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Association between outdoor air pollution during *in vitro* culture and the outcomes of frozen–thawed embryo transfer

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STUDY QUESTION: Does outdoor air pollution differentially affect the outcomes of frozen–thawed embryo transfer (FET) and fresh transfer in IVF treatment?

SUMMARY ANSWER: Increased SO_2 and O_3 levels at the site of IVF unit were significantly associated with lower live birth rates following FET but did not affect the contemporary fresh transfer outcomes.

WHAT IS KNOWN ALREADY: Ambient air pollution has been associated with human infertility and IVF outcomes. However, most of the studies excluded FET cycles.

STUDY DESIGN, SIZE, DURATION: A retrospective cohort study of 11148 patients contributing to 16290 transfer cycles between January 2013 and December 2016 was carried out.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The average age of the cohort was 31.51 ± 4.48 years and the average BMI was 21.14 ± 2.37 kg/cm^2. Inverse distance weighting interpolation was used to estimate the daily ambient exposures to six pollutants ($PM_{2.5}$, PM_{10}, SO_2, NO_2, CO, O_3) at an IVF clinical site, according to the data from fixed air quality monitoring stations in the city. The exposures of each cycle were presented as average daily concentrations of pollutants from oocyte retrieval to embryo transfer/cryopreservation. Exposures were analyzed in quartiles. A generalized estimating equation was used to evaluate the association between pollutants and IVF outcomes, adjusted for important confounding factors including maternal age, infertility diagnosis, BMI, endometrial status and embryo transfer policy.

MAIN RESULTS AND THE ROLE OF CHANCE: The clinical pregnancy rate and live birth rate of the cycles was 55.1% (8981/16290) and 47.1% (7672/16290), respectively. Among the included cycles, 4013 patients received 5299 FET cycles, resulting in 2263 live births (42.7% per ET), whereas 9553 patients received 10991 fresh transfer cycles, resulting in 5409 live births (49.2% per ET). SO_2 and O_3 levels were significantly associated with live birth rates in FET cycles, whereas none of the pollutants were significantly associated with IVF outcomes in contemporary fresh transfer cycles. The FET cycles in the highest quartile of SO_2 and O_3 exposure had significantly lower live birth rates (adjusted odds ratio (OR) 0.63, 95%CI 0.53–0.74; 0.69, 95% CI 0.58–0.82, respectively) in comparison with those in the lowest quartile. Models involving all transfer cycles and interaction terms (FET×exposures) suggested that FET significantly enhanced the effects of SO_2 and O_3 exposure on IVF outcomes ($P < 0.001$). Multi-pollutant models gave consistent results for the association between SO_2 and live birth in FET cycles. Accounting for all six pollutants, women in the highest quartile of SO_2 still had the lowest live birth rates (OR 0.61, 95%CI 0.47–0.80).

LIMITATIONS, REASONS FOR CAUTION: The study was limited by its retrospective nature. The exposure data were estimated according to monitoring data rather than measured directly from the IVF unit. Unknown confounding factors may skew the results.

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WIDER IMPLICATIONS OF THE FINDINGS: Our data implied that embryos undergoing FET may be more vulnerable to a suboptimal environment than those undergoing fresh transfer. In heavily polluted sites or seasons, fluctuation in FET outcomes may be partially explained by the dynamic changes of ambient gaseous air pollutant.

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Key words: pollution / sulfur dioxide / ozone / live birth / frozen–thawed embryo transfer / IVF / embryo culture / cryopreservation

Introduction

Air pollution is an ongoing challenge around the world. A suspected causal relationship between air pollution and various health problems, including cardiovascular diseases, lung cancer and asthma, has been highlighted in numerous studies (Guan *et al.*, 2016; Newell *et al.*, 2017). Ambient air pollutants also showed an impact on human reproduction, with increased risk of miscarriage and reduce birth weight (Vizcaino *et al.*, 2016; Carre *et al.*, 2017a, 2017b). Both spermatogenesis in the male and spontaneous fecundability in the female have been negatively associated with concentration of ambient air pollutants, bringing awareness of concerns regarding reproductive health (Carre *et al.*, 2017a, 2017b).

For infertile patients, ART provides an effective option for achieving pregnancy. For women receiving ART, it was also shown that ambient air pollutant concentrations during the period of follicular growth or early pregnancy were significantly associated with ovarian response, embryo quality, implantation and miscarriage (Legro *et al.*, 2010; Carre *et al.*, 2017a, 2017b; Choe *et al.*, 2018). Interestingly, the work of Legro *et al.* (2010) suggested that outdoor air pollution at IVF sites was significantly associated with live birth rates. This observation may suggest additional risks of potential *in vitro* exposure of gametes and embryos to ambient air pollutants. However, the data in this regard are still limited and a recent report by Choe *et al.* (2018) did not find any significant association between the average concentrations of major air pollutants during the period from oocyte retrieval to embryo transfer and intrauterine pregnancy.

While the data are limited, most of the studies regarding the association between ambient air pollution and reproductive outcomes in the IVF population excluded the frozen–thawed transfer (FET) cycles (Legro *et al.*, 2010; Carre *et al.*, 2017a, 2017b; Choe *et al.*, 2018). Because the number of FET has increased rapidly over the past decades, simply excluding the FET cycles may suggest a considerable loss of information. Excluding FET cycles from analyses also excluded patients who had all their embryos cryopreserved or patients who achieved pregnancy through supernumerary frozen embryo transfer. This approach may introduce potential biases, especially when one considers the patients undergoing repeated treatment. Although embryo cryopreservation has been accepted as a safe approach and comparative studies have demonstrated comparable outcomes between FET and fresh transfer cycles (Wong *et al.*, 2017), evidence suggested that cryopreservation is associated with extensive disruption of cell membranes, alteration in metabolic status and damage to genomic integrity (Kopeika *et al.*, 2015). The association between FET and pregnancy-related complications and perinatal outcomes (Wong *et al.*, 2017) implies that cryotechnology may still affect the fate of embryos.

The present study was designed to explore the association between the average concentrations of six major air pollutants ($PM_{2.5}$, PM_{10}, SO2, CO, NO_2 and O_3) at an IVF site and the outcomes of FET cycles. Additionally, the association between air pollutants and live birth in FET cycles was compared to that in fresh cycles.

