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Administration of minute quantities of 17β-estradiol on the nasal area terminates early pregnancy in inseminated female mice

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Abstract

It is well established that chemical emissions of novel males disrupt intrauterine implantation of fertilized ova in inseminated female mice, but the specific nature of these chemicals is not known. Given that novel males excrete androgens and estrogens in their urine and feces, the current experiments were designed to determine whether nasal application of these steroids could disrupt pregnancy. Nasal application of testosterone propionate to females during early pregnancy had no impact on gestation. However, nasal application of 17β-estradiol terminated all pregnancies in females at all doses greater than or equal to approximately 1 μg/day. Nasal application of 17β-estradiol benzoate similarly terminated all pregnancies in females at very low doses. In subcutaneous administration, 17β-estradiol is also the most potent steroid in disrupting pregnancy compared to other estrogens and androgens. These data suggest the possibility that males' emission of estrogens is among factors mediating the Bruce effect.

Keywords: 17β-estradiol; Testosterone; Pregnancy; Pheromones; Bruce effect; Implantation; Estrogen

1. Introduction

Early pregnancy, particularly the process of intrauterine implantation of fertilized ova, can be vulnerable to major environmental and social changes such as physical restraint, temperature extremes, nutritional deprivation, predator exposure, human handling, and other apparent stressors (deCatanzaro and MacNiven, 1992). Pharmacological administration of adrenal catecholamines and corticosteroids to inseminated females generally fails to disrupt pregnancy, but direct administration of adrenal androgens and estrogens does (deCatanzaro and Graham, 1992; deCatanzaro et al., 1991; Harper, 1967, 1969; Trend and Bruce, 1989). Radioimmunoassay of blood samples from pregnant restraint-stressed rats during implantation indicates elevated 17β-estradiol (MacNiven et al., 1992). Administration of exogenous antibodies to 17β-estradiol increases the capacity of females to bear litters in the face of restraint stress (deCatanzaro et al., 1994). High plasma estradiol levels can produce lysis of the corpus luteum (Greenwald, 1964), alter the timing of arrival of fertilized ova in the uterus (Burdick and Whitney, 1937), and produce suboptimal endometrial receptivity (Harper, 1992; Hunt and Roby, 1994; Suginami, 1995). Estrogen activity at the ventromedial hypothalamus is known to induce the onset of estrus (Pfaff, 1980).

Novel males, those other than the sire, are also well known to disrupt implantation, a phenomenon known as the Bruce effect (Bruce, 1960). This is thought to be mediated via vomeronasal stimulation of females by urinary excretions produced by the novel males. Male mouse urine is a source of chemical signals that affect female reproductive parameters (Bronson and Whitten, 1968; Vandenbergh, 1969). Lesioning of the vomeronasal organ of females has been reported to prevent the Bruce effect (Bellringer et al., 1980; Lloyd-Thomas and Keverne, 1982; Rajendren and Dominic, 1984). Application of male urine to the nasal area of inseminated females may disrupt pregnancy (Dominic, 1965), especially if urine comes from males housed near females (deCatanzaro et al., 1999). Urinary proteins

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extracted from male urine may similarly disrupt implantation when applied to the nasal region of inseminated females, which may be due to unknown appended smaller molecules rather than the proteins per se (Marchlewska-Koj, 1981).

Males' capacity to induce the Bruce effect is clearly androgen dependent. Castration can eliminate this capacity (Bruce, 1965), while testosterone or 17β-estradiol injections can restore it (deCatanzaro et al., 1995b; Dominic, 1965). Nevertheless, surgical removal of major androgen-dependent male accessory glands, the preputials and vesicular-coagulating complex, does not reduce males' capacity to disrupt pregnancy (deCatanzaro et al., 1996; Zacharias et al., 2000). Stimulus females that have been given androgens can disrupt pregnancy in proximate inseminated females (deCatanzaro et al., 1995c; Dominic, 1965). Collectively, these data suggest that some direct metabolite of androgens could be responsible for the Bruce effect (see also Rajendren and Dominic, 1988). It is well known that 17β-estradiol is a metabolite of testosterone; females given antibodies to 17β-estradiol can resist pregnancy disruption by novel males (deCatanzaro et al., 1995a).

