Nicotine does not affect vascularization but inhibits growth of freely transplanted ovarian follicles by inducing granulosa cell apoptosis

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BACKGROUND: There is clear support for an association between smoking and decreased female fecundity and fertility. Cigarette smoke appears to have adverse effects along a continuum of reproductive processes. We therefore studied the effect of nicotine on follicular growth and vascularization of freely transplanted ovarian follicles. METHODS: We used the skinfold chamber model in Syrian golden hamsters, which allows the in vivo microscopy of follicular grafts. Animals were treated daily with nicotine at doses mimicking low-rate and high-rate smokers (0.2 and 1.0 mg/kg body weight subcutaneously). Saline-treated animals served as controls. To further evaluate the effect of nicotine on angiogenesis, an in vitro aortic ring assay was used. RESULTS: The re-vascularisation rate of follicles was similar in nicotine-treated animals and controls. During the 7 days after transplantation, nicotine further caused a dose-dependent inhibition of follicular growth. In contrast, the vascularized area and microvessel density were not affected by the nicotine exposure. In vitro aortic ring assays confirmed that nicotine does not influence sprouting and microvessel formation. However, immunohistochemistry for cleaved caspase-3 revealed a large extent of granulosa cell apoptosis within transplanted follicles of high-dose nicotine-treated animals. CONCLUSIONS: Nicotine as one toxic component of cigarette smoke does not affect vascularization, but adversely influences follicular growth by an increase in apoptotic cell death. As follicular growth is a crucial step in normal ovulation and fertilization, nicotine-induced cell apoptosis may represent one of the mechanisms underlying the well-established link between smoking and fertility disorders.

Key words: aortic ring assay/apoptosis/growth/ovarian follicles/vascularization

Introduction

Approximately 30% of women of reproductive age in the USA still smoke cigarettes (Stillman et al., 1986), although it is widely recognized that smoking is a major risk factor for the development of severe pulmonary and cardiovascular diseases and the induction of a variety of neoplasias. Beside these effects, smoking also affects female fecundity and fertility because of its adverse action along a continuum of reproductive processes, including gamete production and function, ovulation and cyclicity, fertilization, embryonic transport and implantation (Stillman et al., 1986; Bezerra de Mello et al., 2001; Practice Committee of the American Society for Reproductive Medicine, 2004).

In the past, it has been suggested that smoking may affect fertility rates because of its influence on the angiogenic process (Shiverick and Salafia, 1999). Angiogenesis, which is defined as the development of new capillaries from pre-existing blood vessels, is involved in a number of physiological processes within the female reproductive system, such as folliculogenesis and corpus luteum formation (Shimizu et al., 2003; Tamanini and De Ambroggi, 2004). Correspondingly, vascular endothelial cells of ovarian tissue exhibit mitotic rates equal to or greater than those observed for tumour endothelial cells (Gaede et al., 1985). Previously, in vivo studies have shown that exposure of hamsters to smoke at doses equivalent to those received by human smokers causes a significant decrease in the number of blood vessels within corpora lutea (Magers et al., 1995). This decreased vascularization could influence steroid hormone production and may contribute to the increased incidence of spontaneous abortions observed in human smokers (Stillman et al., 1986; Saraiya et al., 1998). However, because cigarette smoke is a complex mixture of over 4000 different chemicals, it is not yet known which components are important inhibitors of physiological angiogenesis within the female reproductive system.