Materials and Methods

Study subjects

The retrospective study included all patients who underwent FET or fresh transfer in the affiliated Chenggong Hospital of Xiamen University in the period between January 2013 and December 2016. Due to the availability of air quality data, patients undergoing oocyte retrieval before 18 January 2013 were excluded (1175 cycles). Additionally, 423 cycles were excluded due to incomplete record of cycle information. Institutional Review Board approval for this retrospective study was obtained from the Ethical Committee of the Medical College Xiamen University. The study involved only a retrospective review of anonymous medical records, therefore informed constant was not required.

IVF procedures

In oocyte retrieval cycles, the majority of the patients (97.6%) were treated with conventional protocols using FSH or hMG, as previously described (Cai *et al.*, 2017), whereas the rest the patients were treated with ovarian stimulation protocols without GnRH analogs. Oocytes were inseminated using either conventional IVF or ICSI. All embryos were cultured in traditional incubators (C200, Labotect, Göttingen, Germany) at 37°C, 6% CO_2, 5% O_2.

Top-quality embryos for transfer were defined as the following: the embryos with <10% fragmentation and on-time cell size on Day 3 and with a good inner cell mass (regularly formed with numerous, tightly packed cells) and good trophectoderm (with many cells arranged in regular epithelium) on Day 5.

For all FET cycles, a vitrification protocol, employing 15% dimethyl sulfoxide, 15% ethylene glycol and 0.6 M sucrose as cryoprotectants, was used for cryopreservation, as described previously (Cai *et al.*, 2018). For blastocysts, blastocoelic volume was reduced before cryopreservation using a laser system (SATURN, RI, Falmouth, UK).

Laboratory air quality control

The IVF laboratory was located on the top floor of a 23-floor building. A centered high-efficiency particulate Air (HEPA) system was used for supplying filtered air to the IVF laboratory and related critical areas. The HEPA

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filter was replaced annually. A CODA tower unit was used for controlling volatile organic compounds (VOCs). No incubator filter box was used.

Exposure estimation

The exposure of transfer cycles to air pollutant was presented as average daily concentrations of pollutants during the duration of laboratory culture (from oocyte retrieval to fresh transfer or cryopreservation). With consideration of the spatial distribution of pollutants, estimation of pollutant concentrations at the IVF site was based on an inverse distance weighting interpolation modeling method, which is commonly used in spatial interpolation to model air pollutant distribution based on data from fixed monitoring stations (Eberly *et al.*, 2004). The estimated daily concentration at the IVF site was calculated as the average air pollutant concentration at monitoring stations weighted by $1/d^2$, where d refers to distance between the IVF site and monitoring stations. The calculations were based on all three state-controlled monitoring stations in the city, which were responsible for the release of hourly air quality data for PM_{10}, $PM_{2.5}$, CO, SO_2, NO_2, and O_3. Data were obtained from daily reporting system of the Ministry of Environmental Protection of China (http://106.37.208.233:20035). Daily concentrations of O_3 were presented as an 8-h rolling average of the value.

Statistical analysis

We analyzed the association between air pollutant concentrations and embryo transfer outcomes using the generalized estimating equation (GEE) model, which allows accounting for correlations between repeated measurements for the same subject. The base model of the analyses was adjusted for known factors related to IVF outcome according to published data and our experience, which included maternal age (continuous), BMI (underweight, normal weight and overweight), order of embryo transfer (ET) (continuous), primary infertility (primary versus secondary), duration of infertility (continuous), diagnosis of tubal problem (with versus without), polycystic ovary syndrome (with versus without) and endometriosis (with versus without), basal antral follicle count (continuous), starting dose of gonadotrophin (continuous), type of GnRH analog (agonist, antagonist and none), endometrial thickness (continuous) and pattern (Zhao *et al.*, 2014) (type A, B and C), oocyte yield (continuous), number of embryos transferred (continuous), stage of embryos transferred (cleavage versus blastocyst), presence of top-quality embryos transferred (yes versus no) and distance from catheter tip to fundal. For FET cycles, type of endometrial preparation (natural, artificial and GnRH agonist) and indicators for FET (freeze-all versus surplus) were also considered. The GEE model was also used to evaluate the association between the pollutant concentrations and embryo parameters, making adjustment for age, order of ET, gonadotrophin dose, oocyte yield and ICSI. The embryo parameters included cell number, non-synchronized cleaving (Sela *et al.*, 2012) and the presence of compact embryos at Day 3, morphological score of inner cell mass and trophectoderm at Day 5 and cryosurvival after thawing. Models evaluating the association between embryo parameters and air pollutants were adjusted for confounders that related to the timing of embryonic development (Kirkegaard *et al.*, 2016), including BMI, maternal age, FSH dose and number of previous cycles.

Because non-linearity was expected for some pollutants (Supplementary Table SI), exposure data were divided into quartiles (Q1–Q4) and introduced into the model as categorized data. Linear trend across quartiles was tested by including the median of each quintile range in the model. For each single pollutant, its association with IVF outcomes was evaluated separately in FET and in fresh transfer cycles with adjustment for the previously mentioned confounders. The difference in exposure–outcome relations between FET and fresh transfer cycles were compared according to the effect size and trends. More formally, an interaction term (single pollutant × FET) was introduced to a multivariate model that included all transfer cycles.

Sensitivity analyses were conducted to test the robustness of results of single-pollutant analyses. Because reduced live birth rates in patients with poor prognosis might be related to unknown risk factors that are unrelated to air pollution, we conducted further sensitivity analyses in a subgroup with good prognosis (age < 35 years, ET order=1 and transfer at blastocyst stage).

Besides the single-pollutant model, multipollutant models were also constructed to assess the association between pollutants and IVF outcomes in all FET cycles and freeze-all cycles. Correlations between pollutants were evaluated with Spearman correlation coefficients. Multicollinearity of pollutants was examined using the variance inflation factor (VIF). A VIF < 4 was considered acceptable.

All calculations were performed with SPSS (version 19; IBM, Armonk, NY, USA) and Stata (version 12; StataCorp, College Station, TX, USA).

