



# Semen quality and sperm DNA methylation in relation to long-term exposure to air pollution in fertile men: A cross-sectional study<sup>☆</sup>

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## ARTICLE INFO

### Keywords:

Air pollution

PM<sub>10</sub>

Semen quality

Sperm motility

5 mC

5-hmC

## ABSTRACT

Some studies have examined the association between air pollution and semen quality. While it is less of evidence on the sperm quality after long-term air pollution exposure, especially the co-exposure of different air pollution components. Additionally, the role of DNA methylation in it hasn't been confirmed. This study aimed to investigate whether long-term exposure to air pollution was associated with semen quality, as well as to explore the effect of sperm DNA methylation in such association. From 2014 to 2016, 1607 fertile men were enrolled to evaluate 14 parameters of semen quality. Exposure window was defined as one-year before semen sampling. Multivariable linear regression and weighted quantile sum (WQS) regression model were used to investigate the association between six air pollutants co-exposure and semen quality. Sensitivity analysis regarding at the normal semen quality group was also conducted. Semen samples were randomly selected from 200 participants to detect the genomic 5-methylcytosine (5 mC) and 5-hydroxymethylcytosine (5-hmC) levels in sperm. In the total population, PM<sub>10</sub>, PM<sub>2.5</sub>, SO<sub>2</sub>, and NO<sub>2</sub> were negatively associated with sperm total motility (PM<sub>10</sub>:  $\beta = -2.67$ ,  $P = 0.009$ ; PM<sub>2.5</sub>:  $\beta = -2.86$ ,  $P = 0.004$ ; SO<sub>2</sub>:  $\beta = -2.32$ ,  $P = 0.011$ ; NO<sub>2</sub>:  $\beta = -2.21$ ,  $P = 0.012$ ). Results of the normal semen quality group were consistent with those from the whole population. WQS regression results indicated significant decreasing sperm total motility after the co-exposure of the six air pollutants ( $\beta = -1.64$ ,  $P = 0.003$ ) in whole participants. Wherein, PM<sub>10</sub> accounted for largest proportion (43.4%). The 5-hmC level was positively associated with PM<sub>10</sub> exposure ( $\beta = 0.002$ ,  $P < 0.001$ ). Long-term exposure to PM<sub>10</sub>, PM<sub>2.5</sub>, SO<sub>2</sub>, and NO<sub>2</sub>, as well as co-exposure to six air pollutants, reduced semen quality in fertile men. As the most significant contributor of air pollutant, PM<sub>10</sub> exposure decreased sperm DNA methylation.

## 1. Introduction

Male semen quality has been reportedly decreased globally over the past several decades (Huang et al., 2017; Virtanen et al., 2017). There is no precise reason currently understood for this decline. Exposure to environmental toxicants like air pollution has been regarded as a possible risk factor (Mao et al., 2017), although the mechanisms are still

unclear. At present, six prominent air pollutants including carbon monoxide (CO), sulfur dioxide (SO<sub>2</sub>), nitrogen dioxide (NO<sub>2</sub>), ozone (O<sub>3</sub>), and particulate matter (PM<sub>2.5</sub>, PM<sub>10</sub>) are monitored routinely in China to evaluate air quality (Zhou et al., 2021).

Several studies have investigated the association between air pollutants and semen quality (Sun et al., 2020; Yang et al., 2021; Zhang et al., 2020). However, the conclusions remain inconsistent, most of

<sup>☆</sup> This paper has been recommended for acceptance by Wen Chen.

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them have verified that exposure to air pollution was associated with lower total sperm count, semen volume, sperm concentration, and sperm motility (Chen et al., 2020; Nobles et al., 2018b; Sokol et al., 2006; Zhou et al., 2014). However, the majority of these studies focused on short-term exposure (90 days before semen collection) of air pollution (Guan et al., 2020; Sun et al., 2020). It is still less of evidence on the sperm health after long-term low-dose air pollution exposure, especially the co-exposure of different air pollution components. Existing research suggests that chronic, low-dose exposure can lead to clinically impairments of spermatogenesis (Gabrielsen and Tanrikut, 2016).

DNA methylation plays a vital role in the control of gene expression and chromosome structure, which are crucial factors during the process of spermatogenesis and are essential for sperm function (Santana et al., 2020). Genome-wide reduction of DNA methylation has been in association with decreased male fertility (Aston et al., 2015; Jenkins et al., 2016a; Jenkins et al., 2016b). In particular, methylation of cytosine at the fifth carbon (5-methylcytosine, 5 mC) and modified form of cytosine (5-hydroxymethylcytosine, 5-hmC) are the essential epigenetic biomarker linked to the spermatogenesis (Gao et al., 2019). It has been reported that 5 mC and 5-hmC were involved in the male rat germline progression (Rose et al., 2014), as well as the development in mammalian germ lines and early embryos on both mice and humans (Chen and Zhang, 2020). Several studies have pointed out air pollutants would result in aberrant DNA methylation (Jiang et al., 2019; Shou et al., 2019; Tantoh et al., 2020). While few studies analyzed the associations between air pollution and semen quality from the perspective of sperm DNA methylation modifications.

A better comprehension of these relationships will help us to fully understand the role of air pollutants and their effort on male reproductive health. Taking advantage of the comprehensive air quality monitoring data and complete laboratory analysis of the sperm samples from the participants of 1607 fertile men, we investigated the association between long-term air pollutants exposure and semen quality in China through a cohort study, and to explore the roles of DNA methylation play in such association.

## 2. Methods

### 2.1. Study population

The cohort study was conducted among 1607 fertile men whose partners participated in Nanjing Medical University Longitudinal Investigation of Fertility and the Environment (NMU-LIFE) study from January 1st, 2014 to December 31st, 2016. Pregnant women who came to Nanjing Medical University Affiliated hospital for the antenatal registration have been consulted to join this study. The eligible participants should be permanent residents in Nanjing, with age range of 20–45 years old and they didn't have fertility treatments. When they joined the program, each participant gave written informed consent, as well as permission for their husbands and children agreed to be recruited. The NMU-LIFE study collected basic demographic information and disease information as well as biospecimen including placenta, blood, urine, and semen.

A total of 21 men with incomplete semen reports, 17 males with unclear examination dates, and 15 men with missing addresses were excluded. Figure S1 shows the including/excluding procedure and a total of 1554 participants involved in our following analysis. For the 1554 participants, they were healthy fertile men whose partners had given birth to at least one child over the past year. None of them had been treated for infertility or received assisted reproductive therapy. The participants were approached for an interview by the trained medical researchers to collect the following information: age, ethnicity, height, weight, residence address, education, family income, smoking status, and alcohol consumption. The number of abstinence days from ejaculation were also recorded. All of them voluntarily donated semen samples for research analysis. The study was approved by the

Institutional Ethics Committee of Nanjing Medical University.

