Ambient outdoor air pollutants and sex ratio of singletons born after in vitro fertilization: the effect of single blastocyst transfer

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Objective: To evaluate the impact of air pollution on the sex ratio in singletons after IVF treatment and to evaluate the influence of the number of and the developmental stage of transferred embryos on the sex ratio.
Design: Retrospective cohort study.
Setting: University-affiliated IVF unit.
Patient(s): A total of 7,004 singletons born after fresh transfer or frozen-thawed embryo transfer (FET) between January 2013 and December 2017.
Intervention(s): None.
Main Outcome Measure(s): Male-to-female ratio in live-born singletons.
Result(s): The estimated medians (interquartile range) of particle matter $(PM)_{10}$, $PM_{2.5}$, CO, NO_2, O_3, and SO_2 at the IVF site were 51.4 (39.5–64.6), 27.7 (20.7–37.4), 0.62 (0.5–0.72), 32.5 (25.4–40.1), 79.6 (63.3–96.6), and 11.9 (9.3–15.9) $\mu g/m^3$, respectively. Multivariate analysis indicated that SO_2 was the only pollutant clearly associated with sex ratio. In singletons from single blastocyst transfer (SBT), as indicated by the generalized additive model, the SO_2 concentration and sex ratio showed an inverted-U-shape association. In singletons after non-SBT, a monotonic decreasing in the sex ratio was observed with increased SO_2 concentration. Compared with the referent category ($SO_2 < 7.57\ \mu g/m^3$), the sex ratio at the 5th decile of SO_2 (10.81–11.94 $\mu g/m^3$) was increased by 2.1-fold (95% confidence interval [CI], 1.3–3.14) after adjusting covariates. In singletons born from non-SBT, the sex ratio significantly decreased only in the 9th (odds ratio = 0.69; 95% CI, 0.53–0.90) and 10th (OR = 0.74, 95% CI, 0.56–0.98) deciles.
Conclusion(s): Low concentrations of SO_2 showed an association with increased sex ratio in singletons of SBT, while in singletons born from another ET system the sex ratios did not show an association at low concentrations of SO_2. (Fertil Steril® 2020;113:140–8. ©2019 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Air pollution, in vitro fertilization, sex ratio, single blastocyst transfer, SO_2

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The sex ratio of newborns after spontaneous conception is considered to be constant and slightly biased toward men, with a male-to-female ratio of approximately 1.05–1.07 (1). However, changes in the ratio of male to female have been reported in the population born after assisted reproductive technology (ART) (2–4). The proportion of males significantly increased in offspring born from IVF and blastocyst transfer. Patient etiologies such as semen quality (5) and paternal obesity (6) and ART procedures such as sperm preparation (7), in vitro culture (8), and embryo selection (9) have been proposed as underlining mechanisms of the skewed sex ratio. Among these factors, the in vitro

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culture condition is a unique exposure factor for IVF population. It is well known that male and female embryos may respond differently to in vitro culture systems, and extended culture is more favorable for male embryo development (10). Subtle changes in culture conditions, such as the composition of the culture media, may have an impact on embryonic development, neonatal outcomes, and sex ratio at birth (8, 11, 12). Since current ET strategies rely heavily on embryonic morphology and developmental assessment, embryo selection may have a synergistic effect with embryonic culture environment/condition to impact the sex ratio.

In IVF laboratories, the embryo culturing environment could be affected by several factors, such as medium (11) and incubator parameters (13) and environmental conditions such as temperature, humidity (14), and concentration of air pollutants (15–17). Seasonal changes in outdoor temperature and humidity (14) or human activities in areas around IVF locations, such as pesticide use, traffic emissions, and road resurfacing (15, 16), may affect laboratory air quality and consequently reduce the success rate of IVF. More specifically, Legro et al. (17) pointed out that outdoor air quality, measured as six standard pollutants at the IVF site, is significantly associated with live birth rates. Moreover, in previous studies, we have demonstrated that there is a link between air pollution and embryonic developmental parameters (18). The presence of higher concentrations of air pollutants at an IVF unit may result in faster embryo cleavage, while the blastocysts tend to show delayed development (18). The data indicate that air pollution at the IVF site has an impact on in vitro culture conditions. From this result, we hypothesize that the effects of air pollutants in the IVF unit site may further affect subsequent embryo selection and could skew the sex ratio. In this study, we aimed to investigate the association between estimated air pollution at the IVF site and the sex ratio after ET and to assess whether this association was modified by the embryo selection.

MATERIALS AND METHODS

Study Subjects

All patients who underwent fresh transfer or frozen-thawed ET (FET) in the Affiliated Chenggong Hospital of Xiamen University in the period between January 2013 and December 2017 were accessed for potential inclusion. None of the patients received preimplantation genetic testing. Patients who gave birth to a singleton were included, whereas patients with multiple pregnancies, incomplete neonatal outcomes, or an ovum delivery before January 18, 2013 (due to air quality data being unavailable before January 2013) were excluded. Institutional Review Board approval for this retrospective study was obtained from the Ethics Committee of the Medical College of Xiamen University.

Laboratory Protocol

The patient underwent a conventional stimulation protocol or a mild protocol without a GnRH analog, as described elsewhere (19). The physician adjusted the starting dose based on the patient's age, body mass index (BMI), and ovarian reserve. BMI was measured in the physician`s office. When at least one follicle reached an average diameter of 18 mm as determined by ultrasound, hCG was administrated. Oocytes were recovered 34–36 hours after hCG administration and underwent conventional IVF or intracytoplasmic sperm injection (ICSI) treatment.

Semen parameters were evaluated with a semen analyzer (SQA-V, MES) as well as microscopic observation. For standard IVF or ICSI, semen was collected by masturbation and prepared by centrifugal fractionation (350 *g*, 10 minutes) using a sperm isolation medium (Isolate, Irvine Scientific). For patients with azoospermia, testicular or epididymal sperm aspiration was used to produce the sperm.

All embryos were cultured in traditional incubators (C200, Labotect) without an additional incubator filter box at 37°C, 6% CO_2, 5% O_2. We used Cook IVF media (Cook Medical) for both cleavage-stage embryos (K-SICM) and blastocysts (K-SIBM) cultures.