Results

The present study included 11 148 patients contributing to 16 290 transfer cycles. The average age of the cohort was 31.51 ± 4.48 years and the average BMI was 21.14 ± 2.37 kg/cm^2. The clinical pregnancy rate and live birth rate of the cycles were 55.1% (8981/16290) and 47.1% (7672/16290), respectively. Among the included cycles, 4013 patients received 5299 FET cycles, resulting in 2263 live births (42.7% per ET), whereas 9553 patients received 10 991 fresh transfer cycles, resulting in 5409 live births (49.2% per ET). The detailed characteristics of the cycles are shown in Table I. In FET cycles, patients were younger and had a higher oocyte yield but the median endometrial thickness was significantly lower. Most of the FET cycles were in natural cycle (n = 2637) or hormone replacement cycles (n = 2201), while a small fraction of them were in cycles with GnRH agonist (n = 461). About half of the FET cycles (48.1%) were due to canceled fresh transfer, whereas others were carried out after a failed transfer (n = 2748).

During the period of embryo culture, the medians of estimated mean daily concentration of six air pollutants ($PM_{2.5}$, PM_{10}, SO_2, CO, NO_2, O_3) at IVF sites were 30.26, 51.99, 12.86, 0.66, 30.79 and 82.6 μg/m^3, respectively.

When associating each single pollutant with clinical pregnancies and live births in fresh transfer cycles, none of the pollutants showed significant association with IVF outcomes, except that SO_2 reduced clinical pregnancy rates in the highest quartile (Table II). However, the size of association was relatively small (odds ratio (OR) 0.88, 95%CI: 0.78–0.99) and the significance diminished when associating SO_2 concentrations with live births. In contrast, multivariate analyses showed that SO_2 and O_3 concentrations were significantly associated with clinical pregnancy rates and live birth rates in FET cycles (P < 0.001). The live birth rates in the third and fourth quartiles of SO_2 were 0.8 (95% CI: 0.68–0.94) and 0.63 (95%CI: 0.53–0.74) fold than the first quartile. Similarly, O_3 levels were associated with reduced live birth rates in the third and fourth quartiles (adjusted OR 0.72 and 0.69, respectively). For each interquartile range increase of exposure to SO_2, the OR was 0.86 (0.81–0.91); for each interquartile range increase of exposure O_3, the OR was 0.87 (0.83–0.92) (Table II). The P-values for trend were all significant (P < 0.001).

Sensitivity analyses were performed in a subgroup of patients who aged <35 years, undergoing their first transfer with embryos at the blastocyst stage. The analysis included 521 fresh cycles and 1170 FET

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Table I Patient characteristics and cycle parameters.

	Fresh	FET	Absolute difference (95%CI)
ET cycles, *n*	10 991	5299	
Primary infertility (%)	5106/10 991 (46.5)	2643/5299 (49.9)	−3.47 (−5.1, −1.83)
Second child	1926/10 991 (17.5)	722/5299 (13.6)	3.9 (2.7–5.1)
Maternal age, year	31 [7]	31 [6]	0 (0, 1)
Number of embryo transfer attempts	1 [0]	2 [1]	−1 (−1, −1)
BMI			
Normal weight	9086/10 991 (82.7)	4185/5299 (79)	3.69 (2.4, 5.01)
Underweight <18.5 kg/m^2	1346/10 991 (12.2)	890/5299 (16.8)	−4.55 (−5.74, −3.38)
Overweight, ≥25 kg/m^2	559/10 991 (5.1)	224/5299 (4.2)	0.86 (0.16, 1.52)
Diagnosis			
Tubal (%)	6678/10 991 (60.8)	3280/5299 (61.9)	−1.14 (−2.73, 0.46)
Endometriosis (%)	1229/10 991 (11.2)	490/5299 (9.2)	1.93 (0.94, 2.9)
PCOS (%)	649/10 991 (5.9)	423/5299 (8)	−2.08 (−2.95, −1.24)
Duration of infertility, year	4 [4]	4 [4]	0 (0, 0)
Basal AFC	7 [5]	8 [5]	−1 (−1, −1)
Basal FSH, IU/l	7.09 [2.49]	6.72 [2.11]	0.37 (0.31, 0.43)
Basal LH, IU/l	4.18 [2.47]	4.41 [2.61]	−0.28 (−0.34, 0.43)
Basal E_2, pg/ml	40 [26]	40 [26]	0 (−0.71, 1)
Ovarian stimulation characteristics			
GnRH analogs			
Agonist (%)	8628/10 991 (78.5)	4566/5299 (86.2)	−7.68 (−8.88, −6.46)
Antagonist (%)	2137/10 991 (19.4)	571/5299 (10.8)	8.67 (7.54, 9.77)
No analogs (%)	226/10 991 (2.1)	162/5299 (3.1)	−1 (−1.56, −0.49)
Total dose of stimulation, IU	2250 [900]	2250 [825]	75 (0, 75)
Duration of stimulation, day	11 [2]	11 [2]	0 (0, 0)
Starting dose of stimulation, IU	225 [37.5]	225 [75]	0 (0, 0)
Oocyte number	8 [7]	13 [10]	−5 (−5, −5)
Embryo transfer characteristics			
Endometrial thickness, mm	10.5 [3.1]	8.9 [2.2]	1.4 (1.3, 1.5)
Endometrial patterns			
A	2053/10 991 (18.7)	212/5299 (4)	14.68 (13.77, 15.57)
B	7786/10 991 (70.9)	4460/5299 (84.2)	−13.33 (−14.61, −12.01)
C	105/10 991 (1)	45/5299 (0.8)	0.11 (−0.22, 0.4)
Unclassified	1045/10 991 (9.5)	580/5299 (10.9)	−1.44 (−2.46, −0.45)
Blastocyst transfer	916/10 991 (8.3)	3810/5299 (71.9)	−63.57 (−64.86, −62.23)
At least one top-quality embryo transferred	1894/10 991 (17.2)	1052/5299 (19.9)	−2.62 (−3.92, −1.35)
Catheter tip to fundal distance, cm	1.1 [0.4]	1 [0.4]	0.1 (0.1, 0.1)
Number of embryos transferred	2 [1]	2 [1]	0 (0, 0)
Average daily concentration of air pollutants			
$PM_{2.5}$, μg/m^3	30.26 [14.53]		-
PM_{10}, μg/m^3	51.99 [20.59]		-
SO_2, μg/m^3	12.86 [8.69]		-
CO, μg/m^3	0.66 [0.18]		-
NO_2, μg/m^3	30.79 [13.26]		-
O_3, μg/m^3	82.6 [36.03]		-

ET, embryo transfer; PCOS, polycystic ovary syndrome; AFC, antral follicle count; FET, frozen–thawed embryo transfer; E_2: estradiol.
Data are median [interquartile range] or count (percentage).

