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Effects of cigarette smoking and age on the maturation of human oocytes

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We investigated whether cigarette smoking, measured by follicular fluid concentrations of cotinine (a major metabolite of nicotine), affects the maturity of oocytes from women undergoing in-vitro fertilization (IVF) and embryo transfer. In 234 women, follicular fluid samples were assessed for cotinine and their 2020 oocytes were assessed for maturity stage. Data on individual proportions of oocytes which were mature (OM) and were fertilized (OF) were analysed by regression in relation to age and follicular fluid cotinine. OF gave an independent assessment of oocyte maturity. Both age and follicular fluid cotinine entered the OM and OF regressions and were significant. The age-adjusted regression coefficients for log cotinine were positive; greater cotinine concentrations usually accompanied greater OM and OF. The cotinine effect on OM was positive in younger women, but it became negative (decreased OM with increasing cotinine concentrations) in older women (40 years). We further found in older women an average reduction of ~50% in the number of mature oocytes; this reduced number was lower than the number of embryos usually transferred. Smoking can reduce the number of mature oocytes even further, therefore risking a negative IVF–embryo transfer outcome. This may be the reason why the negative effects of smoking become clinically detectable in older women.

Key words: cigarette smoking/cotinine/fertilization/follicular fluid/oocyte maturation

Introduction

In a recent study on the effect of cigarette smoking on the chromosome status of unfertilized oocytes (Zenzes *et al.*, 1995), we found that the proportion of oocytes in metaphase II, namely those which were mature and gave cytogenetic data, was significantly higher in smoking women than in non-smokers. The proportion of such analysable oocytes in light smokers (1–10 cigarettes per day) was 55.0% (33/60), and in heavy smokers (>10 cigarettes per day) was 54.1% (33/61). In contrast, in non-smokers this proportion was 37.8% (197/521). Thus, the smoking groups had proportionally more mature oocytes ($P = 0.0003$) suitable for cytogenetic analysis than the non-smokers. Confirming this finding in a larger body of data and with more sensitive methods was the aim of the present study.

A majority of studies on women undergoing in-vitro fertilization (IVF) therapy that analysed the effects of smoking found no effect on the number of oocytes retrieved (Harrison *et al.*, 1990; Elenbogen *et al.*, 1991; Pattinson *et al.*, 1991; Hughes *et al.*, 1992, 1994; Rosevear *et al.*, 1992; Sharara *et al.*, 1994; Sterzik *et al.*, 1996). There is, however, no information available on whether the quality of oocytes is affected. This question is addressed in the present study, since the quality of retrieved oocytes may affect the outcome of IVF–embryo transfer.

For this analysis, data on stage of oocyte maturity and fertilization were tested for correlation with age and with concentrations of cotinine in follicular fluid samples. Cotinine, a major metabolite of nicotine, is a reliable indicator of recent smoking exposure and dose (Benowitz *et al.*, 1983). It is present in follicular fluid samples of smokers (Weiss and Eckert 1989; Rosevear *et al.*, 1992; Sterzik *et al.*, 1996; Zenzes *et al.*, 1996) and has also been detected in most passive smokers and a large proportion of women self-reported as non-smokers (Zenzes *et al.*, 1996).

Materials and methods

Subjects

This study included 234 couples participating in a hospital-based IVF–embryo transfer programme for whom follicular fluid cotinine values were determined. Each couple was represented by only one IVF cycle. Each couple signed a consent form approved by the Committee for Research in Human Subjects of The Toronto Hospital. The overall mean age of women (±SE) was 33.9 ± 0.3 years (range 24–43). The distribution of types of infertility was tubal factor only, $n = 114$; unexplained, $n = 6$; endometriosis, $n = 18$; polycystic ovary, $n = 2$; other single causes (e.g. pelvic inflammatory disease), $n = 18$; two or more causes, $n = 76$.