Nicotine is the major active component of cigarette smoke. Interestingly, nicotine has recently been shown to stimulate angiogenesis in different pathological settings including wound healing, tumour growth and vascularization of atherosclerotic plaques (Heeschen et al., 2001; Jain, 2001; Jacobi et al.,
experiments. Animals were kept on water and standard laboratory chow ad libitum. The experiments were conducted in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

Materials and methods

Animals

Six- to 8-week-old female Syrian golden hamsters (body weight 50–80 g; Charles River Laboratories, Sulzfeld, Germany) were used for all experiments. Animals were kept on water and standard laboratory chow ad libitum. The experiments were conducted in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

Preparation of the hamster dorsal skinfold chamber

The chamber technique and its implantation procedure have been described previously in detail (Endrich et al., 1980). In brief, under intraperitoneal pentobarbital sodium anaesthesia (50 mg/kg body weight), two symmetrical titanium frames were implanted on the extended dorsal skinfold of the animals, so that they sandwiched the double layer of skin. One layer of skin was then removed in a circular area of 15 mm in diameter, and the remaining layers, consisting of striated skin muscle, subcutaneous tissue and skin, were covered with a removable coverslip incorporated into one of the titanium frames (Figure 1). After preparation, the animals were allowed to recover from anaesthesia and surgery for at least 48 h. As previously reviewed (Menger et al., 2002), the dorsal skinfold chamber enables continuous observation and repetitive analysis of the microcirculation in awake animals for more than 14 days using trans- and epi-illumination microscopy.

Follicle isolation and transplantation

For follicle isolation, pentobarbital sodium-anaesthetized Syrian golden hamsters were laparotomized. Ovaries were aseptically removed and placed in Petri dishes filled with warm (37°C) RPMI medium (10% fetal calf serum, 1% penicillin/streptomycin). After removing the surrounding tissue, ovaries were microdissected using 27-gauge needles under a stereo-microscope. This handpicking procedure guaranteed single connective tissue-free follicles for transplantation. The isolated follicles were visually collected and transferred into another Petri dish containing warm (37°C) RPMI medium. For follicle transplantation, the cover glass of the dorsal skinfold chamber was removed and three follicles (diameter range ∼360–580 μm) were placed onto the striated muscle within each chamber, as described in previous studies by our group (Vollmar et al., 2001; Laschke et al., 2002, 2004). Upon transplantation, follicular grafts retain their three-dimensional structures despite the fact that some pressure is exerted via this chamber technique. Moreover, intact histomorphology of grafts, as demonstrated previously (Laschke et al., 2002) and in the present study, suggests a lack of chamber-associated harmful effects.

Donor and recipient animals

Four donor animals were pretreated with pregnant mare’s serum gonadotrophin (PMSG) (Sigma, Deisenhofen, Germany) dissolved in phosphate-buffered saline (1000 U/ml) in order to increase the number of tertiary follicles for transplantation. PMSG was given subcutaneously at 2 p.m. at a single dose of 2 U/10 g body weight. Follicles were harvested 48 h after PMSG treatment. In parallel, age-matched female hamsters (n = 12) fitted with a dorsal skinfold chamber were also treated with a single subcutaneous injection of PMSG (2 U/10 g body weight) for hormonal synchronization with the donors. Follicles were transplanted 48 h after PMSG treatment.

After follicle transplantation, recipient animals received daily a subcutaneous injection of either 1.0 mg/kg body weight nicotine (nicotine hydrogen tartrate salt dissolved in physiological saline; Sigma), 0.2 mg/kg body weight nicotine or physiological saline (2 μl/g body weight). Doses and mode of application of nicotine were chosen in accordance with the study of Keith evaluating blood nicotine levels in hamsters after smoking and subcutaneous nicotine application (Keith, 1988). Being aware that subcutaneous application is not the normal route of nicotine exposure, we still preferred this mode, as exposure of hamsters to cigarette smoke is difficult to standardize, induces stress, and may not achieve appropriate nicotine levels due to only passive smoking.

