

Cigarette smoking during early pregnancy reduces the number of embryonic germ and somatic cells

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BACKGROUND: Cigarette smoking during pregnancy is associated with negative reproductive consequences for male fetuses in adult life such as reduced testicular volume and sperm concentration. The present study evaluates the number of germ and somatic cells present in human embryonic first-trimester gonads in relation to maternal smoking.

METHODS: The study includes 24 human first-trimester testes, aged 37–68 days post-conception, obtained from women undergoing legal termination of pregnancy. A questionnaire was used to obtain information about smoking and drinking habits during pregnancy. Validated stereological methods were used to estimate gonadal cell numbers in histological sections. Results were also evaluated in the context of previously published data on ovaries from our laboratory.

RESULTS: A significant reduction in the number of germ cells by 55% [95% confidence interval (CI) 74–21% reduction, $P = 0.004$] and somatic cells by 37% (95% CI 59–3%, $P = 0.023$) was observed in testes prenatally exposed to maternal cigarette smoking, compared with unexposed. The effect of maternal smoking was dose-dependent being higher in the heavy smokers and remained consistent after adjusting for possible confounders such as alcohol and coffee consumption ($P = 0.002$). The number of germ cells in embryonic gonads, irrespective of gender, was also significantly reduced by 41% (95% CI 58–19%, $P = 0.001$) in exposed versus non-exposed embryonic gonads.

CONCLUSIONS: Prenatal exposure to maternal cigarette smoke reduces the number of germ and somatic cells in embryonic male and female gonads. This effect may have long-term consequences on the future fertility of exposed offspring. These findings may provide one potential cause of the reduced fertility observed during recent years.

Key words: germ cells / human embryos / first-trimester pregnancy / smoking / PAH

Introduction

The frequency of malfunctions observed in the male reproductive system is reported to increase during recent years (Wohlfahrt-Veje *et al.*, 2009). The cause(s) have not yet been determined, but some studies suggest a common fetal origin (Wohlfahrt-Veje *et al.*, 2009).

Benzo[a]pyrene (BaP) belongs to the polycyclic aromatic hydrocarbons (PAHs) and is one of the main toxic and hormone-modulating components of cigarette smoke (MacKenzie and Angevine, 1981; Smith *et al.*, 2000). In a mouse model, reduced testicular weight,

induced atrophy of the seminiferous cords, and altered spermatogenesis in male progeny of BaP-exposed mothers have been reported (MacKenzie and Angevine, 1981). Studies of men exposed to maternal cigarette smoke *in utero* showed reduced sperm concentration and count and reduced testis size (Storgaard *et al.*, 2003; Jensen *et al.*, 2004, 2005; Ramlau-Hansen *et al.*, 2007; Fowler *et al.*, 2009). PAH from maternal cigarette smoke has been suggested to act directly on human fetal germ cells inducing apoptosis and, as a consequence, result in reduced spermatogenesis in adult life (Coutts *et al.*, 2007).

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Further, a 48% decrease in the sperm concentration was found among young men prenatally exposed to heavy smoke from their mothers compared with unexposed men (Storgaard et al., 2003). In addition, two other studies reported around 20% lower sperm concentration and 25–38% lower total sperm count in men *in utero* exposed to smoke from their mothers (Jensen et al., 2004; Ramlau-Hansen et al., 2007). Furthermore, smaller testis size among men exposed to maternal cigarette smoking *in utero* was reported and found to be dose-dependent (Jensen et al., 2004, 2005). However, these results are not consistently reported; one study found no association between maternal smoking during pregnancy and the semen concentration in adult sons (Ratcliffe et al., 1992). However, women included in both case and control groups of this study had been exposed to diethylstilbestrol (DES) during pregnancy, and since DES itself may reduce semen quality (Gill et al., 1979), it is difficult to deduce the origin of the effect on sperm quality.

Human chorionic gonadotrophin (hCG) stimulates fetal testosterone secretion (Huhtaniemi et al., 1977), which exerts a major function in testes development, masculinization and descent. Decreased hCG levels were found in human aborted fetuses exposed to maternal cigarette smoke *in utero*. However, the decreased level of hCG was not mirrored in a reduced testosterone output (Fowler et al., 2009).

Taken together, the available data suggest that maternal smoking during pregnancy impacts on the fertility of their sons later in life and may, to some extent, explain the increased incidence of malfunctions observed in the male reproductive system.

