Smoking impairs angiogenesis during maturation of human oocytes

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Objective: This study determines whether smoking influences ovarian vascularization which thus may impair follicular development.

Design: Prospective laboratory study of follicular fluids and granulosa cells from patients undergoing in vitro fertilization.

Setting: University Hospital Aachen, Germany.

Patient(s): Fifty smoking women and 50 nonsmoking women.

Intervention(s): Cultivation of human granulosa cells. Cultivation of human umbilical vein endothelial cells (HUVECs) with either granulosa cell–conditioned medium or follicular fluid. Determination of clinical parameters.

Main Outcome Measure(s): Quantification of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) and cotinine.

Result(s): Mean sVEGFR-1 concentration in follicular fluid of smokers was 499.6 pg/mL compared with 159.2 pg/mL in nonsmokers. Correspondingly, supernatant of HUVECs cultured with follicular fluid from smoking and nonsmoking women showed, respectively, 1,174.1 pg/mL versus 794.2 pg/mL sVEGFR-1. The HUVECs incubated with conditioned medium from smokers’ granulosa cells at culturing days 5, 9, 13, and 17 secreted, respectively, 1,712.4, 1,560.6, 1,619.0, and 1,635.0 pg/mL sVEGFR-1, whereas nonsmokers showed, respectively, 1,147.6, 1,067.2, 1,135.9, and 1,206.3 pg/mL sVEGFR-1. Mean cotinine concentration in smoking women was 83.9 ng/mL and in nonsmoking was 2.8 ng/mL. In all four comparisons, differences between groups reached statistical significance.

Conclusion(s): This study showed that smokers secrete significantly higher amounts of sVEGFR-1 than nonsmokers, which may result in decreased ovarian vascularization and reduced oocyte maturation. (Fertil Steril 2006; 86:186–91. ©2006 by American Society for Reproductive Medicine.)

Key Words: Smoking, ovarian vascularization, follicular development, oocyte quality, sVEGFR-1, cotinine

Numerous scientific studies that examined how smoking interferes with human reproduction support the conclusion that smoking has an adverse effect on female fertility. First, time to pregnancy is longer in smokers than in nonsmokers. Delay of conception increases with the daily number of cigarettes smoked (1–3). Second, natural menopause, and therefore the loss of reproductive ability, occurs one to four years earlier in smoking than in nonsmoking women (1, 4).

Third, smoking can reduce the number of mature oocytes, and therefore, more in vitro fertilization (IVF) cycles and embryo transfers to conceive are required (4–6). Also, gametogenesis appears to be vulnerable to chromosomal damage from smoke exposure, which may result in the disruption of early embryo development and very likely in spontaneous miscarriage (1, 7).

Optimal follicular and consequently also oocyte maturation requires an adequate vascularization in the human ovary for oxygen and nutritional supply (8). Vascular endothelial growth factor A (VEGF-A) is a potent inducer of angiogenesis, vasculogenesis, and lymphangiogenesis (9, 10). It exists in five isoforms, generated by alternative splicing of the mRNA to result in proteins of 121, 145, 165, 189, and 206 amino acids in length (9, 11). Produced by the ovarian theca interna and granulosa cells, VEGF-A regulates vascularization during follicular development and corpus luteum formation (12, 13). It mediates its effects through the two transmembrane receptors VEGFR-1 (fms-like tyrosine receptor 1 [Flt-1]) and VEGFR-2 (kinase-inserted tyrosine domain receptor 2 [KDR]) (12, 14, 15). Both VEGFRs are tyrosine kinases and rather specific for endothelial cells (12, 14, 15).

In addition, a soluble truncated form of VEGFR-1 (sVEGFR-1, or sFlt-1) is known that is secreted from endothelial cells into the vicinity and circulation. By binding of VEGF-A, the soluble receptor reduces the bioavailability of this growth factor and thus acts as antagonist of VEGF-A activity (13, 14). In addition to VEGF-A production, granulosa cells decrease the endothelial sVEGFR-1 secretion by a paracrine mechanism supporting angiogenesis in a double
way (unpublished observations). The aim of our study was to investigate the influence of smoking on sVEGFR-1 concentration in follicular fluid as well as on the paracrine activity of granulosa cells to inhibit endothelial sVEGFR-1 production and, finally, on ovarian vascularization supporting follicular development and oocyte maturation.