Intravitale fluorescence microscopy

For in vivo microscopic observation, pentobarbital-anaesthetized animals were injected intravenously with 0.15 ml of 5% fluorescein isothiocyanate (FITC)-labelled dextran 150 000 (Fluka, Buchs, Switzerland), which guaranteed contrast enhancement by staining of plasma. Subsequently, the hamsters were placed in a Plexiglas tube for immobilization and the skinfold preparation was attached to a Plexiglas stage. The stage was placed on a microscope desk, which allowed scanning of each individual follicle. Intravitale microscopy was performed using a Zeiss Axioskop microscope equipped with a 100 W HBO mercury lamp and a blue, green and ultraviolet filter block for epi-illumination (Zeiss, Oberkochen, Germany). The microscopic images were recorded with a charge-coupled device video camera (FK 6990-IQ: Pieper, Berlin, Germany) and were transferred to a video system for off-line evaluation. With the use of 10× and 20× objectives (Zeiss), magnifications of ×204 and ×405 were achieved on a 19-inch video screen.
Microcirculatory analysis
Quantitative off-line analysis of the videotapes was performed by means of a computer-assisted image analysis system CapImage (Zeintl, Heidelberg, Germany) and included the determination of the diameter (μm) and surface area (mm²) of the transplanted follicles, follicular growth (percentage of initial follicular size), the vascularized area of the transplanted follicles (in percent of the actual follicular size), microvessel density, i.e. the length of red blood cell (RBC)-perfused microvessels per observation area (cm/cm²), and the diameters (μm) and RBC velocity (V_RBC, μm/s) of the newly formed follicular microvessels. Volumetric blood flow (VQ, pl/s) of individual microvessels was calculated from V_RBC and diameter (d) for each microvessel as VQ = π × (d/2)² × V_RBC/K, where K (≈1.3) represents the Baker–Wayland factor (Baker and Wayland, 1974), considering the parabolic velocity profile of blood in microvessels.

Experimental protocol
A total of 36 follicles isolated 48 h after PMSG treatment were transplanted into the dorsal skinfold chamber of 12 PMSG-synchronized hamsters. The macroscopic appearance of the skinfold chamber preparations and the implanted grafts were controlled daily by photomicroscopy. Intravital fluorescence microscopic analysis of follicular growth, angiogenesis and microcirculation was performed on days 2, 3, 4 and 7 after transplantation. Measurements of microvessel density and microhaemodynamic parameters included only newly formed microvessels that could be clearly distinguished by their glomerulum-like arrangement from the autochthonous host striated muscle microvessels, which displayed the typical parallel arrangement of the muscle capillaries. Vascular densities were measured in three regions of interest for each graft and observation time point. Microvascular diameters and haemodynamic parameters were determined from 10 microvessels per region of interest. Analysis included a total of 12 transplanted follicles in hamsters treated with 1.0 mg/kg body weight nicotine (4), 12 transplanted follicles in hamsters treated with 0.2 mg/kg body weight nicotine (4) and 12 transplanted follicles in saline-treated hamsters (4).

Proliferating cell nuclear antigen and cleaved caspase-3 immunohistochemistry of transplanted follicles
Skinfold chamber tissue bearing the follicular grafts was harvested, fixed in formalin (4% in phosphate-buffered saline) for 2–3 days at 4°C and embedded in paraffin. To study proliferating cell nuclear antigen (PCNA) and cleaved caspase-3 by immunohistochemistry, 5 μm sections of paraffin-embedded specimens were incubated for 18 h at 4°C (PCNA) or overnight at room temperature (cleaved caspase-3) with a rabbit polyclonal anti-PCNA antibody (1:50; Santa Cruz Biotechnology) and a rabbit polyclonal antibody against cleaved caspase-3 (1:50; Cell Signaling Technology, Frankfurt, Germany). The latter antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3, but not full-length caspase-3. For development of PCNA, an alkaline phosphatase-conjugated goat anti-rabbit IgG (1:20; DakoCytomation) was incubated for 30 min. In the case of cleaved caspase-3, a biotinylated anti-mouse/rabbit immunoglobulin antibody was used as a secondary antibody for streptavidin–biotin complex peroxidase staining (Link, LSAB-HRP; DakoCytomation, Hamburg, Germany). Fuchsin (PCNA) and 3,3′-diaminobenzidine (cleaved caspase-3) were used as chromogens. The sections were counterstained with hemalum.

