PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 333-336 (1979)

# Nicotine Reduces Embryo Growth, Delays Implantation, and Retards Parturition in Rats (40676)

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Cigarette smoking has been implicated in a number of reproductive disorders in women (1, 2), and nicotine, a component of tobacco smoke, interferes with pregnancy in rats. Effects commonly reported are reduced litter size and weight and prolonged gestation (3–6). Studies in our laboratory demonstrate that administration of nicotine to pregnant rats modifies the process of implantation, delaying the attachment of blastocysts by as much as 9 hr (7). The present study was undertaken to determine if the nicotine-induced delay in implantation is associated with altered embryo development. Therefore, the time of zona pellucida loss, growth of the inner cell mass and rate of cell proliferation were determined in embryos retrieved from control and nicotine-treated rats. The effects of the alkaloid on parturition and fecundity were also noted.

*Materials and methods.* Mature, virgin, female rats of Sprague–Dawley strain (Camm Research), 200–350 g body wt were maintained under environmental conditions controlled with respect to room temperature (20–25°) humidity and photoperiod (14-hr light, 10-hr dark, lights on at 6 AM). Animals were housed two per cage and provided free access to Purina Lab Chow and water. Vaginal smears were recorded daily and each rat completed at least two consecutive estrous cycles before use. Females were placed with fertile males on the evening of proestrus; the presence of sperm in the vagina during estrus denoted Day 0 postcoitum. Nicotine solution for injection (10 mg/ml saline) was prepared daily from concentrated nicotine (98%, Eastman Kodak). Animals were randomly assigned to control and experimental groups. Controls received saline injections; experimentals received subcutaneous injections of 5 mg nicotine/kg body wt twice daily (10:00 AM and 3:00 PM) on Days 0 through 5 postcoitum. At selected times each horn was flushed with 2.0 ml of physiological saline in order to retrieve those blastocysts either free within the uterine lumen or loosely attached to the uterine epithelium. This was accomplished by attaching the tubal end of each uterine cornu to a vertically oriented blunt-tipped hypodermic needle (18 gauge). A 70-g weight was attached to the syringe plunger to standardize the force and rate of flow through each uterine horn. A stopcock prevented premature flow until the horn was firmly secured to the needle. The flushing apparatus thereby provided a constant volume, force, and rate of flow of saline through the uterine lumen of each specimen examined so that variations in the number of blastocysts retrieved could be attributed exclusively to the tenacity of blastocyst attachment to the uterine wall (7). Embryos were counted and examined for the presence of a zona pellucida and blastocyst cavity. The cells of each embryo were then dispersed and their nuclei stained and counted (8).

The effects of nicotine on fecundity and time of parturition were determined in a second series of animals receiving either saline or twice-daily nicotine injections (Days 0–5), as above, and allowed to complete pregnancy. On Day 18, animals were placed in an apparatus that recorded the time of onset of parturition (9). Following delivery, young were counted, weighed, and sexed and the mortality rate was calculated. Differences between control and experimental means were assessed by Student's $t$ test (two tailed, significance level $P < 0.05$).

*Results.* The effects of nicotine on implantation as indicated by embryo retrieval are shown in Table IA. Fertilized ova could first be flushed from uteri at 6 PM Day 3 of pregnancy in both control and nicotine-treated rats; the number retrieved did not differ significantly between control ($1.2 \pm 0.8$) and treated ($0.3 \pm 0.3$) groups. Thereafter the number of embryos retrieved increased progressively through 6 AM Day 4 and re-



0037-9727/79/100333-04$01.00/0

TABLE I. EFFECTS OF NICOTINE ADMINISTRATION ON IMPLANTATION AND EMBRYO DEVELOPMENT[a]

| | Day 3 | | Day 4 | | | Day 5 | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 6 PM | 9 PM | 6 AM | Noon | 9 PM | 6 AM | Noon | 6 PM | 9 PM |
| A. Embryo retrieval rate: number of embryos/cornu | | | | | | | | | |
| Control | 1.2± 0.8 (10)[b] | 2.4± 0.8 (14) | 6.5± 0.8 (8) | 5.5± 0.5 (12) | 5.1± 0.5 (18) | 3.8± 0.8 (14) | 0.1± 0.1 (36) | 0 | 0 |
| Nicotine | 0.3± 0.3 (10) | 1.1± 0.5 (16) | 5.3± 0.5 (10) | 5.2± 0.7 (12) | 6.3± 0.8 (10) | 2.6± 0.6 (24) | 3.5± 0.5* (12) | 3.9 ± 0.7 (12) | 1.1 ± 0.5 (16) |
| B. Zona pellucida loss: % embryos with zona | | | | | | | | | |
| Control | 100(10) | 100(34) | 100(52) | 100(66) | 9(92) | 0(53) | 0(3) | | |
| Nicotine | 100(9) | 100(18) | 100(52) | 100(63) | 83(75) | 45(26) | 0(85) | | |
| C. Inner cell mass growth: % embryos with cavity | | | | | | | | | |
| Control | 0(10) | 0(34) | 100(52) | 100(66) | 16(92) | 6(53) | 0(3) | | |
| Nicotine | 0(9) | 0(18) | 100(52) | 100(63) | 46(75) | 43(26) | 73(85) | | 0 (16) |
| D. Embryo cell proliferation: number of nuclei/embryo | | | | | | | | | |
| Control | 12.6± 0.3 (10) | 15.3± 1.5 (9) | 33.6± 4.1 (10) | 42.0± 3.2 (12) | 53.0± 0.7 (8) | 77.0± 1.4 (10) | | | |
| Nicotine | 8.7± 0.3** (9) | 10.0± 1.0* (10) | 16.0± 1.0* (14) | 22.2± 2.7* (10) | 36.0± 1.5* (15) | 60.0± 1.7* (9) | | | |

