Smoking induces oxidative stress inside the Graafian follicle*

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BACKGROUND: A growing body of evidence indicates that pro-oxidant/antioxidant balance inside ovarian follicles plays an important role in folliculogenesis. Over 20% of women of reproductive age in Europe and the USA regularly smoke cigarettes. The impact of tobacco smoking on the intrafollicular markers of oxidative stress has not been fully elucidated. The objective of the present study was to test the hypothesis that cigarette smoking affects the intrafollicular redox milieu. METHODS: In follicular fluid samples originating from 108 IVF patients, lipid peroxidation was assessed by the thiobarbituric reactive substances method and total antioxidative capacity was quantified by the luminol enhanced chemiluminescence method. The level of patients’ exposure to the cigarette smoke was evaluated by measuring the follicular fluid cotinine concentration by means of radioimmunoassay. RESULTS: Intrafollicular exposure to cigarette smoke metabolites was associated with a significant increase in follicular lipid peroxidation intensity (\(P < 0.001\)), which was accompanied by a significant decrease in the local antioxidative potential (\(P = 0.004\)). CONCLUSION: The results indicate that active smoking affects the pro-oxidant/antioxidant balance inside the pre-ovulatory ovarian follicle by inducing intrafollicular oxidative stress. This provides another possible explanation for impaired folliculogenesis in female smokers.

Key words: antioxidants/follicular fluid/lipid peroxidation/oxidative stress/smoking

Introduction

Over 20% of women of reproductive age in Europe and the USA regularly smoke cigarettes (Brandt, 1987). A number of epidemiological studies have shown that smoking reduces the fertility potential of women (Shiverick and Salafia, 1999). Oocytes have been found to be impaired both quantitatively and qualitatively in smoking patients undergoing IVF–embryo transfer (Van Voorhis et al., 1992; Zenses et al., 1995). An inhibitory action of nicotinic alkaloids on granulosa cell steroidogenesis has been suggested as one of the mechanisms for impaired folliculogenesis in smokers (Barbieri et al., 1986). Other possible mechanisms are still being sought. A growing body of evidence supports the idea that pro-oxidant/antioxidant balance within the follicular microenvironment is implicated in the maturation of the oocytes (Paszkowski et al., 1995; Sabatini et al., 1999). Tobacco smoke is a major source of exogenous pro-oxidants; reactive oxygen species (ROS) and free radical generators are present in both its gas and particulate phases (Frei et al., 1991). Smoking results in an elevation of ROS and the depletion of its scavengers in the circulating blood (Frei et al., 1991; Zhou et al., 2000). In this situation, in which the dynamic balance between pro-oxidation and antioxidation is shifted towards the former, oxidative stress occurs. Oxidative stress may lead to cell damage and malfunction through the free radical-mediated decomposition of vital molecules such as DNA, proteins and lipids (Finkel and Holbrook, 2000). Lipid peroxidation is one of the most biologically important free-radical reactions (Yagi, 1994). If unopposed with an efficient local antioxidative defence system, peroxidative injury to the plasma phospholipids may lead to severe cell damage. The capacity of a local antioxidative system depends on the interaction of different antioxidants such as enzymes (superoxide dismutase, catalase, glutathione peroxidase), low molecular weight ROS scavengers (alpha tocopherol, ascorbic acid etc.), transition metal binding proteins (ceruloplasmin, transferrin etc.) and many others.

The relationship between exposure to tobacco smoke and...
oxidative stress markers within the follicular microenvironment remains to be elucidated. The purpose of this investigation was to study this relationship.