Women were classified into three groups according to their smoking habits: (i) non-smokers (NS; husband also a non-smoker; $n = 130$); (ii) passive smokers (PS; wife non-smoker, husband smoker; $n = 30$); (iii) active smokers (AS; husband may or may not smoke; $n = 74$). The mean (±SE) ages of these groups were not significantly different (34.4 ± 0.3, 33.9 ± 0.8 and 33.0 ± 0.4 years respectively).

Ovarian stimulation

All patients had gonadotrophin suppression by gonadotrophin-releasing hormone agonist (Lupron; Abbott, Montreal, Quebec), 1 mg s.c. daily in a long protocol with a luteal phase start. On cycle day 3, if the serum oestradiol concentration was <200 pmol/l in the absence

of ovarian cysts, multiple follicular development was induced using daily administration of 150–300 IU human menopausal gonadotrophins (Humegon from Organon, Toronto, Ontario or Pergonal from Serono, Oakville, Ontario) or highly purified human follicle stimulating hormone (Fertinorm; Serono). The dose of gonadotrophins was altered according to ovarian response, monitored by serial oestradiol concentrations and transvaginal sonography. The final stage of follicular maturation was initiated by injection of 10 000 IU of human chorionic gonadotrophin (HCG, Profasi; Serono) when at least two follicles reached a diameter of 2 cm with a serum oestradiol concentration of ~600–1000 pmol/l.

Follicular aspiration

Follicles were monitored by ultrasound (Bruel & Kjaar, Naerum, Denmark). All follicles were aspirated 36 h after HCG administration using transvaginal ultrasound guidance and local anaesthesia. Follicular fluid samples used for cotinine assay were collected in sterile centrifuge tubes. These were always the first of each ovarian aspirate in order to keep them free of medium and blood contamination. Follicular fluid samples were centrifuged at 400 *g* for 10 min. The supernatants were collected in 1 ml polystyrene cryovials and were frozen at –20°C. Follicular fluid samples were collected between June 1995 and April 1996; they were then thawed and used for cotinine assessments.

Maturity of oocytes

Oocytes were assessed for maturity stage according to conventional morphological parameters (Veeck, 1986) as follows: (i) immature: poorly expanded, dense compact cumulus; compact and adherent not radiating corona; aggregated granulosa cells; oocyte obscured; germinal vesicle observed; cytoplasm may be dark with clumped organelles; (ii) intermediate: expanded cumulus and slightly compact corona; well-dispersed granulosa; oocyte may be visible; (iii) mature: very expanded cumulus and well-dispersed radiating corona, evenly distributed around oocyte; loosely aggregated granulosa; clear zona and ooplasm; polar body visible; (iv) postmature: expanded cumulus with clumps of cells; radiant corona but often clumped; irregular, and incomplete, visible zona; ooplasm may be granular or dark.

Cotinine assay

Cotinine concentrations were assessed by radioimmunoassay, as described in Zenzes *et al.* (1996). The results were expressed as ng/ml of follicular fluid. The sensitivity (lowest detectable amount) of the assay was 0.25 ng/ml. Readings less than this were arbitrarily assigned the value of 0.10. The recovery value for cotinine was 92%.

Data analysis

Separate analyses were performed for oocyte maturity and for fertilization. Data processing and statistical analysis were performed using the StatView Statistical Package (version 4.5; Abacus Concepts, Berkeley, CA, USA) on a Macintosh Performa computer (Apple, Cupertino, CA, USA). All *P* values were two-tailed. χ^2 was used to compare group proportions. One-way analysis of variance (ANOVA) was used for comparing means, and linear regression was used to evaluate relationships between variables. Individual proportions used in regression were first transformed to arc sines (Snedecor and Cochran, 1980) and then weighted by individual sample size, i.e. number of analysed oocytes (Neter *et al.*, 1990). Logarithms (to base 10) of cotinine values were used in statistical calculations because of the extreme non-normality of the cotinine distribution (Zenzes *et al.*, 1996).

Results

Smoking status and follicular fluid cotinine

Table I gives data on the self-reported smoking status of the 74 AS women and the smoking husbands of the 30 non-smoking PS women. AS women reported smoking between one and 30 cigarettes daily, with a mean (±SE) of 9.9 ± 0.7 (SD 6.0). The smoking husbands of the PS women (who presumably were the major source of exposure to nicotine of these women) also smoked between one and 30 cigarettes/day, with a mean of 14.3 ± 1.5 (SD 8.4).