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Table II Association between single pollutants and IVF outcomes.

Exposures		Clinical pregnancy		Live birth	
		Fresh ET *N* = 10 991	FET *N* = 5299	Fresh ET *N* = 10 991	FET *N* = 5299
SO_2, μg/m^3	Q1 (2.97–9.27)	Ref	Ref	Ref	Ref
	Q2 (9.28–13.50)	0.96 (0.86–1.08)	1.03 (0.87–1.22)	0.97 (0.87–1.09)	0.99 (0.84–1.17)
	Q3 (13.51–18.66)	0.93 (0.83–1.04)	0.77 (0.65–0.91)	0.91 (0.81–1.02)	0.80 (0.68–0.94)*
	Q4 (18.67–45.74)	0.88 (0.78–0.99)*	0.64 (0.54–0.75)*	0.90 (0.80–1.01)	0.63 (0.53–0.74)*
$PM_{2.5}$, μg/m^3	Q1 (8.27–23.83)	Ref	Ref	Ref	Ref
	Q2 (23.84–30.26)	1.04 (0.93–1.17)	1.13 (0.95–1.33)	1.03 (0.92–1.15)	1.18 (1-1.39)
	Q3 (30.27–38.36)	0.96 (0.86–1.08)	0.97 (0.82–1.14)	0.96 (0.86–1.08)	1.01 (0.85–1.19)
	Q4 (38.37–108.05)	0.94 (0.84–1.05)	0.86 (0.73–1.02)	0.94 (0.84–1.05)	0.92 (0.78–1.09)
PM_{10}, μg/m^3	Q1 (19.25–43.22)	Ref	Ref	Ref	Ref
	Q2 (43.23–51.99)	1.01 (0.9–1.13)	1.1 (0.93–1.3)	0.99 (0.89–1.11)	1.11 (0.94–1.32)
	Q3 (52.00–63.81)	1.02 (0.91–1.14)	1.04 (0.88–1.22)	0.98 (0.88–1.10)	1.01 (0.86–1.19)
	Q4 (63.82–150.45)	0.91 (0.82–1.02)	0.82 (0.69–0.97)*	0.92 (0.82–1.02)	0.85 (0.71–1.01)
CO, μg/m^3	Q1 (0.31–0.58)	Ref	Ref	Ref	Ref
	Q2 (0.59–0.66)	0.94 (0.84–1.05)	1.01 (0.86–1.2)	0.91 (0.82–1.02)	1.09 (0.92–1.28)
	Q3 (0.67–0.76)	0.98 (0.88–1.1)	0.97 (0.83–1.15)	1 (0.89–1.12)	1.03 (0.87–1.21)
	Q4 (0.77–1.54)	0.91 (0.81–1.02)	0.80 (0.68–0.94)*	0.93 (0.83–1.04)	0.85 (0.72–1.01)
NO_2, μg/m^3	Q1 (10.44–25.01)	Ref	Ref	Ref	Ref
	Q2 (25.02–30.79)	0.97 (0.87–1.09)	1.13 (0.96–1.32)	0.95 (0.85–1.06)	1.13 (0.96–1.33)
	Q3 (30.80–38.27)	0.97 (0.87–1.09)	1.04 (0.88–1.23)	0.96 (0.86–1.07)	1.04 (0.89–1.23)
	Q4 (38.28–94.14)	0.94 (0.84–1.05)	0.85 (0.72–1.01)	0.97 (0.87–1.09)	0.87 (0.74–1.03)
O_3, μg/m^3	Q1 (26.88–66.12)	Ref	Ref	Ref	Ref
	Q2 (66.13–85.43)	0.98 (0.88–1.1)	1.02 (0.86–1.21)	0.96 (0.86–1.07)	1.03 (0.87–1.22)
	Q3 (85.44–104.18)	0.99 (0.88–1.11)	0.72 (0.61–0.85)*	0.97 (0.86–1.08)	0.72 (0.61–0.86)*
	Q4 (104.19–169.43)	0.98 (0.88–1.11)	0.66 (0.56–0.78)*	0.96 (0.86–1.08)	0.69 (0.58–0.82)*

Q1—Q4: quartiles of exposure.
'*' Indicates the difference was significant ($P < 0.05$) in comparison with reference category.
Each model was adjusted for maternal age, BMI, order of ET, primary infertility (primary versus secondary), second child, duration of infertility, diagnosis of tubal problem, PCOS and endometriosis, basal AFC, starting dose of gonadotrophin, type of GnRH analogs (agonist, antagonist and none), endometrial thickness and pattern, oocyte yield, number of embryos transferred, stage of embryos transferred (cleavage versus blastocyst), presence of top-quality embryos transferred and distance from catheter tip to fundal. FET cycles were also adjusted for endometrial preparation protocol and FET indicators (freeze-all versus surplus).

cycles (Supplementary Table SII). The analyses showed that the exposure–outcome associations of SO_2 and O_3 were rather consistent in the subgroup of the patients (P for trend <0.001). In the highest quartile of exposure, the ORs of SO_2 and O_3 for live birth were 0.58 (95% CI: 0.41–0.83) and 0.61 (95%CI: 0.42–0.88), respectively.

In the model including all fresh and FET cycles, we introduced an interaction term (FET × exposure) to quantitatively investigate the effect of FET on the exposure–outcome association (Supplementary Table SIII). The interactions between FET and the exposures of SO_2 and O_3 were significant. In comparison with fresh transfer cycles, ORs for live birth were significantly reduced in the highest quartile of exposures. A similar result was also observed in the subgroup for sensitivity analyses (Supplementary Table SIV).

Accounting for both fresh and FET cycles, 10 232 patients contributing to 11 723 oocyte retrieval cycles had all their embryos used or achieved at least one live birth during the study period. Among these patients, the rates of cumulative live birth per oocyte retrieval were also significantly reduced in the highest quartile of SO_2 and O_3 (Supplementary Table SV).