The chemical constituents of males' urine that are responsible for disruption of implantation are not known. Recent studies have shown that some putative male pheromones (Jemiolo et al., 1986; Schwende et al., 1986) do not have the power by themselves to induce the Bruce effect when applied to the nasal area of inseminated female mice (Brennan et al., 1999). Recent data from our laboratory, derived from ELISA procedures applied to the urine and feces, have shown that there are substantial quantities of 17β-estradiol, testosterone, and other unconjugated steroids in male urine (Muir et al., 2001). On this basis, we decided to inquire whether nasal application of 17β-estradiol or testosterone during the period of intrauterine implantation might have the ability to disrupt pregnancy.

2. Methods

2.1. Subjects

CF-1 strain mice (*Mus musculus*) were obtained from Charles River Breeding Farms of Canada, La Prairie, Quebec, or bred in our laboratory from such stock. Housing involved standard polypropylene cages measuring $28 \times 16 \times 11$ (height) cm with straight-wire tops allowing continuous access to food and water, except where otherwise specified. The colony room was maintained under a reversed 14:10 light/dark cycle at 21°C. This research has been approved by the McMaster University Animal Research Ethics Board, conforming to the standards of the Canadian Council on Animal Care.

2.2. Insemination

CF-1 female subjects between 75 and 100 days of age (27–31 g) were each housed directly with one sexually experienced CF-1 male in a standard cage. Their hindquarters were inspected three times daily for the presence of a sperm plug. Females with sperm plugs were identified as subjects, and the day of detection was designated as Day 0 of pregnancy. Each female remained housed with the inseminating male until the morning after detection of a sperm plug, about the start of the dark phase of the lighting cycle. The female was then removed from the male, housed individually in a clean cage with fresh bedding, and assigned to one of the experimental conditions, counterbalanced across age and date of insemination.

2.3. Nasal application of hormone solutions

Nasal administration of hormones was achieved by giving each female eight applications of the same dose. Each application was achieved by dipping a No. 6 artist's paintbrush (Curry's Series 2600, Hamilton, Ontario) in the solution. The base of the animal's tail was held to restrain its movement, and five strokes were made with the brush aiming for the hairless tip of the snout proximate to the nostrils. Attempts were made to minimize human handling of the animal during this process. Each application took approximately 10–15 s. On Day 1 of pregnancy, 8 h after commencement of the dark phase of the lighting cycle, each female received the first administration. On each of Days 2, 3, and 4 of pregnancy, each female received two additional administrations at 1 and 8 h after commencement of dark phase. On Day 5, at 1 h after commencement of the dark phase, each female received a final administration. The administrator was blind with respect to the dosage of the solutions being applied to particular animals.

Steroids were obtained from Sigma, St. Louis. Concentrations for 17β-estradiol were 18, 6, 2, 0.67, 0.22, or 0 μg 17β-estradiol in 0.05 cc peanut oil. Concentrations for 17β-estradiol benzoate were 54, 18, 6, 2, 0.67, and 0 μg 17β-estradiol benzoate in 0.05 cc peanut oil. Concentrations for testosterone propionate were 202, 67, 22, 7.5, and 0 μg testosterone propionate in 0.05 cc peanut oil.

In order to convert these to approximate doses per animal per application, we subsequently measured weights of the paintbrush before and after the application process. This was applied to samples during the first and second administrations.