The aim of this study was prospectively to investigate whether *in utero* exposure to maternal smoking negatively affected the number of germ cells and somatic cells in the testes of human first-trimester embryos.

Materials and Methods

The present study included a total of 24 human embryonic testes [aged 37–68 days post-conception (pc)] obtained from women who underwent legal termination of pregnancy. Urine and blood samples were obtained preceding the evacuation procedure. All participants answered a questionnaire about their lifestyle during the pregnancy, including smoking and drinking habits.

Participating women

The participating women [aged 19–39 years (mean \pm SD, 28.2 \pm 6.0)] were treated at the Department of Obstetric and Gynaecology, Frederiksberg Hospital, Denmark. Women suffering from any chronic disease or who were dependent on an interpreter were excluded from the study. Each woman received oral and written information according to and approved by 'The Scientific Ethical Committee for the Capital Region' [KF (01) 258206] and consented to participate.

Determination of embryonic sex and age

The chromosomal male sex (XY) was determined by polymerase chain reaction (PCR) of a small piece of snap-frozen embryonic tissue, using X-Y homologous primers (Nakahori et al., 1991). In those testes, in which differentiation had taken place, the sex was verified by the appearance of testicular cords of the histological sections. Fetal age was determined by vaginal ultrasound measurements of the crown rump length (Wisser et al., 1994) and the gestational age was converted to age pc by subtracting 14 days.

Cotinine assay

Cotinine is the major metabolite of nicotine and an appropriate indicator of tobacco exposure, since it can be detected in urine and blood 48 h after exposure (Benowitz, 1996). Serum and urine concentrations were measured using a specific enzyme-linked immunosorbent assay (ELISA) (Cotinine, # CO096D, Calbiotech, CA, USA) and were used to verify the reliability of the women's self-reported smoking habits.

Tissue processing and histology

The embryos were removed from the *uterus* according to routine procedures, with slight modifications in order to prevent damage to the tissue (Lutterodt et al., 2009). Embryos were dissected under a stereomicroscope and the gonadal-mesonephric-duct complexes removed. One randomly chosen left or right testis was isolated and used for stereological sampling. The tissue were fixed in Bouin's solution (Bie & Berntsen, Herlev, Denmark) and processed for paraffin embedding. The gonads were embedded with random orientation and cut into serial sections with a thickness of either 30 or 50 μ m. Sections were stained according to standard methods with Mayer's haematoxylin and periodic acid-Schiff reagent.

Stereology

To estimate the number of cells in the gonad, the optical fractionator technique was used (West et al., 1991). This method provides unbiased estimates of the total cell number in an organ by counting only a fraction of the cells, when the cell population is too large to be counted exhaustively (West et al., 1991). Furthermore, this method ensures that all parts of interest have an equal probability of being sampled (West et al., 1991). However, the method does require that the entire undamaged gonad is available for sampling (Dorph-Petersen et al., 2001). The total cell number in the organ (N) was estimated by multiplying the number of cells sampled ($\sum Q^-$) by the reciprocal of the three sampling fractions: the number of samples measured (ssf), the area of the unbiased counting frame (asf) and the height of the optical disector (hsf) (Dorph-Petersen et al., 2001).

$$N = \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{hsf} \times \sum Q^-$$

Identification of cell types

The cell types were identified by the morphology of the cell and in particular of the nuclei. In differentiated testes, the morphology of germ cells and Leydig cells appeared similar. Thus, germ cells were defined as situated inside the testicular cords and Leydig cells outside, as described by Bendtsen et al. (2003). One person only, who was unaware of the fetal exposure information, performed the stereological sampling to eliminate interpersonal variation. In order to verify that the sampling technique was reproducible with an acceptable intra-personal variation, the germ cells of one randomly chosen testis were counted three times and showed a variation of 4.8% [21 137 \pm 1021 (mean \pm SD)], which was considered satisfactory.

Counting rules and precession of the estimate

For counting, the unbiased optical disector counting rules, based on the original physical disector counting rules, were used (Gundersen et al., 1988; West et al., 1991; Dorph-Petersen et al., 2001). In stereology, the results of the estimation are dependent on the precision and accuracy

of the estimator, which is measured by estimates of the coefficient of error (CE). A CE, calculated according to [Dorph-Petersen et al. \(2001\)](#), <0.1 was considered acceptable. In the present study, the CE was calculated to be 0.05 and 0.06 in the unexposed and exposed group, respectively, and was achieved by counting ~100 observations in a systemic random manner in each testis. One hundred observations are considered satisfactory for estimation according to the standard counting rules ([Gundersen et al., 1988](#)).

