Smoking habits of parents and male: female ratio in spermatozoa and preimplantation embryos

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BACKGROUND: Previous observations have addressed a decreased male:female ratio associated with smoking. Our aim was to assess whether this effect is observed at the spermatozoa or at the early embryo development.

METHODS: We retrospectively assessed smoking intake habits of 56 couples included in our preimplantation genetic diagnosis (PGD) program. Three groups were established according to male or female cigarette consumption per day: non-smokers, smokers (1–19 cigarettes per day) and heavy smokers (>20 cigarettes per day). Fluorescence in-situ hybridization (FISH) was performed on ejaculated sperm samples to analyse chromosomes X and Y. On day 3, embryos were also analysed. Additionally, sperm samples from four heavy smoking and four non-smoking donors were prospectively analysed before and after capacitation.

RESULTS: FISH on spermatozoa revealed no statistical differences in the Y:X ratio between the three groups. However, in the PGD study, in male heavy smokers, the XY:XX embryo ratio was decreased compared with non-smokers (22:47 versus 80:71; P = 0.0057). The smoking condition of the female partner had no significant effect on embryo XY:XX ratio, but for non-smoking females with a heavy smoking partner, the ratio was decreased (P = 0.0018) compared with non-smoking males. In heavy smoking donors a decreased Y:X ratio was observed after swim-up with a statistically significant difference of ratios (P = 0.021).

CONCLUSIONS: Smoking habits of males do not have an effect on the percentage of X- and Y-bearing spermatozoa on ejaculated samples. However, male heavy smokers produce an increased incidence of female embryos that could be related to an enrichment of X spermatozoa after swim-up in patients with high tobacco consumption.

Key words: cigarette/fluorescence in-situ hybridization/preimplantation genetic diagnosis/sex ratio/sperm

Introduction

There is evidence that the male:female ratio has declined in many industrialized countries in the last decades (Moller, 1996; Davis et al., 1998). Although the reason for this trend is not fully understood, exposure to toxic environmental agents (Mocarelli et al., 2000; Sakamoto et al., 2001) and stress (Fukuda et al., 1998) have been pointed out as possible causes.

Some recent reports have also related this decrease to cigarette smoking. Fukuda et al. (2002) assessed the periconceptional smoking habits of >5000 couples and found the lowest male:female ratio among children whose mothers and fathers both smoked >20 cigarettes per day. Mills et al. (2003) studied >26,000 women over a 15 year period and found a significant decrease in the male:female ratio, but no association with the smoking habit of the mother, indicating that the effect of smoking on sex ratio could not be attributed to maternal or environmental effects.

There are controversial reports in the literature, some of them showing a detrimental effect of cigarette smoking on sperm quality (Künzle et al., 2003), with a decrease in sperm density, total sperm count, motility, morphology, citrate concentration and pH (Vine et al., 1994; Vine et al., 1996; Shi et al., 2001; Künzle et al., 2003; Zitzmann et al., 2003). However, other authors have published that cigarette smoking did not appear to affect sperm quality in fertile men (Chia et al., 1998). Martini et al. (2004) did not find statistical differences in seminal parameters according to the degree of alcohol or tobacco consumption in a large population of men attending an andrology laboratory. However, when patients with these two habits were compared with those without, a significant reduction in seminal volume, sperm concentration and percentage of motile spermatozoa was detected.

The mechanism of the effects of tobacco on sperm is not known, but given that it contains more than 30 chemical agents known to be mutagens, aneugens or carcinogens in model systems, direct deleterious effects on male germ cells or embryos are plausible (Zenzes, 2000). As a consequence,
male smokers have decreased success in assisted reproduction technologies (ART) (Zitzmann et al., 2003). Moreover, it has been also reported that the likelihood of achieving an ongoing pregnancy at 12 weeks is significantly reduced in couples in whom the males were smokers, suggesting an increase in miscarriages (Joesbury et al., 1998).

