Interovarian Differences in Levels of Cotinine, a Major Metabolite of Nicotine, in Women Undergoing IVF Who Are Exposed to Cigarette Smoke

MARIA TERESA ZENZES[1,3] and T. EDWARD REED[2]

Submitted: July 3, 1997
Accepted: September 29, 1997

Purpose: *Our purpose was to determine whether there is variation in levels of follicular fluid (FF) cotinine between the two ovaries of women undergoing IVF-ET who are exposed to cigarette smoke.*
Methods: *In 61 women, there were two to four determinations of FF cotinine levels for each of two follicles, one from each ovary. For each woman a t test for significant difference between the means of both ovaries was done to test for interovarian variation.*
Results: *Thirty-seven nonsmokers, 8 passive smokers, and 16 active smokers differed greatly ($P < 0.0001$) in mean FF cotinine levels: 13.0, 91.1, and 420.3 ng/ml, respectively. Fourteen women had significant differences, at the $P = 0.025$ level or below, between their two ovaries. Five of them had differences significant at the 0.001 level. Even so, the correlation between the cotinine levels of the two ovaries was high.*
Conclusions: *Cotinine uptake between the two ovaries of a woman may differ approximately one-fourth of the time. In spite of these differences, the overall correlation between ovaries is high. The clear distinction in levels of FF cotinine among active, passive, and nonsmokers demonstrates the reliability of FF cotinine testing. Detection of cotinine in a large proportion of nonsmokers shows how pervasive nicotine is in the environment.*

KEY WORDS: cotinine; follicular fluid; ovaries; cigarette smoking; in vitro fertilization–embryo transfer.

[1] Department of Obstetrics and Gynaecology, University of Toronto, Toronto, Ontario, Canada.
[2] Department of Zoology and Anthropology, University of Toronto, Toronto, Ontario, Canada.
[3] To whom correspondence should be addressed at Division of Reproductive Science, The Toronto Hospital Research Center, Room CCRW 1-813, 101 College Street, Toronto, Ontario M5G 1L7, Canada.

INTRODUCTION

Cigarette smoking has deleterious effects on the reproductive health of women. Epidemiologic evidence has shown that women who smoke experience more delays in conceiving, a higher frequency of spontaneous abortions, and an earlier-age onset of natural menopause than do nonsmokers (reviewed in Refs. 1 and 2). Women who smoke have higher frequencies of chromosomally abnormal oocytes and zygotes than nonsmokers (3).

Components of cigarette smoke can be detected in several body fluids, including follicular fluid (FF) samples aspirated from women in in vitro fertilization–embryo transfer (IVF-ET) therapy. Cadmium, a heavy metal present in cigarettes, and cotinine, a major metabolite of nicotine, were detected at higher levels in FF samples of women who smoke compared with those of nonsmokers (4–8).

Cotinine has been shown to be a specific marker of cigarette smoke exposure, being more reliable than self-reports on tobacco consumption (9). Cotinine is a stable compound with a half-life of approximately 19 hr, in contrast to nicotine's half-life of approximately 2 hr (10). Because nicotine is so quickly detoxified, the immunoassay for a longer-lived metabolite such as cotinine becomes a suitable marker for cigarette smoke exposure and dose (10).

Because the ovary is highly vascularized, detection of different levels of cotinine among women related to their different exposures to cigarette smoke can readily be demonstrated (8). The aim of the present study was to investigate whether there are significant differences in FF cotinine uptake between the two ovaries of patients in IVF-ET who smoke and whether such interovarian differences are also detectable in passive smokers and self-reported nonsmokers, a group at risk of environmental exposure (8).

1058-0468/98/0200-0099$15.00/0

MATERIALS AND METHODS

Patients

Sixty-one unselected couples in IVF-ET therapy participated in this study. Each couple signed a consent form, approved by the Committee for Research in Human Subjects of the Toronto Hospital. The mean (±SE) age of the 61 women was 33.4 ± 0.6 years (range, 26–43 years). Women were classified according to their self-reported smoking habits as nonsmokers (NS; husband also a nonsmoker; $n = 37$), passive smokers (PS; nonsmoker, husband smokes; $n = 8$), and active smokers (AS; husband may or may not smoke; $n = 16$).

Collection of FF Samples

All patients had gonadotropin suppression by oral contraceptives (Demulen 50; Searle, Oakville, Ontario, Canada) for 3–6 weeks prior to the onset of their IVF-ET cycle, as described by Gonen *et al.* (11). The stimulation protocol was gonadotropin releasing hormone (GnRH) agonist with human menopausal gonadotropin (hMG) as described previously by Zenzes *et al.* (12). Follicles were aspirated 36 hr after human chorionic gonadotropin (hCG) administration, using transvaginal ultrasound (Bruel & Kjaar, Naerum, Denmark) guidance and local anesthesia.