### 2.2. Assessment of individual exposure

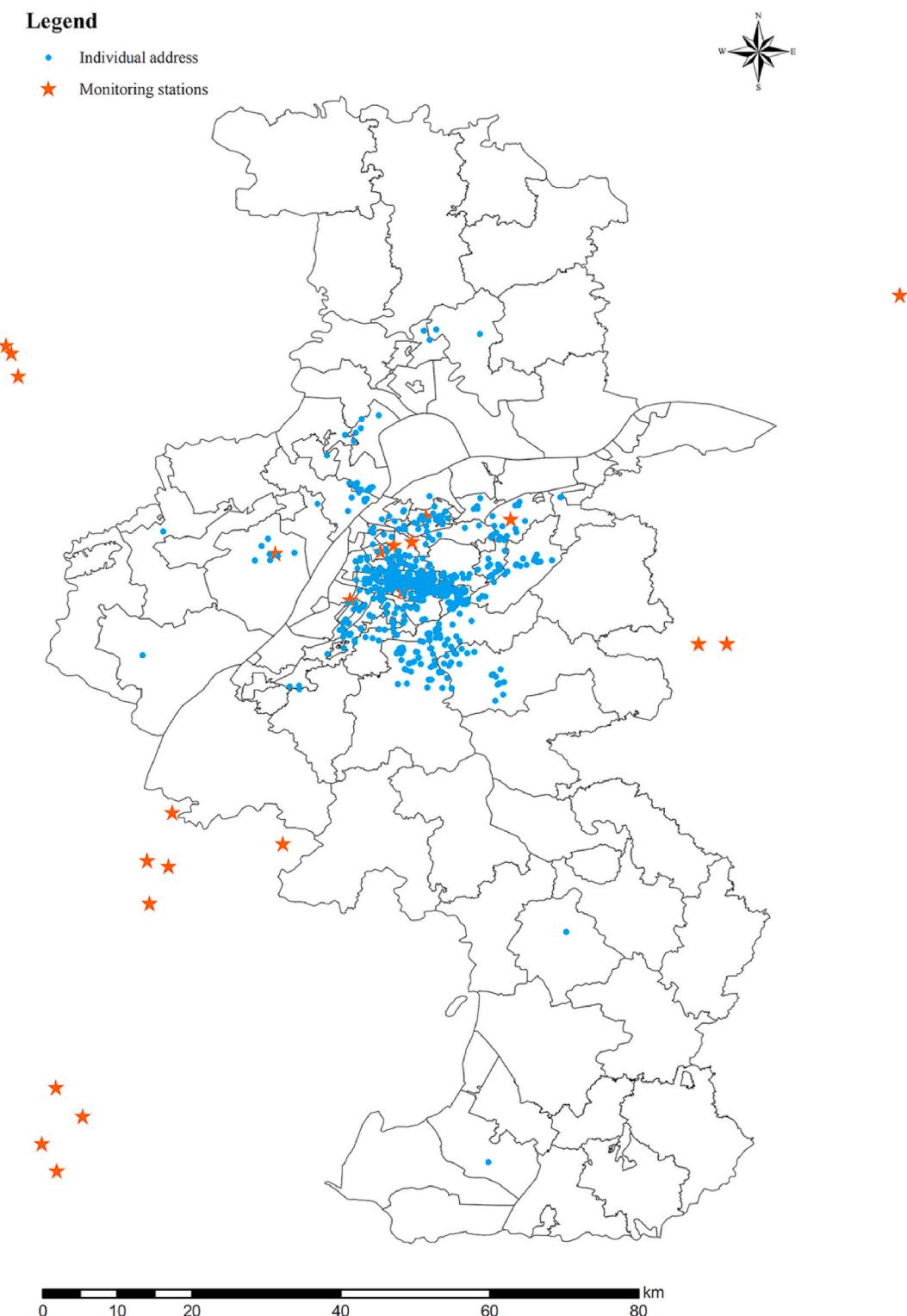
The daily concentrations of air pollutants from January 1st, 2013 to December 31st, 2016 were acquired from 24 state-owned air monitoring stations in Nanjing and surrounding six cities including Zhenjiang, Yangzhou, Jurong, Ma'anshan, Wuhu, and Chuzhou (Fig. 1). These stations could effectively record the air quality data for the whole living area among all the participants. The exposure window was set as 365 days before the day of semen sample collection. Meanwhile, the data of daily ambient average temperature (in Celsius) of Nanjing over the same period was obtained from the China Meteorological Data Service Centre (<http://data.cma.cn/>). The concentration unit of CO (mg/m<sup>3</sup>) was transformed into the µg/m<sup>3</sup> to make it comparable with the other five pollutants (PM<sub>2.5</sub>, PM<sub>10</sub>, SO<sub>2</sub>, NO<sub>2</sub>, and O<sub>3</sub>). Furthermore, the inverse distance weighting (IDW) model (supplement material, Figure S2) was adopted to assess individual exposure. It's a spatial interpolation method to model the distribution of air pollutants using data from the fixed monitoring stations. Briefly, according to the participants' home addresses and the locations of 24 monitoring stations, we firstly converted them into latitude and longitude coordinates. On such basis, IDW modeling method was used to assign six air pollutants exposure levels for each residence address on each day using daily pollutant concentrations from 24 air quality monitoring data. Similarly, individual exposure to temperature was assessed in the same way.

### 2.3. Semen samples collection and analyses for semen parameters

Each participant was given a plastic specimen container and was guided to collect semen samples by masturbation. Then all semen samples were sent to laboratory to liquefy at 37 °C. According to guidelines of the WHO 5th Laboratory Manual for the Examination of Human Semen (Sharma et al., 2016), computer-assisted semen analysis (CASA) was used to analyze aliquots about 30 min after ejaculation. A sterile serological pipette was used to measure semen volume. The following parameters were used for the semen analysis and assessment: total sperm number (million), semen volume (ml), sperm concentration (million/ml), total sperm motility, and progressive sperm motility. Except for the conventional parameters, the remaining nine parameters were also applied for further evaluation: average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), mean angular displacement (MAD), wobble (WOB = VAP/VCL), linearity (LIN = VSL/VCL) and straightness (STR = VSL/VAP). On the basis of WHO guidelines, the male whose semen volume  $\geq 1.5$  ml, sperm concentration  $\geq 15 \times 10^6$ /ml, total sperm number  $\geq 39 \times 10^6$ , total motility  $\geq 40\%$ , and progressive motility  $\geq 32\%$  are defined as normal (Cooper et al., 2010).

### 2.4. Sperm DNA extraction

Power Analysis and Sample Size (PASS) was used to calculate the sample size. When type I error was controlled to 0.05, effect size was selected as 0.15 and the power of the test was 90%, the estimated sample size should be at least 86. Finally, 200 participants were randomly selected to extract sperm DNA. A Percoll (GE Healthcare) gradient which has two concentration layers (40% and 80%) was used to purify motile sperm cells away from immature germ cells, lymphocyte contamination, and epithelial cells. To guarantee the best quality of cell preparations, a microscopic examination of sperm fractions was conducted in advance. Phosphate buffered saline (PBS) was used to resuspend sperm pellets for two times and then centrifuged at 400 g for 10 min (Tang et al., 2018), then the DNA was extracted by QIAamp® DNA Mini Kit (Qiagen). The DNA concentration was determined by the Qubit fluorometer device (Multiskan Sky, Thermo Scientific, USA) (Nakayama et al., 2016).