There is, however, no reference in the literature to a possible effect of tobacco in the testicle, damaging the Y-bearing sperm and resulting in a decreased Y:X ratio, which could also explain the trends found in newborn infants. In addition, no-one as yet has carefully looked at the effect of tobacco on the selection of X and Y sperm cells before and after capacitation, during fertilization or at the early stages of embryo development. In an attempt to clarify how cigarette smoking could alter the male:female ratio, the evaluation of the percentage of X and Y sperm in ejaculated and swim-up-treated sperm samples, as well as fertilization rates and the percentage of male and female embryos on day 3, would be of great interest.

To this end, we have taken advantage of the knowledge gained in ART after the introduction of chromosomal analysis by fluorescence in-situ hybridization (FISH). Employing this tool, we have been able to follow couples subjected to sperm analysis by FISH, in whom the incidence of chromosomal abnormalities and Y:X ratio was investigated before and after swim-up treatment. The chromosomal status of the embryos from couples who underwent a preimplantation genetic diagnosis (PGD) cycle was also assessed, and therefore the incidence of aneuploidies as well as the sex of the embryos was known. This report brings new insights into the consequences of smoking on human reproduction.

Materials and methods

Patients

We included 56 couples from our PGD program, who were asked, after their treatment, about their smoking habits at the time at which the cycle was performed. Most of the couples were included in the PGD program because of recurrent miscarriage and implantation failure. FISH analysis on spermatozoa was performed in the sperm samples of all male partners. Mean ± SEM female age was 33.7 ± 3.5 years and male age was 36.2 ± 5.7 years. Three groups were established according to male cigarette consumption per day: non-smoking males (n = 31), smoking males: 1–19 cigarettes per day (n = 10) and heavy smoking males: ≥20 cigarettes per day (n = 15). Additionally, women were also classified into three similar groups according to their smoking habits in order to separate out the effect of male and female smoking habits on male:female ratio in day 3 embryos.

FISH analysis was also performed prospectively in sperm samples from four heavy smoking (≥20 cigarettes/day) and four non-smoking donors. Each one of these samples was distributed into two tubes. In one tube an aliquot of the sperm sample was directly fixed for FISH analysis. In the other tube, an aliquot of the same sample was processed by means of swim-up (Garrido et al., 2004) and only the selected fractions of spermatozoa were fixed for FISH.

FISH protocol in spermatozoa

The chromosomal status of spermatozoa was assessed by FISH for chromosomes 13, 18, 21, X and Y on two different slides for each patient: triple-FISH for chromosomes 18, X and Y chromosomes in one slide and dual-colour FISH for chromosomes 13 and 21 in a second slide (Aneuvysion; Vysis Inc., Downers Grove, IL, USA). Sperm samples from the donors were analysed by FISH for chromosomes X, Y and 18, before and after swim-up preparation.

Sperm nuclei were decondensed by slide incubation in 5 mmol/l dithiothreitol and 1% Triton X-100. Sperm nuclei scoring was carried out strictly according to the criteria described by Blanco et al. (1996) and Rodrigo et al. (2004). FISH signals were evaluated in a blinded fashion by two independent observers and at least 2000 sperm cells were counted per slide.

PGD protocol

Stimulation, oocyte retrieval and ICSI procedures were performed as described previously (Meseguer et al., 2003). Sperm samples were processed by swim-up before microinjection and ICSI was performed in all PGD cycles. Pronuclear zygote morphology was assessed at 16–18 h post-ICSI and embryo biopsy was performed on day 3 evolutive embryos with five or more nucleated blastomeres and ≤ 25% fragmentation. For the biopsy, embryos were placed on a droplet containing Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free medium (EB-10; Scandinavian IVF, Göteborg, Sweden), and Tyrode’s solution (ZD-10; Scandinavian IVF) was used to perforate the zona pellucida (Rubio et al., 2003). A total of 372 embryos were biopsied and 335 were informative for the FISH analysis for chromosomes 13, 16, 18, 21, 22, X and Y.

