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Controlled Neonatal Exposure to Estrogens: A Suitable Tool for Reproductive Aging Studies in the Female Rat[1]

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ABSTRACT

The present study was designed to determine whether the modification of exposure time to large doses of estrogens provided a reliable model for early changes in reproductive aging. Silastic implants containing estradiol benzoate (EB) in solution were placed into 5-day-old female Wistar rats and removed 1 day (Ei1 group) or 5 days (Ei5) later. In addition, 10 μg EB dissolved in 100 μl corn oil was administered s.c. to another group (EI). Control rats received either vehicle implants or 100 μl corn oil. Premature occurrence of vaginal opening was observed in all three estrogenized groups independently of EB exposure. However, females bearing implants for 24 h had first estrus at the same age as their controls and cycled regularly, and neither histological nor gonadal alterations could be observed at 75 days. Interestingly, they failed to cycle regularly at 5 mo whereas controls continued to cycle. On the other hand, the increase of EB exposure (Ei5, EI) resulted in a gradual and significant delay in the onset of first estrus and in a high number of estrous phases, as frequently observed during reproductive decline. At 75 days, the ovaries of these last two groups showed a reduced number of corpora lutea and an increased number of large follicles. According to this histological pattern, ovarian weight and progesterone (P) content gradually decreased whereas both groups showed higher estradiol (E_2) content than controls. This resulted in a higher E_2:P ratio, comparable to that observed in normal aging rats. The results allow us to conclude that the exposure time to large doses of estrogens is critical to the gradual enhancement of reproductive decline. Furthermore, exposures as brief as 24 h led to a potential early model for aging studies that will be useful to verify whether neuroendocrine changes precede gonadal impairment.

INTRODUCTION

The loss of reproductive function is one of the most characteristic features of old female rodents. Regular estrous cycles of 4–5 days become lengthened and irregular and are eventually replaced by senile deviations (constant estrus [CE], repetitive pseudopregnancies, anestrus) [1–3]. Whereas it is generally accepted that early age-associated reproductive decline is primarily a neuroendocrine event [4], the complex changes occurring at the hypothalamic-pituitary and ovarian levels, although extensively studied [1, 5–7], are still not fully understood. One generality concerning reproductive senescence is that there is a remarkable variability in the transition from cyclicity to CE, which can occur at any time from 6–18 mo [8]. This individual variability emphasizes the need for appropriate models in which the outcome of reproductive decline can be advanced for comparison to age-matched young controls in order to better define the extent to which chronological age and endocrine status influence senescence.

The utility of giving estrogens to young rats to greatly accelerate what appears to be a normal aging process has been substantially supported [9–13]. In addition, perinatal life is the period of greatest sensitivity to the organizing effects of gonadal steroids [8, 14], and large doses of estrogens given s.c. to neonate rats result in neuroendocrine alterations [15, 16] that resemble those observed in spontaneously aging females already in constant estrus [6, 7]. Thus, to reproduce the conditions that cause early reproductive senescence, small doses of steroids have been administered early in life with the result that the animals displayed regular estrus cycles after puberty but soon failed to ovulate [14, 17–19]. This phenomenon has been referred to as delayed anovulatory syndrome (DAS). However, since significant refractoriness to the treatment has been reported [14, 18, 19], long-term studies are required to verify which animals will express DAS in order to be used as an early aging model.

Finch et al. [13] suggested that the dose and the duration of exposure to estradiol (E_2) could vary inversely to induce comparable reproductive alterations. Based on this hypothesis, a first alternative approach was therefore designed to determine whether a controlled neonatal exposure to a large dose of E_2 by means of implants is sufficient to improve the results obtained with low doses by s.c. injections. Thus, to assess the optimal duration of the treatment that will lead to DAS, we examined the effects of gradual reduction of EB exposure time, using a dose (100 μg) previously shown to produce a full anovulatory syndrome when given s.c. [15, 16, 20].

MATERIALS AND METHODS

Animals and Hormonal Treatments

Pregnant female Wistar rats were singly housed and maintained in a light- and temperature-controlled room (lights on from 0700–1900 h; 21–24°C). Tap water and rat laboratory chow were available ad libitum. All litters were

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adjusted to 8 pups on the day of delivery, which was designated Day 1 of life.

On the basis of a pilot experiment carried out to ascertain that implants yielded high levels of plasma estradiol comparable to those reached by injection, the rats received, on Day 5 at 1300 h, one of the following treatments: 1) Implants (1 cm length, Dow Corning Midland, ref. 602-235) filled with estradiol benzoate (EB; 5 mg/ml vehicle; Progynon B Oleoso Fuerte, Schering S.A., Spain) and incubated for 24 h at 37°C in saline were placed s.c. in the forearm and then removed 1 day (Ei1 group) or 5 days (Ei5) later under hypothermia. Solvent-filled implants were placed into controls (Ci). 2) Either 100 μg EB (Steraloids, Wilton, NH) plus 100 μl corn oil (EI group) or 100 μl oil (CI) were injected s.c. A nonmanipulated group (NM) was studied at the same time to explore the effects of surgical stress on the onset of vaginal opening (VO) and first estrus. In order to monitor the dynamics of plasma estradiol levels after treatment, at least 7 animals from each group aged between 5 and 30 days were killed at specific times.

