi and PM_{10} concentration 1-week prior buccal sampling [42].

Our result showed the impact of smoking habit and diets on micronucleus induction. It has been well established that smoking habit, fruit and vegetable consumption affect micronucleus formation [37]. Heavy cigarette smokers ($\geq$ 40 cigarettes per day) related with an increase in MNi frequency [37]. Our study demonstrated that COPD patients who once smoke had more BCMN frequency than those who are still smoking. However, the number of current smokers enrolled in our study (n=9) were too small to see substantial change in BCMN frequency. Particularly, subjects who intake fruit or vegetable daily had a lower BCMN frequencies that those who reported no consumption at all. This result is consistent with several publications demonstrating that fruit and vegetable consumption significantly reduce micronucleus levels [37].

Conclusions

The PM_{10} concentrations exceeded WHO guideline from January (dry season) and reached the peak in March and subsequently decreased by May. Buccal cell with micronuclei and binucleated cells were higher during high pollution (March), particularly in the COPD patients indicating DNA damages and instability. Micronucleus frequency in COPD patients with mild condition was higher than those with severe conditions. However, cells with condensed chromatin detected in the moderate to very severe COPD groups were significantly higher than in mild group indicating the DNA damage cells undergo apoptosis. Exposure to PM_{10} during high pollutant period increased the frequency risk of DNA damage for 295-fold in COPD patients in comparison with the control group. It can be concluded that the COPD patients has more susceptibility to air pollution induced DNA damage than healthy subjects.

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แบบสอบถามสำหรับผู้ป่วยโรคปอดอุดกั้นเรื้อรัง

โครงการวิจัย: ความถี่ของการเกิดไมโครนิวเคลียสในเซลล์เยื่อบุกระพุ้งแก้มของคนไข้โรคปอดอุดกั้นเรื้อรังที่สัมผัสกับมลพิษทางอากาศ

คำชี้แจง ในการตอบแบบสอบถามผู้วิจัยจะเป็นผู้ซักถามและกรอกข้อมูลของอาสาสมัคร โปรดทำเครื่องหมาย ✓ หรือเติมข้อความลงในช่องว่างหน้าข้อความที่ท่านเห็นว่าเหมาะสม

เพศ ❒ ชาย ❒ หญิง

อายุ..... ปี

น้ำหนัก.....กิโลกรัม ความสูง.....เซนติเมตร

อาชีพ

❒ ไม่มี ❒ มี .....

มีโรคประจำตัวอื่นนอกจากโรคปอดอุดกั้นเรื้อรังหรือไม่

❒ ไม่มี ❒ มี เป็นโรค.....

พักอาศัยอยู่ใน อำเภอ..... จังหวัด ❒เชียงใหม่ ❒ ลำพูน เป็นระยะเวลา ..... ปี

ปัจจุบันรับประทานยาประจำหรือไม่

❒ ไม่มี

❒ มี ยาที่ทานประจำ คือ.....รับประทานมาเป็นระยะเวลานาน.....

ท่านสูบบุหรี่หรือไม่

❒ ไม่สูบ

❒ สูบ ปริมาณ.....มวน/วัน ระยะเวลาที่สูบ.....ปี

ชนิดของบุหรี่ที่ท่านสูบ ❒ บุหรี่ขี้โย ❒ บุหรี่ยาสูบ ❒ บุหรี่

❒ เคยสูบ แต่เลิกแล้ว เลิกมาแล้วเป็นเวลา.....ปี

ชนิดของบุหรี่ที่ท่านสูบ ❒ บุหรี่ขี้โย ❒ บุหรี่ยาสูบ ❒ บุหรี่

คนในครอบครัวของท่านสูบบุหรี่หรือไม่

❒ ไม่มีคนสูบ

❒ สูบ จำนวน.....คน

❒ เคยสูบ จำนวน.....คน และเลิกแล้ว เลิกมาแล้วเป็นเวลา.....ปี

ข้อมูลการดื่มแอลกอฮอล์ของท่าน

❒ ไม่ดื่ม

❒ ดื่ม จำนวนครั้งในการดื่มต่อสัปดาห์.....สัปดาห์ ดื่มมาแล้วเป็นระยะเวลา.....ปี

❒ เคยดื่ม แต่เลิกแล้ว เลิกมาแล้วเป็นเวลา.....ปี

ท่านเคยผ่านการ x-ray หรือไม่

❒ ไม่เคย ❒ เคย ครั้งสุดท้ายเมื่อวันที่……เดือน……ปี…….

ท่านเคยได้รับการฉีดวัคซีนหรือไม่

❒ ไม่เคย ❒ เคย ชนิดของวัคซีน ……เมื่อวันที่ ..... เดือน……ปี…….

ท่านมีการรับประทานวิตามินหรืออาหารเสริมหรือไม่

❒ ไม่ได้รับประทาน

❒ รับประทาน ชนิดของวิตามินหรืออาหารเสริม……..

ท่านใช้เตาถ่านในการทำอาหารหรือไม่

❒ ไม่ได้ใช้

❒ ใช้ จำนวนครั้งที่ใช้ .....ต่อสัปดาห์

ท่านรับประทานอาหารประเภทใดมากกว่ากัน

❒ ผักและผลไม้

❒ เนื้อสัตว์

ขอขอบคุณทุกท่านที่ให้ความร่วมมือ

แบบสอบถามสำหรับผู้ป่วยโรคปอดอุดกั้นเรื้อรัง (เพิ่มเติม)

คำชี้แจง ในการตอบแบบสอบถามผู้วิจัยจะเป็นผู้ซักถามและกรอกข้อมูลของอาสาสมัคร โปรดทำเครื่องหมาย ✓ หรือเติมข้อความลงในช่องว่างหน้าข้อความที่ท่านเห็นว่าเหมาะสม

พฤติกรรมและประวัติการดื่มแอลกอฮอล์

❒ไม่เคยดื่ม ❒ 2-3 ครั้งต่อสัปดาห์

❒เดือนละครั้งหรือน้อยกว่า ❒ 4 ครั้งขึ้นไปต่อสัปดาห์

❒2-4 ครั้งต่อเดือน

1.1 ถ้าโดยทั่วไปดื่มเบียร์ ดื่มปริมาณเท่าไหร่ต่อวัน

❒ 1-1.5 กระป๋องหรือ 1/2 -3/4 ขวด ❒ 4.5-7 กระป๋องหรือ 3-4 ขวด

❒ 2-3 กระป๋องหรือ 1-1.5 ขวด ❒ 7 กระป๋องหรือ 4 ขวดขึ้นไป

❒ 3.5-4 กระป๋องหรือ 2 ขวด

1.2 ถ้าโดยทั่วไปดื่มเหล้า ดื่มปริมาณเท่าไหร่ต่อวัน

❒2-3 ฝา ❒3/4 แบน

❒1/4 แบน ❒1 แบนขึ้นไป

❒1/2 แบน

ผลไม้

ไม่กินผลไม้ ❒ กินน้อยกว่า 7 ครั้ง ต่อสัปดาห์

กินผลไม้ทุกวัน ❒ กิน 2-3 ครั้ง ต่อเดือน

โปรดยกตัวอย่างชนิดของผลไม้ที่ท่านรับประทานเป็นประจำ.....