The concentrations of the pollutants highly correlated to each other (Supplementary Table SVI), therefore we performed multipollutant analyses to test the consistency of our results. Colinearitly of pollutants was measured according to VIF, and no VIF values exceeded 4. The dose-dependent effects of SO_2 on live birth rates were consistent among analyses. In the model including all six pollutants, the women in the highest quartile of SO_2 still had lowest live birth rates (OR 0.61, 95%CI 0.47–- 0.80) (Table III). On the other hand, effects of O_3 seemed to be confounded by SO_2. With the presence of SO_2 in the models, the size of association between O_3 and live birth rates was reduced. Additionally, we also noted an interaction between SO_2 and O_3 in the same model (Supplementary Table SVII). A significant association between SO_2 and live birth was also detected in a subgroup analysis with freeze-all using the multipollutant model (Supplementary Table SVIII).

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Table III Multi-pollutant models of live birth following FET ($n = 5299$) associated with quartiles of SO_2 and O_3.

Model	Exposure					
			SO_2, μg/m^3		Per quartile change	*P* for trend
	Q1 (2.97–9.27)	Q2 (9.28–13.50)	Q3 (13.51–18.66)	Q4 (18.67–45.74)		
SO_2+O_3	Ref	1.00 (0.84–1.18)	0.85 (0.70–1.02)	0.69 (0.56–0.85)	0.89 (0.83–0.95)	0.001
SO_2+O_3+NO_2	Ref	0.97 (0.82–1.15)	0.81 (0.67–0.98)	0.63 (0.49–0.81)	0.87 (0.80–0.94)	0.001
SO_2+O_3+NO_2+CO	Ref	0.96 (0.80–1.14)	0.80 (0.66–0.97)	0.63 (0.49–0.81)	0.87 (0.8–0.94)	<0.001
SO_2+O_3+NO_2+CO+$PM_{2.5}$	Ref	0.94 (0.79–1.12)	0.79 (0.65–0.97)	0.62 (0.48–0.80)	0.86 (0.79–0.93)	<0.001
SO_2+O_3+NO_2+CO+$PM_{2.5}$+PM_{10}	Ref	0.94 (0.79–1.11)	0.79 (0.65–0.96)	0.61 (0.47–0.80)	0.86 (0.79–0.93)	<0.001
			O_3, μg/m^3		Per quartile change	*P* for trend
	Q1 (26.88–66.12)	Q2 (66.13–85.43)	Q3 (85.44–104.18)	Q4 (104.19–169.43)		
SO_2+O_3	Ref	1.09 (0.91–1.29)	0.82 (0.69–0.99)	0.90 (0.72–1.11)	0.93 (0.87–1.00)	0.043
SO_2+O_3+NO_2	Ref	1.07 (0.9–1.28)	0.83 (0.69–1.00)	0.91 (0.73–1.13)	0.94 (0.88–1.01)	0.07
SO_2+O_3+NO_2+CO	Ref	1.05 (0.88–1.25)	0.81 (0.67–0.97)	0.9 (0.72–1.12)	0.94 (0.87–1)	0.064
SO_2+O_3+NO_2+CO+$PM_{2.5}$	Ref	1.06 (0.89–1.27)	0.82 (0.68–0.99)	0.91 (0.73–1.14)	0.94 (0.88–1.01)	0.086
SO_2+O_3+NO_2+CO+$PM_{2.5}$+PM_{10}	Ref	1.07 (0.89–1.28)	0.81 (0.67–0.98)	0.9 (0.72–1.13)	0.94 (0.87–1.01)	0.078

Each model was adjusted for maternal age, BMI, order of ET, primary infertility (primary versus secondary), second child, duration of infertility, diagnosis of tubal problem, PCOS and endometriosis, basal AFC, starting dose of gonadotrophin, type of GnRH analogs (agonist, antagonist and none), freeze indicators (freeze-all versus surplus), endometrial preparation, endometrial thickness and pattern, oocyte yield, number of embryos transferred, stage of embryos transferred (cleavage versus blastocyst), presence of top-quality embryos transferred and distance from catheter tip to fundal.

When we explored the association between SO_2 and laboratory parameters, we found that elevated SO_2 levels were negatively associated with utilization and cryosurvial but were positively associated with Day 3 cell number and fragmentation (Fig. 1). In GEE analyses, SO_2 levels were positively associated with incidence of fast-cleaving embryos on Day 3 (embryos that had more than eight cells), increased fragmentation and non-synchronized cleaving on Day 3 (embryos that had even cell number) (Table IV). On the other hand, O_3 levels were positively associated with slow-cleaving embryo on Day 3 (embryos that had fewer than eight cells). For embryos subjected to blastocyst culture, delayed blastocyst development was associated with the exposure of SO_2 and O_3, while the rates of blastulation were not significantly changed across exposure quartiles. When we looked in the proportion of blastocysts derived from embryos with different Day 3 cleaving rates, we found that proportions of blastocysts derived from fast and on-time Day 3 embryos were increased in the highest quartile (Supplementary Fig. S1).

Discussion

Our data demonstrated a negative association between the concentrations of SO_2 and O_3 at the site of IVF and the live birth rate following FET cycles, suggesting a potential effect of ambient pollutants on laboratory environment. The negative association was consistently observed in unselected patients and a subgroup of patient with a good prognosis, adjusted for a series of known predictors of live birth rates. A multipollutant model suggested that the dose–response of O_3 was confounded by the presence of SO_2, while the dose–response of SO_2 was rather consistent with the presence of other monitored pollutants in the model. Other pollutants (CO and PM_{10}) were also associated with decreased pregnancy following FET in the highest quartile, but the significance diminished when evaluating their association with live birth. In contrast to the significant association between SO_2 concentrations and live birth rates observed in the FET cycles, none of the monitored pollutants was significantly associated with live birth rates in fresh cycles, regardless the fact that SO_2 was associated with a decrease in clinical pregnancy in the highest quartile.

Regarding the association between ambient air pollutant at IVF sites and IVF outcomes, Legro *et al.* (2010) showed that NO_2 at IVF laboratory was negatively and significantly associated with live birth. More recently, Choe *et al.* (2018) found that none of criteria pollutant (PM_{10}, NO_2, SO_2, CO, O_3) exposures during the period from oocyte retrieval to embryo transfer was significantly associated with IVF outcomes. In their study, however, the concentration of pollutants at the IVF site was not defined. While the data were limited, different methods of analyses and different sets of confounders adjusted for in the model further contribute to the heterogeneity. In the present study, the analyses were adjusted for a broad range of covariates and showed a negative association between SO_2 and live birth, regardless of differences in oocyte yield and the presence of top-quality embryos at transfer. The data may contribute to further discussion on this issue.