**Fig. 1.** Spatial distribution of twenty-four fixed air quality monitoring stations and residence addresses of the 1554 participants.

### 2.5. Assessment of the levels of 5 mC, 5-hmC

MethylFlash™ Global DNA Methylation (5 mC) ELISA Easy Kit and MethylFlash™ Global DNA Hydroxymethylation (5-hmC) ELISA Easy Kit (Epigentek) were adopted to measure the genomic levels of 5 mC and 5-hmC in sperm DNA from the 200 participants. Firstly, 100  $\mu$ l Binding Solution was placed in each well of a 96-well plate. Then different volumes of each DNA sample were added in the well according to

different concentrations. When the solution was coated well, the plate was incubated at 37 °C for 60 min. Then each well was washed with 150  $\mu$ l of the Diluted Washing Buffer each time for three times, followed by adding 50  $\mu$ l of the 5 mC (5-hmC) Detection Complex Solution. Additional incubation, washing, and developer/stop solution were applied in the final procedure for detection through a spectrophotometer (Infinite M200 Pro, Tecan, CN) at 450 nm.

## 2.6. Statistical analysis

Descriptive statistics was conducted to describe the demographic characteristics for participants, air pollution exposure and semen parameters. Continuous variables and categorical variables were measured by means  $\pm$  standard deviation and frequencies separately. The box-cox conversion was used to normalize semen parameters (Smarr et al., 2018). Detailly, the covariates related to semen quality based on previous studies were as follows: age (continuous), ethnicity (Han and others), education (middle school and below, high school and secondary school, college degree and above), body mass index (BMI, categorized into <18.5, 18.5–24.0, 24.0–28.0, ≥28.0), family income (<100,000 yuan/year, 100,000–200,000 yuan/year, ≥ 200,000 yuan/year), smoking status (never smoker and ever smoker), drinking status (never drinker and ever drinker), abstinence days (<3 days, 3–5 days, ≥ 5 days) (Keihani et al., 2017), seasons of sample collection (winter, spring, summer, fall) (Levitas et al., 2013), and temperature (Wang et al., 2020). Aiming to fit exposure-response curves between temperature and sperm quality, natural cubic splines with degrees of freedom as 3 was used in our generalized linear model.

Multiple linear regressions were utilized to investigate the association between individual exposure to each air pollutant and 14 semen parameters through single-pollutant model, in which all of the above covariates had been adjusted. Regression coefficients were calculated based on every interquartile (IQR) increment for all the six air pollutants (Zhou et al., 2021). Considering that abnormal semen quality can be caused by a variety of factors, we therefore excluded the population with abnormal semen quality to re-analyze the association between air pollution and semen parameters in normal group. In addition, the sensitivity analysis was also conducted to verify whether the results were consistent in the total population and non-smoker, non-drinker groups. The weighted quantile sum (WQS) regression models were conducted to evaluate effects of co-exposure of six air pollutants (Stafoggia et al., 2017; Zhang et al., 2019c) and to identify the most weighted pollutant. Furthermore, the correlations between the most weighted air pollutant and the level of 5 mC, 5-hmC were evaluated via the multiple linear regression.

All analyses were executed using R software (Version 4.0.2, R Core Team, 2020). The Benjamini & Hochberg (BH) procedure was applied to assess the false discovery rate (FDR) for multiple correction of all P-values, and the total number of hypotheses tested was 14. A two-sided P value  $< 0.05$  was defined as the statistically significant level.

## 3. Results

### 3.1. General characteristics of the study population

A total of 1554 fertile men were finally included in the analysis, with an average age of  $30.9 \pm 4.2$  years. Smoking and alcohol drinking rates were 37.7% and 44.5% separately. It is identified that 57.0% participants were overweight, and 66.8% ones were well educated. The abstinence period for 45% of the participants was 3–5 days. In total population, 962 had normal semen quality while 592 were abnormal. No differences were found across BMI, ethnicity, education, family income, smoking status, and drinking status in the two groups by univariate analyses (Table 1). In addition, the basic characteristics of 200 participants who were randomly selected to detect the levels of 5 mC and 5-hmC was presented in Table S1. Except for education, no significant association was found between these 200 participants and the whole population.

### 3.2. Distribution of temperature and air pollutants

Daily average concentrations of six air pollutants during the study period are displayed in Fig. 2, and the concentrations varied seasonally was identified. PM<sub>10</sub>, PM<sub>2.5</sub>, SO<sub>2</sub>, NO<sub>2</sub>, and CO showed lower levels in

**Table 1**

Characteristics of all fertile men participating in the study and two groups.

Characteristics	All participants (n = 1554)	Normal semen quality group (n = 962) <sup>a</sup>	Abnormal semen quality group (n = 592) <sup>b</sup>	p-value
Age (years)*				<0.001
Mean (SD)	30.9 (4.2)	30.6 (4.0)	31.4 (4.3)	
Ethnicity, n (%)				0.752
Han	1507 (97.0)	932 (96.9)	576 (97.3)	
Other	47 (3.0)	30 (3.1)	16 (2.7)	
BMI (kg/m <sup>2</sup> ), n (%)				0.699
<18.5	25 (1.6)	18 (1.8)	7 (1.2)	
18.5–24.0	643 (41.4)	393 (40.9)	250 (42.2)	
24.0–28.0	652 (42.0)	403 (41.9)	249 (42.1)	
≥28.0	234 (15.0)	148 (15.4)	86 (14.5)	
Education, n (%)				0.629
Middle school and below	19 (1.2)	10 (1.0)	9 (1.5)	
High school and secondary school	497 (32.0)	304 (31.6)	193 (32.6)	
College degree and above	1038 (66.8)	648 (67.4)	390 (65.9)	
Family income (yuan/year), n (%)				0.159
<100,000	558 (35.9)	343 (35.7)	215 (36.3)	
100,000–200,000	706 (45.4)	452 (47.0)	254 (42.9)	
≥200,000	290 (18.7)	167 (17.3)	123 (20.8)	
Smoking status, n (%)				0.346
Never smoker	968 (62.3)	590 (61.3)	378 (63.9)	
Ever smoker	586 (37.7)	372 (38.7)	214 (36.1)	
Drinking status, n (%)				0.990
Never drinker	862 (55.5)	533 (55.4)	329 (55.6)	
Ever drinker	692 (44.5)	429 (44.6)	263 (44.4)	
Days of abstinence, mean (SD)*				<0.001
<3	375 (24.1)	168 (17.5)	206 (34.8)	
3–5	699 (45.0)	476 (49.5)	224 (37.8)	
≥5	480 (30.9)	318 (33.0)	162 (27.4)	
Season of sperm collection, n (%)*				0.006
Spring	457 (29.4)	310 (32.2)	146 (24.7)	
Summer	358 (23.0)	217 (22.6)	144 (24.3)	
Autumn	371 (23.9)	208 (21.6)	161 (27.2)	
Winter	368 (23.7)	227 (23.6)	141 (23.8)	

Note: SD, standard deviation; BMI, body mass index.