ผัก

❒ ไม่กินผัก ❒ กินน้อยกว่า 7 ครั้ง ต่อสัปดาห์

❒ กินผักทุกวัน ❒ กิน 2-3 ครั้งต่อเดือน

โปรดยกตัวอย่างชนิดของผักที่ท่านรับประทานประจำ.....

ปลา

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❒ กินปลาทุกวัน ❒ กิน 2-3 ครั้งต่อเดือน

เนื้อสัตว์

❒ ไม่กินเนื้อสัตว์ ❒ กินน้อยกว่า 7 ครั้ง ต่อสัปดาห์

❒ กินเนื้อสัตว์ทุกวัน ❒ กิน 2-3 ครั้งต่อเดือน

Output

1. Publication

   1.1. PM_{10}-related DNA damages and abnormal buccal cells in the chronic obstructive pulmonary disease (manuscript preparation for Journal Environmental Science and Pollution Research, Impact factor 2.8)

2. การนำผลงานวิจัยไปใช้ประโยชน์

   - *เชิงพาณิชย์* (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
     ยังไม่มีการนำไปใช้

   - *เชิงนโยบาย* (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
     ยังไม่มีการนำไปใช้

   - *เชิงสาธารณะ* (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     ประชาชนทั่วไปมีความรู้ความเข้าใจ เกิดความตระหนักถึงผลกระทบของการเผาป่าและผลกระทบของฝุ่นละอองขนาดเล็กต่อสุขภาพ ซึ่งนำไปสู่การเปลี่ยนวิธีคิด พฤติกรรมการเผาป่า และส่งเสริมคุณภาพสิ่งแวดล้อม

   - *เชิงชุมชนและพื้นที่*
     ส่งผลให้ประชาชนเกิดความตระหนักถึงโทษของการเผากากเหลือจากผลผลิตทางการเกษตร และกระตุ้นให้ชุมชนมีการป้องกัน หรือลดการเผาป่า

   - *เชิงวิชาการ* (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)
     นำผลงานวิจัยไปต่อยอดงานวิจัยสำหรับนักศึกษาปริญญาโท เพื่อสร้างนักวิจัยใหม่ และนำเทคนิคที่ใช้ในการทำวิจัยเป็นหัวข้อในการเรียนการสอนกระบวนวิชา Research Methodology and Analysis in Toxicology ซึ่งเป็นกระบวนวิชาในหลักสูตรวิทยาศาสตรมหาบัณฑิต สาขาวิชาพิษวิทยา

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)
   ไม่มี

Journal Environmental Science and Pollution Research, Impact factor 2.8

PM_{10}-related DNA damages and abnormal buccal cells in the chronic obstructive pulmonary disease

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Abstract

Exposure to PM_{10} generated by biomass burning can result in reduction of lung functions and induction of DNA damage especially in Chronic obstructive pulmonary disease patients. This present study investigated the frequency of DNA damage cells and different types of cell death using buccal micronucleus cytome assay (BMCyt) as well as determined the correlations between DNA damage cells and lung function. The changes in DNA damage and cell death in March (PM_{10} > 50 μg/m^3) and August (PM_{10} <50 μg/m^3) pollutant periods were evaluated to explore whether PM_{10} exposure increases genotoxic damages in COPD patients when the PM_{10} concentration reached the peak in March. Fifty-eight COPD patients and 26 control subjects living in Chiang Dao district, Chiang Mai, Thailand were recruited in this study. The results revealed that buccal cells with micronuclei (BCMN), micronuclei (MNi) and binucleated cells (BN) observed in March were higher than those found in August in both study groups. A weak negative correlation between BCMN, MNi and COPD severity were demonstrated showing more DNA damage cells in patients with mild conditions. The excessive exposure of PM_{10} increased the risk of DNA damage in COPD patients for 295.23-fold.

Keywords: Chronic obstructive pulmonary disease, buccal micronucleus cytome, micronuclei, PM_{10}, DNA damage, biomass burning, buccal cells

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Introduction

The incidence of new chronic obstructive pulmonary disease (COPD) patients has become more concerned by Ministry of Public Health, Thailand over the decade (Ministry of Public Health Thailand 2015). Cigarette smoking is one of the significant risk factors for COPD, however, air pollution has now become more recognized as one of the risk factors as well (Ceylan et al. 2006, Hu et al. 2015, Pothirat et al. 2016, Yang and Omaye 2009). The pathogenesis of COPD and its severities are complicated due to the interactions of several mechanisms including genetic susceptibility, inflammation, oxidative stress, chromatin modifications, DNA damage, apoptosis and defective DNA repair (Hu et al. 2015, Neofytou et al. 2012, Yang and Omaye 2009). A number of reports have revealed an increase in numbers of patients with respiratory problems and hospital admissions or emergency room visits after exposure to excessive level of air pollution (Hu et al. 2015, Pengchai et al. 2009, Pothirat et al. 2016, Sezer et al. 2006, Vinitketkumnuen et al. 2002, Wiriya et al. 2013, Yang and Omaye 2009).