The association between exposures and several embryo development parameters, including Day 3 cleavage patterns and blastocyst growth, may support our hypothesis that outdoor air pollutant adversely affects the embryo culture environment. While overall viability of embryos, as measured by the utilization rate on Day 3, was only slightly affected, higher SO_2 exposure was associated with increased

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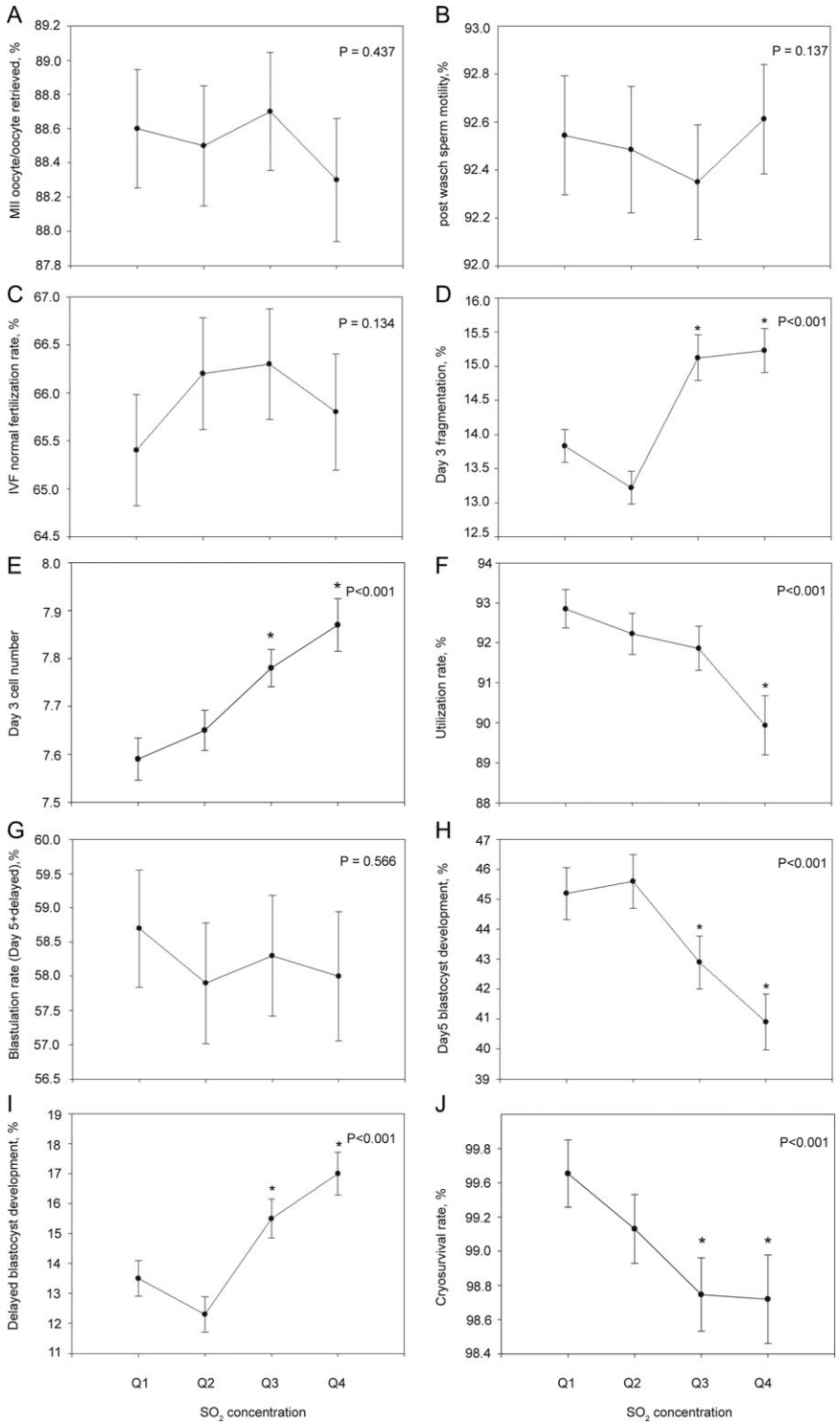

Figure 1 Changes of laboratory indicators and embryo developmental parameters across SO_2 quartiles (Q1–Q4). (**A**) maturation rates (n = 126 958,oocytes), (**B**) post-wash sperm motility (n = 16 290, cycles), (**C**) IVF normal fertilization rate (n = 93 294, oocytes), (**D**) Day 3 fragmentation (n = , embryos), (**E**) Day 3 cell number (n = 79 110, embryos), (**F**) utilization rate (n = 83 519 2PN zygotes), (**G**) blastulation rate (n = 46 976, embryos cultured), (**H**) proportion of Day 5 (n = 27 422, blastocysts) and (**I**) Day 6 blastocyst (n = 27422, blastocysts) and (**J**) cryosurvival (n = 5299, cycles). Proportion data were analyzed with Chi square test with Bonferroni adjustment. Continuous data were analyzed with Kruskal–Wallis H test. The data are mean (95% CI) or proportion (95% CI). * Indicates significant difference versus referent category (Q1). Utilized embryos were cleavage embryos that are considered suitable for ET, cryopreservation or blastocyst culture.

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Table IV Association between embryo parameters and quartiles of SO_2 and O_3.