\*P < 0.05 when normal semen quality group comparing with abnormal semen quality group.

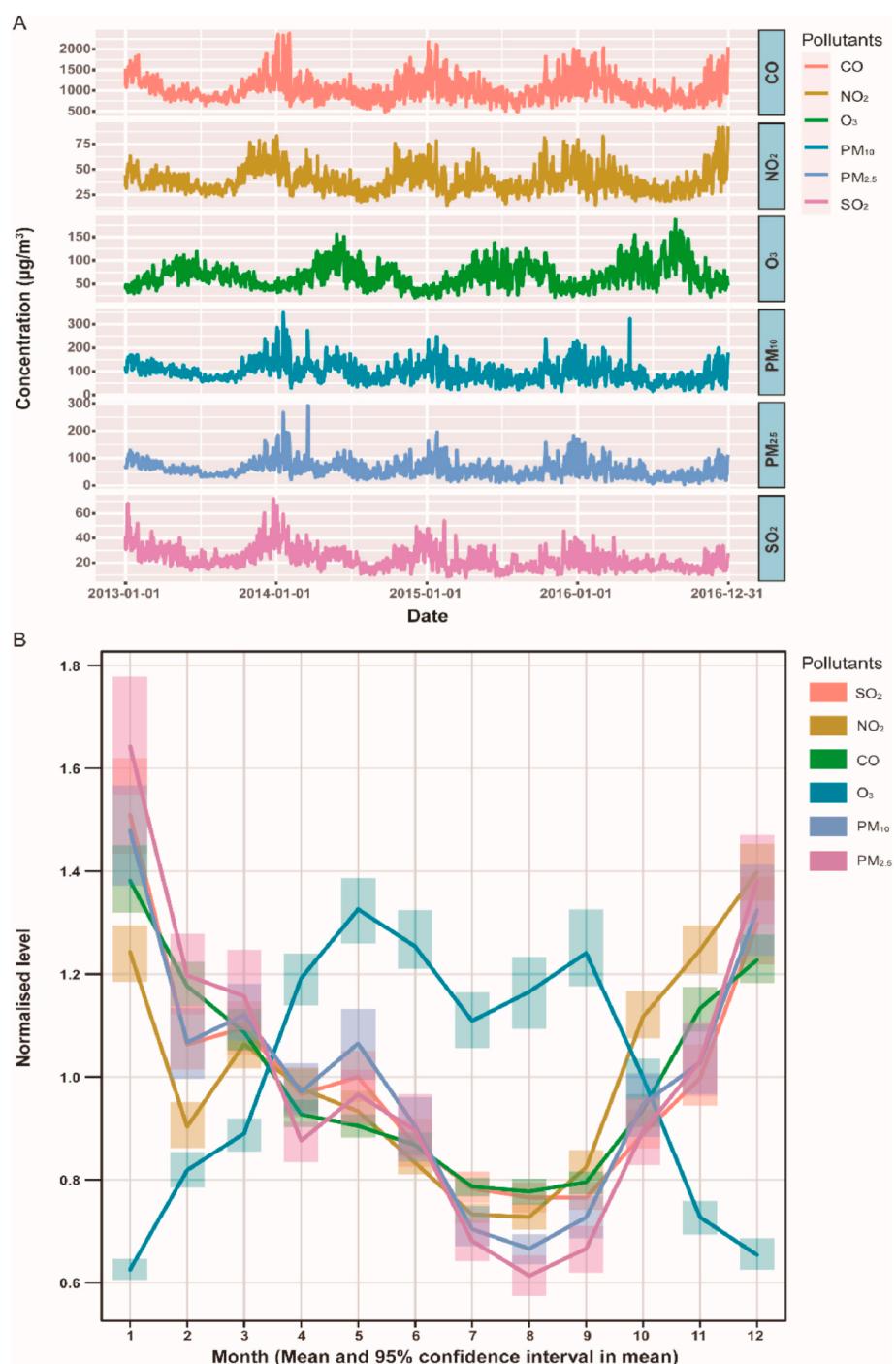
<sup>a</sup> Group defined by semen volume  $\geq 1.5$  ml, sperm concentration  $\geq 15 \times 10^6/\text{ml}$ , total sperm number  $\geq 39 \times 10^6$ , total motility  $\geq 40\%$ , and progressive motility  $\geq 32\%$ .

<sup>b</sup> Group defined by at least one abnormal semen parameter (semen volume  $< 1.5$  ml, sperm concentration  $< 15 \times 10^6/\text{ml}$ , total sperm number  $< 39 \times 10^6$ , sperm motility  $< 40\%$  or progressive motility  $< 32\%$ ).

summer and higher levels in winter, while O<sub>3</sub> was on the contrary. Table 2 demonstrated the descriptive statistics for estimated 1-year individual exposures to the six air pollutants. The average concentrations of CO, O<sub>3</sub>, NO<sub>2</sub>, SO<sub>2</sub>, PM<sub>10</sub>, and PM<sub>2.5</sub> were 1041  $\mu\text{g}/\text{m}^3$ , 91.80  $\mu\text{g}/\text{m}^3$ , 54.46  $\mu\text{g}/\text{m}^3$ , 23.52  $\mu\text{g}/\text{m}^3$ , 109.69  $\mu\text{g}/\text{m}^3$ , and 64.67  $\mu\text{g}/\text{m}^3$  respectively. Figure S3 shows the average daily temperatures from 2014 to 2016. The lowest temperature was  $-6.6^\circ\text{C}$  in winter and the highest one appeared at  $34.1^\circ\text{C}$  in summer. As shown in Figure S4, the Pearson correlations between the 6 air pollutants also indicated that O<sub>3</sub> was negatively correlated with the other 5 pollutants.

### 3.3. Distribution of semen parameters

The semen characteristics of all participants are presented in Table 3. The median (IQR) of the total sperm number, semen volume, sperm concentration, sperm total motility, and progressive motility were 142.60 (152.10)  $\times 10^6$ , 2.00 (1.00) ml, 58.30 (47.25)  $\times 10^6/\text{ml}$ , 56.18% (27.27%), and 44.06% (24.08%) respectively. Manne Whitney U test results showed that the values of 14 semen parameters, except MAD (P



**Fig. 2.** Distribution of six air pollutants from 2013 to 2016. (A) The daily concentrations of six air pollutants from 2013 to 2016; (B) Monthly variation of normalized concentrations of six air pollutants 1 year before semen collection.