MATERIALS AND METHODS

Subjects

Follicular fluid samples and granulosa cells were obtained during oocyte retrieval from women aged 22 to 46 years (mean age 33 years) undergoing IVF treatment at the Clinic of Gynecologic Endocrinology and Reproductive Medicine at the University Hospital Aachen, Aachen, Germany. Patients were selected randomly for this study. The first 50 incoming smoking and nonsmoking women were included. The etiologies of infertility were andrologic abnormalities (69%), tubal diseases (15%), hormonal abnormalities (7%), endometriosis (4%), and idiopathic causes (5%). Patients were classified into three ethnic groups: Caucasian (96%), Hispanic (2%), and Asian (2%).

The protocol for ovarian stimulation has been described previously (12). Briefly, after pituitary down-regulation with nafarelin (Synarel; Pharmacia, Karlsruhe, Germany) follicular development was induced with 2,300 IU of recombinant hFSH as a mean dosage per cycle (Gonal F; Serono, Unterschleißheim, Germany) and continued until follicular maturity. Thirty-six hours after ovulation induction by administering 5,000 or 10,000 IU hCG (Predalon; Organon, Oberschleißheim, Germany) to the patients, oocytes were retrieved from follicular aspirates. Oocytes were inseminated by either conventional IVF or intracytoplasmic sperm injection (ICSI) with sperm from ejaculates or testicular sperm extraction. Eighteen hours (± 1 h) after insemination, zygotes with a high pronucleus score and thus a prospective high developmental potential were identified and cultivated until transfer (16).

According to the German law, the selection of a maximum of three zygotes for transfer is permitted. embryo quality was assessed immediately before transfer on the second or third day using an embryo scoring system (17). Data concerning the clinical parameters were gathered from the patient files. The study was approved by the Ethics Committee of the University Hospital Aachen. Written informed consent was obtained from patients individually.

Isolation and Culture of Granulosa Cells

Follicular aspirates were centrifuged at 1,000 rpm for 5 min to separate granulosa cells from follicular fluids. The individual follicular fluids were stored at −20°C. Granulosa cells were isolated as described previously (18). Briefly, cells separated from follicular fluid were washed several times with Ca²⁺- and Mg²⁺-containing phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany) and pelleted at 1,000 rpm for 5 min. Subsequently, they were purified over a 50% percoll gradient (Biochrom) with 2,000 rpm for 10 min and washed once with Ca²⁺- and Mg²⁺-free PBS (PBS w/o) afterwards.

The purified granulosa cells were digested with 20 IU hyaluronidase (Sigma-Aldrich, Taufkirchen, Germany) for 10 min at 37°C. After enzyme inhibition with medium M199 (Biochrom) supplemented with 10% fetal calf serum, 5 mmol/L L-glutamine, and 10,000 IU/10,000 μg/mL penicillin/streptomycin (Biochrom), they were centrifuged, resuspended, and counted. The granulosa cells were plated in 24-multiwell plates (Becton Dickinson Labware, Frankfurt Lakes, NJ) at a concentration of 200,000 cells per well and cultivated up to 17 days at 37°C and 5% CO₂. Culture medium M199 was discarded 24 h after cell preparation. Afterwards, every fourth day the conditioned medium was removed and stored at −20 °C.

Culture of Human Umbilical Vein Endothelial Cells

Cryopreserved pooled human umbilical vein endothelial cells (HUVECs) (Bio Science, Verviers, Belgium) were thawed at room temperature, resuspended in endothelial cell culture medium EBM-2 (Cambrex Bio Science, Verviers, Belgium), and grown until confluency. The confluent cell layer was trypsinized (0.05% Trypsin/0.02% EDTA (Biochrom) in PBS w/o) for 3 min at 37°C. After enzyme inhibition with EBM-2 medium, HUVECs were centrifuged at 1,200 rpm for 10 min, washed with PBS w/o, resuspended, and counted. Endothelial cells were then plated in 24-multiwell plates at a concentration of 100,000 cells per well and cultivated at 37°C and 5% CO₂. Culture medium EBM-2 was discarded 24 h after cell preparation.