The follicular fluid cotinine and log follicular fluid cotinine concentrations (means, SE, SD and ranges) for the 130 NS, 30 PS and 74 AS women, and the totals are also given in Table I. Both mean cotinine and mean log cotinine concentrations differed greatly among the smoking groups, as shown by ANOVA, as also did pairwise comparisons. The correlation coefficient between the log follicular fluid cotinine value and the number of cigarettes smoked/day was 0.724 ($P < 0.0001$). Table I also shows that the ranges of the cotinine and log cotinine distributions for the three smoking groups overlapped. This is seen more clearly in Figure 1, which shows the frequency distributions for log cotinine.

Oocyte numbers

Of 2183 oocytes retrieved, 2020 were analysable; thus, 7.5% of the total number of retrieved oocytes could not be assessed for maturity stage. Of the oocytes analysed, 58.7% (1186/2020) were assessed as mature by morphological characteristics. The numbers of retrieved oocytes and of mature oocytes, and the proportions of oocytes which were mature, did not differ significantly among the infertility groups.

A woman's age significantly affected the number of both retrieved and mature oocytes; both values decreased with increasing age. The correlations between these numbers and age were –0.201 ($P = 0.0020$) and –0.207 ($P = 0.0014$) respectively. Using the regression equations, the number of retrieved oocytes at age 24 years was estimated to be ~12.3, reducing to ~7.0 at age 42 years. For mature oocytes, the values were ~7.1 and 3.4 respectively. In contrast, the log follicular fluid cotinine value was not correlated with total or mature oocyte number, with or without age in the regression.

Maturity stage

Table II gives the distribution of the four stages of maturity of oocytes by three log cotinine groups (<–0.4, –0.4 to +1.2 and >1.2) for two age groups [<35 ($n = 129$) and ⩾35 years ($n = 105$)]. For the younger age group, the χ^2 for the four maturity stages by log cotinine value was significant ($P = 0.0029$). This was largely due to a consistent trend for increasing proportion of oocytes which were mature with increasing cotinine concentration, and, concomitantly, to a consistent trend for decreasing proportions of oocytes which were of intermediate maturity with increasing cotinine. There was no such significant relationship in the older age group.

To examine the effect of cotinine and age more exactly, at the level of the individual woman, weighted proportions of oocytes which were mature (OM), from individual women,

Table I. Smoking status and follicular fluid cotinine concentrations (means, SE, SD and ranges) for 234 women

Variable	Status*	*n*	Mean ± SE	SD	Range
Cigarettes smoked/day	AS	74	9.9 ± 0.7	6.0	1–30
Husbands of PS women[a]		30	14.3 ± 1.5	8.4	1–30
Cotinine concentration	All[b]	234	154.8 ± 24.6	377.0	0.1–3000
(ng/ml)	NS[c]	130	8.4 ± 3.7	42.7	0.1–337.7
	PS	30	64.5 ± 37.0	202.4	0.1–964.3
	AS	74	448.4 ± 64.2	552.5	0.1–3000
Log(cotinine concentration)	All[d]	234	0.60 ± 0.09	1.42	–1–3.48
	NS	130	–0.28 ± 0.07	0.77	–1–2.53
	PS	30	0.34 ± 0.20	1.12	–1–2.98
	AS	74	2.25 ± 0.10	0.87	–1–3.48

*Smoking status of women: NS = non-smoker (husband also non-smoker), PS = passive smoker (wife non-smoker, husband smoker), AS = active smoker.
[a]To show the major source of cotinine in PS women.
[b]Significant differences among the smoking groups: NS versus AS and PS versus AS, $P < 0.0001$ by Fisher's probability of least significant difference (PLSD); NS versus PS, not significant.
[c]Of the NS women, 83 (63.8%) had detectable cotinine (>0.25 ng/ml).
[d]Significant differences among the smoking groups (all three pairwise comparisons were significant at $P < 0.0001$ by Fisher's PLSD).