Statistical methods

To study the association between maternal smoking and the number of germ and somatic cells, multiple linear regression models were used on the log-transformed cell counts. The main interest was in the effect of smoking adjusted for age and gender. The model assumptions were checked using, among other things, residual plots and outliers and influential observations were identified. The potential confounders, alcohol and coffee consumption, were included in the models to see whether the effect of maternal smoking was robust. To test whether the effect of smoking was similar for germ and somatic cells, the two cell types were modelled simultaneously using a multivariate linear regression allowing for correlation between log-transformed cell counts from the same

fetus. All analyses were conducted using STATA version 11 (StataCorp LP, College Station, TX, USA).

Results

Cotinine levels exceeding 13.7 ng/ml in plasma have previously been accepted as a cut-off value for discriminating between smokers and non-smokers ([Jarvis et al., 1987](#)). According to this standard, the self-reported smoking status was considered to be reliable (Table 1).

The two groups of participating women, smokers and non-smokers, were similar in regard to age, height and body mass index (BMI); the group who smoked during pregnancy aged 26.6 ± 6.1 years (mean \pm SD), were 1.70 ± 0.06 m high and had a BMI of 23.1 ± 2.8 and the non-smokers aged 31 ± 5 years (mean \pm SD), were 1.69 ± 0.05 m high and had a BMI of 22.1 ± 2.0 .

The two groups of male embryos, exposed and unexposed to cigarette smoke, were similar with regard to age; exposed: 47.9 ± 8.5 days pc and unexposed 47.9 ± 7.1 (mean \pm SD) (Table 1).

The mean (\pm SD) number of germ and somatic cells in exposed testes was estimated to $18\,500 \pm 19\,900$ and $679\,100 \pm 652\,600$

Table 1 Embryonic age in days pc, the estimated number of germ, somatic and Sertoli cells per testis.

Age in days pc	Number of germ cells/testis	Number of somatic cells/testis	Number of Sertoli cells/testis	Number of cigarettes/day	Cotinine in serum (ng/ml)
Exposed					
37	1.288	74.201	—	21–25	60
37	1.660	129.894	—	16–20	77
40	678	85.222	—	11–15	11
40	2.523	98.446	—	o.e.*	<1
43	9.194	236.762	—	1–5	36
44	2.536	152.867	—	1–5	75
44	8.797	526.923	179.453	o.e.*	<1
50	10.653	580.289	155.069	11–15	148
50	6.413	300.706	118.034	16–20	204
51	22.189	1.045.858	362.212	o.e.*	7
51	38.392	956.859	345.532	16–20	187
51	24.818	952.371	435.276	1–5	59
56	54.798	1.426.302	398.253	6–10	4
57	33.525	1.282.620	310.006	11–15	321
68	60.421	2.336.739	451.604	11–15	58
Unexposed					
40	3.464	133.689	—	—	<1
41	12.575	403.733	—	—	<1
44	20.751	894.466	364.559	—	<1
44	6.315	293.988	108.123	—	<1
45	12.559	249.454	119.034	—	<1
46	14.375	433.092	130.906	—	<1
56	65.237	2.102.026	439.184	—	<1
57	98.319	2.709.792	837.295	—	<1
58	49.904	1.841.449	499.038	—	<1

The daily maternal cigarette consumption and the maternal serum cotinine concentration on the evacuation day are presented.

*o.e., occasionally exposed.

per testis, respectively, versus $31\,500 \pm 32\,600$ and $1\,006\,900 \pm 958\,200$ per testis in unexposed testes. The cell numbers were estimated in an average of 8.1 and 9.5 sections per testis in the unexposed and exposed groups, respectively. An average of 152 germ cells and 236 somatic cells were counted per testis versus 125 and 283 in the unexposed group, respectively.

The doubling time of male germ cells was calculated to be 4.7 days [95% confidence interval (CI) 3.6–5.9] and that of somatic cells was 5.5 days (95% CI 4.3–6.6).