Air pollutions have been implicated as causes of serious health effects especially during high polluted season which known as open burning season starting from December to April every year in Chiang Mai, Thailand (latitude 18°N and longitude 98°E) (Pothirat et al. 2016, Sillapapiromsuk et al. 2013, Wiwatanadate and Liwsrisakun 2011). The significant sources of air pollution in this area are biomass or agricultural debris burning for land clearing, wildfires, as well as vehicle emissions (Pengchai et al. 2009, Tsai et al. 2013, Wiriya et al. 2013, Wiwatanadate and Liwsrisakun 2011). This haze has been concerned as a major health risk in Northern Thailand as

well as neighbor countries (Chantara et al. 2012, Wiriya et al. 2013, Wiwatanadate and Liwsrisakun 2011). According to the Pollution Control Department of Thailand (PCD), 89% of the pollutants found in Chiang Mai originate from forest fires, 5.4% from solid waste burning, and 2.3% from burning of agricultural waste (Kim Oanh and Leelasakultum 2011). Diesel combustion and industry sources contributed only less than 4% (Kim Oanh and Leelasakultum 2011, Sirimongkonlertkul and Phonekeo 2012, Tsai et al. 2013). The dust and ash material from the burning materials, or airborne particulate matter (PM) with diameter less than 10 (PM_{10}) have become the significant threats to local population (Sirimongkonlertkul and Phonekeo 2012). The PM_{10} measured in Chiang Mai ambient air contained several inorganic and organic species, including nitrogen oxides (NO_x), sulfur oxides (SO_x), PM_{10}-bounded ions and polycyclic aromatic hydrocarbons (PAHs) (Chantara et al. 2012, Gadde et al. 2009, Pengchai et al. 2009, Simoneit 2002, Tsai et al. 2013, Wiriya et al. 2013). These components, especially PAHs, are carcinogens, which can induce DNA damage and respiratory distress (Danielsen et al. 2011, Pengchai et al. 2009, Shosuke et al. 1989, Stohs and Bagchi 1995).

The pattern of PM_{10} concentrations in Chiang Mai, Thailand are similar every year since 2010. The high levels of PM_{10} concentrations can be found at the beginning of December and peaks in March. Subsequently, the levels of particulate ions and PM_{10} started to decrease significantly compare to the previous months in July or August (Chantara et al. 2012, Punsompong and Chantara 2018). Chiang Dao, Mae Rim and Mae Chaem districts are the dominant areas where rice and maize were produced (Sillapapiromsuk et al. 2013, Wiriya et al. 2016). Even though Mae Chaem district was reported to be the area that had high rate of agricultural residual burning during the harvest season, biomass burning from Chiang Dao district seem to emit more chemicals ions into the air (Sillapapiromsuk et al. 2013).

This study hypothesized that weather DNA damages and cell deaths in buccal cells of COPD patients and heathy subjects would be high during burning season due to excessive pollutant exposure. In addition, an increase in DNA damage-micronuclei and cell deaths may correlate with the severity of COPD. Therefore, we aimed to evaluate an increase in DNA damage using the Buccal Micronucleus Cytome (BMCyt) assay as an indicator in different stage of COPD during biomass burning season or high polluted period (PM_{10} >50 $\mu g/m^3$) in comparison with low pollution period (PM_{10} <50 $\mu g/m^3$) as per WHO Air quality guidelines for particulate matter (WHO

2006). Moreover, the relationship between abnormal buccal cell types and severity of COPD status were determined.

Materials and methods

Subjects

This study was conducted in 58 patients with mild to very severe COPD stages and 29 healthy control subjects lived in Chiang Dao, Chiang Mai Thailand. The COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease guideline (GOLD) (Pothirat et al. 2007). The inclusion criteria as follow: 1) Subjects aged over 40 years old who have been living in Chiang Dao for more than 1 year. 2) The post bronchodilator FEV_1/FVC ratio is less than 0.7 for the COPD group and more than 0.7 for the healthy control group. 3) The chest radiograms reveal no other cardiopulmonary diseases related to their symptoms such as tuberculosis, bronchiectasis, lung abscess, interstitial lung diseases, lung mass, and left ventricular failure. The patients were grouped according to COPD stage as mild, moderate, severe or very severe as per GOLD standard (Rabe et al. 2007, Vestbo et al. 2013). This study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethic Committee of Faculty of Medicine, Chiang Mai University, Thailand (Study code: FOR-2559-03852, FOR-2558-03434 and MED-2558-0303). The subjects were informed about the study and signed the consent form according to the guidelines of the Faculty of Medicine ethical committee. All individuals were interviewed face-to-face using demographic data questionnaire and COPD Assessment Test (CAT) to evaluate the health-related quality of life as well as a detailed personal questionnaire containing individual characteristics, potential cofounders such as smoking status, alcohol-drinking habits, diet, medication, and exercise habits were also obtained before sampling. High CAT score indicates COPD worsen severity of airflow limitation in stable COPD patients (Ghobadi et al. 2012).

COPD GOLD stage

This study classified the **s**everity of COPD according to the GOLD 2017 Guidelines (GOLD 2017) as follows: stage 1 mild FEV_1 > or equal to 80% predicted; stage 2 moderate FEV_1

between 50 and 80% predicted; stage 3 severe FEV_1 between 30 and 50% predicted; stage 4 very severe FEV_1 < or equal to 30% predicted or FEV_1 < 50% predicted plus chronic respiratory failure.

Sample collection

The buccal cells from each subject were collected two times in March 2016 (burning season with PM_{10} >50 μg/m^3) (Chantara et al. 2012, Wiriya et al. 2013) and August 2016 (PM_{10} <50 μg/m^3). The stage and severity of COPD at the sampling time were verified in order to evaluate the relationships between DNA damage and COPD status. The buccal samples or oral mucosa were collected and processed in accordance with Thomas et al. (Thomas et al. 2009). Briefly, buccal cells were collected in a circular motion from inside of the left and right cheek wall using wood spatula. Then, the collected cells were placed into the fixative containers containing Saccomanno's fixative solution. Afterward, the cell suspension was centrifuged at 4,000 rpm for 15 mins and the supernatant was removed. The cell suspension was washed twice using 500 μl of the fixative solution and centrifuged at 13,000 rpm for 10 min. Next, the supernatant was removed and replaced with 500 μl of the fixative solution. The final suspension was vortexed and then 100 μl of the cell suspension was placed directly onto a glass microscope slide. The slides were air-dried overnight and then placed in methanol: glacial acetic acid (3:1) for 10 mins before stained with Feulgen reaction solution before counterstaining with Fast-green reagent.

Buccal Micronucleus Cytome Assay

The slides were coded before scoring by two investigators (double-blinded scoring) and were examined at ×1,000 magnification using a good-quality bright field. The frequency of all the various cell types was scored in 2,000 buccal cells including the frequency of buccal cell with micronuclei (BCMN), micronuclei (MNi), bi-nucleated cells (BN), nuclear bud cells (NBUD), pyknotic cells (PY), condensed chromatin cells (CC), karyorrhectic cells (KR) and karyolytic cells (KL). Criteria for identifying and scoring cell types and nuclear abnormalities in the BMCyt assay was performed following Thomas et al. (Thomas et al. 2009) and Bolognesi et al. (Bolognesi and Fenech 2013).

