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Research Paper

Hemolysis of PM₁₀ on RBCs *in vitro*: An indoor air study in a coal-burning lung cancer epidemic area

Mengyuan Zhang^a, Longyi Shao^{a,*}, Tim Jones^b, Ying Hu^{a,c}, Rachel Adams^d, Kelly Bérubé^e

^a State Key Laboratory of Coal Resources and Safe Mining, and College of Geoscience and Surveying Engineering, China University of Mining & Technology, Beijing 100083, China

^b School of Earth and Environmental Sciences, Cardiff University, Museum Avenue, Cardiff CF10 3YE, UK

^c Guangdong Province Nuclear Industry Geological Bureau, Guangzhou 510800, China

^d School of Health Sciences, Cardiff Metropolitan University, Llandaff Campus, Western Avenue, Cardiff CF5 2YB, Wales, UK

^e School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, Wales, UK

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ABSTRACT

Epidemiological studies have suggested that inhalation exposure to indoor ambient air from coal-burning environments is causally associated with respiratory health risks. In order to explore the toxicological mechanisms behind the adverse health effects, the hemolytic activity of PM₁₀ (particulate matter with an aerodynamic diameter of 10 μm or less) samples collected from homes burning coal in the recognized China “cancer village” Xuanwei were evaluated and matched against their trace elemental contents. The results demonstrated that the hemolytic activity of indoor PM₁₀ in coal-burning environments ranged from 4.28% to 5.24%, with a clear positive dose-response relationship. Although low dose samples exhibited a reduced hemolytic activity, PM₁₀ could have a toxic effect upon people in a coal-burning indoor environment for extended time periods. The concentrations of analyzed trace elements in PM₁₀ samples ranged from 6966 to 12,958 ppm. Among the analyzed elements, Zn, Ti, Ni, Cu, Pb, Ba, Mn, Cr and V were found at higher concentrations and accounted for over 95% of the total elements. The concentrations of total analyzed elements in the PM₁₀ samples revealed a significant positive correlation with PM₁₀ hemolytic activity. Of the analyzed elements, Zn, Pb and Cs positively correlated with hemolysis, while Li, U and V negatively correlated with the hemolysis of human red blood cells (RBCs). Therefore, the heavy metal elements could be one of the main factors responsible for the hemolytic capacity of indoor PM₁₀ in coal-burning environments.

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1. Introduction

In recent years, the impact of airborne particulate matter 10 μm or less (PM₁₀) on human health has attracted widespread attention (Li et al., 2020; Silva et al., 2020a; Xing et al., 2020). Epidemiological investigations have indicated a link between increase of the mass levels of airborne particles and adult morbidity and mortality (Kim et al., 2016; Li et al., 2017; Hamanaka and Mutlu, 2018). However, the biological mechanisms underlying the adverse health effects of airborne particles still remains unclear. Many methods have been used to assess the toxicity of PM₁₀, including animal tests (Marchini et al., 2016; Jin et al., 2018), single-cell gel electrophoresis (SCGF; Velali et al., 2016; Bahadori et al., 2018), Ames fluctuation test (Klobučar, 2011; Du et al., 2019), plasmid DNA assay (Greenwell et al., 2002; Feng et al., 2020) and the hemolysis assay (Quintana et al., 2011; Faraji et al., 2019).

The hemolysis assay is a practical and rapid method to investigate the cytotoxicity and hemolytic properties (*i.e.* biological membrane damage capacity) of particulate matter (PM) (Hadnagy et al., 2003; Mesdaghinia et al., 2019). Particles dissolved in water exhibit a strong free radical signal of reactive oxygen species (ROS) (Tong et al., 2001), which is known to be cytotoxic (Nash et al., 1966) and interacts with the cell membrane inducing an increase of membrane fluidity and permeability. This interaction may exhaust the protective mechanisms of the cell, and thereby, causes lipid peroxidation of RBCs and their cell components, leading to cell membrane rupture.