The regression lines for both genders (Fig. 1) were parallel and allowed for the use of the statistical analysis. This also illustrates that the growth rate and the effect of smoking affected gender in the same way. A significant reduction in the number of germ cells by 55% (95% CI 74–21% reduction, $P = 0.004$) and that of somatic cells by 37% (95% CI 59–3%, $P = 0.023$) was observed in testes prenatally exposed to cigarette smoking compared with unexposed, when adjusted for age. The effect of maternal smoking was dose-dependent with a higher effect in the heavier smokers (Table II). Two observations obtained from exposed testes had a remarkably low number of germ cells in relation to age and were considered as potential high-influence observations. Excluding these two observations temporarily from the analyses, the number of germ cells was still significantly reduced ($P = 0.009$). On the number of somatic cells, the effect of smoking was no longer significant after excluding two high impact observations from the data set in relation to age from the test ($P > 0.05$) (Fig. 1).

Germ cell number in testes and ovaries in relation to smoking exposure

The human embryonic male gonads of the present study were originally collected by Lutterodt et al. (2009), who studied the numbers of oogonia and somatic cells in female embryos and fetuses in relation to maternal smoking exposure. We have analysed the data from the present study together with those from our previously published study (Lutterodt et al., 2009). Thus, the whole data set used for the present statistics consisted of 28 embryonic ovaries (Lutterodt et al., 2009) and 24 embryonic testes. The smoking exposure of male and female embryos was not identical, as the male conceptuses had been exposed to a median level of 11–15 cigarettes a day, where the median female exposure was only 'occasionally exposed' (data not shown). The difference was, however, not statistically significant (Fisher's exact test, $P = 0.14$).

Analysing the effect of maternal smoking on the number of germ cells in human embryonic gonads adjusting for gender and age showed that the number of germ cells was significantly reduced by 41% (95% CI 58–19%, $P = 0.001$) when the exposed and non-exposed groups were compared (Table II). This significant negative effect was dose-dependent and was consistent after adjusting for possible confounders such as alcohol and coffee consumption ($P = 0.002$), a result that was maintained even after excluding two potential high-influence observations as discussed above ($P = 0.003$).

The effect of smoking on the number of somatic cells showed a similar significant reduction by 29% (95% CI 45–8%, $P = 0.006$).

We also evaluated whether maternal smoking showed a specific selective negative effect on germ cells when compared with somatic

cells. By analysing the estimated cell numbers of germ and somatic cells together, a statistically significant negative effect on germ cells was observed in relation to the somatic cells ($P = 0.045$).

Discussion

This study demonstrates a significant reduction in the number of germ cells in embryonic gonads obtained from women who smoked cigarettes during the first trimester of pregnancy when compared with those who did not. This effect of maternal smoking was most pronounced in male embryos where the number of germ cells was reduced by 55%, but a highly significant reduction was observed irrespective of the gender. Furthermore, the reduction in germ cell number increased with the number of cigarettes that the mother smoked. Maternal smoking was also observed to significantly lower the number of somatic cells in exposed gonads, irrespective of gender.

Collectively, the present study showed a pronounced negative effect on the growth of the developing gonad by maternal cigarette smoking and suggests that the gonads are susceptible to exogenous factors just around the crucial and critical time of gonadal differentiation.

The present study does not clarify whether the reduction in cell numbers (i.e. somatic and germ cells) is permanent or reflects a growth delay, which may be compensated for later in life. It is possible that germ and somatic cells are more sensitive to smoke in very young embryos (e.g. aged 40–50 days pc), compared with older embryos, since the magnitude of growth restriction did not increase with age, and thereby the exposure time, in exposed embryos. It will be of importance to determine the mechanism of the reduced cell numbers, since one of the major determinants of the mature testis size is the final Sertoli cell complement and only secondarily germ cell numbers. When sufficient germinal stem cells are present, the available evidence is that under Sertoli cell control, germ cells replicate to the final carrying capacity dictated by Sertoli cell numbers. However, in the present study, both germ and somatic cell numbers were significantly reduced and it cannot be determined whether the observed effects are permanent or temporary.

The fact that maternal smoking is known to cause fetal growth restrictions may have lead to an underestimation of the embryonic age, and thereby, the estimated cell numbers in relation to age will also be underestimated and the effect of smoking may be more pronounced than the present results show.

The reliability of the stated smoking habits was verified by cotinine analysis, which is a derivate from the nicotine metabolism (Shipton et al., 2009) that can be detected in plasma up to 48 h after exposure (Benowitz, 1996). The undetectable cotinine levels obtained from two women, who claimed to smoke occasionally, may be explained by exposure having taken place more than 48 h before the blood sample was obtained.