PM_{10} data

The PM_{10} data used in this study was retrieved from the Thailand's air quality and situation reports of Pollution Control Department (PCD) Ministry of National Resources and Environment database (http://air4thai.pcd.go.th/webV2/) from January to December 2016 at the Chiang Mai City Hall station, Thailand. The PM_{10} concentrations were measure performed using beta-ray attenuation operated by the PCD. The data was reported as daily average concentrations of PM_{10}.

Statistical analysis

Results with numerical values were expressed as mean ± SD. Categorical data were demonstrated as absolute frequencies and percentages. The statistical analysis was performed on GraphPad Prism software Version 5.01 and IBM® SPSS® Statistics Version 22. The statistically significant difference was considered when $p \leq 0.05$. As the data was not normal distributed, the non-parametric test was employed. The Wilcoxon matched-pairs signed rank test was used to compare the means of variables of the same subjects between low and high pollution season. Chi-square test or Fisher's Exact test were used to compare the proportional categorical data. Statistical differences between the COPD and control group were tested using the non-parametric Mann Whitney U Test. The correlations between different variables were determined using the Spearman Rank Correlation Test. The Poisson log-linear model were applied to the data to estimate the association between BCMN, MNi and factors that involving micronucleus induction including PM_{10} levels, disease status, host factors, smoking habit, drinking habit, diet. Adjustments with dependent covariates were made for age, gender, FEV_1/FVC ratio, FEV_1, COPD severity, CAT score.

Results and discussions

PM_{10} data

The monthly PM_{10} concentrations from January to December is shown in Fig. 1 indicating the PM_{10} levels were exceeded the WHO Air quality guidelines (50 $\mu g/m^3$) from January to April with the peak level of 187.33 $\mu g/m^3$ in March. Consistent with the previous work performed by Punsompong and Chantara (Punsompong and Chantara 2018) stating that the monthly PM_{10} concentrations from year 2010 to 2015 were similar showing the high levels of PM_{10} in dry season (January to April) and lower concentrations in wet season (May to October). The PM_{10} levels in

2016 showed the same pattern as the previous observation which peak levels of PM_{10} in March resulting from active biomass burning (Punsompong and Chantara 2018). Moreover, Chiang Mai is surrounded by mountains causing high accumulation of PM_{10} in dry season because of the low air-flow and inversion temperatures (Sillapapiromsuk et al. 2013, Wiriya et al. 2013).

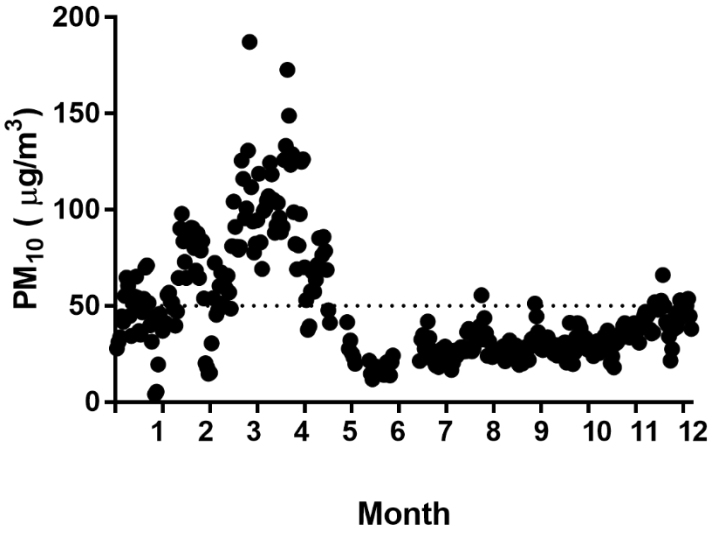

Fig. 1 The PM_{10} concentrations from January to December 2016.

Subjects

Fifty-eight COPD patients and 26 healthy subjects lived in agricultural area, Chiang Dao, Chiang Mai, Thailand were enrolled in this study. General characteristic of the study population (COPD patients and healthy control subjects) including gender, age, lung function, smoking and alcohol drinking status are shown in Table 1. The subjects from control group were 10 years younger than the COPD group. The control group had normal lung function according to GOLD standard. More than 50 percentages of the subjects were former smokers and most of the subject did not consumed alcohol. The post bronchodilator FEV_1/FVC ratio during polluted season was significantly lower than that measured during the low pollution in COPD patients as well as % predicted FEV_1 (Table 1). This finding confirms that PM_{10} levels involved in lung function status and disease progression as same as previous studies showing a strong correlation between PM_{10} exposure and exacerbations or COPD disease progression (MacNee and Donaldson 2003, Pope 2000, Pothirat et al. 2016).

Table 1 Demographic data and general characteristic of the COPD and the control groups

Demographic data	COPD (n=58)	Control (n=26)
Male (%)	31 (53.45%)	13 (50.00%)
Age (years)	71.45± 7.93[a]	61.31±11.31[a]
FEV_1/FVC (%)		
PM_{10} >50 μg/m^3	57.08±10.26 [a]	82.03±7.32 [a]
PM_{10} <50 μg/m^3	58.58±11.92 [a]	80.78±6.83 [a]
FEV_1 (L)		
PM_{10} >50 μg/m^3	1.20±0.48 [a,b]	2.26±0.62 [a]
PM_{10} <50 μg/m^3	1.29±0.54 [a,b]	2.20±0.71 [a]
% predicted FEV_1		
PM_{10} >50 μg/m^3	64.78±24.46 [a,b]	103.80±20.46 [a]
PM_{10} <50 μg/m^3	70.80±24.65 [a,b]	102.16±21.47 [a]
FVC (L)		
PM_{10} >50 μg/m^3	2.09±0.68 [a,b]	2.74±0.66 [a]
PM_{10} <50 μg/m^3	2.20±0.69 [a,b]	2.70±0.79 [a]
CAT		
PM_{10} >50 μg/m^3	11.76±7.20 [a]	6.23±5.11 [a]
PM_{10} <50 μg/m^3	11.00±6.28 [a]	5.92±6.18 [a]
GOLD severity stage (n, %)		
Stage 1 Mild	14 (24.14%)	-
Stage 2 Moderate	17 (29.31%)	-
Stage 3 Severe	13 (22.41%)	-
Stage 4 Very severe	14 (24.14%)	-
Cigarette smoking (n, %)		
Never	8 (13.8%)	8 (30.8%)
Former	41 (70.7%)	14 (53.8%)
Current	9 (15.5%)	4 (15.4%)

Alcohol drinking (n, %)		
Never	32 (55.2%)	10 (38.5%)
Former	19 (32.8%)	7 (26.9%)
Current	7 (12.1%)	9 (34.6%)

Mean ± SD, FVC = Forced Vital Capacity, FEV_1= Forced Expiratory Volume in first second, [a] Mann Whitney U Test (COPD vs Control), [b] Wilcoxon matched-pairs signed rank test (within subjects, PM_{10} >50 μg/m^3 vs PM_{10} <50 μg/m^3), significant *p* value < 0.05.