**Table 2**  
The distribution of 1-year individual exposure to six air pollutants.

Pollutants ( $\mu\text{g}/\text{m}^3$ )	Mean	SD	Min	25 <sup>th</sup>	50 <sup>th</sup>	75 <sup>th</sup>	Max	IQR
PM <sub>2.5</sub>	64.67	9.80	47.05	56.41	59.79	71.68	83.57	15.27
PM <sub>10</sub>	109.69	15.89	80.74	96.78	101.01	120.94	145.41	24.16
SO <sub>2</sub>	23.52	4.30	14.59	20.65	21.89	25.54	35.47	4.89
NO <sub>2</sub>	54.46	4.50	33.82	51.28	53.68	56.90	67.83	5.63
CO	1041	81.26	846.12	980.25	1026	1086	1339	105.77
O <sub>3</sub>	91.80	9.81	45.32	86.72	93.22	99.16	114.95	12.45

Note: SD, standard deviation; IQR, interquartile range.

**Table 3**

Distribution of semen parameters for all fertile men (n = 1554).

Semen parameters	All participants (n = 1554)								Normal group (n = 962) <sup>a</sup>		Abnormal group (n = 592) <sup>b</sup>	
	Mean	SD	Min	25 <sup>th</sup>	50 <sup>th</sup>	75 <sup>th</sup>	Max	IQR	Median	IQR	Median	IQR
Semen volume (ml)	2.72	1.33	0.20	2.00	2.00	3.00	10.00	1.00	3.00	1.00	2.00	2.00
Sperm concentration ( $10^6/\text{ml}$ )	62.05	31.98	1.03	37.10	58.30	84.35	240.00	47.25	63.00	43.48	50.10	52.73
Total sperm number ( $10^6$ )	172.25	129.89	1.90	79.20	142.60	231.30	1200.00	152.10	166.50	136.50	93.70	154.43
Total motility (%)	55.15	19.26	1.46	42.01	56.18	69.28	100.00	27.27	62.96	20.52	37.44	23.79
Progressive motility (%)	43.81	16.86	0.79	31.85	44.06	55.92	100.00	24.08	49.55	18.00	28.43	18.64
VCL ( $\mu\text{m}/\text{s}$ )	47.71	8.86	6.74	42.11	47.16	53.27	79.76	11.16	48.31	10.61	45.37	12.25
VSL ( $\mu\text{m}/\text{s}$ )	29.78	6.06	3.20	25.79	29.58	33.58	68.33	7.79	30.56	6.89	27.37	7.32
VAP ( $\mu\text{m}/\text{s}$ )	33.52	6.15	7.99	29.42	33.36	37.46	52.59	8.03	34.45	7.13	30.82	7.81
BCF (Hz)	5.12	0.71	3.10	4.64	5.06	5.50	12.67	0.86	4.95	0.77	5.27	1.03
ALH* ( $\mu\text{m}/\text{s}$ )	3.64	1.05	0.50	2.90	3.58	4.26	8.67	1.36	3.61	1.26	3.54	1.52
LIN (%)	61.35	7.49	28.00	56.16	61.21	66.37	93.07	10.21	62.18	9.90	59.76	10.42
STR (%)	85.23	4.09	53.71	82.95	85.60	87.95	99.44	5.00	85.94	4.53	84.88	5.75
WOB (%)	70.31	6.36	31.01	65.71	70.18	74.64	91.92	8.93	70.79	8.65	69.04	9.18
MAD* (°)	56.64	7.88	0.68	51.95	57.21	62.14	78.96	10.19	57.16	9.89	57.25	10.90

Note: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; MAD, mean angular displacement; SD, standard deviation; STR, straightness; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; WOB, curvilinear path wobble.

<sup>a</sup> Group defined by semen volume  $\geq 1.5$  ml, sperm concentration  $\geq 15 \times 10^6/\text{ml}$ , total sperm number  $\geq 39 \times 10^6$ , total motility  $\geq 40\%$ , and progressive motility  $\geq 32\%$ .

<sup>b</sup> Group defined by at least one abnormal semen parameter (semen volume  $< 1.5$  ml, sperm concentration  $< 15 \times 10^6/\text{ml}$ , total sperm number  $< 39 \times 10^6$ , sperm motility  $< 40\%$  or progressive motility  $< 32\%$ ).

= 0.342) and ALH ( $P = 0.072$ ), were of significant differences between the normal semen group and the abnormal group ( $P < 0.001$ ). In addition, all the parameters related to semen quality were higher in normal group compared to the abnormal group, excluding the parameter of VAP.

### 3.4. Associations of air pollutants exposure with semen quality

The results of the single-pollutant analysis have been summarized in Fig. 3. After adjusted for age, ethnicity, BMI, education, family income, smoking status, drinking status, days of abstinence, and seasons of semen collection, the significant associations between single air pollutant exposure and semen parameters were found except CO. Among all semen parameters, total motility was the only one that associated with all the other 5 pollutants after the adjustment of FDR. The results showed that PM<sub>2.5</sub>, PM<sub>10</sub>, SO<sub>2</sub>, NO<sub>2</sub> were negatively associated with total motility (PM<sub>2.5</sub>:  $\beta = -2.85$ , 95%CI: (-4.55, -1.15); PM<sub>10</sub>:  $\beta = -2.65$ , 95%CI: (-4.38, -0.92); SO<sub>2</sub>:  $\beta = -2.29$ , 95%CI: (-3.63, -0.95); NO<sub>2</sub>:  $\beta = -2.21$ , 95%CI: (-3.56, -0.86)), while O<sub>3</sub> shown a positive association with it (O<sub>3</sub>:  $\beta = 1.45$ , 95%CI: (0.25, 2.66)). Additionally, PM<sub>2.5</sub>, PM<sub>10</sub> were positively associated with BCF, LIN, STR, WOB, but these parameters showed negative association with O<sub>3</sub>. Moreover, positive associations between O<sub>3</sub> and total sperm number ( $\beta = 0.10$ , 95%CI: (0.01, 0.19)), sperm concentration ( $\beta = 0.35$ , 95%CI: (0.06, 0.65)) were observed. Oppositely, the inverse associations were found between NO<sub>2</sub> and total sperm number ( $\beta = -0.13$ , 95%CI: (-0.22, -0.03)), sperm concentration ( $\beta = -0.35$ , 95%CI: (-0.67, -0.02)), and ALH ( $\beta = -0.02$ , 95%CI: (-0.04, -0.01)). The results of sensitivity analysis for normal semen quality group are shown in Figure S5. The results from normal group were consistent with the outcome from the analysis based on the whole population. The results of sensitivity analyses in non-smoker and non-drinker are also presented in Figure S6 and Figure S7. They were consistent with the whole population in total motility. In addition, the results also shown that the associations between air pollution and progressive motility were significant in non-smoker and non-drinker groups.

Fig. 4 presents the results of WQS regression models. We found that sperm number, total motility, VSL, VAP, WOB were negatively associated with WQS indexes. Wherein, the association between air pollution and sperm total motility was also consistent with the results through the general linear regression model. Figure S8 demonstrates the estimated weight for WQS index of six air pollutants. The results shown that PM<sub>10</sub>

contributed the most (index weight = 43.4%).