Epidemiological investigations have reported that PM pollution induced decreased human red blood cells (RBCs) counts and even anemia (Nikolic et al., 2008; Bahrami et al., 2013; Kargarfard et al., 2015). To further investigate the mechanism, Faraji et al. (2019) analyzed chemical compositions of PM₁₀ in two air pollution conditions (*i.e.* dust storms and inversions) and evaluated the toxicological effects of PM₁₀ collected in these conditions on the hemolysis of human RBCs *in vitro*. They have identified the significant positive relationship between concentration of PM and hemolytic activity. In addition, Quintana et al. (2011) compared

* Corresponding author.

E-mail address: ShaoL@cumtb.edu.cn (L. Shao).

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the PM collected at an urban-emitting site and a suburban-receptor site and found that the suburban-site induced greater hemolysis. All of these studies indicated that the hemolysis effect is related not only to the concentrations of PM, but also to the composition.

At present, the use of coal for heating and cooking is still the main source of indoor air pollution in rural areas (Zheng and Bu, 2018; Liu and Mauzerall, 2020). Especially in Xuanwei county, Yunnan Province, the mortality and morbidity rate of lung cancer is the highest among the Chinese cities (Mumford et al., 1987). According to the “2012 Chinese Cancer Registry Annual Report”, the mean morbidity rate of lung cancer in Xuanwei was 92 per 100,000 which is 4–5 times the national rate (Hao et al., 2012). A number of studies have indicated that lung cancer in Xuanwei was highly associated with the domestic burning of local bituminous coal (Xiao et al., 2012; Seow et al., 2014). The coals for residents in Xuanwei are from the C1 coal seam situated geologically at the end-Permian mass extinction (Large et al., 2009; Shao et al., 2015; Wang et al., 2018) which is characterized by relatively enriched Si together with elevated contents of heavy metals including Zn, Pb, Cd and Cu, in comparison to Chinese coal averages and world averages (Shao et al., 2015). The unusual properties for this coal may result in the special characteristics of compositions and toxicity of the coal-burning particles.

Indoor PM emitted from coal burning in Xuanwei includes high concentration of heavy metals (Shao et al., 2013), crystalline silica (Tian et al., 2008; Large et al., 2009), and polycyclic aromatic hydrocarbons (PAHs; Mumford et al., 1995; Lui et al., 2016). These toxic components of PM can enter the respiratory and circulatory systems and be retained in the body (Pinkerton et al., 2000; Chuang et al., 2016), potentially leading to pneumonia, asthma, cardiovascular disease, and even lung cancer (Zhou et al., 2015; Lin et al., 2016). Different methods have been utilized to better understand the toxicity of indoor particulate matter in coal-burning environments. Mumford et al. (1990) compared mouse skin tumorigenicity induced by indoor air particles from coal combustion and wood combustion in Xuanwei and linked this to human lung cancer. Their results showed that, compared to the particles emitted from wood combustion, the particles emitted from coal combustion were more active in tumor initiation; these findings agreed with the epidemiological data, which showed that the residents using ‘smoky’ bituminous coal as their major fuel in homes had a higher lung cancer mortality rate (Mumford et al., 1990). Li et al. (2012) measured cell viability by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method and concluded that the particles emitted from the combustion of bituminous coal had great cytotoxicity. Shao et al. (2013) used the plasmid scission assay to investigate the bioreactivity of PM₁₀ in coal-burning environments. The results showed that indoor PM₁₀ caused higher oxidative damage than corresponding outdoor PM₁₀, and heavy metals could be one of the main factors responsible for the high oxidative capacity of indoor PM₁₀. Since few studies have been undertaken on the cytotoxicity of coal-burning particles, understanding hemolysis damage induced by indoor coal-burning PM₁₀ in lung cancer village has an important role in studying the toxicity of particles.