Aortic ring sprouting assay
To further evaluate the effect of nicotine on angiogenesis, an ex vivo angiogenesis assay was used. For this purpose, aortas from 10-week-old female Syrian golden hamsters were excised, immediately placed into Dulbecco’s modified Eagle’s medium (DMEM) and cut into cross-sectional rings of ~1.5–2.0 mm in length. Rings were embedded in 200 μl growth factor-reduced Matrigel (BD Biosciences, Bedford, MA, USA) in 48-well tissue culture grade plates and allowed to polymerize for 30 min at 37°C and 5% CO₂. The wells were then overlaid with 800 μl of DMEM supplemented with increasing doses of nicotine (0.15–1.20 μmol/l). The rings were maintained at 37°C and 5% CO₂ for 7 days with a medium change every 2 days. All assays were done in duplicate in a total of three animals. Vascular sprouting from each ring was examined daily on a conventional light microscope (Leica DM IL; Leica, Bensheim, Germany), and digital images were obtained (Canon PowerShot A80; Canon, Krefeld, Germany). By means of a computer-assisted image analysis system (CapImage; Zeintl, Heidelberg, Germany), quantitative analysis was performed using images from day 7 and included the determination of the length (μm) and area (mm²) of the outer aortic sprouting.

Statistics
All data are expressed as mean ± SEM. Data were first analysed for normal distribution and equal variance. Differences between groups were then calculated by analysis of variance followed by the Dunnett test for multiple comparisons. The criterion for significance was taken to be P < 0.05. Statistics were performed using the software package Sigma Stat 3.0 (Jandel Corporation, San Rafael, CA, USA).

Results
Follicular graft transplantation
In saline-treated animals all ovarian follicles showed adequate vascularization after transplantation onto the striated muscle of the dorsal skinfold chamber (Figure 2). In both nicotine-treated groups, 11 of the 12 follicular grafts showed revascularization, reflecting a take rate of 91.7%.

Grafted follicles of nicotine-treated hamsters had initial diameters of 475 ± 18 μm (0.2 mg nicotine/kg body weight daily) and 463 ± 16 μm (1.0 mg nicotine/kg body weight daily), which did not differ significantly from follicles of the saline-treated controls (466 ± 17 μm). As previously shown in histological studies, these grafts correspond to tertiary follicles, which are characterized by antrum formation and a well-developed multilayered thecal shell (Roy and Greenwald, 1985). Sequential analysis of follicular growth revealed a slight increase in saline-treated hamsters, with a final size of 113 ± 14% of baseline at day 7, whereas nicotine treatment resulted in the reduction of the initial follicular size, indicating follicular regression after transplantation (Figure 3A).

With regard to follicle vascularization, initial angiogenesis could be observed at day 2 after transplantation. The development of new blood vessels was characterized by sinusoidal sacculations, capillary budding and sprout formation. Subsequently, these sprouts interconnected with each other, finally resulting in a newly formed microvascular network, which showed a glomerulum-like arrangement of the capillaries (Figure 2). Of interest, angiogenesis in nicotine-treated follicles did not differ from that in controls.

Calculation of the size of the vascularized area in relation to the actual size of the follicular graft revealed ~80% revascularization at day 4 and almost completion, to approximately 95%, at
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day 7 after transplantation. Although on days 2 and 3 follicles in animals with low and, particularly, high nicotine exposure presented with somewhat smaller areas of vascularization, there was no significant difference compared with saline-treated controls over the entire observation period (Figure 3B). Further, microvessel density of the newly formed microvascular networks was constantly about 400–500 cm/cm² and did not show significant differences between the three experimental groups (Table 1 and Figure 2). These data suggest no major effect of nicotine on new vessel formation.

