

Recent cigarette smoking and assisted reproductive technologies outcome

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Objective: To assess the association between recent cigarette smoking (CS) in female and male partners and assisted reproduction technology (ART) outcomes.

Design: Cohort prospective study.

Setting: University ART program in Chile.

Patient(s): One hundred sixty-six couples seeking pregnancy through ART.

Intervention(s): Follicular fluid (FF) and serum cotinine concentrations were measured in female partners. Self-reported CS data were collected through personal interviews.

Main Outcome Measure(s): The association between female recent smoking, assessed by FF and serum cotinine concentrations, and ART outcomes, such as number of ova retrieved and implantation rates, and the association between self-reported male recent smoking and live birth rates.

Result(s): A significant age-adjusted association between increased FF cotinine level and decreased number of ova retrieved was found. The male partner's smoking habit significantly decreased the live birth rate from 21.1% to 7.8%. Serum cotinine concentrations paralleled those of FF.

Conclusion(s): The hypothesis of a detrimental effect of recent female smoking over implantation rates is rejected. However, recent male smoking is associated with significantly decreased live birth rates even after adjusting for confounders. Female recent smoking was significantly associated with decreased number of retrieved ova. (Fertil Steril® 2010;93:89–95. ©2010 by American Society for Reproductive Medicine.)

Key Words: In vitro fertilization, cigarette smoking, number of retrieved ova, male recent smoking

Cigarette smoking (CS) is widely recognized as hazardous to health and is a major cause of preventable mortality. Many epidemiologic studies show that smoking in women of reproductive age present with increased time to conception and decreased fecundity. There is a strong association between CS and both reduced fertility and earlier mean age of menopause. Overall, the literature supports a small but significant negative impact of female CS on time to conception in spontaneous menstrual cycles. This is especially true among heavy smokers, although the subject remains controversial; several authors who postulate that CS has no effect on spontaneous fertility (1–4).

Despite being different from natural conception, in vitro fertilization (IVF) represents an interesting model in which

to evaluate the precise effect of CS on fertility. Female CS has been associated with lower fertilization rates (5, 6), decreased numbers of ova retrieved (7), lower clinical and term pregnancy rates (8–11), and increased rates of spontaneous abortion. It has been suggested that the negative effect of CS on IVF outcomes is mediated by a decrease in oocyte number and quality, possibly resulting in a high frequency of spontaneous abortion (12, 13).

Conflicting results have been reported in studies that have evaluated the effect of female CS on couples undergoing ART. A recent study demonstrated no measurable effect of past female CS on ART outcome (14). On the other hand, most of the studies are based on self-reported smoking status with almost no research accounting for cigarette exposure during the cycle days of the ART procedure. Keeping this in mind, we performed a prospective cohort study to evaluate the effect of recent cigarette exposure (active or passive), as established by cotinine levels in the follicular fluid, on ART outcome measures. Our hypothesis was that female recent smoking would be associated with a decreased number of ova available for fertilization and a poor implantation rate.

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METHODS

Patients

A cohort of 166 patients undergoing a first cycle of IVF or intracytoplasmic sperm injection (ICSI) treatment were selected at our center between July 2004 and April 2005. Patients undergoing their first attempt at ART were defined to be in the first cycle. The criteria of selection included only those women who had reached the follicular aspiration step. Owing to the experimental question, treatment cycles involving ovarian hyperstimulation syndrome and oocyte donation were excluded from analysis. Follicular fluid and serum were collected on the same day as follicular aspiration and then frozen until cotinine concentrations were determined for each female patient. The study was previously approved by the Ethics Board of the San Borja-Arriarán Hospital in Santiago.

The CS data were collected by conducting personal interviews on the day of hospitalization for oocyte retrieval. The questionnaire given by a midwife also included information about the quantity of alcohol and caffeine imbibed. The serum cotinine concentrations were not determined in male partners.

CS Status

There were two groups formed according to the concentrations of cotinine measured in the follicular fluid: 1) recent smoker women (RSW), who had cotinine concentrations >10 ng/mL in the follicular fluid; and 2) nonsmoking women (NSW) who denied smoking during the ART cycle and showed undetectable levels of cotinine in their follicular fluid. The CS status of male participants was determined only through their questionnaire answers.