Cotinine Assay

The smoking status was not known to the persons taking FF samples and performing the cotinine assay. Two FF samples, each from a different ovary, were obtained from each patient. The samples were not marked "right" or "left" by the operating physician; for this reason we called them A or B. The samples were always the first of each ovarian aspirate and were taken with an unused needle to keep them free of medium and interfollicular blood contamination. Samples were collected in sterile centrifuge tubes and were centrifuged at 800*g* for 10 min. The supernatants were dispensed into 1-ml polystyrene cryovials and were frozen at −20°C. Samples were then thawed and assessed for cotinine levels by radioimmunoassay, as described previously (8). Briefly, isogel Tris buffer (trimethamine hydrochloride, 0.01 n*M*; sodium chloride, 0.14 m*M*; and 0.1% gelatin; pH 7.4), tritiated cotinine, rabbit antiserum, and goat anti-rabbit γ-globulin were used to separate the antibody-bound cotinine from the free analysate. For quantification we used a standard cotinine (0.2 to 20 ng/ml). The results are expressed as nanograms of cotinine per milliliter of FF. Two to four determinations per sample were performed. The sensitivity (lowest detectable amount) of the assay was 0.25 ng/ml; readings below this level were arbitrarily assigned the value of 0.10 ng/ml. The recovery value for cotinine was 92%.

Data Analysis

Data processing and statistical analysis were performed using the StatView Statistical Package (Version 4.5; Abacus Concepts, Berkeley, CA) on a Macintosh Performa computer (Apple, Cupertino, CA). All *P* values are two-tailed. For each woman a *t* test for a significant difference in mean cotinine values between her two ovaries was done. ANOVA was used for comparing means of the three smoking groups. When comparing cotinine levels of all women, logarithms (to base 10) were used because of the extreme nonnormality of the cotinine distribution (8).

RESULTS

The mean ages (±SE) for each group of women were as follows: NS, 34.0 ± 0.6 years; PS, 32.9 ± 2.0 years; and AS, 32.1 ± 1.1 years. These means do not differ significantly. Table I summarizes the data about self-reported smoking status, means for FF cotinine for each ovary [designated FF(A) and FF(B)], and mean absolute difference (the difference ignoring the sign, + or −) between the cotinine means of the two ovaries [FF(A) − FF(B)].

The cotinine levels of FF(A) and FF(B), as expected, are similar within smoking groups. After combining

Table I. Mean FF Cotinine Levels in Both Ovaries, and Their Mean Absolute Difference (Abs), in 61 Women[a]

Smoking status	Number	FF(A) (ng/ml)	FF(B) (ng/ml)	Abs[FF(A) − FF(B)] (mean ± SE)
Nonsmokers	37	12.64	11.93	8.06 ± 4.82
Passive smokers	8	90.00	92.10	19.84 ± 11.05
Active smokers	16	457.09	384.11	115.72 ± 34.46*
Total	61	139.36	120.06	37.85 ± 11.15*

[a] Both ovaries of 61 women were tested for cotinine. The cotinine level of each woman's ovary is based on two to four determinations. One ovary is designated "A," and the other "B" (information about whether left or right was not available from the physicians). The absolute difference between ovaries in cotinine levels ignores the sign (+ or −) of the difference.

* Significantly different from zero at the 0.005 level.

the two mean FF cotinine values, the overall means are as follows: NS, 12.3 ± 7.9; PS, 91.0 ± 76.0; and AS, 420.6 ± 177.0. In the nonsmokers 26 (70%) of the women had cotinine levels above detection levels, ranging between 0.35 and 260.1 ng/ml, while all women in the other two groups had cotinine values above the detection level. The three groups differ very significantly in overall mean FF cotinine levels ($P < 0.0001$); all three pairwise comparisons are significantly different ($P < 0.011$ or less) by Fisher's protected least-significant difference test.

The mean absolute difference between ovaries increases with increasing smoking exposure: NS, 8.06; PS, 19.84; and AS, 115.72 (Table I). These absolute differences for NS and PS do not differ significantly from zero, but that for AS does differ at the 0.005 level. For all 61 women the absolute differences are very significantly and positively correlated with the overall mean cotinine value ($P < 0.0001$).