In this paper, the coal-burning indoor PM₁₀ samples collected in the high lung cancer village in Xuanwei, Yunnan Province, and the hemolysis assay is used to assess the toxicity of these indoor PM₁₀. Trace element compositions of PM₁₀ were examined by inductively coupled plasma mass spectrometry (ICP-MS). The relationship of the heavy metal compositions with the toxicity of these inhalable particles was further discussed.

2. Methods

A sampling campaign was carried out in the Xuanwei area during 2013 and 2014. PM₁₀ samples were collected from two homes in Hutou Village, which represents the high lung cancer mortality area in China (Mumford et al., 1987). The sampling site in Home#1 was in

the kitchen, which had a chimney. Except cooking with coal during daytime, no daily activity took place in this room. The sampling site of Home#2 was in a connected space between the living room and kitchen. The residents of Home#2 used induction cookers for cooking during daytime and portable stoves burning coal for heating at night in the winter. The two houses both used bituminous coal purchased from Yantang coal mine about 1.4 km away from the village. PM₁₀ selective inlet heads (C-30, Thermo Fisher Scientific, USA) and polycarbonate filters (47 mm diameter, 0.67 mm pore size, Millipore, UK) were used to collect PM₁₀ at a flow rate of 30 L/min. The sampling points were at the average breathing heights (1.5 m above the floor). Detailed sample information is shown in Table 1.

The hemolysis assay was undertaken in the School of Biosciences, Cardiff University and the School of Health Sciences, Cardiff Metropolitan University. The PM₁₀ samples were incubated with isolated RBCs and analyzed by the Tecan Infinite® 200 PRO plate reader at λ 540 nm. Initially 10 mL of blood was drawn from a 40 year old female donor. The blood samples were centrifuged at 3750 rotations per minute (rpm) at 4 °C for 10 min; the supernatant and a thin layer of platelets were removed from the blood and discarded. The blood samples were re-suspended with 0.9% saline to make them back up to 10 mL; this was repeated two more times. Four 1 mL aliquots were stored in Eppendorf tubes and centrifuged again at 1700 rpm for 5 min. The final working solution of diluted RBCs was made by adding 200 μ L from each of the four Eppendorf tubes to 7.2 mL of 0.9% saline to make a total volume of 8 mL. The PM₁₀ was suspended in the phosphate buffered saline (PBS, Sigma, UK) and diluted to 5 doses: 2 mg/mL, 1 mg/mL, 500 μ g/mL, 250 μ g/mL, 50 μ g/mL. The PM₁₀ solution (125 μ L) was added to the prepared blood (125 μ L) in a 96-well (Cole-Palmer, UK) plate which was then sealed with adhesive plastic and placed on a platform shaker (ThermoFisher Scientific, USA) for 10 min. The plate was then centrifuged at 200 rpm for 5 min at 4 °C, 100 μ L of supernatant was removed and placed into a corresponding well of a new 96-well plate. The negative control contained 0.9% PBS and the positive control contained 0.1% Triton X-100 mixed with the prepared blood. The plate containing the supernatant had its optical density read using the Tecan Infinite® 200 PRO plate reader at 540 nm. The absorbance readings in optical density from the plate reader was converted into percentage of hemolysis with the following formula: % hemolysis = [(sample OD-control OD)/(standard OD-control OD) \times 100] (Dobrovol'skaia et al., 2008).

The trace elements in the PM₁₀ samples were analyzed by ICP-MS using a Thermo Elemental X Series (X7; ThermoFisher Scientific, USA). The solution of the whole sample was prepared by digesting pooled samples of polycarbonate filters using concentrated nitric acid (Fisherbrand™ Precision Specific Gravity 1.48). Digestions were carried out in a CEMMDS-200 microwave system, using CEM advanced composite vessels with Teflon liners. The digested samples were then concentrated by evaporating the nitric acid and by re-dissolving in 2 mL of 10% nitric acid. Samples were diluted to 20 mL volume using de-ionized (>18 MU) water. One milliliter of each sample was combined with a 50 ryam ppb thallium standard (1 mL) and this solution was made up to 10 mL with 2% nitric acid to be analyzed in the ICP-MS. The samples were run with an international basalt standard as a positive control. The detection limits of the elements were in the range of 1 ppt

Table 1
Sampling information.