Assays

The HUVECs (passes 3–10) were incubated with EBM-2 culture medium containing 30% follicular fluid or 30% granulosa cell conditioned medium for four days at 37°C and 5% CO₂ in 24-multiwell plates. The amount of sVEGFR-1 was examined both in supernatant of HUVECs treated with follicular fluid or granulosa cell–conditioned medium and in native follicular fluid by ELISA (Bender MedSystems, Vienna, Austria). Cotinine, one of the major metabolites of nicotine, was quantified in native follicular fluid of both test groups also by ELISA (BíoQuant, Ann Arbor, MI). Cotinine is known to accumulate in body fluids proportionally to the daily number of cigarettes smoked and was used to confirm the patients’ smoking or nonsmoking habits (1, 19). Both ELISA tests were accomplished according to the manufacturer’s protocol and analyzed by using the automated microplate reader HTII (Anthos Labtec Instruments, Salzburg, Austria) set at 450 nm.

Statistical Analysis

Data were analyzed by using either a Microsoft Excel spreadsheet or the SPSS statistical software, version 12.0.
Results are shown as mean ± SEM. Independent t-tests were used to compare group mean values. Two-tailed P values < 0.05 were considered statistically significant.

RESULTS

Cotinine Concentration in Follicular Fluid

According to their indicated 1 to 25 daily smoked cigarettes, smoking women showed cotinine values ranging from 4.3 to 335.4 ng/mL, with a mean value of 83.9 ± 10.5 ng/mL (Fig. 1). The mean number of cigarettes smoked per day was 14. In most smokers, cotinine accumulated in proportion to the amount of smoked cigarettes. In comparison, follicular fluids of nonsmokers contained lower amounts of cotinine, between 0.0 and 5.9 ng/mL and a mean of 2.8 ± 0.2 ng/mL, which is attributable to passive smoking. Consequently, smokers showed on average a 30-fold higher cotinine concentration in follicular fluid than nonsmokers.

Clinical Parameters

Smoking and nonsmoking women were comparable concerning age (32.3 vs. 33.7 years) and body mass index (23.4 vs. 23.9 kg/m²), as shown in Table 1. No significant differences between smokers and nonsmokers were found with regard to stimulation dose (2,113.6 vs. 2,414.6 IU, respectively), days of stimulation (12.2 vs. 12.3), E₂ (5,118.4 vs. 6,037.1 pmol/L), and hCG levels (6,875.0 vs. 6,521.7 IU). Furthermore, both patient groups showed no significant differences concerning the number of follicles on the day of hCG (12.0 vs. 11.9), the number of oocytes retrieved (8.7 vs. 10.8), the number of mature oocytes obtained from ICSI cycles (7.6 vs. 8.8), and the fertilization rate (43.2 vs. 45.5%). Smokers and nonsmokers had comparable qualities of embryos on day 2 (10.3 vs. 10.0) or day 3 (16.1 vs. 16.9), numbers of embryos transferred (2.0 vs. 1.9), and pregnancy rates per transfer (32.4 vs. 30.0%). Also, no significant differences concerning the infertility diagnosis and ethnic affiliation were seen between patient groups.

Amount of sVEGFR-1 in Follicular Fluid

In the follicular fluid of smokers, sVEGFR-1 was found to be between 0.0 and 2,479.0 pg/mL with a mean value of 499.6

### Table 1

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<th>Clinical parameters of smokers and nonsmokers in mean values.</th>
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a MII oocytes obtained from ICSI cycles.

Nonsmokers showed 0.0 to 948.0 pg/mL sVEGFR-1 with a mean concentration of 159.2 ± 29.8 pg/mL (Fig. 2). The sVEGFR-1 content in follicular fluid samples of smokers was on average significantly three times higher than in follicular fluid of nonsmoking women. Furthermore, no significant correlation between the sVEGFR-1 concentration in follicular fluid samples of smokers and the number of cigarettes smoked was observed. Human serum albumin was used as a negative control and did not react with the ELISA sVEGFR-1 antibody.