Fourteen of the 61 women had significant differences, at the $P = 0.025$ level or below, between their two mean FF cotinine values. This is shown in Table II. Five of the differences were at the 0.001 level. Perhaps the most convincing demonstration of a large interovary difference was in a woman (patient 9; Table II) who had four cotinine determinations for each ovary: the cotinine levels were 302.57, 285.16, 345.60, and 337.99 (mean ± SE: 317.83 ± 14.37) and 110.02, 136.00, 156.30, and 123.58 (mean ± SE: 131.48 ± 9.83). These means differ at the 0.001 level of significance (actually at $P = 0.0004$). Other patients had greater interovary differences but fewer cotinine determinations.

Table II. Significant Differences in Mean FF Cotinine Levels Between the Two Ovaries of 14 (of the 61) Women[a]

Patient No.	Smoking status	FF(A) (mean)	FF(B) (mean)	t	df	$P <$
1	NS	4.47	41.50	19.56	3	0.001
2		0.61	3.73	15.77	4	0.001
3		2.51	2.22	6.56	2	0.025
4		0.81	0.38	6.80	2	0.025
5		1.16	0.37	15.80	2	0.005
6	PS	91.50	22.00	11.50	2	0.010
7	AS	99.13	188.28	6.25	2	0.025
8		697.50	405.03	8.28	2	0.025
9		317.83	131.48	10.70	6	0.001
10		506.20	1.39	21.76	2	0.005
11		236.56	10.28	17.26	4	0.001
12		177.25	217.25	14.03	2	0.005
13		337.65	182.74	50.04	2	0.001
14		67.54	242.95	4.66	4	0.025

[a] Means are from two to four determinations per ovary. For any one woman, "A" represents one ovary, and "B" the other ovary (see footnote *a*, Table I). All differences are based on the t value and its degrees of freedom (df) and are significant at the 0.025 level or below. Nine are significant at levels lower than $P < 0.01$.

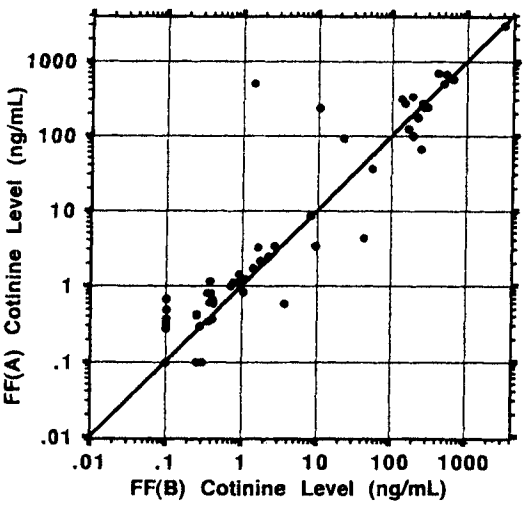

Fig. 1. Scattergram of mean FF cotinine level of one ovary (A) vs. mean FF cotinine level of the other ovary (B); log scale. The diagonal line shows ovary pairs with no difference in cotinine levels.

In spite of these significant differences between the cotinine value of follicle A and that of follicle B in approximately one-quarter of the patients, the two cotinine values are still highly correlated. Using logs, the correlation is 0.930 ($P < 0.0001$). Figure 1 shows the scattergram for log FF(A) cotinine vs log FF(B) cotinine. This figure shows that most pairs of values lie close to the line indicating no cotinine difference, but several pairs are far from this line, indicating a considerable difference.

DISCUSSION

To our knowledge, this study is the first to investigate interovarian differences in cotinine uptake of women in IVF therapy who are exposed to cigarette smoke. The major finding of this study is that approximately one-fourth of women exposed to cigarette smoke have ovaries that differ significantly in their FF cotinine levels. These differences occurred in 5 (14%) of 37 self-reported nonsmoking women, 1 (13%) of 8 passive smoking women, and 8 (50%) of 16 active smoking women. Because exposure to

cigarette smoke is much higher in the latter group, as shown by the significantly higher levels of FF cotinine, this may explain the higher interovarian variation in this group.

The reasons for the interovarian differences in cotinine levels may be anatomical, such as differences in vascularization between ovaries or differences in follicle size as a result of ovarian hyperstimulation. Multiple follicular growth, stimulated by gonadotropins in women undergoing IVF-ET treatment, is known to be asynchronic among ovarian follicles (13), leading to follicles of different size at the time of aspiration. In a recent cytochemical study on cotinine immunoreactivity of granulosa–luteal cells from FF samples, we detected significant variations in cotinine uptake among cells in a follicle and between follicles (14). The difference in cotinine uptake by these cells may reflect not only differences in cell cycle and metabolic rate, but also differences in cotinine accumulation in the follicles during follicular growth. Weiss and Eckert (5) did not find a correlation between follicle volume and cotinine levels in eight follicles. This number may be too small to allow detection of significant differences.