Ovarian Stimulation

Every woman participating in this study underwent gonadotropin suppression with the GnRH agonist leuprolide at a dose of 1 mg/day SC starting during the luteal phase of the last menstrual cycle before ART. On day 3 of the subsequent cycle, ovulation induction was accomplished using a daily administration of 150–300 IU recombinant FSH. Human menopausal gonadotropin (hMG) dose (150–225 IU) was adjusted according to the E_2 production and follicular development. The final stage of follicular maturation was initiated by injection of 10,000 IU of hCG when at least three follicles reached a diameter of 18 mm on ultrasound examination.

Follicular Aspiration

Follicles were monitored by transvaginal ultrasound. All follicles were aspirated 36 h after hCG administration using transvaginal ultrasound guidance and regional anesthesia. All follicular fluid samples used for the cotinine assay were the first aspirate and were collected in sterile centrifuge tubes to avoid contamination by medium and blood. Follicular fluid samples were centrifuged at 400g for 10 min. The supernatants were collected in 1-mL polystyrene cryovials and frozen at -20°C .

Percentage of High-Quality Embryos

Embryo quality was assessed on the same day as embryo transfer (3 days after follicular aspiration). The percentage of high-quality embryos was calculated for RSW and NSW by dividing the number of embryos of optimum quality by the total number of embryos in each group. The characteristics of a top-quality embryo were the absence of multinucleated blastomeres, a count of six to eight blastomeres, and a level of fragmentation $<14\%$ (of the embryo volume), thereby allowing an adequate spatial relationship between the blastomeres within an embryo.

Cotinine Assay

The cotinine concentrations were established by a competitive chemiluminescent immunoassay in a solid phase (Immulate LKNM1; DPC, Los Angeles, CA). Concentrations of cotinine were expressed as ng/mL. The sensitivity of the assay (lowest detectable value) was 0.25 ng/mL. Readings less than this were arbitrarily assigned the value of 0.10, following Zenzes et al. (15).

Statistical Analyses

Based on an implantation rate of 15% in our IVF program, the total sample size necessary to detect a 40% effect of smoking on implantation rates was 160 subjects, assuming $\alpha = .05$ and $(1 - \beta) = 80\%$.

The normal distribution of continuous variables was established through the Kolmogorov-Smirnov test. Relative risk and 95% confidence interval (CI) and t test were used where appropriate. Multiple regression analysis was used to test the relationship between cotinine concentrations in follicular fluid and the number of retrieved oocytes, age, body mass index (BMI), and coffee and alcohol consumption. Logistic regression was used to compare rates adjusting for confounders.

Fertilization rate was calculated as the number of ova fertilized divided by the number of ova inseminated. A positive pregnancy test was established with an elevated β -hCG 14 days after transfer and then was confirmed if this value was twofold higher 3 days later. Clinical pregnancy was confirmed with a positive fetal heartbeat by ultrasound 6.5 weeks after a positive pregnancy test (β -hCG). The number of fetal sacs with a positive heartbeat divided by the total number of embryos transferred was used to calculate implantation rate.

RESULTS

Population

The overall prevalence of history of CS (ever having smoked) among the 166 couples was close to 58% in male partners and 56% in female partners. According to cotinine levels in the follicular fluid samples, there were 33 (19%) RSW and 133 (80.1%) NSW. The age of RSW was significantly higher than that of NSW (35.12 ± 0.66 years vs. 33.17 ± 0.30 years, respectively, $P < .05$; Table 1). There were no differences in BMI and alcohol and coffee consumption between the two

TABLE 1**Baseline characteristics according to smoking status.**

	Detectable cotinine (n = 25)	Undetectable cotinine (n = 133)	P value
Age (yrs)	35.12 ± 0.66	33.17 ± 0.30	< .05
Duration of infertility (yrs)	5.28 ± 0.41	5.31 ± 2.07	NS
Body mass index (kg/m ²)	25.41 ± 0.50	26.04 ± 0.21	NS
E ₂ per follicle (pg/mL)	134.17 ± 22.0	111.0 ± 8.22	NS
Oocytes retrieved (n)	7.78 ± 0.81	9.55 ± 0.46	NS
Duration of HOS (days)	11.48 ± 0.21	11.80 ± 0.17	NS
Basal FSH (mIU/mL)	7.42 ± 0.35	6.74 ± 0.17	NS

Note: Values are expressed as mean ± SEM. HOS = hyperovulation stimulation.