The quality of the blastocysts was assessed by the Gardner scale (20). Only cleavage-stage ($\geq$grade 3) and blastocysts (>4CC, expanded blastocyst with grade C inner cell mass [ICM] and grade C trophectoderm [TE]) with acceptable morphological scores were considered for transfer. Grade 1 embryos on day 3 (with <10% fragmentation and on-time cell size) or AA blastocyst on day 5 (blastocysts with grade A ICM and grade A TE) were defined as top-quality embryos. We determine the type of embryo to be transferred based on the patient's preferences, with written consent.

In FET, the embryos were selected based on their morphology before cryopreservation. A vitrification protocol, using 15% dimethyl sulfoxide, 15% ethylene glycol, and 0.6 M sucrose as cryoprotectants, was used (21). For blastocysts, blastocoelic volume was reduced before cryopreservation using a laser system (Saturn, RI).

Laboratory Air Quality Control

The IVF laboratory was located on the top floor of a 23-floor building. The air quality of the laboratory was controlled by a centralized high-efficiency particulate air (HEPA) filtration system to reduce the concentration of particles in the air and a stand-alone Coda tower system unit to control the concentration of volatile organic compounds. The HEPA filter was replaced annually.

Exposure Estimation

The exposure to air pollutants at the IVF site was evaluated as daily concentrations of pollutants during laboratory culture (from oocyte retrieval to fresh transfer or cryopreservation) (18). The pollutant concentration at the IVF site was estimated by accessing air-quality data from all the three state-controlled monitoring stations in Xiamen, Fujian, People's Republic of China. These data were obtained from the 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 8-hour rolling averages of the value. The environmental protection agency releases hourly air-quality data for particle matter (PM)$_{10}$, $PM_{2.5}$, CO, SO_2, NO_2, and O_3 according to an inverse distance weighting interpolation modeling method, which considers the spatial distribution of pollutants and is commonly used to estimate spatial air pollutant distribution based on data from fixed monitoring stations (22). 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.

Statistical Analysis

The sex ratio was the primary outcome, and it was determined by the ratio of males to females in a population. The secondary outcome was implantation in the single blastocyst transfer (SBT) subgroup, which is determined by the presence of gestational sac (under ultrasound scan) 4 weeks after ET. Miscarriage was defined as an intrauterine pregnancy failing to reach 22 weeks of pregnancy.

Because nonlinear dose response is often observed in toxicological studies, the generalized additive model (GAM) was used to estimate the association of the sex ratio with air pollution levels. GAMs are regression models involving a sum of smooth functions of covariates instead of linear covariates. We fitted GAM as follows:

$$Logit(y) = X\theta + s_1(X_1) + s_2(X_2) + \cdots + Interception$$

Smooth functions $s(X)$ are penalized cubic regression splines modeling the contribution of estimated levels of the six criteria air pollutants. The term $X\theta$ represents the linear components of the model. The linear covariates were selected based on previous knowledge, including maternal age, paternal age, male BMI (6), sperm motility (5), duration of in vitro culture (day 3, day 5, and day 6), and insemination protocol (IVF, ICSI with ejaculated sperm, and ICSI with nonejaculated sperm) (8). We also adjusted for ovarian stimulation protocol (agonist, antagonist, and other), male smoker, year of delivery, type of transfer cycle (fresh and FET), and quality of embryo transferred (at least one top-quality embryo transferred) because of their potential confounding.

There were four ET strategies in the study: SBT, double blastocyst transfer (DBT), single cleavage-stage ET (SET), and double cleavage-stage ET (DET). To compare trends in the differences between ET and selection strategies, GAMs were also used to estimate trends in the sex ratios at ET and selection strategies. The fitted smooth functions were compared for selected ET strategies via the use of a prediction matrix (23). We also analyzed air pollutants as categorical data. The estimated ambient concentrations of air pollutants were categorized into deciles and fitted into a logistic regression model. All statistical analyses were performed with the R statistical software, version 3.5.1 (24) and the mgcv package (25).

RESULTS

Patients who received IVF-ET treatment during the period between January 18, 2013, and December 31, 2017, in our clinic gave birth to 7,695 singletons. From this, we excluded cases with vanishing twin or embryo reduction (n = 688) and incomplete record (n = 3). All singletons included (n = 7,004) were derived from a single gestational sac. The male-to-female ratio in the population was 1.19. The DET and SET contributed to a sex ratio of about 1.06, while SBT and DBT contributed to a sex ratio biased toward males. The detailed characteristics of the population and the estimated medians [interquartile range] of six air pollutants (PM_{10}, $PM_{2.5}$, CO, NO_2, O_3, and SO_2) at the IVF site are shown in Table 1. The cycles leading to male singletons seem to be exposed to higher levels of PM_{10} and SO_2 (Table 1).

Using GAM models, we demonstrated the dose-dependent relationship between the air pollutants levels at the IVF site and the male-to-female ratio in singletons after IVF-ET. The sex ratio in the overall IVF-ET population showed no significant association with any of the six pollutants (Fig. 1 and Supplemental Fig. 1). However, in the singletons derived from SBT, an inverted-U-shape relationship was observed between SO_2 and sex ratio (Fig. 1). In the population without SBT, the sex ratio tends to decrease with exposure to higher SO_2 concentration. On the other hand, there was no significant association between pollutants other than SO_2 and sex ratio in populations with or without SBT (Supplemental Fig. 1). As the concentration of SO_2 increases, the estimated smoothed sex ratio of SBT is significantly different from non-SBT populations (Supplemental Fig. 2). In non-SBT populations, there was no significant sex ratio difference in trends between ET types (Supplemental Fig. 3). Accordingly, we combined the types of ET in the non-SBT population to increase the efficiency of the statistical analyses.

When SO_2 concentrations were categorized into deciles, the change of the sex ratio across the SO_2 categories was similar to what was shown in GAM (Table 2). When SO_2 concentrations were $<12\ \mu g/m^3$, the sex ratio tended to be elevated at higher SO_2 categories in the SBT population. However, the size of the association between the sex ratio and SO_2 tended to decrease when SO_2 concentrations were $>12\ \mu g/m^3$. In the non-SBT population, the sex ratio tended to be decreased at higher SO_2 concentrations. In the 9th and 10th decile concentrations, the sex ratio was significantly decreased. The crude sex ratios across SO_2 deciles with respect to different types of ET strategies are shown in Supplemental Table 1.