Buccal Micronucleus Cytome Assay

For the Buccal Micronucleus Cytome Assay, the photomicrographs of scored abnormal cell types are shown in Fig. 2. The mean frequencies of abnormal cells (PY, CC, KR and KL) and different types of DNA damage cells (BCMN, MNi, BN, NBUD) are shown in Fig. 3. The BCMN and MNi cells were not statistically significant different between the COPD and control groups during both high and low PM_{10} levels. The MNi detected in the COPD and control groups during low pollution were used as a baseline MNi since spontaneous MNi can be found ranging from 0.05-11.5 MNi/1,000 cells even in healthy population (Holland et al. 2008).

A significant increase in DNA damage cells from their baseline were detected in both populations as presented in Fig.3. The mean frequency of BCMN observed in the COPD group during high-polluted period was significantly higher than that found in low pollution period (Fig. 3 a), with the mean frequencies of 1.09±1.95 and 0.29±0.64 (p value = 0.027), respectively. The MNi frequency (Fig. 3 b) found in the control group when the $PM_{10} > 50$ μg/m^3 was significantly higher than that found during $PM_{10} < 50$ μg/m^3 (1.39±2.12 and 0.8±1.05, respectively). The BCMN as well as MNi frequencies are the markers of chromosome loss or fragmentation due to the genotoxic exposure (Holland et al. 2008). An increase in DNA or chromosome damages during high pollution demonstrated in our findings may possibly link to an excessive exposure of PM_{10} from agricultural waste burning in dry season. Several studies indicated that air pollution especially from biomass burning cause severe genotoxic effects on COPD patients as much as cigarette smoking (Caramori et al. 2011, Ceylan et al. 2006, Danielsen et al. 2011, Hu et al. 2015, Yang and Omaye 2009). Furthermore, Ceretti et al. stated that an increase in 10 μg/m^3 unit of PM_{10} was significantly related to the elevation of MNi, CC cells and KR cells (Ceretti et al. 2014).

Micronucleus formation occur originally in the basal layer after systemic exposure of genotoxic substances during cell division. The cells with MNi differentiate into the stratum spinosum layer (the prickle cell layer) and the stratum corneum layer (the keratinized superficial layer) and then exfoliate into the buccal cavity. The time frame of cellular migration from basal layer to the keratinized superficial layer is between 7 to 10 days (Holland et al. 2008, Thomas et al. 2009). Meanwhile, some of the cells containing DNA fragments may undergo program cell death and turn into cells with condensed chromatin, karyorrhectic cells, pyknotic nuclei or karyolitic cells.(Holland et al. 2008). However, the epithelial tissues have a rapid turnover rate of about 7-21 days. Consequently, the genotoxic effects observed in our study could be a result of

PM_{10} – induced DNA damage 1 to 3 weeks before buccal cell collection (Holland et al. 2008, Majer et al. 2001).

Moreover, the frequency of the BN cells (Fig. 3 d) was dramatically larger during high pollution than that detected during low pollution in both COPD (11.43±18.68 and 1.60±1.3, *p* value = 0.004) and control groups (7.77±12.76 and 1.00±1.17, *p* value = 0.012). Binucleated cells are an indicator of defective of cytokinesis due to aneuploidy (Bonassi et al. 2011). Aneuploidy reflects gain or loss of whole chromosomes as well as non-balanced rearrangements of chromosomes, including deletions, amplifications or translocations of large regions of the genome (Orr et al. 2015). Moreover, aneuploidy cells or chromosomal instability cells have an elevated rate of DNA mutations and chromosome missegregation which can lead to micronucleus formation (Varetti et al. 2014). In addition, the elevation of binucleate:mononucleate cell ratio can be used to identify a cytokinesis failure caused by higher-than-normal rates of aneuploidy which is related with cancer risk (da Silva et al. 2013, Thomas et al. 2009). Therefore, the high frequency of BN observed in our study during open burning season, and subsequently return to baseline in wet season, could be a result of cytokinesis failure during cell division induced by biomass burning exposure. Our finding is in agreement with Mondal et al. stating that chronic exposure to biomass fuel not only causes chromosomal and DNA damage resulting MNi induction but also induced cytokinesis defect leading to higher frequency of binucleated cells (Mondal et al. 2010). The frequency of BN was also slightly associated with air pollution as reported by Ceretti et al. The studied stated that an increase in the PM_{10} concentration every 10 $\mu g/m^3$ unit resulting in the elevation of BN for 0.01 unit (Ceretti et al. 2014).

Furthermore in our study area, there are three main types of the biomass including rice straw, maize residue and leaf litter that emitted high concentration of PM10-bound PAHs after burning (Wiriya et al. 2016). The PAHs profiles from Wiriya et at. study revealed that high molecular weight PAHs high molecular weight PAHs, for example benzo[a]pyrene, benzo[k]fluoranthene, benzo[b]fluoranthene, chrysene, benzo[a]anthracene, pyrene, fluoranthene, phenanthrene were the most abundant pollutants (Wiriya et al. 2016). These PAH species involve in DNA and oxidative damage (Xue and Warshawsky 2005), consequently, initiate and promote cancer (Armstrong et al. 2004). The chromosome break or cytokinesis dysfunction demonstrated in our study could be an impact of PAH exposure. Nonetheless, the limitation of this study was

the lack of individual pollutant exposure data as a real-time particulate matter measurement for each subject have not been performed.

The frequency of CC cells was higher during low pollution period in both COPD (132.40±104.30 and 230.20±87.35, *p* value = 0.006) and control groups (168.10±119.90 and 267.78±97.66, *p* value = 0.012) indicating the cells undergoing early stages of apoptosis when the pollution decreased. This phenomenon may indicate repair and elimination process of DNA damage in buccal cells (Fig.3 e) (Thomas et al. 2009). In addition, a lower frequency of CC cells found in our present work may suggest the reduction of regenerative capacity of epithelial tissue during high pollution (Bolognesi et al. 2015). Similar results were reported in the study in open-cast coal mine workers by Rohr et al. A frequency of CC cells in the exposed group was lower than in non-exposed group. They concluded that an increase of this cell type in non-exposure group occurred randomly (Rohr et al. 2013).