A corollary of the above finding is that approximately three-fourths of women in IVF treatment have ovaries that do not differ significantly—at least in our sample—in cotinine level. This results in a high correlation (0.93) between ovaries in cotinine level. A between-ovary correlation in cotinine levels was also found by Weiss and Eckert (5) in four patients. Based on this and our findings above, we feel that testing two ovaries is better than testing one, but if only one is available and there are two or more cotinine determinations (as is usual for us), this is acceptable.

In the present study we found that a large majority of nonsmoking women are actually environmentally exposed to tobacco smoke; these women have detectable levels of FF cotinine. Furthermore, we were able to detect interovarian differences in cotinine levels among the two groups of passively exposed women, albeit at levels much lower than those in active smokers. These results demonstrate that these individuals are regularly exposed to enough environmental tobacco smoke for it to get into the bloodstream and be metabolized.

Our findings in the present study and in a previous study (8) of detectable FF cotinine in a large majority of nonsmokers agree with those in a recent large-scale (n = 10,642) U.S. survey of serum cotinine (15); this study found that 88% of self-reported nonsmokers had detectable levels of cotinine in their blood. Our study provides further evidence that the reproductive systems of almost all women, regardless of whether or not they smoke, are exposed to metabolites of environmental tobacco smoke.

ACKNOWLEDGMENTS

This study was supported by Grant MT-12871 from the Medical Research Council of Canada to M.T.Z. (Ottawa, Ontario, Canada). We thank the patients for their willingness to participate in this study. We also thank Robert F. Casper, M.D., Director of the IVF-ET Program at The Toronto Hospital, for providing follicular fluid samples; Julia Klein, M.Sc., for cotinine assessments in FF; Ryszard Bielecki, D.V.M., for assistance in the preparation of the manuscript; and the nurses and technicians of the IVF-ET program for assistance.

REFERENCES

1. Zenzes MT: Cigarette smoking as a cause of delay in conception. Reprod Med Rev 1995;4:189–205
2. Midgette AS, Baron J: Cigarette smoking and the risk of natural menopause. Epidemiology 1990;1:474–480
3. Zenzes MT, Wang P, Casper RF: Cigarette smoking may affect meiotic maturation of human oocytes. Hum Reprod 1995; 10:101–105
4. Zenzes MT, Krishnan S, Krishnan B, Zhang RF, Casper RF: Cadmium accumulation in follicular fluid of women in in vitro fertilization-embryo transfer is higher in smokers. Fertil Steril 1995;64:599–603
5. Weiss T, Eckert A: Cotinine levels in follicular fluid and serum of IVF patients: Effect on granulosa-luteal cell function in vitro. Hum Reprod 1989;4:482–485
6. Rosevear SK, Holt DW, Lee TD, Ford WCL, Wardle PG, Hull MG: Smoking and decreased fertilisation rates in vitro. Lancet 1992;340:1195–1196
7. Sterzick K, Strehler E, De Santo M, Trumpp N, Abt M, Rosenbusch B, Schneider A: Influence of smoking on fertility in women attending an in vitro fertilization program. Fertil Steril 1996;65:810–814
8. Zenzes MT, Reed TE, Wang P, Klein J: Continine, a major metabolite of nicotine, is detectable in follicular fluids of passive smokers in in vitro fertilization therapy. Fertil Steril 1996;66:614–619
9. Benowitz NL, Kuyt F, Jacob P III, Jones RT: Cotinine disposition and effects. Clin Pharmacol Ther 1983;34:604–611
10. Langone JJ, Gjika HB, Van Vunakis H: Nicotine and its metabolites. Radioimmunoassays for nicotine and continine. Biochemistry 1973;12:5025–5030
11. Gonen Y, Jacobson W, Casper RF: Gonadotropin suppression with oral contraceptives before in vitro fertilization. Fertil Steril 1990;53:282–287

12. Zenzes MT, Reed TE, Casper RF: Effects of cigarette smoking and age on the maturation of human oocytes. Hum Reprod 1997;12:1736–1741
13. Laufer N, Tarlatzis BC, DeCherney AH, Masters JT, Haseltine FP, MacLusky N, *et al.*: Asynchrony between human cumulus-corona cell complex and oocyte maturation after human menopausal gonadotropin treatment for in vitro fertilization. Fertil Steril 1984;42:366–372
14. Zenzes MT, Puy LA, Bielecki R: Immunodetection of cotinine protein in granulosa-lutein cells of women exposed to cigarette smoke. Fertil Steril 1997;68:76–82
15. Pirkle JL, Flegal KM, Bernert JT, Brody DJ, Etzel RA, Maurer KR: Exposure of the US population to environmental tobacco smoke: The Third National Health and Nutrition Examination Survey, 1988 to 1991. JAMA 1996;275:1233–1240