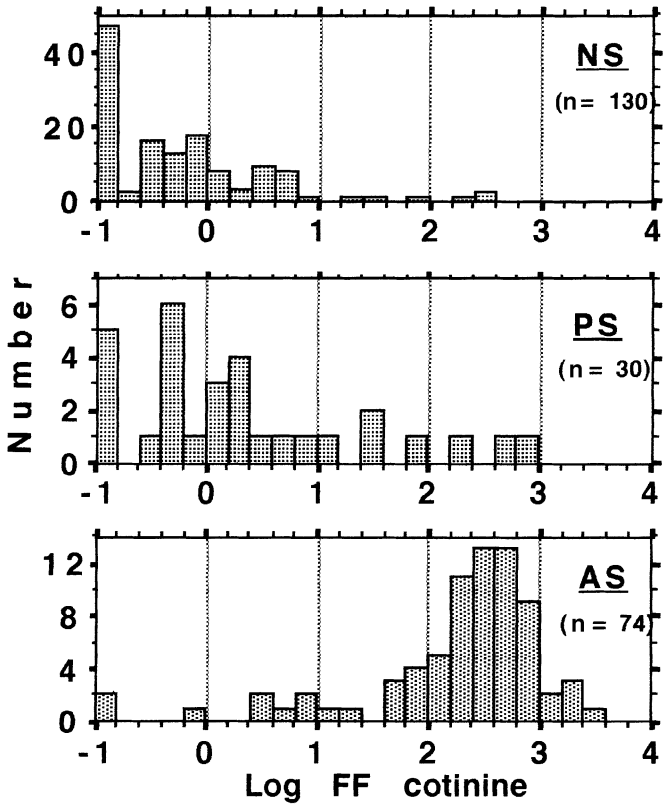

Figure 1. Distributions of log follicular fluid (FF) cotinine values for the three smoking groups. NS = non-smokers, PS = passive smokers, AS = active smokers. Note the extensive overlap in distributions.

were regressed separately on age and on log follicular fluid cotinine. Each regression was significant. The correlation with age was 0.69 ($P < 0.0001$) and with log cotinine 0.23 ($P = 0.019$). Therefore, the OM increased with increasing age or increased follicular fluid cotinine. Stepwise weighted individual regression of OM on both age and log follicular fluid cotinine included both variables in the regression; both coefficients were significant, age at $P < 0.0001$ and log cotinine at $P = 0.0005$. The correlation coefficients of each were again positive; increasing age and increasing cotinine concentration were correlated with greater OM.

Age × cotinine interaction effect on OM

Because the age effect was so strong, another term, age×log cotinine, was added to the above regression to test for possible interaction between age and cotinine. Age was again significant ($P < 0.0001$), with almost the same regression coefficient (1.362) as before (1.377); log cotinine was significant ($P = 0.006$), with a positive coefficient (15.241), and the interaction term was significant ($P = 0.020$), with a negative coefficient (–0.389). Thus the cotinine effect on OM depended partly on age. In particular, using the two cotinine coefficients (log cotinine and age×log cotinine) and noting that their effects were in opposite directions, it was possible to calculate that, when the age is about 15.241/0.389 = 39.2 years, their combined effects on OM will cancel out. Below this age, the combined cotinine effect is positive (OM increases with increasing cotinine) and above this age the combined effect is negative (OM decreases with increasing cotinine). This cotinine effect on OM steadily decreases with increasing age, becoming negative at about age 39 years. This confirmed the finding above (Table II) of a positive cotinine effect on OM only for women aged <35 years.