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groups. When both partners shared the CS habit, the men smoked a significantly greater number of cigarettes per week, with a mean of 42 ± 5.28 cigarettes compared with the women's 17.8 ± 8.94 ($P < .05$). Basal FSH levels on day 3 of the menstrual cycle were similar in the two studied groups (RSW: 7.42 ± 0.35 mIU/mL; NSW: 6.7 ± 0.17 mIU/mL). Similarly, no significant difference was observed in the levels of per-follicle serum E₂ on the day of hCG administration among the two groups (RSW: 134.17 ± 22.16 pg/mL; NSW: 11 ± 8.22).

Table 1 shows that there were no differences in the two studied groups in the following parameters: duration of infertility, BMI, proportion of women with ongoing IVF or ICSI, basal FSH levels, per-follicle E₂ concentration; duration of ovulation induction, mean number of ova retrieved, percentage of high-quality embryos, fertilization rate, clinical pregnancy rate, and term pregnancy rate.

Causes of Infertility

As shown in Table 2, altered tubal patency was the most prevalent indication for ART in this cohort, with 58 affected individuals (35%). Alteration of seminal parameters was the second most common cause of infertility, affecting 50 individuals (30%), followed by endometriosis in 25 individuals (15%), unexplained infertility in 23 individuals (14%), ovulation disorder in 10 individuals (6%).

No significant difference regarding infertility causes was found between the two groups ($P > .05$). The statistical power analysis for this calculation was 72%.

Patients undergoing ICSI had a significantly higher frequency of altered male factors than those undergoing traditional IVF (57% vs. 16%, respectively), and IVF patients had a significantly higher frequency of altered tubal patency than ICSI patients (51% vs. 7%, respectively).

Duration of Infertility

No significant difference was found between RSW and NSW regarding duration of infertility. However, the mean number of years of infertility was significantly lower among women who ended in a live birth compared with those who did not deliver a child (4.18 ± 0.28 years vs. 5.56 ± 0.18 years, respectively; $P < .05$).

CS Status and Follicular Fluid Cotinine Levels

There were 33 women with detectable cotinine levels in the follicular fluid. Within this group, 25 women were active smokers (range 12.7–299.0 ng/mL), and 8 were passive smokers (range 13–69.5 ng/mL) (Fig. 1). From the interviews and self-reported smoking status, it had been established that 27 women smoked the day before follicular aspiration, and

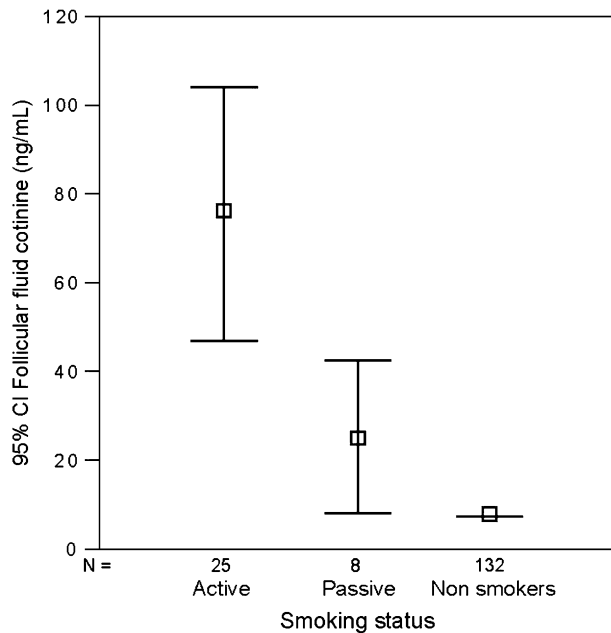
TABLE 2**Indications for ART.**

Cause	Active female smokers	Passive female smokers	Female nonsmokers	Total, n (%)
Tubal patency	12	5	41	58 (35)
Male factor	5	1	44	50 (30)
Endometriosis	2	0	23	25 (15)
Unexplained	6	2	15	23 (14)
Anovulation	0	0	10	10 (6)
Total	25	8	133	166 (100)

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FIGURE 1

Cotinine follicular fluid concentrations are depicted according to smoking status. Values are expressed as mean \pm 95% confidence intervals (CI). Each group is significantly different from each other group at $P < .05$.



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among those only 25 had detectable levels of cotinine in the follicular fluid.

Cotinine concentration in serum correlated positively with cotinine concentration in the follicular fluid (Fig. 2) ($r^2 = 0.95$; $P < .001$). There was a direct and significant correlation between follicular fluid and serum levels of cotinine concentration and the number of cigarettes smoked by the female partner ($r = .754$).