The ET strategy-specific association led to the suspicion that the association between SO_2 exposure and sex ratio was related to the criteria of blastocyst selection. We evaluated the difference in trend between parameters with respect to embryo selection, including blastocyst quality (good vs. fair), ICM (grade A vs. non–grade A), and TE score (grade A vs. non–grade A). Although degree of expansion is also an important morphological parameter for blastocysts, most (98.1%) singletons after SBT were derived from blastocysts with grade 4 expansion. Therefore, it is impossible to compare the difference between different degrees of expansion.

There was no significant difference of association between blastocysts of good quality and fair quality and grade A ICM and non–grade A ICM. However, the association

TABLE 1

Patient characteristics, cycle parameters, and concentrations of air pollutants at the IVF site in cycles that resulted in male or female singletons.

Variable	All	Female	Male	*P* value	Male to female (95% confidence interval)	
n (%)	7,004 (100)	3,198 (45.7)	3,806 (54.3)		1.19	(1.15–1.23)
PM_{10}, $\mu g/m^3$	51.4 [39.5–64.6]	50.9 [39.3–63.9]	52 [39.9–65]	.028	—	—
$PM_{2.5}$, $\mu g/m^3$	27.7 [20.7–37.4]	27.6 [20.6–37.1]	28 [20.9–37.8]	.063	—	—
CO, $\mu g/m^3$	0.617 [0.503-0.718]	0.618 [0.504-0.716]	0.616 [0.502-0.722]	.606	—	—
NO_2, $\mu g/m^3$	32.5 [25.4–40.1]	32.1 [25.2–39.7]	32.7 [25.6–40.6]	.058	—	—
O_3, $\mu g/m^3$	79.6 [63.3–96.6]	79 [63.1–95.9]	80.1 [63.7–97.1]	.089	—	—
SO_2, $\mu g/m^3$	11.9 [9.3–15.9]	11.8 [9.2–15.6]	12.1 [9.3–16.1]	.015	—	—
Maternal age	30 [28–33]	30 [28–33]	30 [28–34]	.875	—	—
Paternal age	32 [29–36]	32 [29–36]	32 [29–36]	.862	—	—
Maternal BMI	20.9 [19.4–22.7]	20.8 [19.4–22.6]	21 [19.4–22.8]	.934	—	—
Paternal BMI	23.6 [21.3–25.9]	23.6 [21.3–25.8]	23.6 [21.3–25.9]	.519	—	—
Paternal sperm motility, %	47.8 [29.1–60.4]	47.7 [28.7–60.2]	47.9 [29.7–60.5]	.135	—	—
Paternal smoker, n (%)	3,404 (48.6)	1,542 (48.2)	1,860 (48.9)	.586	—	—
Year of delivery, n (%)				.744		
2013	142 (2)	67 (2.1)	75 (2)		1.12	(0.89–1.41)
2014	941 (13.4)	450 (14.1)	491 (12.9)		1.09	(1–1.19)
2015	1,193 (17)	545 (17)	645 (16.9)		1.18	(1.09–1.28)
2016	1,455 (20.8)	665 (20.8)	793 (20.8)		1.19	(1.11–1.28)
2017	1,807 (25.8)	817 (25.5)	990 (26)		1.21	(1.13–1.29)
2018	1,466 (20.9)	654 (20.5)	812 (21.3)		1.24	(1.15–1.34)
Stimulation protocol						
Agonist	6,046 (86.3)	2,749 (86)	3,297 (86.6)	.314	1.20	(1.16–1.24)
Antagonist	853 (12.2)	406 (12.7)	447 (11.7)		1.10	(1–1.21)
Other	105 (1.5)	43 (1.3)	62 (1.6)		1.44	(1.09–1.91)
Insemination protocol, n (%)				.579		
IVF	5,090 (72.7)	2,305 (72.1)	2,785 (73.2)		1.21	(1.16–1.26)
ICSI + ejaculated sperm	1,518 (21.7)	710 (22.2)	808 (21.2)		1.14	(1.06–1.22)
ICSI + nonejaculated sperm	396 (5.7)	183 (5.7)	213 (5.6)		1.16	(1.01–1.34)
Transfer cycle, n (%)				.002		
Fresh	4,427 (63.2)	2,085 (65.2)	2,342 (61.5)		1.12	(1.08–1.17)
Frozen-thawed	2,577 (36.8)	1,113 (34.8)	1,464 (38.5)		1.32	(1.24–1.39)
Duration of in vitro culture, day				.001		
3	4,288 (61.2)	2,056 (64.3)	2,232 (58.6)		1.09	(1.04–1.13)
5	2,502 (35.7)	1,059 (33.1)	1,443 (37.9)		1.36	(1.29–1.44)
6	214 (3.1)	83 (2.6)	131 (3.4)		1.58	(1.29–1.93)
Type of transfer, n (%)				.362		
Single ET	1,095 (15.6)	523 (16.3)	572 (15.0)		1.09	(1.01–1.19)
Double ET	3,193 (45.6)	1,533 (47.9)	1,660 (43.6)		1.08	(1.03–1.14)
Single blastocyst transfer	1,905 (27.2)	795 (24.9)	1,110 (29.2)		1.40	(1.31–1.49)
Double blastocyst transfer	811 (11.6)	347 (10.9)	464 (12.2)		1.34	(1.21–1.48)
At least one top-quality embryo transferred, n (%)	5,542 (79.1)	2,515 (78.6)	3,027 (79.5)	.025	1.20	(1.16–1.25)

Note: Data are presented as median [interquartile range] or count (percentage). BMI = body mass index; ICSI = intracytoplasmic sperm injection.