### 3.5. Associations between PM<sub>10</sub> exposure and 5 mC, 5-hmC methylation

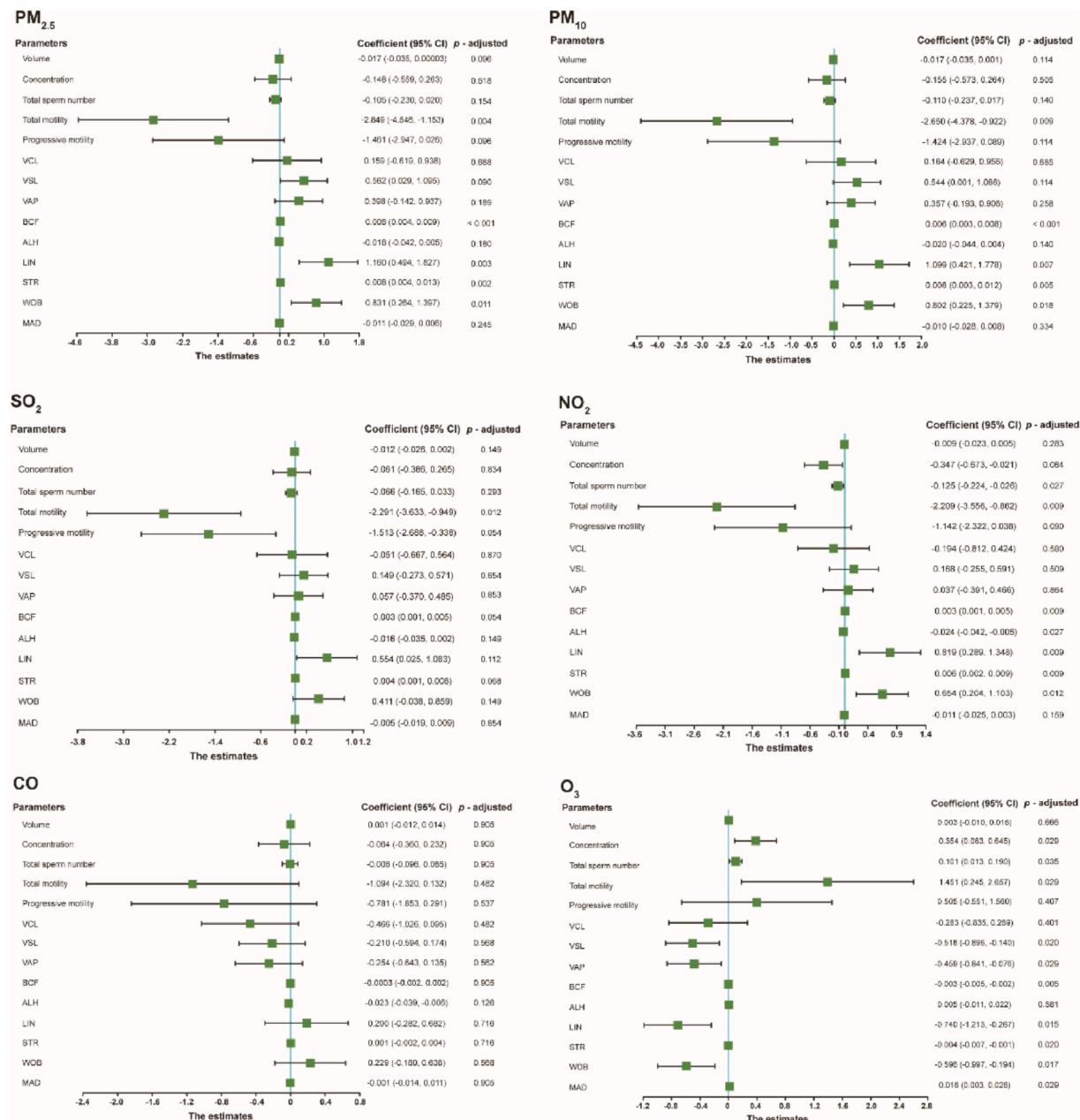
Based on the findings from WQS regression, the most weighted pollutant, PM<sub>10</sub>, was chosen to analyze the effects of specific pollutant exposure on 5 mC, 5-hmC methylation (Fig. 5). After adjusting for smoking and drinking status, PM<sub>10</sub> exposure was observed in a positive association with 5-hmC levels ( $\beta = 0.002$ , 95% CI: (0.001, 0.003)), however, no significant association was found between the genomic level of 5 mC and PM<sub>10</sub> exposure level ( $\beta = -0.002$ , 95% CI: (-0.006, 0.002)).

## 4. Discussion

In this study, we conducted analysis based on a birth cohort to examine the associations between single or multiple long-term air pollution exposure and semen quality among 1554 fertile men, as well as the genomic methylation levels of 5 mC and 5-hmC in sperm. To the best of our knowledge, the study was the first to evaluate the effects of long-term exposure to 6 air pollutants on the 14 semen parameters via both single-pollutant model and co-exposure model. We finally observed an elevated risk of decreasing total motility associated with exposure to PM<sub>2.5</sub>, PM<sub>10</sub>, SO<sub>2</sub>, NO<sub>2</sub> in single-pollutant models after adjustment of crucial confounders, as well as a persistent outcome from the co-exposure of pollutants. It indicated that main air pollutants exposure might affect the total motility of semen among fertile men through the epigenetic modification.

Most previous studies focusing at the 90-day exposure period before semen sampling (Huang et al., 2019; Liu et al., 2017; Santi et al., 2016; Zhang et al., 2019b), due to the reason that 90 days was recognized a circle of spermatogenesis commonly. However, it is still less of evidence on the effects from long-term exposure to air pollution. Lao et al. reported that 2-year PM<sub>2.5</sub> exposure had inverse association with sperm normal morphology but positive association with sperm concentration (Lao et al., 2018), which was consistent with what we found from 1-year exposure period. Differently, their study demonstrated a persistent result from short-term exposure as well as the long-term exposure, while it was not observed in our study. We only found a negative association between PM<sub>2.5</sub> and total motility instead.

Furthermore, it is interesting to observe that total motility in our study was in a negative association with PM<sub>2.5</sub>, PM<sub>10</sub>, SO<sub>2</sub>, and NO<sub>2</sub>, but



**Fig. 3.** The associations between per interquartile range (IQR) exposure to air pollution and semen parameters.

was positively associated with O<sub>3</sub> in single pollutant model. From a study conducted in Huai'an, China with different exposure time, they found that PM exposure would decrease sperm total motility especially during 45–59 days before semen collection (Guan et al., 2020). As for SO<sub>2</sub>, the negative association with total motility was in line with a study conducted in Wuhan, China. Sun et al. reported that exposing to SO<sub>2</sub> during 0–5th weeks of sperm development weeks would decrease the total motility (Sun et al., 2020). Also, Chen et al. proposed NO<sub>x</sub> exposure was one of risk factors for decreased sperm motility (Chen et al., 2020). In addition, the WQS regression model in our study also illustrated significant association between air pollution and total motility.