Number	Sampling season	Site	Sample weight (μ g)	PM ₁₀ concentration (μ g/m ³)
1	Winter	Home#1	3445	148.5
2		Home#2	2311	169.6
3	Summer	Home#1	1008	331.4
4		Home#2	935	261.5

(parts per trillion) to 1 ppb (parts per billion). The final results were reported as the ppm (parts per million) or ppb of each element in the analyzed PM₁₀.

Statistical evaluation was performed by SPSS Statistics 17.0. Pearson correlation tests was used to assess the relationship between the concentrations of trace elements and the hemolysis under the dose of 500 µg/mL. $P < 0.05$ and $P < 0.1$ was regarded as significant for analysis.

3. Results

The values of hemolysis at the five doses; 50, 250, 500, 1000, 2000 µg/mL are shown in Table 2. The highest doses were 2000 µg/mL in winter and 1000 µg/mL in summer. Hemolysis ranged from 4.28% to 5.24%, with Home#1 having the lowest value in winter, with 4.28% at 250 µg/mL, and Home#2 having the highest value in summer, with 5.24% at 1000 µg/mL. Hemolysis in Home#2 [(4.66 ± 0.34)%] was higher than Home#1 [(4.5 ± 0.20)%], but this difference was not significant ($p = 0.45$).

The concentrations of 42 trace elements in the PM₁₀ were analyzed by ICP-MS (Table 3). Fig. 1 shows the mean value of trace element concentrations (over 10×10^{-6}) under the different sampling conditions (Ramirez et al., 2020). Total trace elements concentrations of indoor PM₁₀ in Xuanwei ranged from 6966 to 12,958 ppm, averaged 9462 ppm. Zn, Ti, Ni, Cu, Pb, Ba, Mn, Cr, V had the highest concentrations, and accounted for over 95% of the total trace elements. Zn had the highest concentrations ranging from 1926 to 7720 ppm, with the mean value 3494 ppm. The trace element concentrations of PM in Xuanwei showed a significant higher value than other cities (Silva et al., 2020b; Zhang et al., 2020; Lima et al., 2021). Compared with airborne particles in Beijing (Shao et al., 2019), the trace element concentrations of indoor PM₁₀ in Xuanwei showed 3–5 times higher. It should be noted that Sc and W were not found in Beijing. The heavy metals, such as Ti, V, Co, Ni, Eu, Cu, Cd, Pr, Nd, and In, had noticeably higher concentrations, being 5–10 times higher in Xuanwei than in Beijing.

4. Discussion

The results of linear fitting (Fig. 2) showed that the hemolysis was positively correlated with the doses of PM₁₀ ($R^2 = 0.77-0.90$), except the sample collected during winter in Home #2. A similar result in Iran has been reported that hemolysis raised with increased PM concentration (Faraji et al., 2019). The positive dose-response relationships between hemolysis and the sample concentrations indicated that the indoor airborne particles in Xuanwei could induce toxic effects if the particulate matter reached a high enough mass concentration. Previous studies have shown that particle matter, once inhaled, cannot be completely removed from the body in a short term, and some may be retained in the lungs (Oberdörster, 1988). Although low dose samples showed a low hemolytic activity, it can be inferred that if people are exposed to a higher concentration of coal-burning emissions for a longer time, the toxic effects of particles to human health would become significance.

A number of studies have shown that heavy metal elements in particulate matter have strong oxidative capacity (Huang et al., 2015;

Table 2 Quantification of hemolysis induced by indoor PM₁₀ in the Xuanwei coal-burning environments.