	SO_2, μg/m^3				*P* for trend
	Q1 (2.97–9.27)	Q2 (9.28–13.50)	Q3 (13.51–18.66)	Q4 (18.67–45.74)	
[a]Non-synchronized cleaving on Day 3 (n = 79 110)	Ref	1.12 (1.06–1.18)	1.11 (1.05–1.17)	1.1 (1.02–1.19)	0.001
On-time eight-cell embryo on Day 3 (n = 79 110)	Ref	0.92 (0.87–0.97)	0.94 (0.89–1)	0.92 (0.85–0.99)	0.01
[b]Fast-cleaving embryo on Day 3 (n = 79 110)	Ref	1.11 (1.03–1.19)	1.12 (1.04–1.22)	1.24 (1.11–1.38)	0.001
[c]Slow-cleaving embryo on Day 3 (n = 79 110)	Ref	1.02 (0.96–1.08)	0.98 (0.92–1.05)	0.94 (0.86–1.03)	0.383
Compact on Day 3 (n = 79 110)	Ref	1.1 (0.95–1.26)	1.29 (1.11–1.51)	1.34 (1.09–1.66)	0.008
Fragmentation>20% (n = 79 110)	Ref	1.08 (0.94–1.23)	1.87 (1.59–2.2)	2.73(2.28–3.28)	<0.001
Delayed blastocyst (n = 27 422)	Ref	0.89 (0.82–0.98)	1.17 (1.07–1.27)	1.31 (1.20–1.43)	<0.001
	O_3, μg/m^3				*P* for trend
	Q1 (26.88–66.12)	Q2 (66.13–85.43)	Q3 (85.44–104.18)	Q4 (104.19–169.43)	
[a]Non-synchronized cleaving on Day 3 (n = 79 110)	Ref	1.03 (0.98–1.09)	1.05 (1.00–1.11)	1.07 (1.00–1.14)	0.211
On-time eight-cell embryo on Day 3 (n = 79 110)	Ref	1.01 (0.96–1.06)	1.03 (0.98–1.09)	1.09 (1.02–1.16)	0.067
[b]Fast-cleaving embryo on Day 3 (n = 79 110)	Ref	1.04 (0.97–1.12)	1.12 (1.04–1.22)	1.13 (1.03–1.24)	0.016
[c]Slow-cleaving embryo on Day 3 (n = 79 110)	Ref	0.97 (0.91–1.02)	0.90 (0.84–0.96)	0.85 (0.79–0.92)	<0.001
Compact on Day 3 (n = 79 110)	Ref	1.17 (1.02–1.35)	1.1 (0.95–1.28)	1.12 (0.94–1.32)	0.191
Fragmentation >20% (n = 79 110)	Ref	1.31 (1.14–1.51)	1.82 (1.56–2.13)	3.03(2.54–3.61)	<0.001
Delayed blastocyst (n = 27 422)	Ref	1.04 (0.95–1.14)	1.16 (1.06–1.26)	1.34 (1.23–1.46)	<0.001

[a]Non-synchronized cleaving embryo was defined as embryo with even cell number at the time of observation.
[b]Fast-cleaving embryo was defined as embryo with more than eight cells at the time of observation.
[c]Slow-cleaving embryo was defined as embryo with less than eight cells at the time of observation.

fragmentation, a more unsynchronized cleavage pattern and more fast-cleaving embryos. It is supposed that a too slow or too fast embryo cleavage rate may have a negative impact on embryo quality (Alpha Scientists in Reproductive and Embryology, 2011). Embryos in the early development stages from fertilization to compaction are supposed to be more susceptible to stresses from the environment than the morulae and blastocysts because they are undergoing the transition from maternal to embryonic genetic control and lack the regulatory transport systems created at later stages (Gardner and Kelley, 2017). It is known that, small changes in culture environment, such as culture media and oxygen concentration, may lead to changes in the timing of embryo development during early cleavage (Ciray *et al.*, 2012; Kirkegaard *et al.*, 2013). This may make the Day 3 cleavage patterns sensitive to the exposure to ambient pollutants.

The exposure was also related to an increased proportion of delayed blastulation, although the overall blastulation rates were not significantly affected. The delayed blastulation might suggest a reduced development competence. The timing of blastulation, on the other hand, might also correlate to the cleavage events in earlier developmental stages. As the morphokinetic studies have shown, a trend in development potential increased with cell number on Day 3, but fast embryos resulting from direct cleavages dividing one blastomere into to three or more may lead to a reduced developmental potential (Kong *et al.*, 2016). In our data, we found that the proportions of blastocysts derived from slow, fast and on-time embryos were significantly changed across SO_2 quartiles. On-time embryos contributed to more delayed blastocysts in higher SO_2 quartiles, which may suggest changes in cleaving events in response to exposure. While the current work is limited by the discontinuous nature of the routine morphological observation, future studies would benefit from the use of time-lapse imaging to show the association between morphokinetic events and ambient environment changes.

In vitro exposure of sodium bisulfite, the physiological form of SO_2, could lead to a decrease in cytochrome c oxidase activity, mitochondrial membrane potential, ATP content, mitochondrial DNA (mtDNA) content and mRNA expression of complexes IV and V subunits encoded by mtDNA (Ku *et al.*, 2016; Qin *et al.*, 2016). Interestingly, a recent report showed that Day 6 blastocysts have lower mtDNA contents than Day 5 blastocysts (Klimczak *et al.*, 2018), which may support the idea that mitochondrial dysfunction is correlated to delayed blastulation. Other *in vitro* effects contributing to increased viability, migration, F-actin intensity and contractility of smooth muscle cells were also documented (Song *et al.*, 2014). The effects of SO_2 on *in vitro* cell models may be helpful in understanding the potential effects of ambient SO_2 on culturing embryos. However, SO_2 is also a hydrophilic gas, with only low solubility to medical mineral oil. The direct exposure of embryos to SO_2 is yet to be determined and it may relate to the type of overlay and the time to achieve equilibrium concentration.

As a potent oxidant, O_3 may interact with various biological and environmental factors. Because the half-life of O_3 in liquid and solid media is negligible, O_3 might not easily penetrate the oil overlay of embryo culture. However, the heterogeneous reactions of O_3 with indoor material surfaces and the gas phase reactions of O_3 with indoor pollutants might produce secondary pollutants that could penetrate oil overlay. For instance, a report showed that indoor concentrations of formaldehyde may increase with the increasing outdoor O_3 concentrations (Mélanie *et al.*, 2003). Formaldehyde is toxic to the embryo

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(Thrasher and Kilburn, 2001) and may penetrate oil due to its oil solubility. Even though the source of VOC emission is carefully controlled in IVF laboratories, various species resulting from human body/O_3 interactions could still change the chemical processing indoors (Kruza *et al.*, 2017). In the outdoor ambient air, on the other hand, ground-level O_3 is a secondary pollutant produced by the interaction of sunlight with nitrogen dioxide and VOCs (Krzyzanowski, 2008). When co-existing with other pollutants, such as SO_2 and ambient particles, O_3 may take part in reactions which generate products such as ketone and sulfate that lead to serious environmental issues (He *et al.*, 2017). The reactions indoors and outdoor may relate to the interaction between O_3 and SO_2 in our study and the confounding of its effect by the presence of other pollutants. The mode of actions of O_3 may also explain the conflicting conclusions regarding the effects of O3 on IVF outcomes in previous studies.