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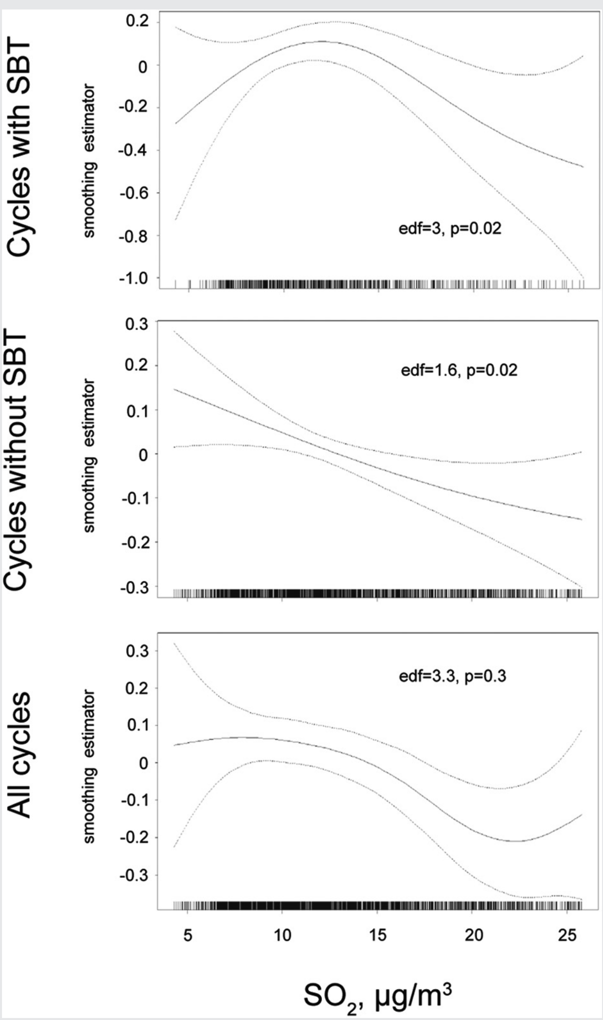
between sex ratio and SO_2 was significantly different between singletons after SBT with grade A TE and non–grade A TE (Fig. 2). The inverted-U-shape relationship was only observed in singletons with non–grade A TE of SBT. In singletons of SBT with grade A TE, the association between SO_2 and sex ratio was insignificant and showed a curve similar to that of the non-SBT population. The patterns of implantation and pregnancy loss changing with increased SO_2 exposure were similar between singletons after SBT with grade A and non–grade A TE (Fig. 2).

DISCUSSION

This study demonstrated that the outdoor air concentration of SO_2 at the IVF site showed an association with the male-to-female sex ratio. Additionally, the association was affected by the strategy of ET. In the SBT population, the low concentration of SO_2 was associated with an increased sex ratio. GAM analyses showed an inverted-U-shape smooth trend indicating the concentration of SO_2 exerted an effect on the sex ratio of the SBT population. On the other hand, in non-SBT populations the increase of SO_2 concentration was associated with a decrease of the sex ratio. The observed differences among ET strategies suggested that environmental factors may interact with laboratory techniques in determining the fate of the next generation after ART.

The observation of the present study is supported by our previous study (18), in which we reported that outdoor air pollutants, particularly SO_2, are associated with embryo development and live birth rate in IVF treatment (18). Nevertheless, most data on SO_2 concentrations were lower than the

FIGURE 1



Association between concentrations of SO_2 and male-to-female ratio at birth in multipollutant generalized additive models in singletons conceived through single blastocyst transfer (SBT) and non-SBT cycles. All models were adjusted for paternal body mass index, paternal sperm motility, paternal smoker, year of delivery, insemination protocol, transfer cycle, at least one top-quality embryo transferred, and concentrations of CO, NO_2, O_3, $PM_{2.5}$, and PM_{10}. The smoothing estimator indicates the contribution of the covariate fitted to a given smooth function to the response variable; edf indicates the estimated degree of freedom of the smooth function. Solid lines indicate response curves, and dotted lines indicate 95% confidence intervals.

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guideline values provided by the World Health Organization (26). Determined graphically based on the estimated smooth trend, the inflection point of the inverted-U-shape dose-response curve of SO_2 was approximately 12–14 $\mu g/m^3$, which is much lower than the annual and seasonal average concentration of SO_2 in many regions of China (27). If the analysis is performed in a more contaminated area, the dose-response curve can move to the right and show a downward trend.

The biased sex ratio after SBT is known to be associated with sex-differential morphological and developmental characteristics of blastocysts. Male blastocysts may have better morphological scores and grow faster than female blastocysts. Comprehensive chromosome screening data suggest that transferring advanced blastocysts may increase the proportion of males (28). This implies that the currently used embryo scoring system accounts for a large portion of the sex ratio skew at birth. Although sibling embryos are affected by the same culture environment, only one embryo is selected for transfer in SBT. Therefore, the effect of SO_2 on sex ratio among embryos with different scoring parameters was examined (Fig. 2). The examination indicated that there is no association between SO_2 and sex ratio in singletons derived from blastocysts with grade A TE. On the other hand, in those conceived through transfer of blastocysts with grade B or C TE, the sex ratio was significantly associated with SO_2 concentrations. It appears that the effect of SO_2 on sex ratio is only obvious when blastocysts with grade B or C TE are selected. The finding may also support our hypothesis that the difference in sex ratio was the embryo origin.

Embryos cultured in vitro may respond to stress in a sex-differentiated manner. A recent study on the effect of 2, 2`-Azobis (2-amidinopropane) dihydrochloride and menadione showed that male embryos are more resistant to oxidation than female embryos in the media supplemented with fetal calf serum (29). Male blastocysts under induced oxidative stress had a higher total cell count and lower apoptotic rate than female blastocysts (29). This may suggest that male blastocysts are largely preferred if one blastocyst is selected from the embryo cohort under induced oxidative stress based on embryonic scoring criteria based on TE and ICM cell numbers. Our previous study showed that an elevated SO_2 concentration was associated with a higher proportion of delayed blastocysts during in vitro culture while the overall blastulation rate was unaffected. This suggests SO_2 may also induce stress that delays the development of blastocysts. In in vitro studies, SO_2 is known to decrease cell viability via inducing oxidative stress (30, 31). The induced oxidative stress may derive from an impairment in the reactive oxygen species–scavenging system, such as glutathione-S-transferase. Disruption in the glutathione-S-transferase system may not only affect the detoxification of xenobiotics but may also result in mitochondrial dysfunction and alternations in signal cascades that regulate apoptosis and cell survival (32). A differential effect of SO_2-induced stress on male and female blastocysts could also be expected if the same mechanisms that respond to the sex-differential effects of oxidative stress in bovine blastocysts are also applicable in humans.