Analysis of microhaemodynamic parameters, including diameter, $V_{RBC}$ and VQ, of newly formed microvessels did not show relevant differences between the three experimental groups (Table 2). Diameters of the follicular grafts’ capillaries showed a decrease from $\sim 13 \mu m$ at day 2 to $\sim 11–12 \mu m$ at day 7, while $V_{RBC}$ increased progressively from $\sim 30–40 \mu m/s$ at day 2 to $\sim 100–150 \mu m/s$ at day 7. Correspondingly, calculation of VQ revealed an increase throughout the observation period. Interestingly, follicles of recipient animals treated daily with 1.0 mg nicotine/kg body weight presented with the highest values of VQ at the end of the experiment ($17.8 \pm 2.5 \text{ pl/s}$), which was due to increased capillary diameter and in particular increased $V_{RBC}$ when compared with the animals treated with 0.2 mg/kg nicotine and saline-treated controls.

**PCNA and cleaved caspase-3 immunohistochemistry of transplanted follicles**

PCNA and cleaved caspase-3 staining was studied in follicular grafts at day 7 after transplantation, serving as parameters of proliferation and apoptotic cell death, respectively. PCNA staining did not differ significantly between groups. Follicles of saline-treated controls failed to show cleaved caspase-3 staining (Figure 4A), while follicles of low-dose nicotine-treated animals presented with some cells positive for cleaved caspase-3 (Figure 4B). Most notably, follicular grafts of high 

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**Figure 2.** Intravital fluorescence microscopy of follicular grafts (borders are indicated by double arrows) directly after transplantation (A and D) as well as at day 2 (B and E) and day 7 (C and F) after transplantation into the dorsal skinfold chamber of a saline-treated (A–C) and high-dose nicotine-treated (D–F) PMSG-synchronized Syrian golden hamster. Note the lack of nutritive capillaries within the freshly isolated and transplanted graft (A and D). At day 2 after transplantation (B and E) newly formed microvessels create a network of capillaries, although a substantial part of the follicular graft still lacks vascularization (asterisk). At day 7 after transplantation (C and F) the follicular graft exhibits a complete glomerulum-like microvascular network with multiple intercapillary anastomoses (arrows) between the follicular capillaries and the striated muscle capillaries of the dorsal skinfold preparation. There were no marked differences in kinetics of vascularization and microvessel density within the follicular grafts among groups. Blue-light epi-illumination with contrast enhancement by 5% FITC-labelled dextran 150 000 intravenously. Scale bars represent 130 μm.
nicotine-treated animals showed positive staining of a considerable number of granulosa cells, indicating pronounced apoptotic cell death within these grafts (Figure 4C).

**Aortic ring sprouting assay**

Incubation of Matrigel-embedded aortic rings with increasing concentrations of nicotine (0.15–1.20 μmol/l) did not affect the nature and extent of vascular sprouting, as values of the length and area of the outer aortic sprouting were comparable to those in control rings without nicotine exposure (Figure 5).

**Discussion**

In the present study we analysed the growth and vascularization of ovarian follicles which had been freely transplanted into dorsal skinfold chambers of either nicotine- or saline-treated PMSG-synchronized animals. For this purpose, we used tertiary follicles with a well-developed theca interna, because this cell layer has been shown to be a major prerequisite for the development of new blood vessels after transplantation (Vollmar et al., 2001; Laschke et al., 2002).

The doses of nicotine chosen in the present study may correspond to a blood nicotine level of approximately 50 and 200 ng/ml, as studied by Keith in smoking and subcutaneous nicotine application (Keith, 1988). Though blood nicotine levels cannot be directly transferred from experimental animals to humans because of differences in smoke exposure and nicotine catabolism, as well
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as variations in smoking pattern and cigarette strength (Isaac and Rand, 1972; Herning et al., 1983; Feyerabend et al., 1985), mean peak levels of ∼50 ng/ml have been reported in humans shortly after smoking several cigarettes (Russell et al., 1980). As measurements from hamsters with similar cigarette strengths were found to be almost three times the human values (Keith, 1988), the doses used here, of 0.2 and 1.0 mg/kg nicotine, may ideally mimic low-rate (∼10 cigarettes/day) and high-rate (∼30 cigarettes/day) smokers respectively.

