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RAPID COMMUNICATION

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## Components of Cigarette Smoke Inhibit Expansion of Oocyte-Cumulus Complexes from Porcine Follicles

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### Summary

The role of alkaloids in cigarette smoke was investigated in the cumulus expansion of oocyte-cumulus complexes (OCC) isolated from large antral porcine follicles. Suppression of the cumulus expansion stimulated by FSH was observed in the presence of different concentration of cadmium, anabasine and nicotine but not its metabolite cotinine. There were comparable inhibitory effects of cadmium and nicotine on the synthesis and accumulation of hyaluronic acid in the cell/matrix compartment of OCC. The inhibitory effect of tested compounds on the cumulus expansion was accompanied by decreased progesterone synthesis by cumulus cells during 42 h incubation of OCC with FSH. The results suggest that cigarette smoking may affect intrafollicular processes, which are responsible for normal ovulation and fertilization.

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### Key words

Porcine ovary • Cigarette alkaloids • Cumulus expansion • Hyaluronic acid • Progesterone

Smoking cigarettes, one of the most common habits in the world, is a widely recognized health hazard. Smoking also affects reproductive health. Approximately one-third of women smoke. The link between smoking and fertility disorders, including poor embryo development following *in vitro* fertilization treatment and even infertile offspring is well established (Zenses 2000). Smoking has been associated with delayed conception (Baird and Wilcox 1985) and reduced number of retrieved oocytes, leading to premature menopause (Bolumar *et al.* 1996). Studies of *in vitro* fertilization treatment have shown that the spontaneous abortion rate

of smokers is twice as high as in non-smokers (Pattinson *et al.* 1991) and that smoking increases the occurrence of babies with low birth weight and premature delivery (Li *et al.* 1993). The effects of constituents that appear in cigarette and fuel smoke may explain the infertility problems related to smoking. Cadmium, anabasine, nicotine and its metabolite cotinine are the most abundant compounds in tobacco and smoke (Zenses 2000). Because the reproductive system is complex, many sites, from the hypothalamic-pituitary-axis to the germinal cells, may be vulnerable to disruption of reproduction. The oocyte and companion granulosa cells comprising

the follicular unit maintain a close association throughout development from primordial to preovulatory stages. At the time of follicular antrum formation, the granulosa cell population becomes divided into mural layers, inner layers of granulosa cells and cumulus cells surrounding the oocyte. In response to the ovulatory surge of gonadotropins, the cumulus cell-oocyte complex (OCC) undergoes mucification and expands by depositing an extensive extracellular matrix between the cumulus cells

enriched in hyaluronan (Salustri *et al.* 1989). Extracellular matrix components appear to be important for ovulation and sperm-egg interaction, thereby contributing to successful fertilization (Chen *et al.* 1993). In the present studies, the effects of cadmium, nicotine, cotinine and anabasine on FSH-induced cumulus expansion of OCC isolated from porcine follicles were evaluated.

**Table 1.** Effect of cadmium, anabasine, nicotine and cotinine on FSH-induced cumulus expansion and synthesis of progesterone of the oocyte-cumulus complexes (OCC) isolated from large (5-8 mm) porcine follicles.