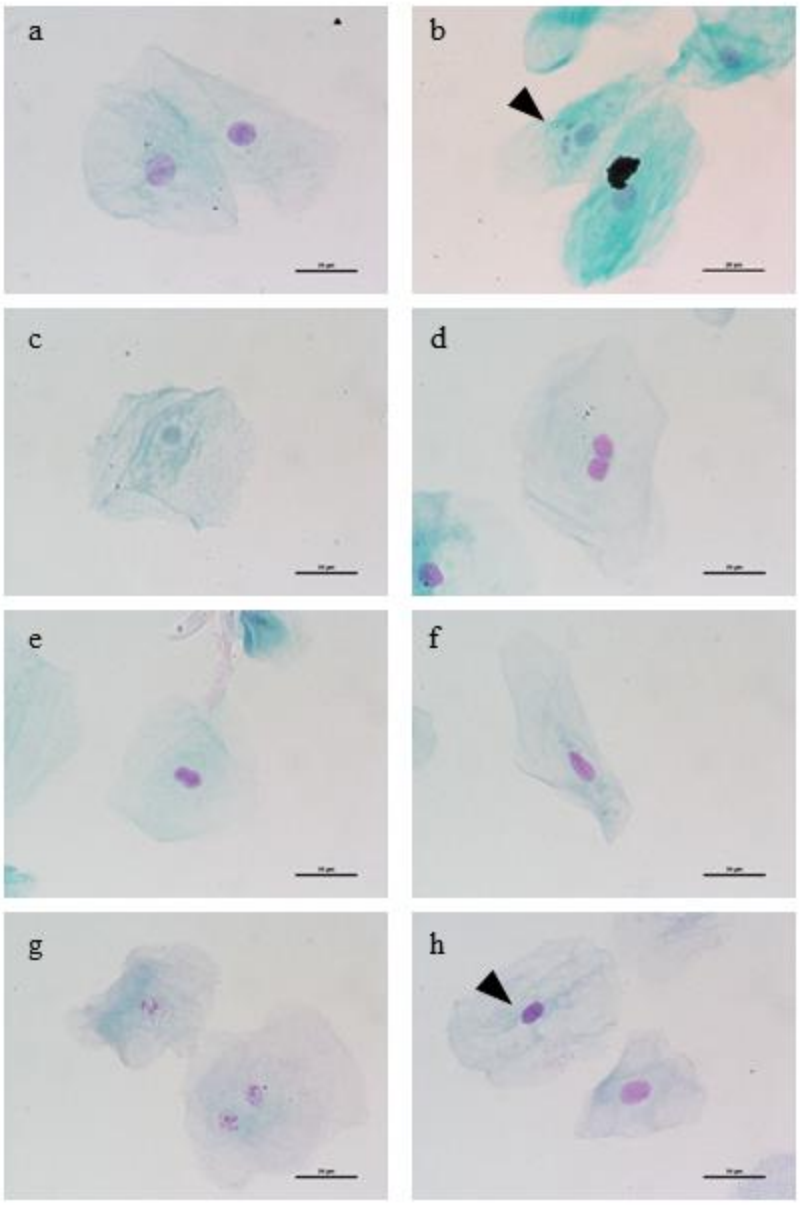

Fig. 2 Images of the different cell types stained using Feulgen and Light Green viewed by transmitted light microscope. **a** Normal differentiated cell, **b** Buccal cell with micronuclei, **c** Karyolitic cell, **d** Binucleated cell, **e** Nuclear bud cell, **f** Condensed chromatin cell, **g** Karyorrhectic cell, **h** Pyknotic cell. All images were taken at ×1,000 magnification, scale bar 20 μm.