The sex ratio at birth may also be affected by a sex-selective embryo loss during pregnancy (1). Blastocysts with better-looking TE are known to be associated with higher implantation (33). However, we found that the effect of SO_2 on implantation and pregnancy loss was similar between SBT cycles transferred with grade A TE blastocysts and non–grade A TE blastocysts. Interestingly, the dose-response curve for implantation and pregnancy loss also appeared to be biphasic: implantation and miscarriage decrease and increase, respectively, when SO_2 concentrations are $>$12–14 $\mu g/m^3$ (Fig. 2).

TABLE 2

Multivariate analyses for male singleton associated with SO_2 deciles.

SO_2 deciles	Adjusted odds ratio for male singleton (95% confidence interval)			Crude male-to-female ratio (95% confidence interval)		
	All	SBT	Non-SBT	All	SBT	Non-SBT
D1 (<7.57 μg/m^3)	Ref.	Ref.	Ref.	1.25 (1.12–1.39)	1.07 (0.88–1.31)	1.32 (1.17–1.5)
D2 (7.57–8.7 μg/m^3)	1.01 (0.81–1.25)	1.57 (1.02–2.42)	0.83 (0.64–1.08)	1.22 (1.10–1.35)	1.49 (1.22–1.82)	1.12 (0.99–1.27)
D3 (8.71–9.77 μg/m^3)	1.09 (0.87–1.37)	1.62 (1.05–2.5)	0.93 (0.71–1.21)	1.33 (1.19–1.48)	1.43 (1.19–1.73)	1.28 (1.12–1.45)
D4 (9.78–10.81 μg/m^3)	1.01 (0.79–1.26)	1.47 (0.94–2.33)	0.81 (0.62–1.05)	1.18 (1.06–1.31)	1.32 (1.09–1.6)	1.13 (0.99–1.28)
D5 (10.82–11.94 μg/m^3)	1.17 (0.92–1.50)	2.10 (1.3–3.41)	0.86 (0.65–1.15)	1.36 (1.22–1.51)	1.66 (1.36–2.02)	1.24 (1.09–1.41)
D6 (11.95–13.2 μg/m^3)	0.89 (0.69–1.15)	1.44 (0.86–2.41)	0.70 (0.52-0.95)	1.04 (0.93–1.15)	1.36 (1.11–1.67)	0.94 (0.83–1.06)
D7 (13.21–14.96 μg/m^3)	1.16 (0.89–1.51)	1.89 (1.09–3.29)	0.89 (0.65–1.22)	1.36 (1.22–1.51)	1.82 (1.49–2.22)	1.19 (1.04–1.35)
D8 (14.97–17.1 μg/m^3)	0.95 (0.72–1.26)	1.34 (0.73–2.46)	0.82 (0.59–1.14)	1.11 (0.99–1.23)	1.35 (1.07–1.69)	1.05 (0.93–1.18)
D9 (17.2–20.65 μg/m^3)	0.89 (0.66–1.20)	1.25 (0.66–2.39)	0.66 (0.46–0.94)	1.10 (0.99–1.22)	1.31 (1.04–1.65)	1.05 (0.93–1.18)
D10 (>20.71 μg/m^3)	0.85 (0.60–1.21)	0.93 (0.43–2.06)	0.72 (0.47–1.10)	1.03 (0.93–1.14)	1.06 (0.81–1.38)	1.02 (0.91–1.15)

Note: All models were adjusted for maternal age, paternal age, paternal body mass index, paternal sperm motility, paternal smoker, year of delivery, ovarian stimulation protocol insemination protocol, transfer cycle, at least one top-quality embryo transferred, and concentrations of $PM_{2.5}$, PM_{10}, CO, NO_2, and O_3. D = decile; SBT = single blastocyst transfer; Ref. = referent category.

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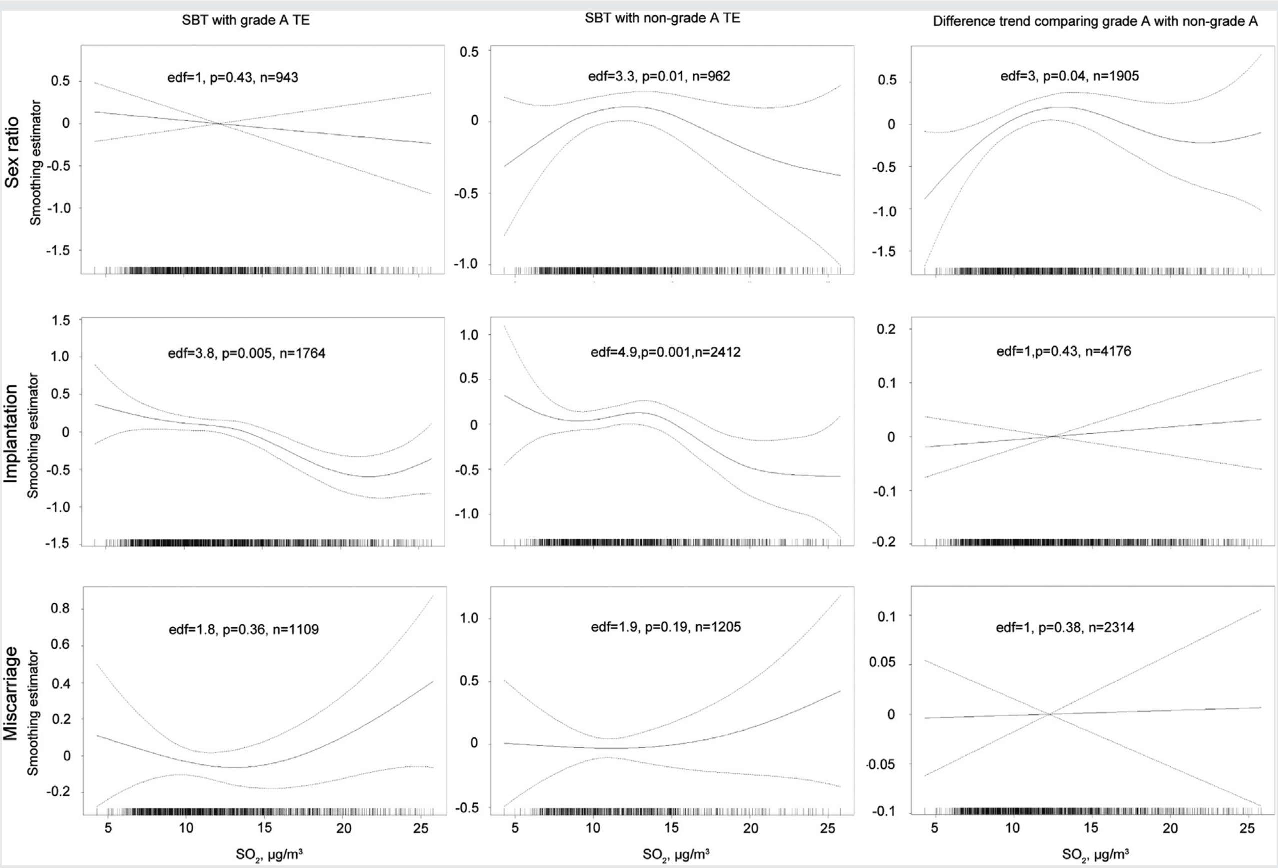
FIGURE 2