The composition of air pollutants and the mode of their interaction may vary from area to area. For instance, SO_2 concentrations were always lower than the detectable level in the study of Carre *et al.* (2017a, 2017b) and ranged from ~5 to 60 parts per billion in two other studies (Legro *et al.*, 2010; Choe *et al.*, 2018). Notably, the SO_2 concentrations in the study of Legro *et al.* (2010) were higher than those in the present study. However, the negative association between SO_2 and O_3 in their data may imply a different mode of interaction between pollutants than in the present study. To our knowledge, there are limited data regarding the effect of ambient pollutants on FET outcomes in other IVF sites with a different composition of air pollutants. Further studies are warranted to confirm our findings.

The differential effect of outdoor air pollution on fresh and FET cycles might relate to the stress caused by the cryotechnologies. The physical and chemical stress during cryopreservation is supposed to result in a series of adverse effects, including destruction of cellular membranes, redistribution of the cytoskeleton, organelle dysfunction, pH shifts and general protein denaturation (Kopeika *et al.*, 2015). The structural integrity and functional viability of mitochondria were significantly disrupted in cryopreserved animal gametes and embryos, following either slow freezing or vitrification (Ahn *et al.*, 2002; Zhao *et al.*, 2009; Somoskoi *et al.*, 2015; Nohales-Corcoles *et al.*, 2016; Amoushahi *et al.*, 2017; Sefid *et al.*, 2017; Sowinska *et al.*, 2017; Succu *et al.*, 2018). In human embryos, the oxygen consumption and mitochondrial activity are greatly reduced during the procedure of freezing–thawing and take hours to recover after thawing (Yamanaka *et al.*, 2011). Although most of the cryopreserved embryos may survive, and, as some embryologists believe, may even benefit from the process due to a supposed hormesis effect (Vladimirov *et al.*, 2017), it may also make the embryos vulnerable during the process. For embryos with an impaired precryopreservation mitochondrial activity, the mitochondrial activity after thawing might decrease to below the minimal threshold that allows implantation. As previous studies have shown, ambient pollutants, such as SO_2, and secondary pollutants, such as formaldehyde, might all contribute to impaired mitochondrial activity (Zerin *et al.*, 2015; Ku *et al.*, 2016; Qin *et al.*, 2016), and thus, potentially affect developmental potential after embryo thawing. In fresh cycles, in contrast, the mitochondria dysfunction could be compensated for by the increase in the mitochondrial number during later development, and transfer of embryos to the female reproductive tract may provide further protection against associated detrimental effects, such as oxidative stress (Ng *et al.*, 2018). Although our data could not provide direct evidence, the decreased cryosurvival in the highest exposure quartile may support the hypothesis.

Consistent with several previous studies, the study was based on regional monitoring data rather than a direct measurement of exposure. This was a significant limitation because the monitor data are not only associated with the exposure at IVF sites but also reflect the exposure background of the general population in a given region during the certain period. SO_2 levels have fallen in most parts of the world, including China, where a decreasing trend of SO_2 pollutant has been observed since 2011 (Liu *et al.*, 2017). The improved IVF outcomes with decreasing SO_2 levels may relate to the reduced exposure of the population and/or the improved air quality at the IVF site. Nevertheless, the observation that air pollutants correlated to live birth following FET but not fresh embryo transfer still implied an interaction between the ambient environment and *in vitro* procedures.

The study was also limited by its retrospective nature, as the demonstrated association might be confounded by known or unknown factors. Due to the limitation of our Electronic Medical Records, we could not analyze the information regarding the patients' smoking history or alcohol use routinely. However, according to our experience and published data, the prevalence of smoking and drinking is very low in Chinese women (Chen *et al.*, 2015; Millwood *et al.*, 2017). Therefore, it is reasonable to hypothesize that not adjusting for smoking and alcohol use may not skew the result significantly. Also, lack of reproducibility of the observations might relate to detailed culture conditions that may vary among clinics, such as the type of incubator and the preparation of oil overlay.

Although a strength of the current study is its robust sample size, we still found some associations were of marginal significance. Pollutants such as CO and PM_{10} were significantly associated with clinical pregnancy but not live birth. Significant associations between live birth and these pollutants may be detected with a larger sample size.

In summary, our data have demonstrated a significant association between the concentrations of ambient air pollutants at/near the IVF site and live birth following FET, whereas no significant effect of air pollutants on the live birth rates following fresh ET was observed. While inhalation is the only route of exposure for many ambient pollutants in the general population, our observation highlighted that additional *in vitro* procedures may introduce additional risks with regard to health effects of air pollutants. For decades, cumulative observations from various IVF laboratories highlighted the role of air quality control in achieving optimal IVF outcomes. VOCs and air particles were of major concerns. Modern IVF laboratories use HEPA filtration, a dedicated air handling unit or a dedicated supply duct to control airborne contaminants (Mortimer *et al.*, 2018). However, it is not clear whether the same mechanisms can effectively handle inorganic ambient pollutants, such as SO_2. A previous study showed that the inside laboratory SO_2 concentration declined from 1.07 to 0.29 mg/m^3 after installation of a specially treated honeycomb matrix media-fitted air filtration system, whereas the traditionally used CODA system seems to make little improvement in the same laboratory (Khoudja *et al.*, 2013). However, the study compared the data collected from different months within a year and the effect of seasonal change in SO_2 concentrations could not be excluded, especially when a decrease in SO_2 was also observed in the hallway outside the laboratory at the same time. Our observations together with other studies imply that additional protection against outdoor pollutants may further improve the embryo culture conditions.

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Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

X.W., J.C. and L.L. contribute to conception and design. X.J., P.L., A.S. and J.R. contribute to acquisition of data. X.W., J.C. and L.L. contribute to analysis and interpretation of data. All authors contribute to drafting the article or revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Conflict of interest

None declared.

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