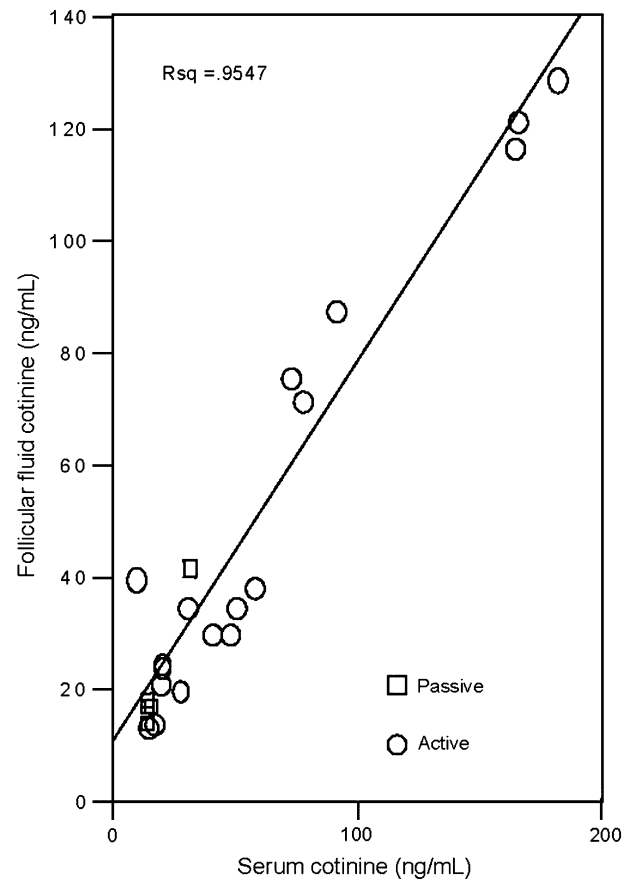
Further analysis was done among women with detectable levels of cotinine in their follicular fluid. There were 17 RSW whose male partners also smoked and 16 whose male partners did not smoke. The cotinine concentration in the follicular fluid was higher for the former group, but the difference was not statistically significant (80.7 ± 18.02 ng/mL vs. 42.6 ± 12.47 ng/mL, respectively; $P > .05$).

Oocyte Number

The mean number of ova retrieved was 7.78 ± 0.81 in RSW and 9.55 ± 0.46 in NSW, although the difference was not statistically significant in the bivariate analysis (t test; Table 1). However, when a multiple linear regression analysis was performed to test associations between follicular fluid cotinine levels and number of ova retrieved during the ART process, adjusting for age, the difference became apparent: Age, coti-

FIGURE 2

The strongly significant correlation between follicular fluid and serum cotinine levels is depicted.



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nine concentration in follicular fluid, BMI, number of alcoholic drinks per week, and number of cups of coffee drunk per week were included in the model. There was a significant association between cotinine concentration in the follicular fluid and age and the independent variable (amount of retrieved ova) with respect to cotinine level ($P < .05$; Fig. 3). The BMI and coffee and alcohol consumption were not associated with the number of ova retrieved.

Embryo Quality

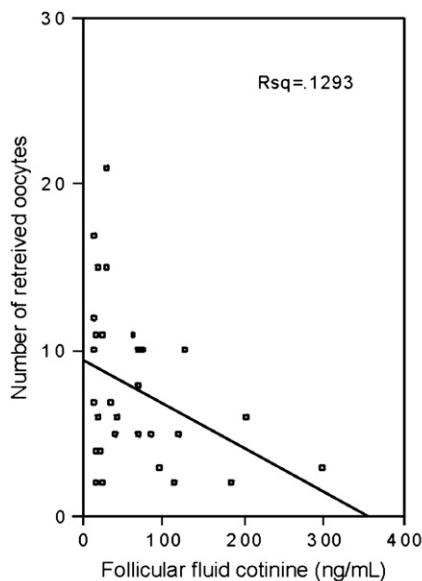
There was no significantly difference in the proportion of high-quality embryos transferred among women in the two studied groups (Table 3).

Clinical and Term Pregnancy and Implantation Rates

Overall, there were 57 women with a positive pregnancy test within this cohort of 166 couples undergoing ART procedures, which indicates a clinical pregnancy rate of 34%. In 36 of these women, a gestational sac with a live embryo was found (22%), and 32 produced a live birth (19%). In

FIGURE 3

The significant regression analysis of age and cotinine follicular fluid levels on the number of retrieved ova is depicted.