[a] The s.c. injection of 5 mg nicotine/kg body wt twice daily (10:00 AM and 3:00 PM) on Days 0 through 5 postcoitum.

[b] Mean ± SEM; significantly different from control at $^{*}P < 0.01$ or $^{**}P < 0.05$ ( ): A No. of cornua; B, C, and D No. of embryos.

mained constant at approximately six per horn through 9 PM Day 4 in both treated and control rats. Subsequently the number of retrievable blastocysts declined in both groups. The decrease in recovery continued in control rats until noon Day 5 when virtually all blastocysts had attained a stage of implantation sufficient to resist dislodgement. By contrast, an average of 3.9 ± 0.7 blastocysts/horn could still be recovered from treated rats at 6 PM Day 5, and 1.1 ± 0.5 blastocysts were retrievable as late as 9 PM Day 5.

All embryos of both control and treated rats were zona encased at noon Day 4 (Table IB). Whereas loss of the zona pellucida was rapid in controls, being completed in most embryos between noon and 9 PM, zona loss was markedly slower in embryos of nicotine-treated animals, the zona being retained by 83% of the embryos at 9 PM Day 4 and by 45% at 6 AM Day 5. Zona loss was not evidenced by all embryos retrieved from treated rats until noon Day 5, i.e., approximately 15 hr later than controls.

In the majority of control embryos, growth of the inner cell mass was sufficient to obliterate the blastocyst cavity by late Day 4, only 6% possessing cavities as late as 6 AM Day 5 (Table IC). As with controls, the number of retrievable blastocysts decreased with time in treated rats, but a higher proportion of them retained a cavity (46, 43, and 73% at 9 PM Day 4, 6 AM and noon Day 5, respectively).

The rate of cell proliferation was reduced in embryos of nicotine-treated rats (Table ID). Upon entering the uterus (6 PM Day 3), embryos of treated rats consisted of fewer cells (8.7 ± 0.3 vs 12.6 ± 0.3; $P < 0.05$). The mean number of cells/embryo remained less ($P < 0.01$) than that of controls at all subsequent times examined.

Control and nicotine-treated rats differed ($P < 0.001$) in mean time onset of delivery (Table II). In controls, parturition occurred from 7 AM to 5 PM Day 22 with a mean time of delivery of 9.8 ± 0.40 hr, whereas treated rats initiated delivery from 6 AM Day 22 to 6 AM Day 25 with a mean time of 17.3 ± 2.0 hr Day 22. Despite delayed delivery, litters of treated rats did not differ significantly from those of controls with respect to size, birth weight, sex ratio, or mortality (Table II).

TABLE II. TIME OF PARTURITION, LITTER SIZE, SEX DISTRIBUTION, MORTALITY, AND BIRTH WEIGHT OF NEONATES BORN TO CONTROL VS NICOTINE-TREATED RATS[a]

| | Parturition | | | | Sex | | % Mortality | | Mean weight (g) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Group | No. rats | Onset: $\bar{X}$ time hr (Day 22) | No. rats | Litter size: pups/litter | % Male | % Female | Male | Female | Male | Female |
| Control | 39 | 9.8 ± 0.4[b] | 19 | 10.4 ± 2.4 | 43.9 | 56.1 | 13.7 | 4.5 | 6.1 ± 0.4 | 6.0 ± 0.4 |
| Nicotine treated | 34 | 17.3 ± 2.0* | 18 | 8.9 ± 1.4 | 49.0 | 51.0 | 9.5 | 9.6 | 6.7 ± 0.4 | 6.5 ± 0.5 |

[a] The s.c. injection of 5 mg nicotine/kg body wt twice daily (10:00 AM and 3:00 PM) on Days 0 through 5 postcoitum.

[b] Mean ± SEM unless indicated otherwise; * significantly different from control at $P < 0.001$ (mean ± SD).