It is possible that the negative effect of maternal smoking during pregnancy may influence on the future fertility of offspring exposed to cigarette smoke prenatally, since the undifferentiated embryonic germ cells are the progenitors of spermatogonia in males and oocytes in females (Byskov and Høyer, 1994). Increased reproductive problems have been observed in the adult male population during the past few decades (Swan et al., 2000; Wohlfahrt-Veje et al., 2009) and

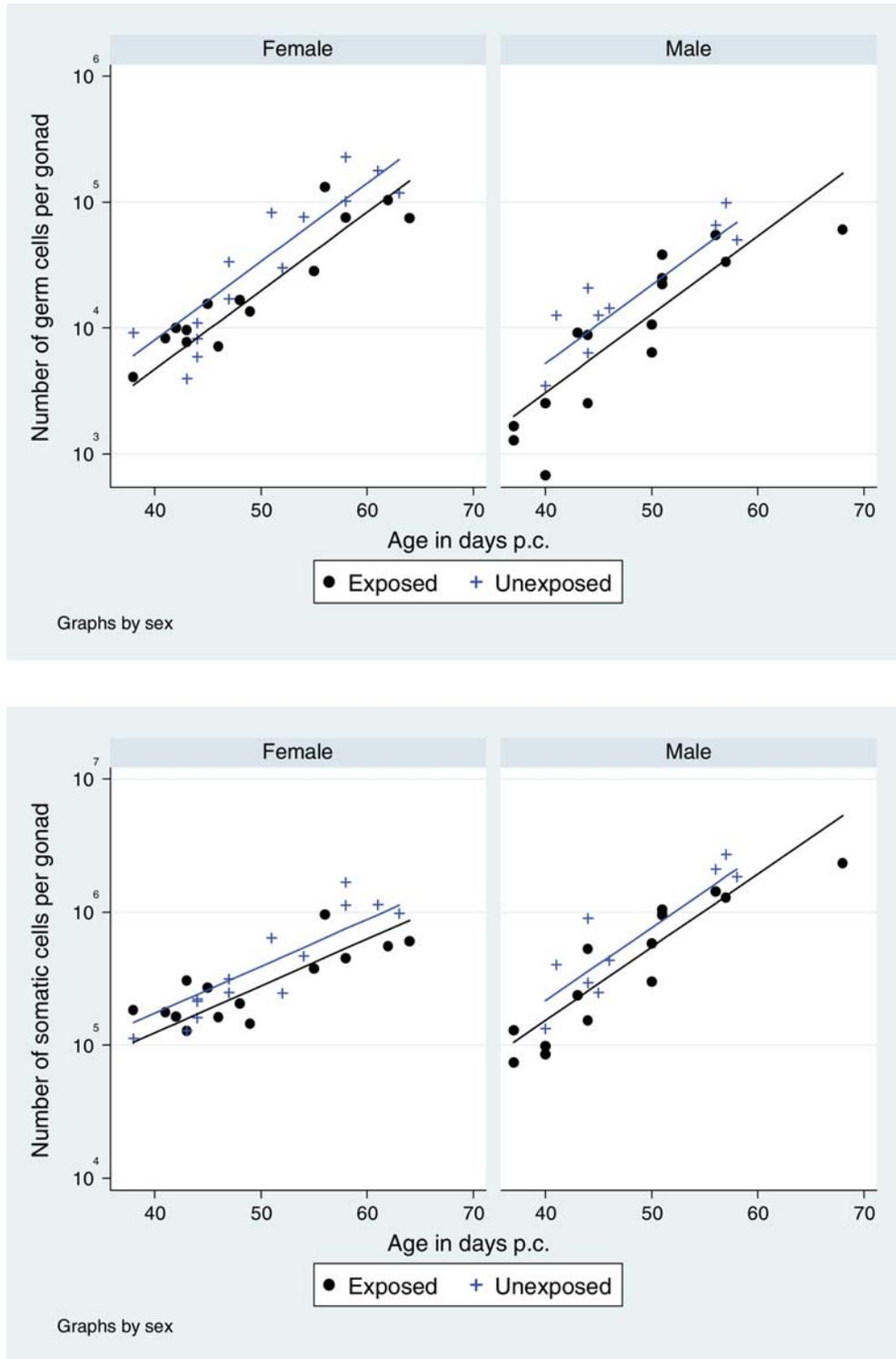


Figure 1 The linear regression lines of log-transformed number of germ and somatic cells per gonad in male and female embryos in relation to age in days pc and smoking exposure. Note that three observations, aged 51 days pc, are included in the graph illustrating the number of somatic cells per gonad in male embryos, giving the impression of only two.

may, at least to some degree, be founded already during embryonic and fetal life through maternal cigarette smoking, by reduction in the germ cell potential together with the supportive foundation.