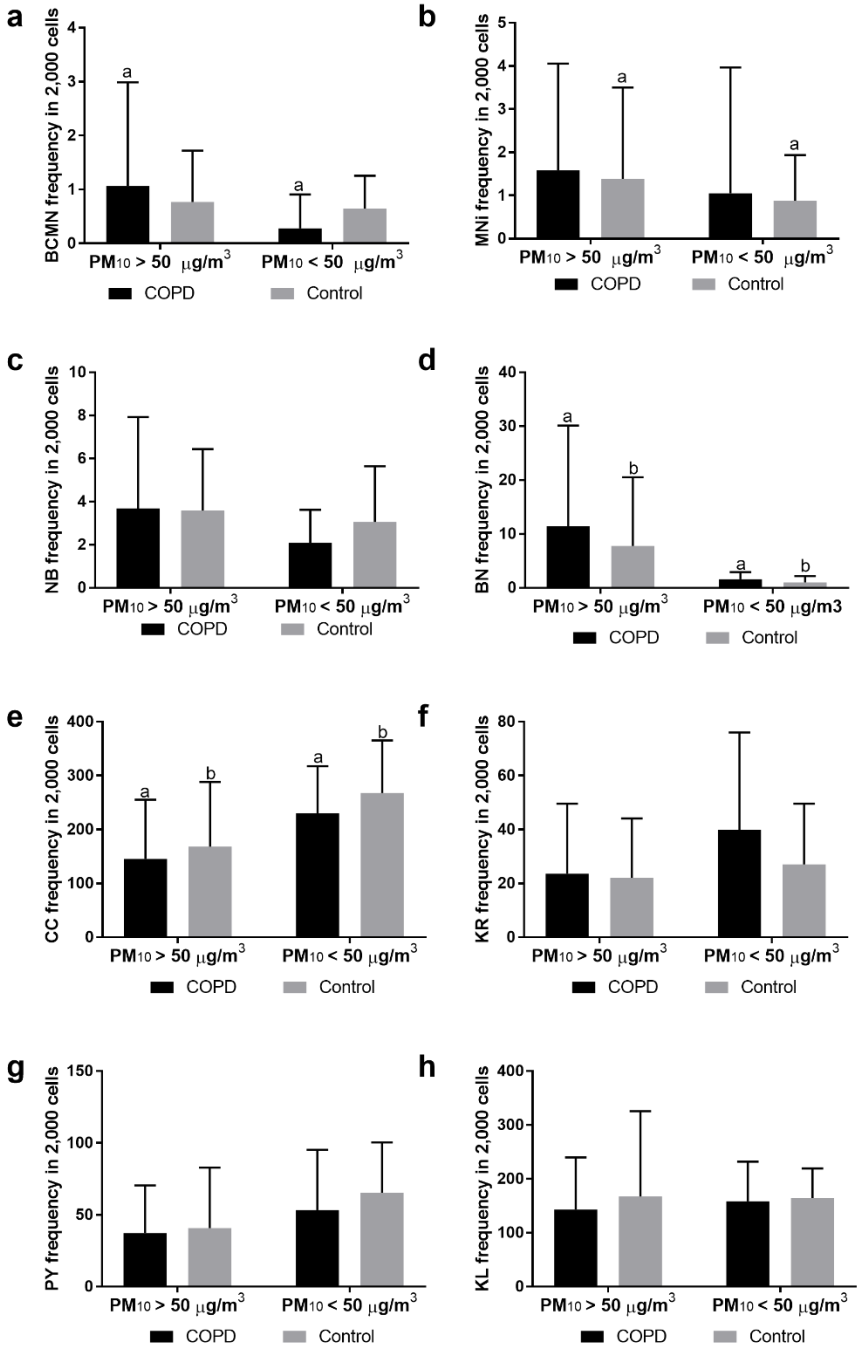

Fig. 3 DNA damage and cell death markers. The frequencies of: **a** Buccal cell with micronuclei, **b** Micronuclei, **c** Nuclear bud cell, **d** Binucleated cell, **e** Condensed chromatin cell, **f** Karyorrhectic cell, **g** Pyknotic cell, **h** Karyolitic cell. Groups not showing the same letter are significantly different from each other.

Only BCMN, MNi and CC cells were significant different among COPD severity groups (Fig. 4). The frequencies of BCMN and MNi (Fig. 4 a and b) in patients with mild symptoms (stage I) were higher than the patients with moderate, severe and very severe stages. On the other hand, the frequency of apoptotic cells or CC cells was found more in patients with stage II to IV (Fig. 4 c). Rohr et al. determined the relationship between genetic damages and cell deaths in open-cast coal mine workers using the buccal micronucleus cytome assay. The results showed that subjects with high MNi frequency had a lower frequency of CC cells (Rohr et al. 2013). In our study, the cells with genetic damages occurring during PM_{10} exposure may be eliminated via the apoptosis resulting in the high frequency of CC cells and less DNA damage cells in the severe COPD groups (Holland et al. 2008).

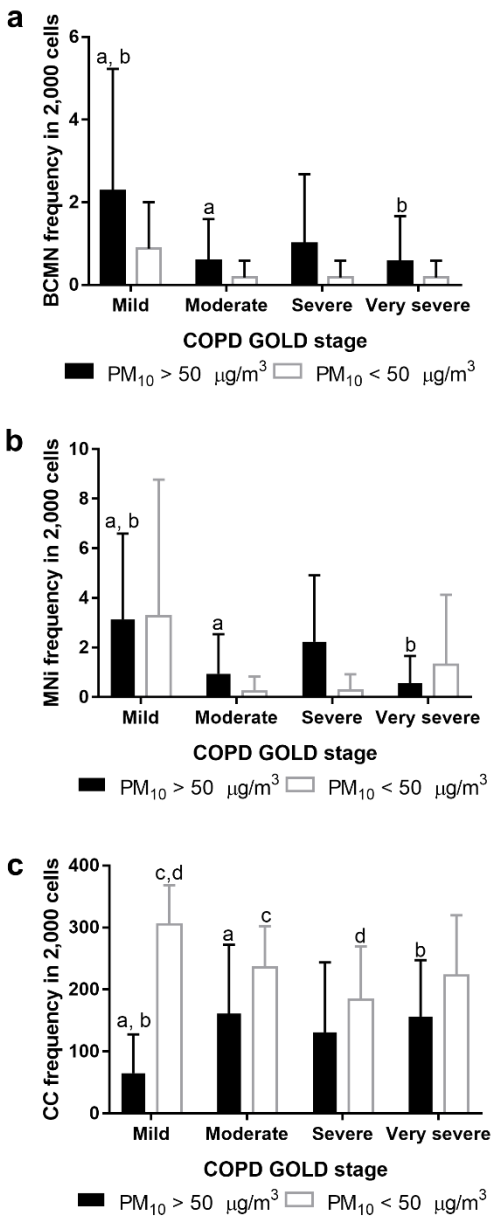

Fig. 4 Frequencies of **a** Buccal cell with micronuclei, **b** Micronuclei, **c** Condensed chromatin cell in COPD group according to COPD severity as defined by GOLD standard. Groups not showing the same letter are significantly different from each other.