Differences between single blastocyst transfer (SBT) cycles with and without embryos with grade A trophectoderm (TE) in the association between SO_2 concentrations and sex ratio and implantation and pregnancy loss. Generalized additive models were adjusted for paternal body mass index, paternal sperm motility, paternal smoker, year of delivery, insemination protocol, transfer cycle, at least one top-quality embryo transferred, and concentrations of $PM_{2.5}$, PM_{10}, CO, NO_2, and O_3. The smoothing estimator indicates the contribution of the covariate fitted to a given smooth function to the response variable; edf indicates the estimated degree of freedom of the smooth function. Solid lines indicate response curves, and dotted lines indicate 95% confidence intervals.

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Within the same dose range, the estimated smooth trend for sex ratio shifted from upward to downward. Taken together, the dose-response curves may reveal a transition of the toxicological mechanism of SO_2: the sex-differential effects of SO_2-induced stress might be overwhelmed by the detrimental effects on embryo viability at higher concentrations.

Epidemiologists use the sex ratio at birth as an indicator of reproductive health risk factors for the general population (34). Exposure to environmental pollutants such as persistent organic pollutants may result in a decrease in the sex ratio at birth (35). With respect to air pollutants, Lin et al. (36) suggested that parental exposure to ambient air pollutants, particularly PM_{10} and SO_2, a few days before conception may lead to a higher chance of giving birth to female babies. The underlying mechanism of such an observation is still not clear. The mechanisms may relate to disrupted parental hormonal condition due to exposure, increased spontaneous abortion and stillbirth, or gender-biased fetal mortality in male infants (36). These theories suggest that male embryos are more susceptible to external exposure than female embryos. The decreasing trend in sex ratio was also observed in our study cohort in both SBT and non-SBT populations exposed to higher concentrations of SO_2. It may be due to either the detrimental effects of culture environment in vitro or background exposure to parents before conception in vivo. In comparison with the general population, in vitro culture in an IVF population may be a potential effect modifier on the association between exposure and sex ratio.

The observed difference between SBT and non-SBT populations may primarily result from embryo selection strategies. In cleavage stages, the transfer of an “on time” embryo is preferred over advanced embryos (37), which minimizes the effect of alternate embryo growth. On the other hand, in DET, there may be some bias or confounders due to the increased complexity of embryo selection and interembryo interaction. The transfer of a high-score embryo along with a low-score one may adversely affect the high-score embryos (38, 39), suggesting that the surviving embryos in DET are the consequence of an in utero interaction between transferred embryos. It is not clear whether sex dimorphic embryo development or sex-specific pregnancy loss plays a role in such observations. Therefore, singletons derived from SET may be a more appropriate model to discuss the underlying factors that influence the sex ratio at birth.

In our study, only singletons derived from a single gestational sac were analyzed to minimize the effect of vanishing twin or multifetal pregnancy reduction. Therefore, this population is biased compared with the general IVF population. If multiple pregnancies are considered, either twin births may be associated with a decrease in sex ratio (40) or multiple births are not associated with sex ratios (3). The inconsistencies may indicate that the proportion of sex in the overall population conceived by IVF is more affected by unadjusted factors such as obstetric factors associated with multiple pregnancies.

To the best of our knowledge, this study is the first large-scale cohort study to investigate the effects of air pollution on sex ratio in IVF babies. The finding is also fortified by a clear dose response demonstrated by GAM analyses, which allows a simple interpretation of a causal relationship. Retrospective design is a major limitation of this research. A retrospective study is influenced by the selection bias of the patients, and the data are limited to records that have already been collected. The study lacked prospectively collected exposure data or data on the sex of the transferred embryo. Since the sex ratio at birth is related not only to the sex of the transferred embryo but also to spontaneous abortion or other sex-selective fetuses during pregnancy, it cannot be ruled out that parental background exposure leads to a skewed sex ratio.

We found no significant association between sex ratio and air pollutants other than SO_2. This may suggest that these pollutants have no visible effect on sex ratio within the range of concentration evaluated in the study. However, it could also be interpreted as a variation of a lab’s air purification ability. While HEPA removes most of the particulate material (0.3 microns) in the air, its effects on other pollutants is less clear. Khoudja et al. (40) observed that with a change of the HEPA air filter, the NOx concentration in the lab was reduced by almost half, while the concentration of SO_2 remained stable. The nature of a pollutant also affects its ability to penetrate the oil overlay and become present in the culture medium. Future investigation on the presence of pollutants in the medium is needed to confirm the relationship between air quality at the IVF site and IVF outcomes.

The reproducibility of our results could be affected by many factors including laboratory settings, population background, and the composition of air pollutants at the IVF site. Among these factors, the variability of culture media used is known to be related to the alternating sex ratio. Even from the same manufacturer, embryos cultured in G5 PLUS appear to have a lower sex ratio than embryos cultured in G5 (8). Animal studies have shown that the composition of culture media may alter the effects of environmental stress (29). When the culture media is changed from fetal calf serum supplemented medium to bovine serum albumin with a mixture of insulin, transferrin and selenium supplemented medium, the skewed sex ratio induced by oxidative stress in cultured bovine embryos may be reduced. Female embryos in BSA-ITS media appear to be more resistant to oxidative stress and exhibit a lower apoptotic rate than male embryos (29). Our data were collected from embryos cultured in Cook media. Thus, it is possible that other types of culture media may change the association between exposure and sex ratio.