Materials and methods

The study group consisted of 108 women treated with IVF for infertility due to male or tubal factor at the Brigham and Women’s Hospital IVF Program (Boston, Massachusetts, USA). All subjects were <40 years old [median (range): 36 (24–39)]. The patients underwent a standard controlled ovarian hyperstimulation with menotrophin (Pergonal; Serono Laboratories, Randolph, MA, USA) following pituitary desensitization (long GnRH agonist protocol) with Lupron (TAP Pharmaceuticals, Deerfield, IL, USA). The details of the stimulation protocol used in this study were described elsewhere (Jackson et al., 1992). A total of 108 uncontaminated follicular fluid (FF) samples originating from co-dominant follicles (>18 mm) were obtained by means of ultrasound-guided transvaginal ovarian puncture ~36 h following the HCG stimulus (Profasi, Serono). The aspirates were examined microscopically and, when the oocyte–corona–cumulus complexes were identified, they were removed from the FF and processed for insemination. The maturity grade of the oocyte–corona–cumulus complex was assessed according to previously described criteria (Veek, 1986). Only the first-puncture clear FF samples free from contamination with blood or medium were included into the study. Immediately after puncture, FF samples were centrifuged at 1000 g and fresh aliquots of cell-free liquid were used for laboratory analyses. All measurements were carried out in samples protected from light.

For the purpose of the present study, tests were chosen which allowed a global approach to estimate oxidative stress inside the Graafian follicle, such as assessments of lipid peroxidation and total antioxidative capacity in FF samples.

Lipid peroxidation in FF was quantified by spectrophotometric evaluation of thiobarbituric acid reactive substances (TBARS) originally described and subsequently improved by Yagi (Yagi, 1994). Although the assay for TBARS does not allow detailed insight into the peroxidation process, especially at its propagation step, it is considered to be a reliable global indicator of oxidative stress (Leferve et al., 1998). This method is based on the fact that final peroxidation products such as malondialdehyde (MDA) react with thiobarbituric acid to yield a red pigment. Since MDA is unstable, tetramethylopropane, which is converted quantitatively (1:1) to MDA in the reaction procedure, was used. To determine specifically the final lipid peroxidation products, they were precipitated along with FF proteins to remove water-soluble TBARS, and the reaction was carried out at pH 3.0 where sialic acid could not react with TBA. The standard procedure is summarized as follows: 20 µl of undiluted FF was mixed with 4.0 ml of 0.08 mol/l sulphuric acid. To this mixture, 0.5 ml of 10% phosphotungstic acid was added and mixed. After standing at room temperature for 5 min, the mixture was centrifuged at 1200 g for 10 min. The supernatant was discarded, and the sediment was mixed with 2.0 ml of 0.08 mol/l H₃SO₄ and 0.3 ml of 10% phosphotungstic acid. The mixture was centrifuged at 1200 g for 10 min. The sediment was suspended in 4.0 ml of distilled water, and 1.0 ml of TBA reagent (a mixture of equal volumes of 0.67% TBA aqueous solution and glacial acetic acid) was added. The reaction mixture was heated at 95°C for 60 min in an oil bath. After cooling with tap water, 5.0 ml of n-butanol was added and the mixture shaken vigorously. After centrifugation at 1200 g for 15 min the n-butanol layer was placed into spectrophotometric cuvettes. The complete spectrum was scanned between 450–600 nm to ensure the absence of interfering peaks and the absorbance was measured at 532 nm using a DU-640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The procedure was calibrated using 0.5 nmol of 1,1,3,3-tetraethoxypropane. All the reagents used in the assay were purchased from Sigma Chemical Co., St Louis, MO, USA.