2.4. Behavior following nasal administration

Behavioral observations were conducted systematically after nasal application of the oil vehicle on a sample of 10 females, for 2 min following the initial application

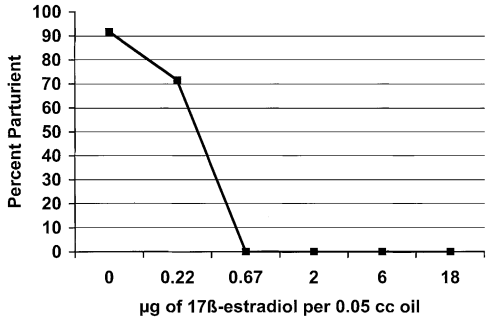

Fig. 1. Percent of inseminated females parturient after nasal applications of varied concentrations of 17β-estradiol on Days 1–5 of pregnancy.

Table 1
Mean (±S.E.) number of pups born and number parturient in inseminated CF-1 females nasally treated with 17β-estradiol during Days 1–5 of pregnancy

µg/0.05 cc oil	Pups/female	Pups/litter	Proportion parturient
0.00	11.6±1.3	12.6±0.8	11/12
0.22	7.1±2.2	10.0±1.7	5/7
0.67	0.0±0.0	–	0/7*
2.0	0.0±0.0	–	0/7*
6.0	0.0±0.0	–	0/8*
18.0	0.0±0.0	–	0/8*

* $P<.001$ by χ^2 test of association comparison to control (0.00 dosage).

then again for 2 min following the second application. The frequency of seven grooming patterns was recorded. These patterns were: face washing using both front paws in a one-time upward sweeping motion from mouth to ears (FWU), face washing using both front paws in a one-time downward motion from ears to mouth (FWD), rapidly repeated face washing involving combined FWU and FWD movements for over a 5-s duration (FWC), licks of the front paws in a hunched position with the weight on the rear legs (PAW), rubbing the face and head on an object or sides of the cage (RUB), rapid repeated head shaking or shivering (SHA), and scratching of the head, especially the ears with one of the hind legs (SCR).

2.5. Subcutaneous administration

We also report here pregnancy outcomes of samples of inseminated females subcutaneously treated with a single daily dosage of 17β-estradiol, estrone, estriol, testosterone propionate, or dihydrotestosterone during Days 1–5 of pregnancy. The dosages followed tripling progressions varied across hormones on the basis of a priori knowledge of the relative potency with respect to other parameters (see deCatanzaro et al., 1991), generally with substantially lower ranges for estrogens as opposed to androgens. Following insemination, females were individually housed in clean cages and each randomly assigned to one dosage group. Hormone concentrations (see Table 5) were each administered subcutaneously in the nape of the neck in 0.05 cc peanut oil. Injections occurred at approximately 4 h after commencement of the dark phase of the lighting cycle. After the final injection on Day 5, females were left undisturbed until pregnancy outcome was observed.

2.6. Dependent measures

After the experimental manipulations ended, subjects were not disturbed until 18 days following sperm plug detection, at which point they were observed twice daily for potential parturition until Day 25 after sperm plug detection. The maximal length of normal gestation in this strain of mice in this laboratory is 22 days. Pregnancy outcome was measured through counts of the number of pups born. The observer was blind with respect to the subjects' treatments.

3. Results

Data on the percent parturient after nasal administration of 17β-estradiol and 17β-estradiol benzoate are given respectively in Figs. 1 and 2. Tables 1, 2, and 3 provide

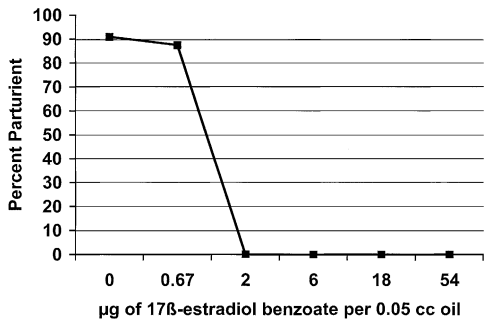

Fig. 2. Percent of inseminated females parturient after nasal applications of varied concentrations of 17β-estradiol benzoate on Days 1–5 of pregnancy.