Thus, total motility is a sensitive parameter for both long-term and short-term exposures which is crucial for male fertility, which is one of the main reasons for infertility (Ben Khelifa et al., 2014; Tang et al., 2017). As one of the most important parameters which associated with the fertilizing ability of spermatozoa, sperm total motility reflects their structural integrity and viability (Nagy et al., 2015). Although all the

participants were fertile in our study, the impaired motility would cause subtle effects on fertility, such as an extended time to pregnancy (Buck Louis et al., 2014). In addition, our study also found the level of 5-hmC in sperm DNA had changed. Namely, air pollution exposure is not only associated with decreasing total motility but also with the level of DNA methylation in sperm. Similarly, Du et al. also revealed that sperm DNA methylation of asthenozoospermic males was different from normozoospermic controls (Du et al., 2016). Additional evidence showed that the epigenetic alterations in the sperm have the potential to promote in subsequent generations the epigenetic transgenerational inheritance of disease and phenotypic alterations (Garrido et al., 2021).

Until now, the associations between O<sub>3</sub> and total motility is still under debatable. Some studies showed that O<sub>3</sub> decreased sperm concentration which contradicted our result (Farhat et al., 2016; Lafuente et al., 2016; Zhang et al., 2019b). Different study regions, populations, and exposure time may cause these discrepancies. Some other studies showed that the annual fluctuation of ozone was opposite to that of

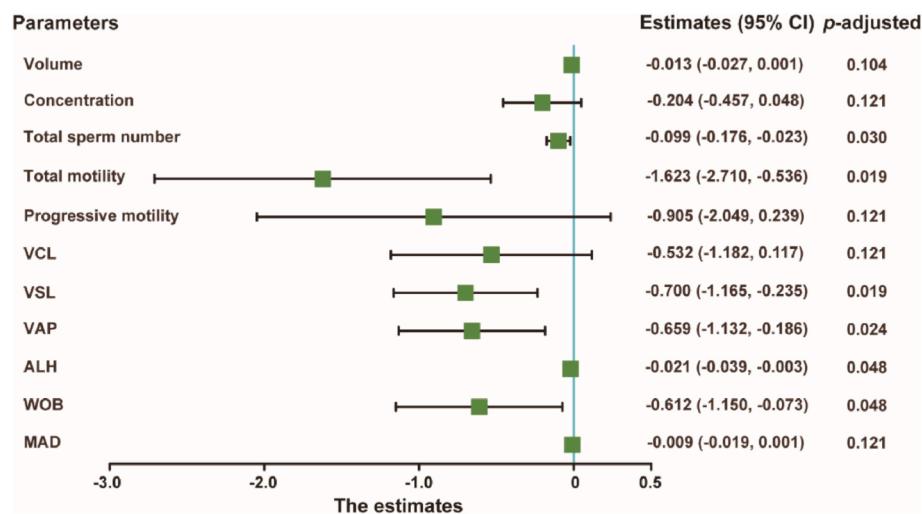


Fig. 4. The associations between air pollution co-exposure and semen parameters by weighted quantile sum regression model.

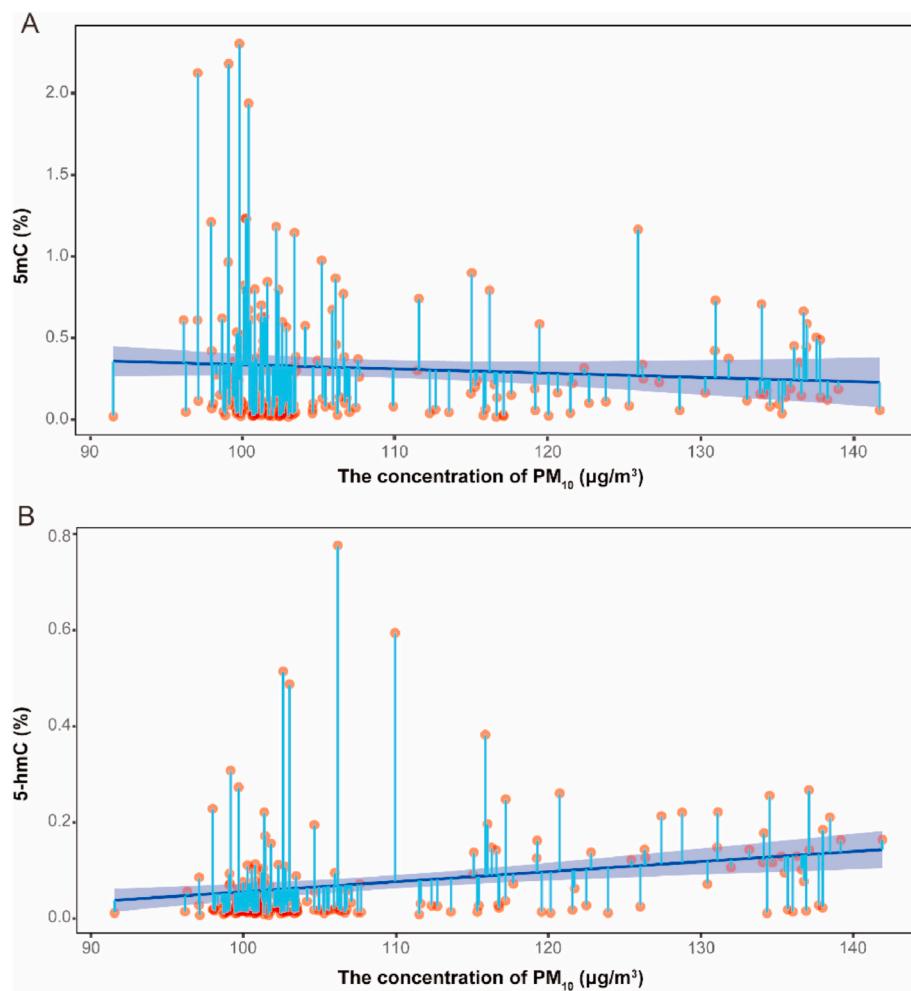


Fig. 5. The associations between PM<sub>10</sub> exposure and 5 mC, 5-hmC levels of sperm DNA. (A) The effects of PM<sub>10</sub> exposure on 5 mC levels; (B) The effects of PM<sub>10</sub> exposure on 5-hmC levels.

other five pollutants (CO, SO<sub>2</sub>, NO<sub>2</sub>, PM<sub>10</sub>, and PM<sub>2.5</sub>) (Huang et al., 2018; Nobles et al., 2018a; Zhang et al., 2019b). Our study also found the same phenomenon of O<sub>3</sub>. Such situation may attribute to the conversion between pollutants. For example, NO<sub>2</sub> can be decomposed by the solar ultraviolet rays into nitric oxide and oxygen atoms, which then

combine with oxygen to form O<sub>3</sub> (Lu et al., 2019; Zhang et al., 2019b). This explained why the concentration of O<sub>3</sub> was high in summer while other pollutants were relatively low. It may be one of reasons why WQS regression demonstrated weaker association between air pollution and total motility when compared to the single pollutant model. In recent

years, the O<sub>3</sub> mixing ratio in China keeps increasing greatly due to the rapid industrial development (Lu et al., 2019). Therefore, we need to pay more attention to the current situation of O<sub>3</sub> pollution and more research are needed to explore its influence on semen quality.