Total antioxidative capacity (TAC) of FF was measured by a luminol-enhanced chemiluminescence method, as previously described (Whitehead et al., 1992). In contrast to the measurements of single antioxidants, the TAC assay quantifies the overall antioxidative activity of the examined body fluid, thus providing a useful oxidative stress indicator. In the enhanced chemiluminescence assay employed in this study, antioxidant capacity is measured by its ability to suppress horseradish peroxidase (HRP)-catalysed oxidation of luminol in the presence of an oxidant (perborate) and an enhancer (p-iodophenol). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; Aldrich, Milwaukee, WI, USA), a water soluble analogue of vitamin E, was used to produce a standard curve. The enhanced chemiluminescence immunoassay signal reagent, containing luminol, p-iodophenol as enhancer and perborate as an oxidant, was purchased from Amersham Life Science Inc. (Arlington Heights, IL, USA). HRP-conjugated sheep anti-mouse immunoglobulin G (whole molecule) was purchased from Sigma. Briefly, 30 µl of signal reagent, 940 µl of distilled water and 30 µl of a 1:250 dilution of the HRP-conjugated antibody were placed in a tube to give a stable light output which depended on the constant production of free radical intermediates derived from p-iodophenol, luminol and oxygen. The addition of antioxidants caused an immediate and deep depression of the light output for a time period that was linearly related to the molar quantity of the antioxidants added. The time (s) until there was a 10% recovery of the initial light output was recorded and compared with the corresponding value for the Trolox calibrant (80 µmol/l).

Light output was quantified using an automated luminometry system containing a BioOrbit (Turku, Finland) 1251 luminometer working on-line with a computer preloaded with S0015 software (BioOrbit) and a printer. To measure FF-TAC, the follicular fluid samples were diluted 1:10 with distilled water and 20 µl were subsequently used in the assay system as for Trolox. Results were expressed as Trolox equivalents.

Cotinine levels in FF (FF-C) were used in the present study as an index of the tobacco smoke exposure. Cotinine is a relatively stable metabolite of nicotine with a plasma half-life of ~19 h (Perez-Stable et al., 1995). Studies have shown that cotinine easily crosses the blood–follicle barrier and that the FF-C provides a reliable test of tobacco smoke exposure among IVF–embryo transfer patients (Zenses et al., 1996). Cotinine concentrations in FF were measured by radioimmunoassay (Double Antibody Nicotine Metabolite RIA Kit—KCTD1; Diagnostic Products Corporation, Los Angeles, CA, USA). The Double Antibody procedure is a liquid-phase radioimmunoassay, wherein I-labelled cotinine competes for a fixed time with cotinine in the patient’s sample for antibody sites. The 50 µl aliquot of undiluted FF was used in the assay. After incubation for a fixed time, the separation of bound from free cotinine was achieved by the polyethylene glycol (PEG)-accelerated double antibody method. Finally, the antibody-bound fraction was precipitated and counted (Cobra II Auto-Gamma; Pacard Instrument Co., Meriden, CT, USA).

The FF-C levels were read from a calibration curve. Exposure to the tobacco smoke was classified according to criteria based on the following cotinine levels in follicular fluid (Sterzik et al., 1996): non-smoker, FF-C <20 ng/ml (conversion factor to SI unit, 5.68); passive smoker, FF-C 20–50 ng/ml; active smoker, FF-C >50 ng/ml.

For the laboratory determinations described above, all samples were analysed in duplicate. The coefficients of variation for the tests were as follows: cotinine, 9.9%; lipid peroxidation end-products, 7.7%; total antioxidative capacity, 5.1%.

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The results were described as medians and ranges. Statistical analysis was performed using the non-parametric Kruskal–Wallis one-way analysis of variance on ranks and Spearman’s rank correlation coefficient. StatView 512+TM software for Macintosh (Brain-Power Inc., Agoura Hills, CA, USA) was used for the statistical processing of the data. P < 0.05 was considered statistically significant.

This study was approved by the Human Research Committee at the Brigham and Women’s Hospital (Boston, MA, USA) for the use of discarded material (protocol number 95-7355-01).