Table 2
Mean (±S.E.) number of pups born and proportion parturient in inseminated CF-1 females nasally treated with 17β-estradiol benzoate during Days 1–5 of pregnancy

µg/0.05 cc oil	Pups/female	Pups/litter	Proportion parturient
0.00	10.4±1.3	11.4±0.9	10/11
0.67	9.7±1.7	11.1±1.1	7/8
2.0	0.0±0.0	–	0/8*
6.0	0.0±0.0	–	0/7*
18.0	0.0±0.0	–	0/8*
54.0	0.0±0.0	–	0/8*

* $P<.001$ by χ^2 test of association comparison to control (0.00 dosage).

Table 3
Mean (±S.E.) number of pups born and proportion parturient in inseminated CF-1 females nasally treated with testosterone propionate during Days 1–5 of pregnancy

μg/0.05 cc oil	Pups/female	Proportion parturient
0.0	12.9±0.7	7/7
7.5	11.3±1.2	7/7
22	12.9±1.3	7/7
67	12.7±1.8	7/7
202	11.3±0.6	7/7

measures of the number of pups born including nonparturient females, the number born excluding these females, and the sample sizes for nasal administration of 17β-estradiol, 17β-estradiol benzoate, and testosterone propionate, respectively. All females were parturient in the experiment involving testosterone propionate. However, females nasally given 17β-estradiol or 17β-estradiol benzoate generally were not parturient, except at the very lowest concentrations examined in each case.

For nasal administration of 17β-estradiol, an overall chi-square test of association comparing conditions to the presence or absence of litters showed significance [$\chi^2(5)=38.34$, $P<.001$]. Individual $\chi^2(df=1)$ comparisons of the control condition to each other condition showed significance for all comparisons except that with the lowest dosage. For nasal administration of 17β-estradiol benzoate, an overall chi-square test of association comparing conditions to the presence or absence of litters showed significance [$\chi^2(5)=42.05$, $P<.001$]. Individual $\chi^2(df=1)$ comparisons of the control condition to each other condition showed significance for all comparisons except that with the lowest dosage. Individual comparisons between the lowest and higher dosages all were significant. For nasal administration of testosterone propionate, given that all females were parturient regardless of dosage concentration, data do not require or merit statistics.

3.1. Estimation of dosages nasally applied per animal

In order to quantify the dosage per animal in nasal application, we weighed the paintbrush before and after application. This was conducted for the lowest concentration of 17β-estradiol that fully disrupted all pregnancies, 0.67 μg/0.05 cc oil. Ten samples of first application of this solution yielded a mean (±S.E.) of 0.0494±0.0034 g deposited on each animal. Following reasoning that the quantity deposited could be diminished in subsequent applications because some oil was conspicuously lingering on the nasal area during repeated administrations, measures were repeated for a second administration of this concentration, yielding values in nine samples of 0.0379±0.0030 g deposited on each animal. Weighing samples of peanut oil, we determined that its specific gravity is 88% of that of water. For the lowest concentration of 17β-estradiol that fully disrupted all pregnancies, 0.67 μg/0.05 cc oil, we accordingly estimate that the dosage of 17β-estradiol was approximately 0.75 μg/animal in the first application and 0.58 μg/animal in the second application. For the 0.22 μg/0.05 cc oil, which partially disrupted pregnancy, the estimates are 0.25 μg/animal in the first application and 0.19 μg/animal in the second application.

3.2. Behavior following nasal application

Behavioral observations after nasal application of the oil vehicle are reported in Table 4. The most frequent observation was FWD movements, which were seen in all cases except for one animal after the second administration. FWU, FWD, and PAW movements were also seen in the majority of cases. There was no apparent difference between the first and second applications in the frequency of grooming patterns observed. The general effect of grooming following nasal administration was to disperse the oil over the head area and to reduce the amount remaining directly on the nasal area and that remaining on the animal.