In conclusion, our study demonstrated an association between air pollutants and the sex ratio of IVF babies. In combination with SBT, exposure to low concentrations of SO_2 at IVF sites may lead to an as high as 2.1-fold increase in the male-to-female sex ratio. With the rising trend of SBT in recent years, the skewed sex ratio associated with types of ET has been discussed fully. By illustrating an interaction between ET and the environment, our finding may contribute to a better understanding of the underlying determinants of the skewed sex ratio after ART.

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Contaminantes del aire exterior y proporción del sexo de nacidos de fecundación in-vitro: el efecto de la transferencia de blastocistos únicos

Objetivo: Evaluar el impacto de la contaminación atmosférica en la proporción de sexos en los recién nacidos únicos después del tratamiento de FIV y evaluar la influencia del número y el estado de desarrollo de los embriones transferidos en la proporción de sexos.

Diseño: Estudio retrospectivo de cohortes.

Marco: Unidad de FIV afiliada a la universidad.

Paciente(s): Un total de 7.004 nacidos únicos tras una transferencia en fresco o una transferencia de embriones congelados-descongelados (FET) entre enero de 2013 y Diciembre de 2017.

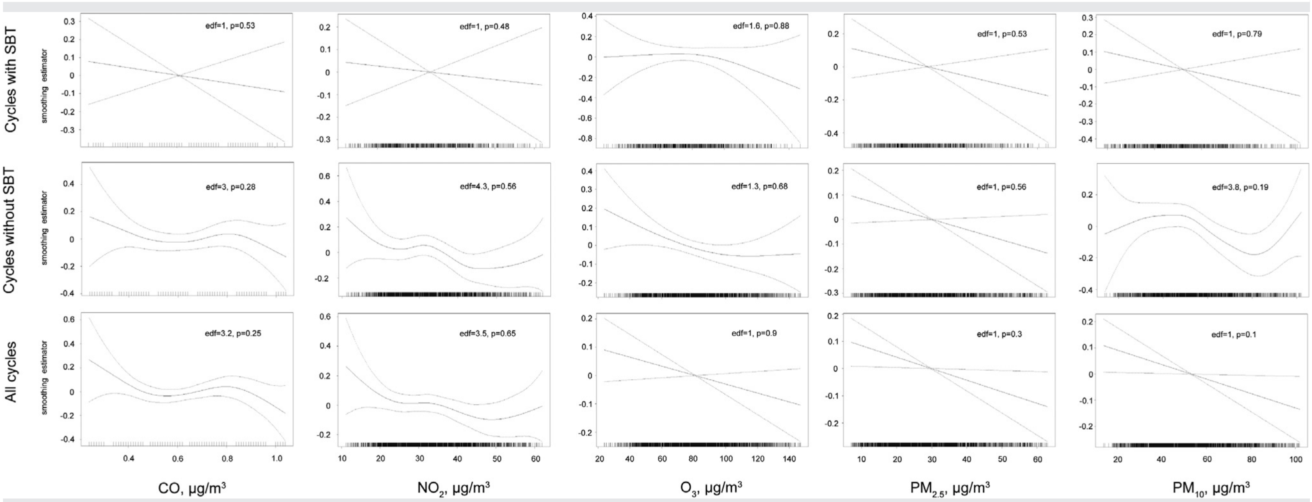
Intervención(es): Ninguna.

Medida(s) de resultado principal(es): La proporción hombre-mujer en los nacidos vivos únicos.

Resultado(s): La mediana estimada (rango intercuartil) de las partículas de materia (PM)10, PM2.5, CO, NO2, O3, y SO2 en el lugar de FIV fue de 51.4 (39,5-64,6), 27,7 (20,7-37,4), 0,62 (0,5-0,72), 32,5 (25,4-40,1), 79,6 (63,3-96,6), y 11,9 (9,3-15,9) mg/m3, respectivamente. El análisis multivariante indicó que el SO2 era el único contaminante claramente asociado con la proporción de sexos. En los hijos únicos de transferencia de un solo blastocisto (SBT), como indica el modelo aditivo generalizado, la concentración de SO2 y la proporción de sexos mostraron una asociación invertida en forma de U. En hijos únicos después de la no-SBT, se observó una disminución monotónica en la proporción de sexos con una mayor concentración de SO2. En comparación con la categoría de referencia (SO2 $<$ 7,57 mg/m3), la proporción de sexos en el 5° decil de SO2 (10,81-11,94 mg/m3) se incrementó en 2,1 veces (95% intervalo de confianza [CI], 1.3-3.14) después de ajustar las covariables. En los niños nacidos únicos non-SBT, la proporción de sexos disminuyó significativamente sólo en el 9° (odds ratio $=$ 0,69; IC del 95%, 0,53-0,90) y 10° (OR $=$ 0,74, IC del 95%, 0,56-0,98) deciles.

Conclusión(es): Las bajas concentraciones de SO2 mostraron una asociación con el aumento de la proporción de sexos en los hijos únicos de SBT, mientras que en los nacidos únicos de otro tipo de ET las proporciones de sexo no mostraron una asociación a bajas concentraciones de SO2.