Fertilization rate

The fertilization rate was used as an independent and objective measure of oocyte maturity. For this analysis the 30 couples with male factor were excluded. The overall proportion of fertilized oocytes was 67.0% (1268/1893). Table III shows the number of fertilized and not fertilized oocytes by log follicular fluid cotinine group and age group, using groupings as described above for oocyte maturity. For the <35 year old group, there was no effect of cotinine concentration on the proportion of oocytes fertilized (OF). For the ⩾35 year old

Table II. Maturity stages by log follicular fluid cotinine concentration and age for 2020 oocytes from 234 women. Data are observed numbers, with percentages in parentheses

Log cotinine	*n*	Maturity stage				
		Immature	Intermediate	Mature	Postmature	Total
Age <35 years[a]						
<–0.4	31	9 (3.4)	68 (25.7)	136 (51.3)	52 (19.6)	265
–0.4–1.2	48	8 (1.5)	92 (17.7)	306 (59.0)	113 (21.8)	519
>1.2	50	4 (1.0)	63 (15.0)	262 (62.4)	91 (21.7)	420
Total	129	21 (1.7)	223 (18.5)	704 (58.5)	256 (21.3)	1204
Age ⩾35 years[b]						
<–0.4	42	10 (2.9)	69 (19.7)	196 (56.0)	75 (21.4)	350
–0.4–1.2	36	3 (1.1)	51 (18.3)	172 (61.6)	53 (19.0)	279
>1.2	27	0 (0.0)	35 (18.7)	114 (61.0)	38 (20.3)	187
Total	105	13 (1.6)	155 (19.0)	482 (59.1)	166 (20.3)	816

[a]χ^2 for maturity stages by log cotinine values = 19.93 (6 df), $P = 0.0029$.
[b]χ^2 for the maturity stages by log cotinine values is 8.57 (6 df), $P = 0.20$.

Table III. Fertilization rate by log follicular fluid cotinine concentration and age for 1893 oocytes from 204 women. Thirty male factor couples were excluded. Data are observed numbers, with percentages in parentheses

Log cotinine	*n*	Fertilization		
		Not fertilized	Fertilized	Total
Age <35 years[a]				
<–0.4	26	76 (30.6)	172 (69.4)	248
–0.4–1.2	42	168 (35.6)	304 (64.4)	472
>1.2	44	128 (32.1)	271 (67.9)	399
Total	112	372 (33.2)	747 (66.8)	1119
Age ⩾35 years[b]				
<–0.4	38	109 (32.5)	226 (67.5)	335
–0.4–1.2	33	110 (38.7)	174 (61.3)	284
>1.2	21	34 (21.9)	121 (78.1)	155
Total	92	253 (32.7)	521 (67.3)	774

[a]χ^2 for the proportions of fertilized oocytes by cotinine concentrations = 2.17 (2 df), which was not significant.
[b]χ^2 for the proportions of fertilized oocytes by cotinine concentrations = 12.86 (2 df), $P = 0.0016$.

group, however, the χ^2 probability was 0.0016 for an effect of cotinine concentration on OF. This effect, however, was not linear, as is shown in Table III.

To explore these effects of cotinine and age more exactly at the level of the individual woman, as above, weighted individual OF values were used in regressions. OF regressed on age alone was significant and positive (increasing age was associated with increasing OF; $P < 0.0001$). The correlation coefficient was 0.68. A similar regression on log follicular fluid cotinine alone, however, was not significant. Stepwise weighted individual regression on both age and log follicular fluid cotinine showed that both entered positively into the regression and both were significant: age, $P < 0.0001$ and log follicular fluid cotinine, $P = 0.0065$. An age×cotinine term, however, was not significant. These regression results for OF showed, after adjusting for age, increased OF coincided with increased cotinine. This agreed with the above regression results for OM.

Discussion

We examined the effects of age and smoking on the proportion of oocytes which were mature (OM) and were fertilized (OF) by two methods: χ^2 analysis of traditional grouped tables and individual weighted regression. The results of the two methods generally agree. When they differ, we note that the regression method should be considerably more reliable; it uses exact ages and cotinine concentrations and weights the proportion by the number of oocytes. This is apparently the first time that the effects of female smoking have been analysed in individual women using exact ages and cotinine concentrations.