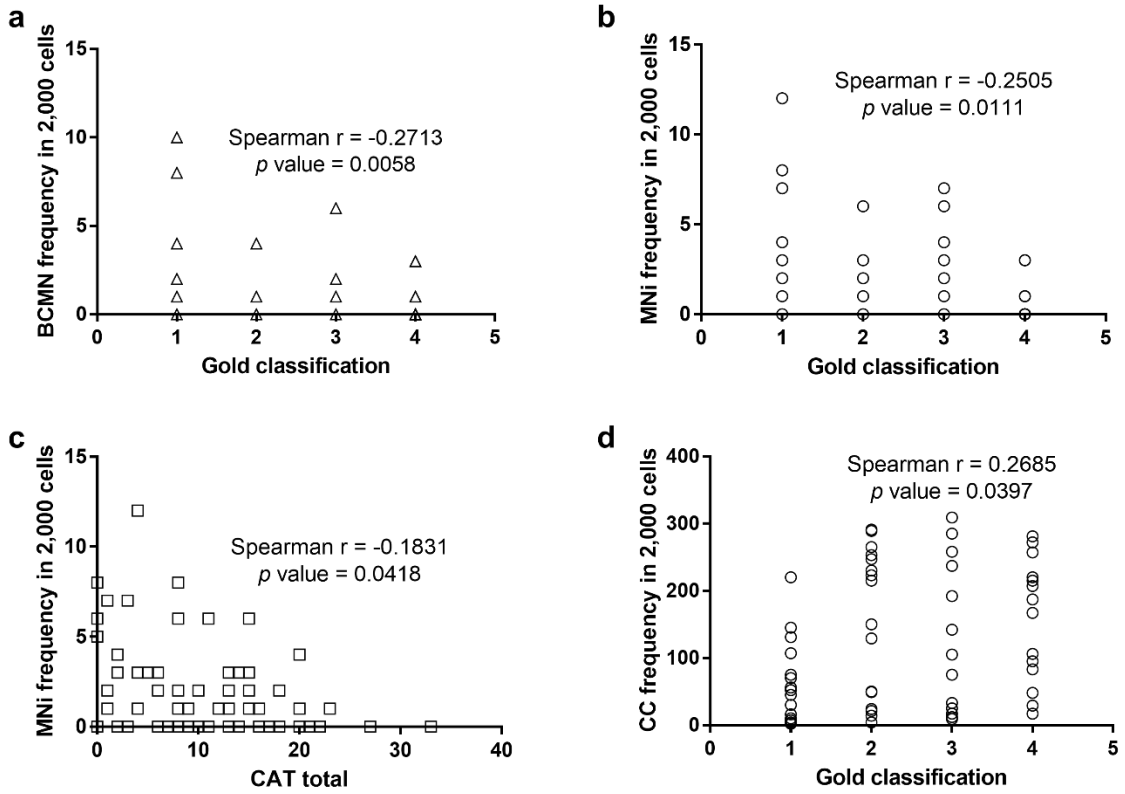

Fig. 5 Correlation between the DNA damage and COPD severity stages as defined by GOLD 1 = mild, 2 = moderate, 3 = severe, 4 = very severe

To evaluate the relationship between DNA damage cells, cell deaths and the COPD severities, Spearman's rank correlation was performed. pulmonary function indicators FEV_1 and FVC was not correlated with any DNA damage or cell death markers. The severity defined by GOLD classes and CAT score correlated negatively with the BCMN and MNi frequencies, however, only a weak correlation was observed with the Spearman r below 0.3 (Fig.5 a-c). This finding confirmed that DNA damage cells presented more in mild to moderate COPD patients. Severe COPD patients on the other hand had more CC cells indicating cells undergo apoptotic cell death program.

Association between DNA damage cells and confounding factors

The confounding factors that may involve in micronucleus induction were taken in to account to remove all bias from the analysis. After adjustment, COPD patients had a higher risk of PM_{10} induced BCMN and MNi formation with the frequency ratio of 295.23 and 64.51 when

the PM_{10} levels were higher than 50 μg/m^3 as shown in Table 2. This indicates that COPD patients has more susceptibility to PM_{10} toxicity when compared to the corresponding control group. In addition, high pollutants enhance the genotoxic effect of PM_{10} in this finding as the frequency's ratio of DNA damages during PM_{10} levels more than 50 μg/m^3 was higher than that in the low pollution period. Ceretti et al. studied the association between the PM_{10} exposure and MNi frequency in children. Linear regression was performed to analyze using air pollutant mean levels at time 0 (buccal cell sampling day), 1, 2 and 3 weeks before exfoliated cell collection. The results showed modest association between MNi and PM_{10} concentration 1-week prior buccal sampling (Ceretti et al. 2014).

Table 2 Risk of BCMN induction in COPD patients and control subjects compare between high and low PM_{10} levels

DNA damage cells	Predictors	Group	PM_{10} > 50 μg/m^3			PM_{10} < 50 μg/m^3		
			FR	95%CI	*p* -value	FR	95%CI	*p* -value
BCMN	Diseases	Control	1	-	-	1	-	-
		COPD	**295.23**	**4.59-18973.26**	**0.007**	**96.51**	**4.95-1881.65**	**0.003**
MNi	Diseases	Control	1	-	-	1	-	-
		COPD	**64.51**	**5.15-807.58**	**0.001**	**29.77**	**1.60-555.06**	**0.023**

[a] FR: Frequency ratio, significant results showed in bold.

The role of confounding factors was also important for micronucleus induction. The association between BCMN and life-style parameters in COPD group were presented in Table 3. The most associated factors links to BCMN cells were smoking and food consumption. The results indicated that former smokers had a possibility to have higher BCMN frequencies for 12.75 times than those who never smoked. Subjects who intake fruit and vegetable daily have less BCMN frequency (FR = 0.036) than those who do not consume with over 96.4% DNA damage reduction.

It has been well established that smoking habit, fruit and vegetable consumption affect micronucleus formation (Bonassi et al. 2011). Heavy cigarette smokers (≥ 40 cigarettes per day) related with an increase in MNi frequency (Bonassi et al. 2011). Our study demonstrated that COPD patients who once smoke had more BCMN frequency than those who are still smoking. However, the number of current smokers enrolled in our study (n=9) were too small to see substantial change in BCMN frequency. Particularly, subjects who intake fruit or vegetable daily had a lower BCMN frequencies that those who reported no consumption at all. This result is consistent with several publications demonstrating that fruit and vegetable consumption significantly reduce micronucleus levels (Bonassi et al. 2011).

Table 3 Effects of smoking habit and food consumption on BCMN frequency

Predictors	Group	$PM_{10} > 50\ \mu g/m^3$			$PM_{10} < 50\ \mu g/m^3$		
		FR	95%CI	*p* -value	FR	95%CI	*p* -value
Smoking	Never	1	-	-	1	-	-
	Former	**12.75**	**2.74-59.85**	**0.001**	**25.33**	**3.21-199.82**	**0.002**
	Current	4.32	0.75-24.78	0.101	4.87	0.53-44.48	0.160
Fruit consumption	Never	1	-	-	1	-	-
	Once a week	**0.046**	**0.00-0.79**	**0.056**	217117.93	0.00-α	0.992
	Every day	**0.036**	**0.00-1.08**	**0.035**	136621.80	0.00-α	0.990
Vegetable consumption	Never	1	-	-	1	-	-
	Once a week	0.077	0.00-0.504	0.156	0.55	0.02-14.20	0.722
	Every day	**0.015**	**0.00-2.66**	**0.019**	0.37	0.01-10.62	0.565

[a] FR: Frequency ratio, significant results showed in bold.

Conclusions

The PM_{10} concentrations exceeded WHO guideline from January (dry season) and reached the peak in March and subsequently decreased by May. Buccal cell with micronuclei, micronuclei and binucleated cells were higher during high pollution (March), particularly in the COPD patients indicating DNA damages and instability. Micronucleus frequency in COPD patients with mild condition was higher than those with severe conditions. However, cells with condensed chromatin detected in the moderate to very severe COPD groups were significantly higher than in mild group indicating the DNA damage cells undergo apoptosis. Exposure to PM_{10} during high pollutant period increased the frequency risk of DNA damage for 295-fold in COPD patients in comparison with the control group. It can be concluded that the COPD patients has more susceptibility to air pollution induced DNA damage than healthy subjects.

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