**Coefficients**

Model		Unstandardized Coefficients		Standardized Coefficients	t	P
		B	Std. Error	Beta		
1	(Constant)	25.429	6.846		3.714	< .05
	Cotinine	-2.510E-02	.011	-.348	-2.223	< .05
	Age	-.457	.193	-.370	-2.361	< .05

a Dependent variable: Number of oocytes retrieved
b Selecting only cases for which cotinine was detectable

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TABLE 3**Relative risks for smokers.**

	RR	(95% CI)
High-quality embryos	1.51	(0.83–2.73)
Implantation rate	0.50	(0.24–1.07)
Biochemical Pregnancy rate	0.86	(0.49–1.51)
LBR for female smoking	2.29	(0.94–5.59)
LBR for male smoking	0.36	(0.14–0.92)

Note: CI = confidence interval; LBR = live birth rate; RR = relative risk.

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Abortion

Overall, there were 57 patients with a positive pregnancy test (34%) and only 36 that progressed to clinical pregnancy (22%). Because 3 of the clinical pregnancies resulted in clinical abortion, only 32 resulted in live births (19%). Thus, 21 patients had a nonclinical pregnancy loss (13%), and 3 had a clinical abortion (1.8%). No significant differences were found in the rate of miscarriage among women with detectable levels of cotinine compared with those who had no detectable cotinine in their follicular fluid.

Ten of 33 (30%) RSW and 47 of 133 (35.3%) NSW achieved a positive pregnancy test. Thus, there was no significant difference in the rate of preclinical pregnancy (Table 3). When analyzing abortions in women whose male partner had smoked the last week before oocyte retrieval, 9 (17%) had a nonclinical pregnancy loss compared with 16 (14%) of those whose male partner had not smoked during that period. The difference was not significant: RR 1.12 (95% CI 0.53–2.34).

Multiple Pregnancies

In the present study, 32 pregnancies ended with at least one live birth; of these, 7 were multiple pregnancies (22%). All of those women belonged to the NSW group. Six of the seven multiple pregnancies were twins, and the remainder was triplets.

DISCUSSION

This study examined the association between CS in women and IVF outcomes, such as number of ova retrieved, embryo quality, fertilization rate, implantation rate, and clinical and term pregnancy rates, and the impact of the male partner's recent exposure to tobacco. A major strength of the present study is that the exposure (smoking) was measured both subjectively (personal interview) and objectively (cotinine). The concentrations of cotinine in follicular fluid correlated strongly with the number of cigarettes smoked and proved to be a reliable dose-dependent marker for recent smoking. Even more important, cotinine concentrations in the follicular

this cohort, there were 5 live births within RSW (15.1%) compared with 27 within NSW (20.3%). There was no significant difference in live birth rate between the two studied groups ($P > .05$; Table 3). The statistical power for data used in this comparison was only 13.3%.

Seven of 94 embryos transferred in the RSW group (7.4%) were successfully implanted, whereas 56 of 378 embryos transferred in the NSW group (14.81%) were successfully implanted. Thus, the implantation rate was lower among RSW than NSW, but the difference was not significant (Table 3). Stratified analysis and logistic regression did not find any significant difference in implantation rates among the studied groups.

A significant difference in live birth rate was found in couples whose male partner smoked during the last week before oocyte pickup, according to self-reported smoking status, compared with couples whose male partner did not smoke during that week (Table 3). Male and female age and female smoking status did not affect this difference.

fluid correlated with serum cotinine levels. This finding indicated that cotinine blood concentrations can be used directly as a marker for recent CS. We found that age and duration of infertility both affected live birth rate, which is in agreement with earlier reports that establish age and duration of infertility as major determinants of success in ART (16).

Two out of the 27 women (7.4%) self-reporting CS the day before follicular aspiration had undetectable levels of cotinine in the follicular fluid and serum. The relatively short half-life of cotinine (18–20 h) may provide an explanation to this unexpected issue. Couples meeting the inclusion criteria in this study were consecutively recruited. Considering that there was a significant difference in female age between the two studied groups, the statistical analysis was conducted adjusting for the effect of this important variable as well as for BMI and alcohol and coffee consumption, which are the main confounding variables for cigarette smoke exposure. Overall, live birth rate in our entire cohort was 19%. Templeton et al. (16), in the largest cohort ever published, reported an overall live birth rate per embryo transfer of 21.6% in 30-year-old patients and 17.6% in 35-year-old patients. Our rate compares well with that of Templeton et al., because the mean age of our present cohort was 33.6 (SEM \pm 0.28) years.