Influence of Follicular Fluid on sVEGFR-1 Secretion in Endothelial Cells

The HUVECs were incubated for four days with follicular fluid from smoking and from nonsmoking women. Production of sVEGFR-1 by endothelial cells was significantly inhibited by adding follicular fluid to the culture medium. However, follicular fluid of nonsmokers led to significantly higher inhibition of sVEGFR-1 secretion compared to follicular fluid of smokers (Fig. 3). In supernatant of endothelial cells cultured with follicular fluid from smokers, sVEGFR-1 concentrations between 111.0 and 2,822.0 pg/mL, with a mean value of 1,174.1 ± 93.8 pg/mL, were detected, whereas in supernatant of endothelial cells incubated with follicular fluid from nonsmokers sVEGFR-1 ranged from 123.0 to 1,806.0 pg/mL, with a mean of 794.2 ± 70.1 pg/mL. The amount of soluble VEGF receptor 1 in cell culture supernatants of smoking patients was on average significantly 1.5 times higher than in the supernatants of nonsmoking patients.

DISCUSSION

We demonstrated that smoking significantly influences sVEGFR-1 content in follicular fluid. Additionally, smoking reduces the ability of follicular fluid as well as of granulosa cell–conditioned medium from human granulosa cells derived from smokers and nonsmokers to inhibit sVEGFR-1 secretion in endothelial cells.
A high level of sVEGFR-1 implies an impaired blood circulation, which could have a negative effect on oocyte maturation. However, as demonstrated before, smokers showed decreased sVEGFR-1 concentrations in plasma compared with nonsmokers, suggesting that sVEGFR-1 accomplishes different tasks in distinct systems and may be regulated in a different way by smoking exposure (22). As shown here, endothelial sVEGFR-1 production is paracrinely regulated in a negative way by a substance which is secreted from native granulosa cells into the surrounding follicular fluid. Incubation of endothelial cells either with follicular fluid or with granulosa cell–conditioned medium led to an inhibition of sVEGFR-1 secretion by the endothelial cells. This inhibitory effect was significantly reduced in smokers.

Smoking cigarettes obviously affects the production of paracrine active substances in granulosa cells and their secretion into the follicular fluid. In consequence, endothelial cells produce more sVEGFR-1 which acts antiangiogenic. Whether this reaction is caused by nicotine or by another component of cigarette smoke remains unclear. Nicotine certainly reduces progesterone production in human luteinized cells and could affect the paracrine activity in granulosa cells in the same way (23). Influence of maternal age or body weight, stimulation dose, and days, as well as E2 level and hCG dosing, could be excluded in this study, because there were no significant differences in these clinical parameters of the two experimental groups.

Because no significant differences in the number of follicles on day of hCG, number of oocytes retrieved, number of mature oocytes, and the fertilization rate were seen, distinctive sVEGFR-1 secretion between smokers and nonsmokers was obviously not caused by these parameters. The same was found for embryo quality at day 2 and 3 as well for the number of embryos transferred and the pregnancy rate per transfer. Also, infertility diagnosis and ethnic affiliation had no influence on sVEGFR-1 secretion. Here we could show that smoking influences the regulation of endothelial sVEGFR-1 production by granulosa cells. Obviously, angiogenic signals from the granulosa cells in smoking women are reduced. This may compromise ovarian blood flow, resulting in reduced oocyte quality.

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REFERENCES

It is known that smoking has an adverse effect on ovarian function and therefore female fertility. On the one hand, various products of cigarette smoke, such as benzopyrene, cadmium, or cotinine, reach the ovarian follicle and probably interfere with gametogenesis (1, 3, 4). On the other hand, the risk of reduced blood circulation in the ovary by exposure to smoke exists, which could result in a decreased quantity of mature oocytes and embryos (5, 21). Vascular endothelial growth factor A is considered to be one of the substances able to increase ovarian blood circulation and therefore improves oocyte maturation, whereas its soluble receptor sVEGFR-1 antagonizes these effects. As demonstrated for native follicular fluid, in smoking patients significantly more soluble VEGFR-1 is secreted into the follicular fluid compared with nonsmokers, regardless of the number of cigarettes smoked per day.