The FISH protocol for screening of aneuploidies was as follows: a first round was performed using locus-specific probes for chromosomes 13 and 21; in a second round and after signal elimination (Vidal et al., 1998) blastomeres were hybridized for chromosomes 16 and 22; finally, in the third round, triple FISH was performed for chromosomes X, Y and 18. More recently, since November 2002, FISH protocol for aneuploidy screening was changed in our laboratory and two rounds are now currently performed: the first round includes chromosomes 13, 16, 18, 21 and 22 and the second round the sex chromosomes (all probes commercially available from Vysis Inc.). Detection washings and signal scoring are carried out following the manufacturer’s instructions.

Embryos were co-cultured on a monolayer of endometrial epithelial cells (Mercader et al., 2003) and euploid embryos were transferred on day 5.

Statistical analysis

To compare basic sperm parameters between the three smoking groups, analysis of variance following multiple post-hoc comparisons in the sperm sex ratio between the donors groups, the Mann–Whitney U-test was employed. Sex ratio comparisons among embryos in the three study groups were performed by χ<sup>2</sup> analysis, and odds ratios and 95% confidence intervals were employed with Fisher’s exact test. The statistical analysis was carried out using the Graphpad Instat v. 2.05a package (Graphpad Software, San Diego, CA, USA) and the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). Statistical significance was defined as P < 0.05.

Results

FISH analysis on patients spermatozoa revealed no statistical differences in the ratio of Y:X spermatozoa between the three groups according to the male smoking condition: non-smoking males, smoking males (1–19 per day) and heavy smoking males (≥20 per day). Sperm parameters were...
similar in the three groups, as well as the incidence of sperm chromosome abnormalities (Table I).

Table II shows the results of the Y:X ratios and the difference of ratios in four sperm samples from heavy smoking donors and four sperm samples from non-smoking donors (control group) with and without previous preparation with swim-up. Comparisons between these two groups showed that in those exposed to tobacco the Y:X ratio after swim-up and the difference of ratios decreased significantly ($P = 0.021$). Therefore, a significant enrichment of X-bearing spermatozoa was observed in the posttreatment aliquots of heavy smoking donors, which finding suggests that tobacco alters selectively the swim-up capacitation effect.

All of these sperm samples had normal sperm concentration and motility according to World Health Organization criteria (World Health Organization, 1999), but in three out of four samples from heavy smoking donors, sperm morphology (Kruger’s strict criteria) was impaired.

In the PGD study (Table III), no significant differences were observed among groups in fertilization and developmental rates. The percentage of abnormal embryos was comparable in the three groups. There were significant differences in the embryos male:female ratio according to the male smoking condition. In the heavy smokers group ($\geq 20$ cigarettes/day), the male:female ratio was significantly decreased compared with the non-smoking males ($P = 0.0057$).

Table IV shows the effect of the smoking condition of the female partner and the embryo male:female ratio. In non-smoking males, no significant differences were observed according to the smoking condition of the female, although in the women smoking 1–19 cigarettes/day there was a trend towards a decreased male:female ratio ($P = 0.0512$). However, in non-smoking females, when the males smoked $\geq 20$ cigarettes per day (heavy smokers), the male:female ratio was significantly decreased compared with non-smoking males ($P = 0.0018$).

**Discussion**

Our study shows that: (i) there are no differences in the Y:X ratio in the ejaculated spermatozoa from non-smoking and heavy smoking patients; (ii) there is a significant enrichment in X-bearing spermatozoa after swim-up preparation in heavy smokers; and (iii) there is a significant increase in the incidence of female embryos in heavy male smokers compared with non-smokers, and it does not seem to be related to the smoking status of the female partner.