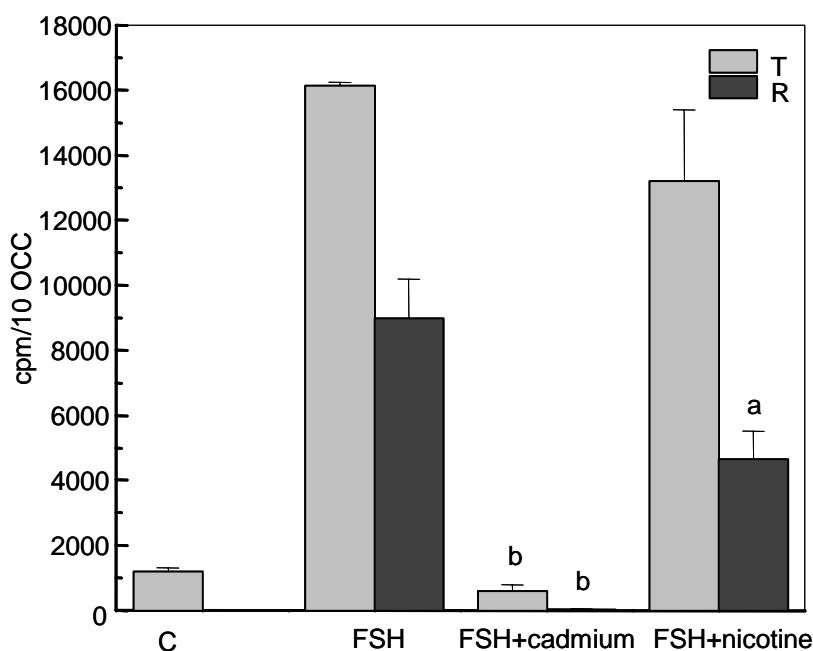
<b>Treatment</b>	<b>n</b>	<b>Degree of cumulus expansion</b>					<b>Progesterone (pg/1 OCC)</b>
		<b>+4</b>	<b>+3</b>	<b>+2</b>	<b>+1</b>	<b>0</b>	
<i>Control</i>	30					30	372 $\pm$ 125
<i>FSH (1<math>\mu</math>g/ml)</i>	70	22	21	15	5	7	6231 $\pm$ 702
<i>FSH+Cd (10<math>^{-6}</math>M)</i>	14	3	1	6	4	0	639 $\pm$ 64 <sup>b</sup>
<i>FSH+Cd (10<math>^{-5}</math>M)</i>	14	0	3	8	2	1	25 $\pm$ 4 <sup>b</sup>
<i>FSH+Cd (0.5x10<math>^{-4}</math>M)</i>	29	0	5	13	7	4	18 $\pm$ 4 <sup>b</sup>
<i>FSH+Nicotine (2x10<math>^{-6}</math>M)</i>	9	1	2	2	2	2	4606 $\pm$ 207 <sup>a</sup>
<i>FSH+Nicotine (2x10<math>^{-5}</math>M)</i>	8	0	2	2	2	2	4052 $\pm$ 364 <sup>a</sup>
<i>FSH+Nicotine (2x10<math>^{-4}</math>M)</i>	8	1	2	1	1	3	3123 $\pm$ 186 <sup>b</sup>
<i>FSH+Anabasine (10<math>^{-6}</math>M)</i>	10	2	1	2	5	0	3702 $\pm$ 229 <sup>a</sup>
<i>FSH+Anabasine (10<math>^{-5}</math>M)</i>	12	0	4	4	1	3	2001 $\pm$ 227 <sup>b</sup>
<i>FSH+Anabasine (10<math>^{-4}</math>M)</i>	11	0	0	3	2	6	1384 $\pm$ 228 <sup>b</sup>
<i>FSH+Cotinine (10<math>^{-6}</math>M)</i>	16	3	4	6	2	1	4071 $\pm$ 418 <sup>a</sup>
<i>FSH+Cotinine (10<math>^{-5}</math>M)</i>	16	3	3	5	3	2	2727 $\pm$ 80 <sup>b</sup>
<i>FSH+Cotinine (10<math>^{-4}</math>M)</i>	16	0	7	8	0	1	3378 $\pm$ 79 <sup>a</sup>

<sup>a</sup> P < 0.05, <sup>b</sup> P < 0.001 versus FSH

Porcine FSH (2039 IU/mg) was generously supplied by NIDDK (NIH Bethesda). Cadmium chloride, nicotine free base, anabasine (neonicotine), cotinine and all other chemicals were from Sigma. The porcine ovaries used for isolation of oocyte-cumulus complexes (OCC) were transported from slaughterhouse to the laboratory in a thermos at 30 °C. The OCC were isolated from 5-8 mm follicles, washed three times in M199 with Earle's salts buffered with 20 mmol/l NaHCO<sub>3</sub> and 6.25 mmol/l HEPES (pH=7) and supplemented with 10 % fetal calf serum, 0.91 mmol/l sodium pyruvate, 1.62 mmol/l calcium lactate, and antibiotics. They were cultured for 24 h at 38 °C in the atmosphere of 5 % CO<sub>2</sub> and 95 % air in the above medium. Groups of 10 porcine OCC were cultured with or without FSH and cigarette smoke

constituents in 24-well dishes using 0.5 ml of media per well (Ježová *et al.* 2001). The degree of expansion was assessed after 24 h incubation according to a subjective scoring system from 0 to +4 as follows: 0: no expansion; +1: separation of only the outermost layer of cumulus cells; +2: further expansion involving the outer half of the cumulus oophorus; +3: further expansion up to, but not including, the corona radiata; +4: complete expansion including the corona radiata cells (Downs 1989).

At the end of the incubation period, the OCC media were collected for progesterone determination. Levels of progesterone in the medium were determined by the [<sup>125</sup>I]-progesterone radioimmunoassay without extraction (Vranová *et al.* 1999).



**Fig. 1.** Hyaluronic acid synthesis (total T and retained R) by follicular OCC incubated for 24 h in the absence (control, C) or presence of FSH (1 µg/ml), cadmium ( $0.5 \times 10^{-4}$  M) and nicotine ( $2 \times 10^{-4}$  M). The values are the means  $\pm$  S.E.M. of three estimations (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  versus FSH).

Hyaluronic acid (HA) synthesis was measured using a procedure described by Eppig (1981) with slight modifications. Porcine OCC were cultured for 24 h at 38 °C in the same experimental conditions as described for culture of OCC in the presence of 2.5 µCi of D-[6-<sup>3</sup>H] glucosamine hydrochloride. The cultures were terminated by adding 10 ml of a solution containing 50 mg/ml pronase and 10 % Triton X-100 in 0.2 mol/l Tris buffer, pH 7.8. The samples were incubated for 2 h at 38 °C and then transferred to Whatman (Clifton, NJ) 3 mm filter paper discs, which were air dried and then washed by three changes of solution containing 0.5 % cetylpyridinium chloride and 10 mmol/l non-radioactive glucosamine hydrochloride for 45 min each and the discs were again dried. Synthesis of HA was measured either in medium plus OCC (total HA) or within the complexes alone (retained HA); this was achieved by simply transferring the complexes through three changes in a culture medium without labeled precursor before addition of the pronase-Triton X-100 solution (Nagyová *et al.* 1999). The data were analyzed by analysis of variance (ANOVA). Significance was assumed when  $P < 0.05$ .