We have performed our analysis of effects of smoking on oocyte maturation by using follicular fluid cotinine, a reliable marker for recent smoking and dose (Benowitz *et al.*, 1983). We found that the mean follicular fluid cotinine values for the three smoking groups differed significantly, as previously reported by Zenzes *et al.* (1996). We also found a great overlap in the distribution of follicular fluid cotinine values of the three smoking groups. A non-smoker may have 100 ng/ml of cotinine in follicular fluid while an active smoker may have 1 ng/ml. These were also the conclusions of our previous study (Zenzes *et al.*, 1996).

Smoking effects on OM and OF

The tables show a strong positive 'cotinine effect' (i.e. an effect of smoking detected by cotinine) on OM for women <35 years of age. This agrees quite well with the regression findings: a strong positive correlation between log cotinine and OM, after correcting for age, for younger women. In contrast, in women aged ⩾40 years, the cotinine effect is negative: the OM decreases with increasing cotinine. This result suggests that a deleterious effect of smoking becomes detectable in older women; this is discussed in detail below.

OF was used as an independent and objective measure of oocyte maturity, since human oocytes that are mature have a higher probability of achieving normal fertilization than immature oocytes (Van Blerkom *et al.*, 1994). We found a significant but non-linear cotinine effect on OF in the older age group,

but no effect in the younger group. The regression findings show an age-corrected cotinine effect. The separate OM and OF results generally agree, showing strong, age-corrected positive effects of cotinine. Perfect agreement is not to be expected since the OF results should also reflect the male contributions. It is noteworthy that the strong effect of age is pervasive in all these analyses.

The effect of cotinine on the proportion of oocytes which are mature, found in the above OM and OF analyses, supports a previous cytogenetic study (Zenzes *et al.*, 1995). This study found a higher proportion of mature oocytes in metaphase II which gave cytogenetic data in smokers, compared with non-smokers. In this study, however, age was not considered in the analysis. A possible mechanism proposed was that the oocytes of smokers have an earlier delay in maturation and, therefore, are less mature at the time of retrieval. At the time of fixation, 44 h later, these oocytes have matured *in vitro*, while those of non-smokers are already degenerating and becoming less suitable for cytogenetic analysis (Zenzes *et al.*, 1995). In the present study, maturity was assessed at the time of retrieval, and the data do not support the above hypothesis. The reasons for an increased OM in younger smoking women are unknown to us and remain to be elucidated.

The increase of OM in younger women due to smoking, found in the present study, was significant but relatively small, i.e. from ~51% to ~62% (Table II). Thus, one would expect that it would have little or no significant effect on embryo quality and pregnancy rates. These women, on average, already have more mature fertilized oocytes than the number of embryos usually transferred, before smoking effects are considered [i.e. ~7.1 oocytes are expected from women aged 24 years; at least 70% (see Table III) of these should be fertilized: $7.1 \times 0.7 = \sim 5$ mature fertilized oocytes per woman]. This expectation agrees with most studies on smoking and IVF–embryo transfer outcome. Three studies reported reduced rates of fertilization in smokers (Elenbogen *et al.*, 1991; Rowlands *et al.*, 1992; Rosevear *et al.*, 1992), but they used relatively low numbers of patients (range 41–71). In contrast, six studies using larger numbers of patients (range 54–650) did not find a reduction (Trapp *et al.*, 1986; Harrison *et al.*, 1990; Pattinson *et al.*, 1991; Hughes *et al.*, 1992; Van Voorhis *et al.*, 1992; Sterzik *et al.*, 1996). Two of these six (Harrison *et al.*, 1990; Hughes *et al.*, 1992), and the present study ($n = 234$), found increased fertilization rates in smokers.

Further support for our present finding is given by a recent study on the effect of follicular fluid cotinine concentration on embryo quality (Zenzes and Reed, 1996), where it was found that follicular fluid cotinine concentrations were positively correlated, in a dose-dependent manner, with embryo quality. The proportion of fragmented embryos decreased with increasing concentrations of cotinine. These results suggest that these embryos developed from oocytes of good quality, thus confirming our present OM finding. There is also support from a majority of studies on smoking and IVF outcome. These report similar rates of pregnancy between smokers and non-smokers (Trapp *et al.*, 1986; Elenbogen *et al.*, 1991; Pattinson *et al.*, 1991; Hughes *et al.*, 1992; Van Voorhis *et al.*, 1992; Sterzik *et al.*, 1996). Only one study found reduced pregnancy rates in smokers (Harrison *et al.*, 1990).