*Discussion.* The present study has established that administration of nicotine during the initial 5 days of pregnancy modifies: (i) the time of zona pellucida loss, (ii) the rate of embryonic cell proliferation, (iii) the time of implantation, and (iv) the time of onset of parturition. The fact that embryos of nicotine-treated rats possessed fewer cells than those of controls upon entering the uterus indicates that nicotine exerts a growth suppressing effect on conceptuses while they reside within the oviduct. Whether cells of the inner cell mass vs trophoblasts differ in susceptibility to the growth-retarding effects of nicotine remains to be established. However, marked differences in cell susceptibility to nicotine action were not readily apparent since growth of the inner cell mass was sufficient to obliterate the blastocyst cavity and support normal organogenesis, and trophoblast invasion was sufficient to result in functional implantation. Indeed, it is possible that, at the time of implantation, blastocysts of nicotine-treated rats do not differ from controls in cell number; they may merely take longer to acquire the number of cells consonant with nidation. On the other hand, perhaps trophoblast cells may be reduced in number without adversely influencing implantation.

This study extends previous observations regarding the effects of nicotine on implantation in the rat (7). Approximately half the embryos present in treated rats remain retrievable at noon Day 5. Such blastocysts continue to reside within the uterus up to 9 hr before becoming sufficiently attached to the uterine wall to resist dislodgement by the standard flushing procedure. Why only approximately 50% of the blastocysts are susceptible to the effects of nicotine remains to be determined. However, since ovulation is completed quickly and ovulation and insemination occur prior to initiation of treatment, it is unlikely that differences in vulnerability to nicotine-induced effects are related to differences in the age of ova and/or embryos. Since all embryos of treated rats entered the uterus at the same time as those of controls, differences in susceptibility to nicotine are not due to differences in duration of exposure to the oviductal and/or uterine environments. The percentage of blastocysts showing retarded growth approximated the percentage of either sex delivered; hence, whether the sex of the blastocyst is a determinant of susceptibility to nicotine action remains to be investigated.

While the factors regulating the duration of gestation in the rat are poorly understood, it is known that the time of parturition is influenced by photoperiod (9) and is related to litter size and fetal bulk (10). The timing of parturition in control rats was comparable to that previously reported (9). Although the cause of prolongation of gestation in nicotine-treated rats remains obscure, it may be related to the delay in implantation experienced by approximately half the blastocysts in rats receiving the alkaloid even though, relative to controls, no differences in litter size or fetal bulk were noted.

Nicotine may modify development by acting directly on the embryo since it is concentrated in uterine secretions (11) and taken up by blastocysts (12). In addition, the alkaloid may act indirectly to modify the environment in which the embryo develops by altering estrogen and/or progesterone secretion via its suppressive effects on luteinizing hormone (13) and/or prolactin (14) secretion. That nicotine reduces uterine competence to un-

dergo decidualization (15), a process dependent on a delicate balance of estrogen and progesterone (16), suggests that ovarian steroid secretion is modified. Similarly, while zona pellucida loss is dependent on trophoblast maturation (17), it is also influenced by estrogen-dependent conditions within the uterus (18). Thus, the effects of nicotine on zona loss may result from altered estrogen levels as well as from retarded trophoblast growth.

Nicotine is a potent vasoactive substance that may indirectly influence the embryo by altering the functional state of the reproductive tract. The normal development of the embryo is dependent on adequate supplies of oxygen and essential nutrients (19). Since, prior to implantation, the conceptus is unattached, all its metabolic needs are met by diffusion of substrates from the oviductal or uterine environment. The availability of oxygen (20) and other metabolic substrates is dependent on nutritive blood flow to the reproductive tract. Occlusion of the uterine blood supply (21) or administration of serotonin, a vasoactive substance, alters uterine–blastocyst interaction (22). Similarly, studies in progress indicate that the nicotine regimen employed in the present study induces a marked and protracted reduction in both oviductal and uterine blood flow that may result in alterations in intrauterine oxygen tension.

Injection of pharmacological doses of nicotine into female rats is clearly not equivalent to inhalation of the alkaloid by women smoking cigarettes (23). Differences in species, route of administration and dosage preclude extrapolating the results of the present study to problems of pregnancy related to tobacco use. However, since nicotine can modify embryo development without being embryotoxic or impairing fecundity, it may be useful for studying embryo–uterine interactions.

That a 31% reduction in cell number occurs in the embryos of nicotine-treated rats prior to their entry into the uterus indicates embryo vulnerability to agents administered to the mother during their sojourn through the oviduct. Among the provocative questions raised by this observation is whether nicotine exerts its growth-suppressing effects by acting directly on the embryo and/or indirectly by modifying the milieu interiéur of the reproductive tract.

*Summary.* Daily injections of nicotine during the initial 5 days of pregnancy reduces embryo growth, delays implantation, and retards the onset of parturition in rats. Nicotine administration does not alter litter size, birth weight, sex distribution, or mortality rates.

The authors thank Dr. Bent G. Boving for his constructive suggestions regarding the manuscript. This study was supported by Grants 1012 and 1012-R1 from The Council for Tobacco Research—USA, Inc.

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Received May 14, 1979. P.S.E.B.M. 1979, Vol. 162.