Currently, the mechanism on the effects from air pollution exposure to the semen quality is still under exploration. Oxidative damage is considered to be one of the important mechanisms. Reactive oxygen species (ROS) could damage spermatogenic cells and cause low semen quality (Wang et al., 2021). Another mechanism demonstrated by some studies was related to the derangement on the integrity of blood-testis barrier. Yan et al. proposed that integrity of the blood-testosterone barrier was disrupted after the long-term exposure to PM<sub>2.5</sub> through an animal experiment. Then the microenvironment of sperm production was altered, followed by the changes on the sperm production and sperm quality (Yan et al., 2016). Cao et al. found impaired blood-testosterone barrier could damage testicular tissue which then greatly reduced male fertility (Cao et al., 2017).

In addition, DNA methylation modifications have been approved to affect the reproduction health from air pollution exposure. In our study, the increased genomic levels of 5-hmC in sperm DNA were observed after the long-term exposure to PM<sub>10</sub>. A study conducted among the Beijing truck drivers proposed that exposure to ambient PM<sub>10</sub> affects 5-hmC over time (Sanchez-Guerra et al., 2015). Another study pointed out exposure to PM<sub>10</sub> would decrease global DNA methylation in whole blood (De Prins et al., 2013). While some studies were inconsistent with our finding (Martin and Fry, 2018). Yauk et al. identified the DNA methylation changes in male mice exposed to particulate air pollution. They reported that sperm DNA of mice was hypermethylated after breathing particulate air (Yauk et al., 2008).

Active demethylation has been considered as one of regulatory features. Although we didn't observe a statistically significant association between PM<sub>10</sub> exposure and 5 mC, yet the trend of negative correlation between them was of biological significance. Ten-eleven translocation (TET) enzymes, a family of α-ketoglutarate-dependent dioxygenases, could oxidize 5 mC to yield 5-hmC (Lio and Rao, 2019). Among them, TET1 gene played a key role in DNA demethylation. As stated above, air pollution exposure would result in the generation of ROS, which also can induce the oxidation of 5 mC to 5-hmC (Chia et al., 2011). A study conducted by Ni et al. found that the level of TET mRNAs in spermatozoa was reduced in spermatozoa of sub-fertile men (Ni et al., 2016). In brief, the activation of TET enzymes would affect the spermatogenesis, with increasing the levels of 5-hmC. A cross-sectional study has also found a positive association between global sperm DNA methylation levels and sperm motility. It suggested that lower methylation levels would be indicative of defective spermatogenesis (Montjean et al., 2015).

DNA methyltransferases (DNMTs) are involved in DNA methylation regulation. In spermatogonia, DNMT1 is highly expressed and DNMT3A, DNMT3B accumulate in spermatocyte and spermatids. Increased levels of 5-hmC indicated that expression of these DNMTs had changed. It would influence the process of spermatogenesis as well as the semen quality (Uysal et al., 2016). In addition, some genes involved in DNA methylation has been also implicated. Houshdaran et al. found that decreased sperm motility was in association with DNA hypermethylation across a number of loci. PAX8, NTF3, SFN, and HRAS were found to be unique sequences to non-imprinted genes. Therefore, the authors proposed that improper DNA methylation outside of imprinted loci would cause infertility (Rajender et al., 2011). Our result supports further study to explore specific mechanism of DNA methylation regulation.

One crucial strength is that this study was based on a cohort study, which provided clinical information and biological samples for our research. The pre-well-designed research protocol ensured the cohort collect enough information so that we could adjust corresponding confounders in the process of statistical analysis. Another feature was that we applied both single pollutant model and multi-pollutants model to examine the influences on semen quality from long-term air pollution

exposure. We focused on six routinely monitored air pollutants that were not available in previous studies. Moreover, we also observed the role of DNA methylation played in the association between air pollution exposure and semen quality.

Several limitations also existed in our study. First, we just considered individual exposure based on residential addresses, but time-activity patterns or occupational exposure were not included. It would underestimate the influence of air pollution as some other studies described (Chen et al., 2020; Sokol et al., 2006). Although such method, IDW, was widely used in many epidemiological studies (Liu et al., 2020; Xie et al., 2021; Zhang et al., 2019a), it may cause different levels of measurement error and ultimately weaken the accuracy of the associations of air pollutants exposure and semen quality. Second, we just detected levels of 5 mC and 5-hmC in sperm DNA. It would be more helpful to identify the specific genes with abnormal methylation by whole-genome bisulfite-sequencing to understand the association identified from epidemiological results. If we could identify which component of PM<sub>10</sub> was most responsible, it would help us better understand the mechanisms involved. Finally, we only had one semen sample which would lead to bias to some extent.

## 5. Conclusion

In summary, this study illustrated a consistent association between long-term exposure to SO<sub>2</sub>, NO<sub>2</sub>, PM<sub>10</sub>, and PM<sub>2.5</sub> and reduced sperm total motility respectively in fertile men. Co-exposure of six air pollutants could also affect the total motility as well. Understanding adverse impacts and epigenetic modification mechanisms from different air pollutants exposure would help to inform public health promotion on the environmental health intervention, as well as strengthen the strategies on the high-quality reproduction development.

## Author statement

**Yuting Cheng:** Conceptualization, Methodology, Formal analysis, Writing - Original draft preparation. **Qiuqin Tang:** Validation, Funding acquisition, Writing - Review & Editing. **Yiwen Lu:** Validation, Data curation, Formal analysis. **Mei Li:** Validation, Data curation, Formal analysis. **Yijie Zhou:** Visualization, Investigation. **Peihao Wu:** Methodology, Data curation. **Jinhui Li:** Review & Editing. **Feng Pan:** Resources, Writing - Review & Editing. **Xiumei Han:** Writing - Review & Editing. **Minjian Chen:** Resources. **Chuncheng Lu:** Resources. **Xinru Wang:** Resources. **Wei Wu:** Conceptualization, Resources, Supervision, Funding acquisition, Writing - Review & Editing. **Yankai Xia:** Resources, Supervision, Funding acquisition, Writing - Review & Editing.

## 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.

## Acknowledgements

We thank all the patients and donors who participated in this study. This work was supported by the Major Project of Natural Science Research in Jiangsu Province Colleges and Universities (20KJA330001), Medical Research Project of Jiangsu Health and Health Commission (Z2019010), National Natural Science Foundation of China (81971405, 81673217), the Major Research plan of the National Natural Science Foundation of China (91943301), and the Priority Academic Program for the Development of Jiangsu Higher Education Institutions (Public Health and Preventive Medicine).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2022.118994>.

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