Although the prevalence of smoking during pregnancy in the industrial countries has declined over the last decade, one in eight mothers continues to smoke throughout their pregnancy (Colman and Joyce,

Table II The effect of cigarette exposure on log-germ cells, adjusted for embryonic age and gender.

	Estimate (95% confidence interval)	P-value
Unexposed	0	0.0142
Occasionally exposed	-0.21 (-0.41, -0.02)	
1–15 cigarettes/day	-0.23 (-0.43, -0.03)	
16–25 cigarettes/day	-0.27 (-0.49, -0.04)	

A dose-dependent effect of cigarette exposure was found.

2003; Egebjerg Jensen et al., 2008). Among Danish women younger than 20 years at delivery, the prevalence of smoking has actually increased to 43% in 2005 (Egebjerg Jensen et al., 2008). This tendency is alarming, and besides the several well-described adverse effects of smoking during pregnancy, this study adds another to the list. In the present study, more than half of the included subjects were current smokers, a higher proportion than the general population. This could potentially indicate that the women of the present study have different socio-economic conditions that may affect the outcome. However, none of the parameters that we assessed seem to suggest that the two groups differ.

Our previously published study by Lutterodt et al. (2009) found a non-significant trend towards a reduced number of oogonia in embryonic ovaries exposed to maternal smoking. Analysing the results from the present study together with the results from Lutterodt et al. (2009) showed a significant reduction in the number of germ and somatic cells by 41 and 29%, respectively. However, the actual exposure to smoking in the exposed group of female embryos was not as strong as in the exposed group of male embryos of the present study. Since the present study found a dose-dependent effect of smoking on gonadal cell number in both the testis group alone and in the combined testis–ovary group, it is likely that the effect of maternal smoking on female gonads was less pronounced due the lower exposure.

Furthermore, we analysed the effect of maternal smoking on embryonic germ and somatic cell number together and found a significantly more pronounced effect on germ cells than somatic cells. This suggests that germ cells are more sensitive to maternal smoking than somatic cells. Since Sertoli cells in the differentiated testis are essential for the growth and survival of germ cells (Byskov and Høyer, 1994; De Gendt et al., 2004; Klönisch et al., 2004), a reduction in the number of embryonic somatic cells may result in insufficient support of the germ cells in the testis, leading to the significant reduction of surviving germ cells as observed in the present study.

The present study does not provide information on the mechanism by which maternal cigarette smoking reduces the number of germ cells in embryonic gonads. However, exposure to BaP and PAH in mice (MacKenzie and Angevine, 1981), rats (Lee and Nagayama, 1980; Ramesh et al., 2001; Archibong et al., 2008) and humans (Coutts et al., 2007) of both adults and *in utero* individuals has demonstrated a number of negative effects on reproductive functions, irrespective of gender. Thus, the negative effect on both germ and somatic cells

in male and female embryos of the present study could also argue for a more general gonadotoxic effect.

Epidemiological studies like this are highly difficult to perform because of the numerous factors that can act on the embryonic testis development, including maternal socio-economic components, lifestyle habits, together with the general health of the mother. We have tried to adjust for some of these potential confounders such as alcohol and coffee consumption and have compared some parameters of the smoking and non-smoking mothers including age, height and BMI and found them to be similar.

In conclusion, maternal cigarette smoking during the first trimester of pregnancy exerts a significant and dose-dependent reduction in the number of embryonic germ and somatic cells that may affect the future fertility potential of the exposed individuals.

Ethical approval

'The Scientific Ethical Committee for the Capital Region' [KF (01) 258206] has given their approval for this study. All participants gave informed consent before taking part and have given written consent to their data can be included in publications.

Authors' roles

L.S.M. was responsible for writing the paper, did the stereological samplings, analysed and interpreted data. M.C.L. collected the human embryos, formulated questionnaires and assisted with the stereological set-up. E.W.A. did the statistical calculations. S.O.S. did the clinical guidance. K.P.S. performed tissue preparation and PCR. C.Y.A. interpreted data, assisted in writing the paper and was responsible for the study design. A.G.B. did the morphological guidance, assisted in writing the paper and was responsible for the study design.

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