Effect of advanced age on OM and OF

In our study, the effect of age on OM and OF shown in the regression analyses is stronger than the cotinine effect; the latter's strong effect is revealed only after correcting for age. These results show that, unless corrected for, the strong age effect can mask the possible deleterious effects of cotinine. The negative effects of cotinine on OM at advanced ages ($\geqslant$40 years) may represent a cumulative effect of long-term smoking.

This finding of an effect of age is supported by studies which analysed age and smoking effects together on IVF–embryo transfer outcome. Sharara *et al.* (1994) reported diminished ovarian reserve, defined as decreased ovarian responsiveness to external gonadotrophins, in active smokers aged between 35 and 39 years, compared to age-matched non-smokers, suggesting that smoking may accelerate this age-dependent process. The earlier natural menopause experienced by women who smoke (Midgette and Baron, 1990) suggests that smoking accelerates follicular depletion and oocyte atresia in older women. Hughes *et al.* (1994) found that female age $\geqslant$35 years had a negative impact on conception; combining all the published data suggested a significant deleterious effect of smoking on time to conception. The study of Sharara *et al.* (1994), together with our present findings, provide strong evidence that advanced age permits the effects of smoking to become clinically detectable.

Effect of age and smoking on number of oocytes

Using the regressions of oocyte number on age, we found that both the numbers of retrieved oocytes and of mature oocytes decreased by approximately 50% between the ages of 24 and 42 years. However, a cotinine effect on the number of retrieved or mature oocytes was not found, even after correcting for age. These appear to be the first published data on the number of mature oocytes in IVF–embryo transfer in relation to age. From our data, we estimate that, on average, the number of mature oocytes drops from ~7.1 at age 24 years to ~3.4 at age 42 years. This marked reduction in the number of mature oocytes in older women has major implications for IVF–embryo transfer outcome, as discussed below.

Our finding of no cotinine effect on the number of retrieved oocytes agrees with a majority of other studies in finding no such effect of smoking (Harrison *et al.*, 1990; Elenbogen *et al.*, 1991; Pattinson *et al.*, 1991; Hughes *et al.*, 1992; Rosevear *et al.*, 1992; Sharara *et al.*, 1994; Sterzik *et al.*, 1996). The study of Van Voorhis *et al.* (1992) found a significant ($P < 0.01$) decrease in the number of oocytes retrieved in smoking women, with no difference in nuclear maturity between smokers and non-smokers; however, this study included only 18 smoking and 36 non-smoking women. Hughes *et al.* (1994) found a significant increase in the number of retrieved oocytes ($P < 0.05$) in their smoking group, but found no increase in the number of follicles $>$10 mm diameter suggestive of mature oocytes.

Proposed mechanism for smoking effects in older women

In our study, women aged ⩾40 years had, on average, ~3.4 mature oocytes, and of these ~70% (Table III) were fertilized: 3.4×0.7 = ~2.4 mature fertilized oocytes per woman. Thus, there was already an average deficit of embryos for transfer in these older women, compared with the younger women, before the additional deficit caused by smoking. More explicitly, the effect of advanced age in drastically reducing the number of mature oocytes in older women is further augmented by the negative effects of smoking on the proportion of mature oocytes. Therefore, older smoking women are doubly deficient in mature oocytes and should have markedly reduced fertility.

In conclusion, our statistical analysis using weighted individual regression is a sensitive and powerful approach. Our results show that smoking, as measured by cotinine concentration in follicular fluid, has a negative (decreasing) effect only in older women. With advanced age, when the number of mature fertilized oocytes is, on average, drastically reduced to below the number of embryos usually transferred, the deleterious effect of smoking becomes detectable.

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