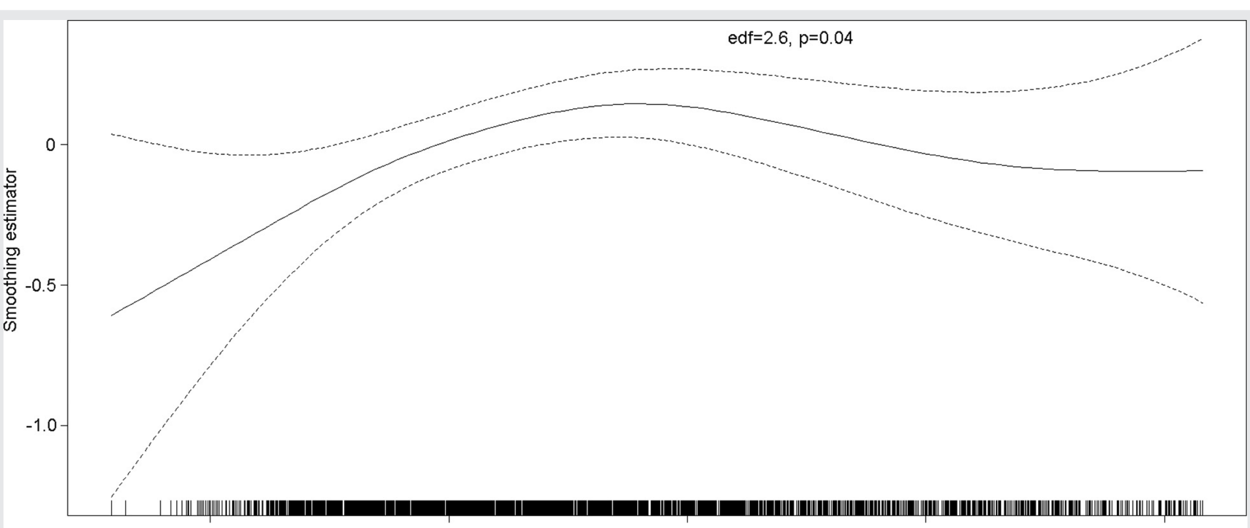
SUPPLEMENTAL FIGURE 1



Association between male-to-female ratio at birth and concentrations of air pollutants (CO, NO_2, O_3, $PM_{2.5}$, and PM_{10}) in multipollutant generalized additive models including SO_2, CO, NO_2, O_3, $PM_{2.5}$, and PM_{10} in singletons conceived through single blastocyst transfer (SBT) and non-SBT cycles. All models were adjusted for paternal body mass index, paternal sperm motility, paternal smoker, year of delivery, insemination protocol, transfer cycle, and at least one top-quality embryo transferred. The smoothing estimator indicates the contribution of the covariate fitted to a given smooth function to the response variable; edf indicates the estimated degree of freedom of the smooth function. Solid lines indicate response curves, and dotted lines indicate 95% confidence intervals.

Wang. Air pollution affects sex ratio. Fertil Steril 2019.

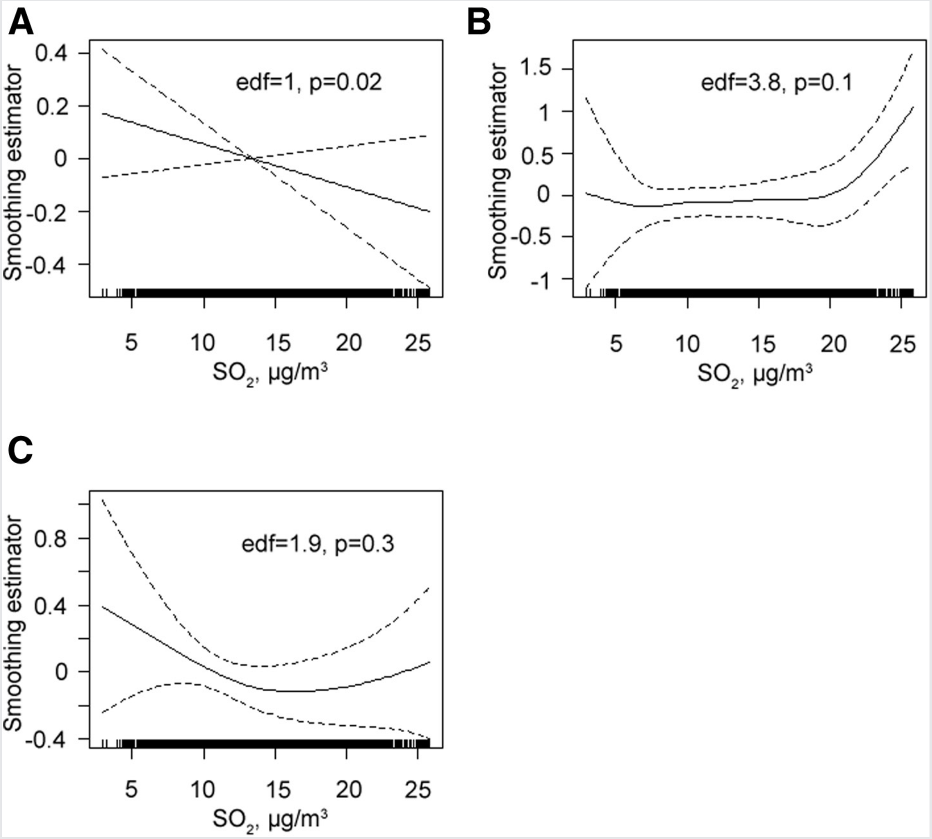
SUPPLEMENTAL FIGURE 2



Differences between single blastocyst transfer (SBT) cycles and non-SBT cycles in the association between SO_2 concentrations and sex ratio. Generalized additive models were adjusted for paternal body mass index, paternal sperm motility, paternal smoker, year of delivery, insemination protocol, transfer cycle, at least one top-quality embryo transferred, and concentrations of $PM_{2.5}$, PM_{10}, CO, NO_2, and O_3. The smoothing estimator indicates the contribution of the covariate fitted to a given smooth function to the response variable; edf indicates the estimated degree of freedom of the smooth function. Solid lines indicate response curves, and dotted lines indicate 95% confidence intervals.

Wang. Air pollution affects sex ratio. Fertil Steril 2019.

SUPPLEMENTAL FIGURE 3



Differences between different types of non–single blastocyst transfer SBT (double ET[A], double blastocyst transfer[B], single ET[C]) in the association between SO_2 concentrations and sex ratio. Generalized additive models were adjusted for paternal body mass index, paternal sperm motility, paternal smoker, year of delivery, insemination protocol, transfer cycle, at least one top-quality embryo transferred, and concentrations of $PM_{2.5}$, PM_{10}, CO, NO_2, and O_3. The smoothing estimator indicates the contribution of the covariate fitted to a given smooth function to the response variable; edf indicates the estimated degree of freedom of the smooth function. Solid lines indicate response curves, and dotted lines indicate 95% confidence intervals.

Wang. Air pollution affects sex ratio. Fertil Steril 2019.