Our finding that recent CS is associated with a decline in the number of ova retrieved during an IVF cycle confirms results from earlier reports (17, 18). A cumulative effect of CS over time on the ovarian reserve has been postulated as being responsible for this result. As far as we know, this is the first study reporting a regression analysis of the number of ova retrieved and cotinine levels in follicular fluid with a modest, but significant, negative age-adjusted effect on oocyte harvest. Thus, recent smoking share with age a negative impact on the number of oocytes retrieved, both associations being statistically significant.

As in other reports (19, 20), no statistically significant association was noted between female smoking and reduced term pregnancy rate. However, when comparing term pregnancy rates between couples in which the male partner smoked with couples in which the male partner did not smoke, independently of the female smoking status, this association of reduced term pregnancy rate was observed, as was reported by Joesbury et al. (13). It is known that sperm DNA is more vulnerable to environmental injury than oocyte DNA, which is believed to have a more effective DNA repair response (21, 22). This difference in DNA vulnerability could be due to a vertical paternal transmission of damaged genetic material to the embryo. However, there was no difference in abortion rate between women exposed to CS and their non-smoking counterparts.

We analyzed the association of recent female CS with the outcomes of ART, including fertilization rates, embryo quality, and implantation rates as covariates. The implantation rate in our cohort was lower among women exposed to cigarette smoke than among those who did not, and the difference was close to significance. New studies with balanced

age among the groups are needed to elucidate this important aspect of the relationship between CS and fertility outcomes. It has been reported that the implantation failure may be due to defects within the embryo or to defects in the endometrial receptivity (23–25).

The largest study on IVF effectiveness was carried out in the U.K. and included 36,961 fertilization attempts (16). No significant difference in live birth rate was found when comparing tubal pathology, endometriosis, and unexplained cervical and uterine subfertilities. The present results on live birth rate correspond to those. The present results also agree with those results regarding duration of infertility and its negative influence on pregnancy rate. However, other reports have found associations between female CS and causes of infertility, such as alterations of seminal parameters and unexplained infertility in ART cycles (26).

Interestingly, we found that CS was not associated with embryo quality. This result agrees with other reported data indicating that CS did not negatively affect embryo quality when assessed by morphologic patterns (23–25). There are several methods available by which to evaluate the quality of embryos by morphologic analysis, with the goal of predicting functional capacity of a particular embryo (or a cohort of embryos) to progress through development and cleavage. In the present study, the percentage of high-quality embryos was used to evaluate overall embryo quality in each group. We demonstrated that embryo quality was similar between women exposed and women unexposed to CS. Thus, the present results suggest that the mechanisms through which CS affects fecundability seem to lie in alterations of the implantational sequence, not in embryo development. The latter is in agreement with the results of a retrospective study by Soares et al. (27). Over 785 cycles of oocyte donations, they demonstrated the disability of accomplishing the implantation process in women who were heavy smokers. The researchers speculated that this could be related to reports of diminished adherence of endometrium in smokers, as has been shown in cultures of endometrial carcinoma cells treated with benzo[a]pyrene (28). They also found a higher frequency of multiple pregnancies among female smokers that was not shown in the present study. To the contrary, we had seven multiple pregnancies, all within nonsmoking mothers.

Finally, the high prevalence of life-time CS history in the present cohort of Chilean women (60%) underscores the need to develop strategies to assist smoking cessation prior to or during ART, as Hughes et al. did in 2000 (29).

In conclusion, combining self-reported data establishing the time sequence of smoking exposure with a biologic marker such as cotinine in plasma and follicular fluid represents a new and accurate method to detect associations of cigarette smoking exposure. Overall, our hypothesis of a detrimental effect of recent female smoking over implantation rates is rejected. However, recent male smoking is associated with significantly decreased live birth rates even after

adjusting for the main confounders. Female recent smoking was significantly associated with decreased number of retrieved ova. Thus, further research is needed to elucidate how smoking interferes with embryo implantation, and the substantial evidence of tobacco interference with ART demonstrates the need for medical or other forms of counseling in couples who smoke during IVF treatment.

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