Dosage(µg/mL ⁻¹)		50	250	500	1000	2000
hemolysis(%)						
Winter	Home#1	4.31	4.28	4.36	4.31	4.51
	Home#2	4.37	4.4	4.39	4.35	4.38
Summer	Home#1	4.53	4.69	4.68	4.87	
	Home#2	4.71	5.02	5.07	5.24	

Table 3 The contents of trace elements in the indoor PM₁₀ in the Xuanwei coal-burning environments (in ppm).

Elements	Summer		Winter		Mean	SD*
	Home#1	Home#2	Home#1	Home#2		
Li	8.51	9.16	12.92	13.70	11.07	2.27
Be	1.47	1.75	2.36	3.02	2.15	0.60
Sc	3.76	2.23	1.77	5.15	3.23	1.33
Ti	1620.00	1237.00	1581.00	3071.00	1877.25	705.14
V	82.30	26.40	134.77	172.00	103.87	54.92
Mn	333.00	663.00	230.67	561.00	446.92	172.81
Cr	128.00	277.00	250.00	536.00	297.75	148.57
Co	51.40	18.90	13.47	30.80	28.64	14.56
Ni	3169.00	1058.00	672.33	835.00	1433.58	1011.25
Cu	757.00	876.00	977.00	226.00	709.00	289.53
Zn	2013.00	7720.00	1926.00	2319.00	3494.50	2443.96
Ga	5.14	9.82	5.62	10.00	7.65	2.27
Rb	53.10	39.30	36.70	27.40	39.13	9.20
Y	5.99	4.24	3.09	12.10	6.35	3.47
Nb	3.83	2.64	2.56	7.16	4.05	1.87
Mo	3.97	6.97	5.38	5.20	5.38	1.07
Cd	66.00	132.00	73.97	120.00	97.99	28.47
Sb	9.92	34.00	18.27	18.80	20.25	8.69
Cs	2.75	4.34	2.08	2.64	2.95	0.84
Ba	298.00	205.00	517.67	172.00	298.17	134.89
La	8.74	6.15	4.39	17.80	9.27	5.16
Ce	16.60	12.50	9.19	35.80	18.52	10.31
Pr	1.95	1.50	1.06	4.35	2.21	1.27
Nd	8.17	5.07	3.47	14.90	7.90	4.38
Sm	1.30	1.51	0.62	3.38	1.70	1.02
Eu	7.12	0.24	0.18	0.64	2.04	2.94
Gd	1.30	0.91	0.67	2.60	1.37	0.74
Tb	0.18	0.14	0.08	0.43	0.21	0.13
Dy	1.49	0.86	0.69	2.80	1.46	0.83
Ho	0.24	0.18	0.10	0.44	0.24	0.12
Er	0.78	0.34	0.36	1.69	0.79	0.55
Tm	0.12	0.05	0.05	0.13	0.08	0.04
Yb	0.68	0.32	0.29	1.38	0.66	0.44
Lu	0.14	0.08	0.07	0.25	0.13	0.07
W	1.67	1.93	1.22	3.16	1.99	0.72
Tl	2.00	2.74	4.58	4.73	3.51	1.17
Pb	390.00	524.00	398.00	343.00	413.75	67.03
Bi	6.76	6.42	5.77	6.32	6.32	0.35
Th	1.91	1.17	1.18	2.94	1.80	0.72
U	1.66	1.24	2.90	2.15	1.99	0.62
Sr	157.00	60.50	62.13	100.00	94.91	39.18
In	0.81	2.54	1.47	3.26	2.02	0.95
Total	9226.74	12,958.14	6966.09	8700.11	9462.77	2184.52

* Standard deviation.