Table I. Indices of ovarian responsiveness in relation to patient’s smoking status. Values are medians and ranges

<table>
<thead>
<tr>
<th>Smoking statusa</th>
<th>n</th>
<th>Age (years)</th>
<th>HMG doseb</th>
<th>Peak serum E2 level (pg/ml)c</th>
<th>No. oocytes retrieved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>72</td>
<td>36 (25–39)</td>
<td>29 (11–94)</td>
<td>1869 (624–3965)</td>
<td>16 (7–38)</td>
</tr>
<tr>
<td>Passive smokers</td>
<td>21</td>
<td>35 (26–38)</td>
<td>32 (11–64)</td>
<td>1720 (625–6794)</td>
<td>12 (2–52)</td>
</tr>
<tr>
<td>Active smokers</td>
<td>15</td>
<td>37 (24–39)</td>
<td>37 (21–81)</td>
<td>891 (603–1970)</td>
<td>8 (2–27)</td>
</tr>
<tr>
<td>P-valued</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS</td>
<td>0.044</td>
<td>0.001</td>
<td>0.010</td>
</tr>
</tbody>
</table>

aFor grouping criteria see text.
bNumber of menotrophin ampoules (75 IU FSH) required to meet the criteria of satisfactory ovarian stimulation (at least two follicles ≥18×12 mm and E2 ≥600 pg/ml).
cConversion factor to SI unit, 3.671.
dP-value as tested by ANOVA on ranks Kruskal–Wallis.
NS = not significant.

Table II. Lipid peroxidation products and total antioxidative capacity measured in follicular fluid of patients with different levels of tobacco smoke exposure. Values are medians and ranges

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>n</th>
<th>FF-C (ng/ml)</th>
<th>FF-TBARS (µmol/l)</th>
<th>FF-TAC (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-smokers</td>
<td>72</td>
<td>3.58 (ND-15.62)</td>
<td>0.49 (0.16–1.75)</td>
<td>440.00 (340.40–544.90)</td>
</tr>
<tr>
<td>passive smokers</td>
<td>21</td>
<td>31.38 (19.50–45.75)</td>
<td>0.50 (0.18–1.00)</td>
<td>395.40 (280.20–800.00)</td>
</tr>
<tr>
<td>active smokers</td>
<td>15</td>
<td>28.569 (6221–59 500)</td>
<td>1.07 (0.41–1.75)</td>
<td>362.30 (254.40–533.30)</td>
</tr>
<tr>
<td>P-valued</td>
<td></td>
<td>0.001</td>
<td>0.001</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Figure 1. Follicular fluid levels of final peroxidation products (FF-TBARS) plotted against cotinine concentration in follicular fluid (FF-C). R = 0.471; P < 0.001.

Results

Of 108 patients examined, 72 woman (66.7%) were classified as non-smokers, 21 (19.4%) as passive smokers and 15 (13.9%) as active smokers. Table I compares the parameters of ovarian responsiveness in patients with different levels of tobacco smoke exposure. The age of patients assigned to the three smoking status groups did not differ significantly. Despite the fact that smokers required a higher dose of gonadotrophin to reach an acceptable ovarian response (P = 0.044), both the peak serum estradiol (E2) and the oocyte yield per cycle were significantly lower in these patients compared with non-smokers (P < 0.001 and P = 0.010 respectively).

Table II characterizes total antioxidative capacity and lipid peroxidation in the FF of active smokers, passive smokers and non-smokers. Patient age did not correlate significantly with the measured oxidative stress markers. Increasing exposure to tobacco smoke was associated with a significant increase in FF TBARS (P < 0.001). This highly significant trend is evidently due to a greater than two-fold increase in final peroxidation products in active smokers as compared with both non-smokers and passive smokers. No significant differences were found in FF-TBARS levels between non-smokers and passive smokers. FF cotinine levels were significantly correlated with the FF-TBARS values (Figure 1).