After 24 h incubation with 1 µg/ml FSH (a positive control group), more than 61 % OCC expanded to the +4 and +3 stage (Table 1). Suppression of cumulus expansion stimulated by FSH was obtained in the presence of different cadmium, nicotine and anabasine but not cotinine concentrations ( $P < 0.05$ ). At the end of the culture period, the mucified cumulus of FSH, FSH+cadmium and FSH+nicotine groups were

dispersed with streptomyces hyaluronidase indicating that hyaluronic acid is an integral component of the expanded cumulus. The amount of HA present in the medium (total, T) and in cell/matrix compartment (retained, R) was determined using <sup>3</sup>H-glucosamine hydrochloride as a metabolic precursor of HA synthesis and distribution (Fig. 1). Treatment of OCC with cadmium resulted in significantly decrease ( $P < 0.01$ ) of total and retained HA. However, nicotine failed to affect total accumulation of HA but significantly decreased ( $P < 0.05$ ) the amount of HA retained within the FSH-treated complex. The inhibitory effect of tested compounds on the cumulus expansion of OCC isolated from large porcine follicles was accompanied by decreased cumulus cell progesterone production during 42 h incubation of OCC (Table 1). Cadmium, nicotine, anabasine, and cotinine caused a significant decrease in FSH-induced progesterone secretion by OCC. At the end of incubation period, oocytes were freed of cumulus cells, mounted on slides and fixed using a mixture of acetic acid and ethanol (1:3) for a period of at least 24 h. Oocytes were then stained with 1 % orcein and observed under a light microscope to assess nuclear maturation. While almost all oocytes of FSH group were reaching the stage of germinal resicle breakdown (GVBD), the presence of cadmium in the highest concentration ( $0.5 \times 10^{-4}$  M) caused complete suppression of oocyte maturation. Nicotine and cotinine did not inhibit the meiotic stage of porcine oocytes (data not shown).

The present data provide the first report of the toxic effects of cigarette smoke constituents on the cumulus expansion of the OCC. Suppression of FSH-induced cumulus expansion of OCC isolated from large antral porcine follicles was obtained in the presence of cadmium, nicotine and anabasine. Cadmium is a heavy metal present in water, foods, solid, air, and in cigarettes; smoking represents a primary source of inhaled cadmium (Staessen *et al.* 1990). Cadmium is a known teratogen and carcinogen that accumulates over a period of years. It is easily incorporated in the reproductive tissues such as gonads and uterus (Pařízek *et al.* 1969, Hamada *et al.* 1998). Acute reproductive effects of cadmium involve ovarian hemorrhage and delayed embryo implantation (De *et al.* 1993). Mice treated with cadmium ovulated fewer oocytes, exhibited an increase in degenerated oocytes and chromosomal anomalies in ovulated oocytes (Watanabe *et al.* 1977). The percentage of oocytes, which reached the stage of metaphase II, decreased in female rats treated with increasing cadmium dose (Píša *et al.* 1990). Nicotine, the most abundant alkaloid in tobacco, is quickly absorbed through the respiratory track. As much as 1 mg of nicotine can be absorbed by smoking a single cigarette (Barbieri *et al.* 1986a). Blackburn *et al.* (1994) demonstrated in PMSG-primed, immature female rats that nicotine caused LH-independent inhibition of ovulation *in vivo* and *in vitro*. Nicotine is metabolized primarily to cotinine. Cotinine has been detected in the follicular fluid of women undergoing assisted conception and was found incorporated into granulosa cells in dose-

dependent relationship with follicular fluid cotinine (Zenzeš *et al.* 1997). In our experiments, cotinine had no significant effect on expansion of cumulus cells of OCC, although it is a more stable compound than nicotine. Another possible mechanism by which cigarette components may modify the female reproductive system, is based upon their effects on steroidogenic function of follicular cells. Cigarette alkaloids, cadmium, nicotine, anabasine, and cotinine, markedly inhibited FSH-induction of cumulus cell progesterone production. *In vitro* studies of smoking on the physiology of granulosa cells are controversial. Aqueous tobacco smoke extracts or alkaloids, nicotine, cotinine and anabasine inhibit granulosa cell aromatase (Barbieri *et al.* 1986b). In contrast, Weiss and Eckert (1989) were unable to find any effect of alkaloids on estradiol or progesterone secretion by granulosa cells in the presence of serum, while in serum-free medium Bódíš *et al.* (1992) found increased secretion of estradiol and decreased progesterone secretion. During reproduction and development, cigarette smoking specifically affects the critical control mechanisms of intrafollicular processes and the present study has shown that the expansion of cumulus cells of OCC is potentially a sensitive and valuable end-point.

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