Song et al., 2015; Gallego-Cartagena et al., 2020; Oliveira et al., 2020). Shao et al. (2013) demonstrated that the concentration of soluble trace elements in the coal-burning indoor PM₁₀ samples in Xuanwei

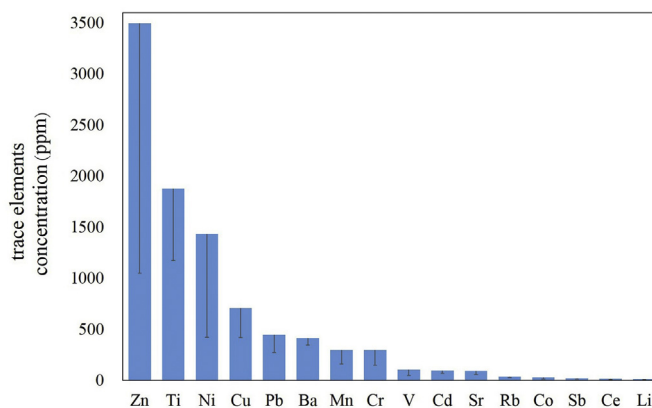


Fig. 1. Histogram showing the concentrations of trace elements in the indoor PM₁₀ in the Xuanwei coal-burning environments.

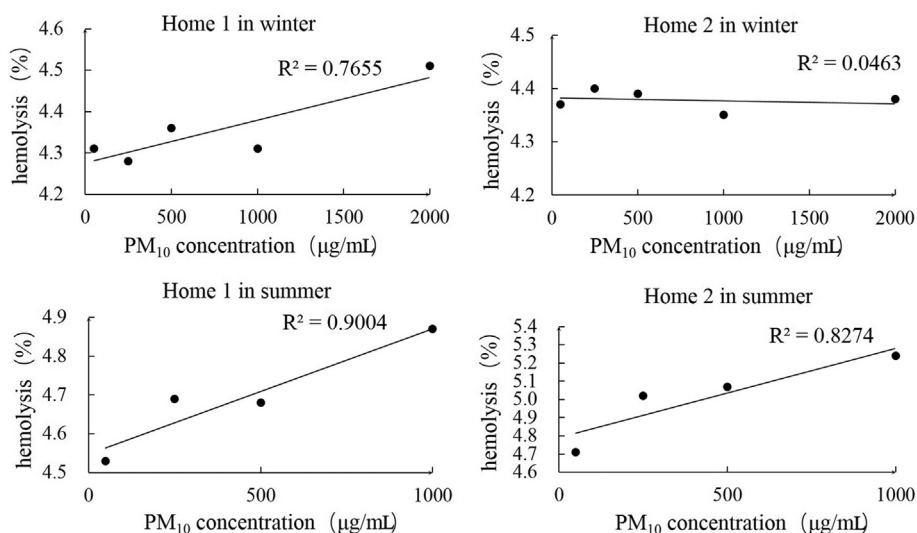


Fig. 2. Correlation analysis between the PM₁₀ concentrations and the hemolysis of PM₁₀ samples from two homes in the Xuanwei coal-burning environments.

Table 4

Correlation coefficients between the concentration of trace elements and the hemolysis of the indoor PM₁₀ in the Xuanwei coal-burning environments.

Li	Be	Sc	Ti	V	Mn	
-0.81*	-0.698	-0.316	-0.622	-0.954**	0.61	
Cr	Co	Ni	Cu	Zn	Ga	
-0.365	0.026	0.234	0.361	0.892*	0.313	
Rb	Y	Nb	Mo	Cd	Sb	
0.404	-0.364	-0.457	0.552	0.448	0.646	
Cs	Ba	La	Ce	Pr	Nd	
0.948**	-0.454	-0.361	-0.371	-0.363	-0.343	
Sm	Eu	Gd	Tb	Dy	Ho	
-0.185	0.09	-0.368	-0.334	-0.397	-0.253	
Er	Tm	Yb	Lu	W	Tl	
-0.475	-0.347	-0.441	-0.421	-0.139	-0.743	
Pb	Bi	Th	U	Sr	In	Total
0.895*	0.527	-0.459	-0.894*	-0.098	0.018	0.955**

* p < 0.1.