3.3. Pregnancy outcome following subcutaneous administration

Comparative potency of three estrogens and two androgens following single daily subcutaneous injection on Days 1–5 of pregnancy are reported in Table 5. This table also gives associated $\chi^2(df=1)$ probabilities comparing the proportion parturient in the particular condition to the proportion parturient in seven vehicle control (0.000 μg/0.05 cc oil) animals run for each hormone. Generally, 17β-estradiol was the most potent substance in disrupting pregnancy; no female was parturient at the 0.33-μg dosage and only one was parturient at the 0.11-μg dosage. Estrone and estriol were second and similar to each other in relative potency for pregnancy disruption, with full elimination of pregnancy at the 27-μg dosage and reduction of the proportion parturient at the 9-μg dosage. Testosterone propionate did not terminate pregnancy below the 27-μg dosage, but no females were parturient at or above this dosage. Only the highest (243 μg) dosage of dihydrotestosterone signifi-

Table 4
Mean frequency (±S.E.) of grooming behavior patterns observed following nasal administration of oil vehicle in the first and second applications. The number of animals (of 10) showing each behavior is given in brackets

	First application	Second application
FWU	1.1±0.5 [4]	1.1±0.4 [6]
FWD	16.8±1.9 [10]	14.6±2.3 [9]
FWC	1.5±0.7 [7]	3.1±0.8 [7]
PAW	2.7±0.9 [6]	5.1±1.1 [9]
RUB	0.5±0.2 [4]	0.8±0.3 [5]
SHA	0.4±0.2 [3]	0.7±0.3 [4]
SCR	1.2±0.7 [3]	0.6±0.5 [2]

Table 5
Proportion parturient of inseminated females subcutaneously treated with a single daily dosage of 17β-estradiol, estrone, estriol, testosterone propionate (TP), or dihydrotestosterone (DHT) during Days 1–5 of pregnancy

	Dosage (μg/day sc)										
	0.000	0.013	0.037	0.11	0.33	1.0	3.0	9.0	27.0	81.0	243.0
17β-Estradiol	7/7	7/7	4/7	1/7*	0/7*	0/7*	0/7*				
Estrone	6/7	7/7	5/6	5/6	7/7	5/7	7/7	1/7**	0/7**		
Estriol	7/7		7/7	7/7	6/6	5/6	4/7	2/7**	0/7*	0/12*	
TP	7/7			6/7	5/7	5/8	5/7	6/8	0/8*	0/7*	0/7*
DHT	6/7						5/7	5/7	5/10	10/11	3/10***

* χ^2 test of association comparison to control (0.000 dosage) outcome: $P < .001$.
** χ^2 test of association comparison to control (0.000 dosage) outcome: $P < .01$.
*** χ^2 test of association comparison to control (0.000 dosage) outcome: $P < .05$.

cantly reduced the proportion of females that were parturient in comparison to controls.

4. Discussion

These data show that nasal application of very low dosages of 17β-estradiol during Days 1–5 of pregnancy reliably terminates gestation in inseminated female mice. Nasal administration of 17β-estradiol benzoate also completely disrupts pregnancy, albeit at somewhat higher dosages, consistent with the fact that the molecular weight of this substance is greater due to benzoate appendage to the natural molecule. On the other hand, relatively high dosages of testosterone similarly applied to the nasal area have no adverse influence on pregnancy. When given by subcutaneous injection, both androgens and estrogens can disrupt pregnancy, but estrogens do so at substantially lower doses. It is clear that 17β-estradiol is the most potent substance for terminating pregnancy during the period of intrauterine implantation of fertilized ova.