Current results about the effects of nicotine on blood vessel development are contradictory. Previous in vitro studies have demonstrated that nicotine in higher doses can inhibit cell division in endothelial cells and induce endothelial cell death (Lin et al., 1992; Villablanca et al., 1998), while nicotine significantly stimulates endothelial cell DNA synthesis and cell proliferation at concentrations lower than those obtained in smokers (Villablanca et al., 1998). The influence of nicotine on endothelial cells seems to be dependent not only on the nicotine concentration but also on the cellular environment (Villablanca et al., 1998). In support of this, it has recently been shown that low, but not high, doses of nicotine stimulate angiogenesis in several pathological states, including wound healing, tumor growth and vascularization of atherosclerotic plaques (Heeschen et al., 2001; Jain, 2001; Jacobi et al., 2002). This proangiogenic effect is probably mediated by nicotinic acetylcholine receptors. Stimulation of these receptors leads to proliferation of endothelial cells and up-regulation and release of growth factors such as basic fibroblast growth factor, transforming growth factor-β1 and vascular endothelial growth factor (Macklin et al., 1998; Villablanca et al., 1998; Cucina et al., 1999; Conklin et al., 2002). In contrast to this promoting role of nicotine in pathologic angiogenesis (Heeschen et al., 2001), revascularization of ovarian follicles remained almost unaffected by daily nicotine exposure, except for increased capillary diameter and microvascular perfusion at the end of the experiment in those animals which received the higher nicotine dose. Nicotine has been shown to stimulate dose-dependently the activity of endothelial nitric oxide synthase and the release of prostacyclin in endothelial cell lines derived from dogs and humans (Boutherin-Falson and Blaes, 1990; Miller et al., 2000; Tonnessen et al., 2000). Though not studied conclusively, microvascular enlargement and increase of blood flow may be attributable to nicotine-induced local release of these mediators. However, the nature and extent of revascularization of follicular grafts were comparable between the three groups studied. In support of this in vivo observation, nicotine also failed to affect ex vivo vascular sprouting significantly.

Of interest, however, we could demonstrate that nicotine dose-dependently inhibits follicular growth, emphasizing the link between smoking and fertility disorders. Nicotine has been reported to exert several toxic effects on intrafollicular processes. Blackburn et al. demonstrated in PMSG-primed, immature female rats that nicotine causes LH-independent inhibition of ovulation in vivo and in vitro (Blackburn et al., 1994). Furthermore, nicotine has been shown to inhibit the induction by FSH of progesterone synthesis in cumulus cells (Vrsanska et al., 2003) and the production of other androgens by theca interna cells (Sanders et al., 2002). We now provide the intriguing finding that nicotine—without affecting proliferation—increases granulosa cell apoptosis within the follicular grafts. This might explain the present observation that the size of follicles grafted in nicotine-treated animals decreased throughout the observation period, because inactive/apoptotic follicles normally undergo regression during the physiological ovarian cycle (Faddy and Gosden, 1995). Correspondingly, cigarette smoke has been shown to impair the growth of choriovallantoic membranes (Melkonian et al., 2002). Moreover, female hamsters that inhaled cigarette smoke for 30 days prior to mating revealed lower numbers of corpora lutea (Magers et al., 1995).

The present study now adds the novel finding that nicotine, as one toxic component of cigarette smoke, does not adversely influence follicular vascularization but alters follicular growth, supposedly by an increase in cell apoptosis. As follicular growth represents...
a mandatory process for normal ovulation and fertilization, nicotine-induced apoptosis of granulosa cells, with the consequent retardation/inhibition of follicular growth, might be partly responsible for the known link between smoking and fertility disorders.

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References

Figure 5. Representative images (A, ×2.5 magnification; B, ×10 magnification) of an aortic ring (arrow) with vascular sprouting after 7 days of incubation. The lower panel displays the quantitative analysis of the area (mm²; insert in A) and length (µm; dotted line in B) of outer aortic sprouting after 7 days of incubation with increasing concentrations of nicotine. Aortic rings without nicotine exposure served as controls (c). Data include a total of three independent experiments with duplicate measurements and are given as mean ± SEM.

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