** p < 0.05.

had a significant positive correlation with DNA damage in the plasmid scission assay, and the heavy metal elements, especially Cd, Pb, Sb, Tl and Zn, were suggested to be the main cause of high oxidative capacity. The plasmid scission assay with the individual soluble metals demonstrated the direct bioreactivity of soluble Cu, Zn, Pb, and Fe (Merolla and Richards, 2005).

Table 5

Heavy metals showing a most significant positive correlation with the oxidative capacity of airborne particles in different coal-burning environments.

Site	Type	Smampling Year	Zn	Pb	Cd	As	Tl	Cu	Mn	Sb	Bi	References
Xuanwei	Indoor PM ₁₀	2007	+	+	+							Shao et al., 2008
		2008	+	+	+		+	+				Zhou et al., 2009
		2011	+	+		+	+			+	+	Wang, 2012
		2013,2014	+	+								
	Indoor and outdoor PM ₁₀	2007	+	+	+	+	+			+		Shao et al., 2013
Laboratory of coal-combustion system	Indoor PM _{2.5}	2016	+	+				+	+		+	Xi, 2018
	Indoor PM	2016,2019	+		+		+	+				Feng et al., 2020
	PM ₁₀	2016	+	+	+	+	+		+	+		Shao et al., 2016
Pingding-shan coal-mining area	Outdoor PM ₁₀	2014	+	+	+	+	+					Song et al., 2015

In this study, the relationship between the concentrations of trace elements and the hemolysis under the dose of 500 µg/mL were analyzed by SPSS Statistics. The correlation coefficients are given in Table 4. The results showed that the correlation coefficient between hemolysis and concentration of total trace elements of indoor PM₁₀ in Xuanwei was 0.955, higher than the critical value 0.878 under the 0.05 significance level. This positive correlation suggested that the trace elements of PM₁₀ in Xuanwei were involved with hemolytic activity. This result implied that the trace elements in particulate matter could not only cause DNA damage (Shao et al., 2013), but also possess a hemolytic effect. A significant positive correlation also existed between the individual elements Zn, Pb, and Cs and the hemolysis, indicating these specific elements could be causing the hemolytic effects of the PM₁₀ samples. A significant negative correlation was observed between the elements Li, U, and V, and the hemolysis, implying that these elements may be able to play an inhibitory role.

Several previous studies investigated the relationships between the heavy metal compositions and the oxidative capacities of the coal-burning particles, including the particles emitted in the coal-burning indoor environment in Xuanwei lung cancer village, in the laboratory coal-combustion system, and also in a coal-mining area. The metal elements which showed positive correlations with the oxidative capacity of the PM were summarized in Table 5. Of the analyzed elements, Zn, Pb, Cd, Cu, As, Tl, Mn, Sb, and Bi showed a significant positive correlation with the oxidative capacity of PM in coal-burning environments. Among them, Zn, Pb, Cd, As and Tl were most frequently identified to be significantly correlated with PM oxidative capacity. Therefore, Zn, Pb, Cd, As

and TI could be regarded as the main toxic elements in PM emitted from coal combustion.

5. Conclusions

- (1) Hemolysis of RBCs by indoor PM₁₀ over a dose range from 50 to 2000 µg/mL ranged from 4.28% to 5.24%. The hemolytic activities of PM₁₀ showed a rising trend with increasing doses. Although low dose samples exhibited a low hemolytic activity, PM₁₀ could have a toxic effect upon people in a closed indoor environment for extended time periods.
- (2) The total trace elements in PM₁₀ had a positive correlation with the hemolytic properties, in which the Zn, Pb and Cs could contribute more obvious hemolysis. In contrast, Li, U and V demonstrated negative correlations with the hemolysis, suggesting these elements may have inhibited the hemolysis of human RBCs. In combination with the results from previous studies, Zn, Pb, Cd, As and TI could be regarded as the main toxic elements in PM emitted from coal-combustion.
- (3) The hemolysis assay as a new, simple method in assessing the particle toxicity could be used in the toxicological study of a variety of environmental pollutants.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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