It is probable that the dosages actually reaching the animal's circulation following nasal administration are lower than those administered. Grooming behavior removed some of the solutions altogether from the females' bodies, produced some ingestion, and displaced the solution from the nasal region. The site or sites of action of 17β-estradiol that led to termination of pregnancy in these experiments is not yet established. There are, however, established mechanisms via which estrogens are known to interfere with intrauterine implantation of fertilized ova. If there were entry into general circulation, which might occur via the exposed vasculature of the nasal cavity, actions could disrupt implantation at the uterus or hypothalamus. Systemically, elevated plasma estradiol levels are known, among other influences, to alter the rate of transport of fertilized ova through the fallopian tubes such that optimal arrival at the uterus is disrupted (Burdick and Whitney, 1937; Greenwald, 1964; Harper, 1992; Hunt and Roby, 1994; Suginami, 1995). Entry into general or local circulation could also induce the onset of estrus via hypothalamic mechanisms, where minute quantities of estradiol can have profound influences through receptors in the ventromedial region (Pfaff, 1980). Most hypotheses about the Bruce effect have focused on pheromonal activity at the vomeronasal organ and accessory olfactory bulb (Bellringer et al., 1980; Lloyd-Thomas and Keverne, 1982; Rajendren and Dominic, 1984). There could conceivably be some more indirect mechanism via as yet not established mechanisms at the vomeronasal organ.

No other pure substance has been established to disrupt pregnancy through this route of administration. Application of male urine to the nasal area of inseminated females may disrupt pregnancy (deCatanzaro et al., 1999; Dominic, 1965). Urinary proteins extracted from male urine may similarly disrupt implantation when applied to the nasal region of inseminated females, which may be due to unknown appended smaller molecules rather than the proteins per se (Marchlewska-Koj, 1981). Brevicomin and thiazole, putative pheromones isolated from male urine, do not have the power by themselves to induce the Bruce effect when applied to the nasal area of inseminated female mice (Brennan et al., 1999).

Testosterone and 17β-estradiol are present in substantial quantities in the urine and feces of both male and female mice (Muir et al., 2001). Some degree of physical contact between males and females may be a necessary condition for the Bruce effect (deCatanzaro et al., 1995c), and the presence of male excretions is required for the transmission of this effect (deCatanzaro et al., 1996; Gangrade and Dominic, 1984; Rajendren and Dominic, 1985). The probability of pregnancy disruption increases with the number of males and quantity of their excretions to which inseminated females are exposed (deCatanzaro et al., 1996). It is thus probable that some estrogens are transmitted from novel males to inseminated females, which could contribute to the disruption of intrauterine implantation of fertilized ova. Clearly, quantitative issues are paramount in the determination of whether males' urinary emissions of estrogens are sufficient to act on a female's pregnancy. Evidence indicates substantial variance among males in the levels of 17β-estradiol in urine, ranging up to values comparable to those of females (Muir et al., 2001; Spironello-Vella and deCatanzaro, 2001). However, whereas females tend to urinate in puddles, males housed in proximity to females

tend to target their urine in discrete sprays at these females (deCatanzaro et al., 1996; Drickamer, 1995; Muir et al., 2001; Reynolds, 1971; Spironello-Vella and deCatanzaro, 2001). Although the total 17β-estradiol relative to urinary creatinine has been quantified, the absolute daily total emitted by a male has not been. In addition, it is probable that, in addition to 17β-estradiol, other estrogens and possibly androgens would summate in influencing females, so that any quantification would have to include some collective amount. Considerations of vehicle also complicate inferences at this point. Accordingly, a substantial amount of additional research is necessary before we could definitively link the current findings to natural processes of male induction of pregnancy failure.

This notion that estrogens might be among agents that act on females accords well with earlier work showing that administration of monoclonal antibodies to 17β-estradiol during implantation can permit pregnancy to be sustained when females are exposed to stimuli that normally disrupt pregnancy, including novel males and restraint-stress (deCatanzaro et al., 1994, 1995a). It may also be consistent with data indicating that novel males' motivational conditions modulate their capacity to disrupt pregnancy, as illustrated by the inability of sexually sated novel males to disrupt pregnancy (Spironello and deCatanzaro, 1999). Males' active direction of excretions at females will expose these females to molecules that alter the females' physiology. Estrogens may be the most potent substances so far detected in males' urine.

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