Tobacco does not directly affect the production of X and Y spermatozoa; however, it seems to have an effect on sperm quality, mostly impairing the quality of Y-bearing spermatozoa. On the other hand, it has been reported that swim-up increases the proportion of spermatozoa with normal morphology in the posttreated aliquots (Menkveld et al., 1990; Englert et al., 1992). Therefore, in heavy smoking males, swim-up technique, with the enrichment of normal morphology spermatozoa, would also increase the proportion of X-bearing sperm, resulting in a higher incidence of female embryos. These results agree with previous studies in newborns infants, suggesting that paternal, rather than maternal,
smoking habits might be the contributing factor to the decrease in the male:female sex ratio (Fukuda et al., 2002). In fact, the processing of sperm in the laboratory by swim-up and other methods has been compared to the capacitation and activation of sperm observed in the female genital tract. Thus, it is not surprising to find similar phenomena in vivo and in vitro, and therefore the results of the present study may have implications for the general population and not only for ICSI patients as included in this study.

In our study, we found a more evident decrease of embryo male:female ratio in heavy smokers (10:27), than did Fukuda et al. (2002) in newborns (2864:2911). These differences could be explained because of the sample size in the two studies, 56 patients in our study and 11 815 newborns in the study by Fukuda et al. (2002), and also by the fact that infertile men undergoing ICSI could have an increased incidence of female offspring compared with the general population, as suggested by Sarraf et al. (1997). On the contrary, other authors did not observe an association between the smoking behaviour around the time of conception and the sex of the resulting offspring (Heron and Ness, 2004). However, this latest study was conducted in the general population attending hospital obstetrics services and the couples were asked about smoking consumption just before getting pregnant, whereas Fukuda et al. (2002) tested smoking consumption from 3 months before the last menstruation to when the pregnancy was confirmed. Mills et al. (2003), evaluated the effect of cigarette smoking on women during the periconceptional period, without finding an association with a decrease of the male:female sex ratio. As an alternative, they did not rule out the possibility that tobacco could damage selectively the Y-bearing spermatozoa, making them more susceptible to the unfavourable changes caused by the cigarette than the X-bearing spermatozoa.

The first question to be addressed was whether smoking affects X or Y sperm production in the tests. We performed FISH analysis on spermatozoa and found a similar proportion of X and Y spermatozoa in heavy smokers (51% versus 49%), confirming previously published work (Rubes et al., 1998). Therefore, the origin of the decrease in the sex ratio of the embryos in heavy smokers could not be attributed to a variation in the proportion of Y:X spermatozoa.

The second question was whether sperm preparation for ART could influence the sex ratio. We tested the effect of swim-up preparation on the proportion of X-bearing spermatozoa in samples from non-smokers and heavy smokers to elucidate whether the negative impact of tobacco in sperm quality could result in enrichment of X-bearing spermatozoa.

FISH analysis in aliquots of four sperm samples from heavy smoking donors, before and after treatment, showed an increased proportion of X-bearing sperm in the treated samples.

In fact, many studies have shown that smoking habits affect sperm parameters. Cigarette consumption is associated with alterations in density, concentration, progressive motility and/or morphology (Wentz, 1986; Saaranen et al., 1987; Vine et al., 1994; Vine et al., 1996; Sofikitis et al., 1995; Merino et al., 1998; Zavos et al., 1998; Wong et al., 2000; Wang et al., 2001; Künzle et al., 2003; Xu et al., 2003; Zitzmann et al., 2003), whereas only a few number of studies concluded that smoking habits did not affect conventional semen parameters (Oldereid et al., 1989; Holzki et al., 1991; Shen et al., 1997; Trummer et al., 2002). Morphology has been found to be one of the parameters mostly affected by cigarette consumption (Wong et al., 2000). In addition, it has been suggested that cigarette smoking is associated with the retention of the sperm cytoplasmic droplet in infertile men, a morphologic characteristic associated with sperm immaturity and impaired sperm function (Mak et al., 2000; Jakab et al., 2003). In our study, concentration and progressive motility were quite similar in the three groups, with a slight impairment in sperm morphology in the group of heavy smokers.