As shown in Table II, total antioxidative potential of FF declined as cotinine concentration in follicular microenvironment increased. This trend was statistically significant (P = 0.004). A significant inverse correlation (r = -0.195;
The effect of smoking on the oogenesis of IVF–embryo transfer patients has been previously shown to be harmful. Our study confirms previously published data on the impaired ovarian responsiveness of smokers undergoing controlled ovarian hyperstimulation (Van Voorhis et al., 1992; Hughes et al., 1994). In unfertilized oocytes originating from smoking IVF–embryo transfer patients, a relatively high incidence of diploidy was observed, suggesting a smoking-related meiotic immaturity of the oocytes (Zenses et al., 1995). An increased risk of trisomy 21 was observed in the offspring of young mothers who smoke cigarettes (Yang et al., 1999). A shift of the pro-oxidant/antioxidant balance inside the ovarian follicle towards oxidative stress may provide another possible explanation of impaired folliculogenesis in female smokers undergoing IVF–embryo transfer. Scarce data published to date suggest that the developmental competence of oocytes retrieved during IVF–embryo transfer procedures is not related to the increased levels of oxidative stress markers in the follicular milieu they originate from (Jozwik et al., 1999; Attaran et al., 2000). Difficulty in establishing such a relationship may be due to the multitude of possible confounding factors affecting the ability of oocytes handled in vitro to be fertilized, as well as the embryo transfer outcome.

In our study, we failed to demonstrate a relationship between follicular oxidative stress markers and the maturity grade of an oocyte originating from the punctured follicle. This result is not surprising, as our analysis concerned only two fertilizable classes of oocyte, namely pre-ovulatory and intermediary. To test the hypothesis that intrafollicular oxidative stress affects oocyte maturation, one should analyse a much greater spectrum of oocyte categories, including immature and post-mature oocytes.

The findings of the present study should be explored further in order to confirm the causal relationship between smoking and free radical-mediated cytotoxicity for oocytes and granulosa cells. Nevertheless, our data support a growing body of data indicating a complex ovariotoxic action of tobacco smoke and provide further evidence for IVF–embryo transfer patients to discontinue cigarette smoking prior to infertility treatment with assisted reproduction techniques.

Acknowledgements
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References

Discussion
Our study suggests that active smoking affects the pro-oxidant/antioxidant balance inside the Graaafian follicle in women undergoing ovulation induction for IVF. These findings show that cigarette smoking, as indicated by the presence of cotinine in pre-ovulatory FF, is associated with an increased intensity of lipid peroxidation inside the mature ovarian follicle, which is accompanied by the depletion of local antioxidative potential. FF levels of TBARS measured in our study were lower than those found by Jozwik et al. who used a different laboratory method (Jozwik et al., 1999). Unfortunately, these authors did not determine the smoking status of their patients.

Our results suggest that in actively smoking patients undergoing IVF–embryo transfer, follicular cells may be exposed to peroxidative stress unopposed by the intrafollicular antioxidative system. Many of the pro-oxidants present in tobacco smoke can trigger a chain-reaction of lipid peroxidation (Frei et al., 1991), which may be harmful for both the oocytes and granulosa cells. In the light of our data, the increased intensity of follicular lipid peroxidation takes place only in patients with a high level of tobacco smoke exposure, i.e. in those with high levels of FF cotinine.

Our findings are in accord with previously published data indicating the depletion of both enzymatic (Paszkowski et al., 1995) and non-enzymatic (Palan et al., 1995) antioxidants in FF of smokers. The decreased antioxidative capacity of FF in smokers is most probably a secondary phenomenon caused by the utilization of antioxidants in defence reactions neutralizing reactive oxygen species originating from, or induced by, the tobacco smoke constituents.

$P = 0.043$ was observed between the FF-TAC and FF cotinine (Figure 2).

Fifty-nine out of total of 108 follicles punctured resulted in oocyte recovery. Of the 59 oocyte–corona–cumulus complexes identified in the follicular aspirates, 35 (59.3%) were graded as intermediary [metaphase I (4N)] and the remaining 24 (40.7%) were assessed as pre-ovulatory [metaphase II (2N)]. Follicular levels of TBARS, TAS and cotinine did not differ significantly between the two oocyte maturity groups.

Figure 2. Total antioxidative capacity of follicular fluid (FF-TAC) plotted against cotinine concentration in follicular fluid (FF-C). $R = -0.195; P < 0.05$. 

$P = 0.043$ was observed between the FF-TAC and FF cotinine (Figure 2).


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