There are many controversies regarding the ability of swim-up to modify the sex ratio of sperm samples. Most authors did not observe any changes in the ratio of X- and Y-bearing sperm following the isolation of motile spermatozoa using the swim-up technique (Han et al., 1993; Lobel et al., 1993; Samura et al., 1997). In contrast, other studies described an increase in the proportion of Y-bearing sperm after swim-up (De Jonge et al., 1997; Li and Hoshiai, 1998; Jakab et al., 2003). In our study, in the aliquots treated by swim-up we found a significant enrichment of X-bearing sperm in heavy smoking donors.

Table III. Results of the PGD cycles according to the number of cigarettes smoked by the male partners

<table>
<thead>
<tr>
<th></th>
<th>Non-smoking males</th>
<th>Smoking males</th>
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<tbody>
<tr>
<td></td>
<td>1–19 per day</td>
<td>&gt; 20 per day</td>
</tr>
<tr>
<td>MII intact oocytes</td>
<td>421</td>
<td>113</td>
</tr>
<tr>
<td>2 PN (%)</td>
<td>302 (71.7)</td>
<td>80 (70.8)</td>
</tr>
<tr>
<td>3 PN (%)</td>
<td>10 (2.4)</td>
<td>4 (3.5)</td>
</tr>
<tr>
<td>1 PN (%)</td>
<td>25 (5.9)</td>
<td>7 (6.2)</td>
</tr>
<tr>
<td>No. of biopsied embryos (%)</td>
<td>214 (70.9)</td>
<td>58 (72.5)</td>
</tr>
<tr>
<td>No. of informative embryos (%)</td>
<td>191 (89.3)</td>
<td>54 (93.1)</td>
</tr>
<tr>
<td>No. of abnormal embryos (%)</td>
<td>120 (62.8)</td>
<td>38 (70.4)</td>
</tr>
<tr>
<td>XY embryos: XX embryos</td>
<td>80:71a</td>
<td>21:22</td>
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</table>

Table IV. Smoking condition of both partners and embryo sex ratio (male:female)
compared with non-smoking donors. These results would indicate that a correlation could exist between the ability of swim-up to provide the spermatozoa with best morphology and motility, and the enrichment of X-bearing spermatozoa in heavy smokers, in which sperm morphology would be impaired more severely owing to the direct effect of cigarette consumption. Furthermore, swim-up has been shown to decrease the proportion of sperm with diminished maturity (Jakab et al., 2003) and cigarette consumption has been also associated to sperm immaturity.

It has been suggested that paternal smoking habits may have deleterious effects on the preimplantation development of the embryo, especially after ICSI (Zitzmann et al., 2003). However, in our study we did not observe statistical differences in fertilization rates, embryo development or the percentage of abnormal embryos in the three studied groups.

Cigarette consumption has been also been proposed to negatively affect sperm chromosome segregation, with an increased incidence of aneuploidies for sex chromosomes (Rubes et al., 1998; Shi and Martin, 2000), but not of diploid sperm (Rubes et al., 1998). Other authors found an increased incidence of disomy for chromosome 13, but not for sex chromosomes and chromosome 21 (Shi et al., 2001), whereas Robbins et al. (1997) showed that there could be an association between smoking and disomy XX, but a significant correlation could not be established. In our study, we did not observe statistical differences in the incidence of disomic and diploid sperm among groups. These results could be due to differences in the population included in each study: infertile couples in our work and fertile men in the others.

In conclusion, we have found an increased incidence of female embryos in couples in which the male partner is a heavy smoker. This effect of tobacco on the sex ratio at the embryo level would be directly related not to the percentage of X and Y spermatozoa at ejaculation, but to the enrichment of X-bearing spermatozoa after swim-up produced only in heavy smoking males. A higher percentage of X-bearing sperm found after swim-up procedure among heavy smokers suggest that smoking may impair the maturation and normal morphological development of Y-bearing sperm disproportionately.

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