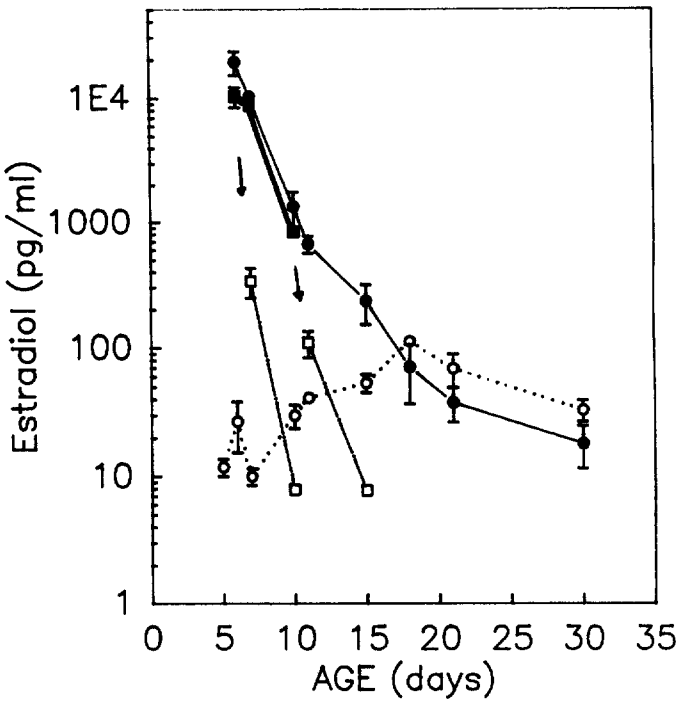

FIG. 1. Pattern of E_2 plasma levels in rats given silastic implants (solid squares) or s.c. injection of EB (solid circles; EI group) on the 5th day of life. E_2 levels were high after treatment (solid squares or circles; note the logarithmic scale) but dropped in both Ei1 and Ei5 groups below control levels (open circles) after implant removal (open squares). They then followed dynamics similar to those of control females (data omitted to simplify the figure). Mean ± SEM.

Experimental Design

Study of VO, first estrus, and cyclicity. The pups were examined from Day 6, and age and weight on VO were recorded. Daily vaginal smears were studied from the Day of VO (Ci, CI groups) or from 29 days of age (Ei1, Ei5, and EI groups) until females were 75 days old (designated as A period), and during Days 75–90 (designated as B period), Days 135–150 (designated as C period), and Days 200–215 (designated as D period). The onset of first estrus was registered.

We assumed that in young rats the frequency of cornified smears is a good biomarker of several subsequent events of reproductive aging [21]. Thus, the alterations in relation to regular cyclicity were expressed, in the four different studied periods, by the number of days in estrus found for each animal and were transformed into percentages. Additionally, the number of regular cycling rats (repeated 4- to 5-day estrous cycles) was noted.

Surgery and histology. On the basis of the above studies, the ovaries of 7–12 previously weighed rats from each group were removed on estrus under ether anesthesia around the age of 75 days, when control and Ei1 groups showed normal regular cycles. The ovaries were dissected, weighed, and briefly sonicated in 500 μl of absolute ethanol. They were then centrifuged, and the supernatant was used to measure progesterone and estradiol. Ovaries from parallel experimental groups were processed to give hematoxylin-eosin-stained preparations. One standard section per animal was examined under a light microscope; and the corpora lutea, follicles between 100 μm and 400 μm diameter, and those larger than 400 μm were separately counted.

Hormone Assays

E_2 and progesterone (P) were measured by commercial RIA kits (Diagnostic Products Corporation, Coat-a-County, Los Angeles, CA). Samples from each experiment were run in the same assay. The intraassay coefficient of variation was 6% for E_2 and 7% for P.

Statistics

Statistical differences between groups were studied by means of Kruskal-Wallis ANOVA by ranks and Mann-Whitney U test post hoc comparisons [22]. A confidence level of $p < 0.05$ was considered significant.

TABLE 1. Effect of neonatal E_2 on vaginal opening (VO), body weight at VO and first estrus (mean ± SEM).

Group[a]	n	Age at VO (days)	Body weight at VO (g)	Age at first estrous (days)
NM	(12)	33.0 ± 0.5	92.0 ± 8.0	41 ± 1
Ci	(12)	33.0 ± 0.3	92.0 ± 6.0	41 ± 1
CI	(7)	33.0 ± 0.4	92.0 ± 5.0	41 ± 1
Ei1	(12)	11.0 ± 0.3***	23.0 ± 1.0***	41 ± 1
Ei5	(12)	11.0 ± 0.2***	22.0 ± 1.0***	50 ± 1***
EI	(7)	10.0 ± 0.2***	19.0 ± 0.3***	53 ± 4*

[a]See Materials and Methods.
*$p < 0.05$; ***$p < 0.001$ vs. controls.

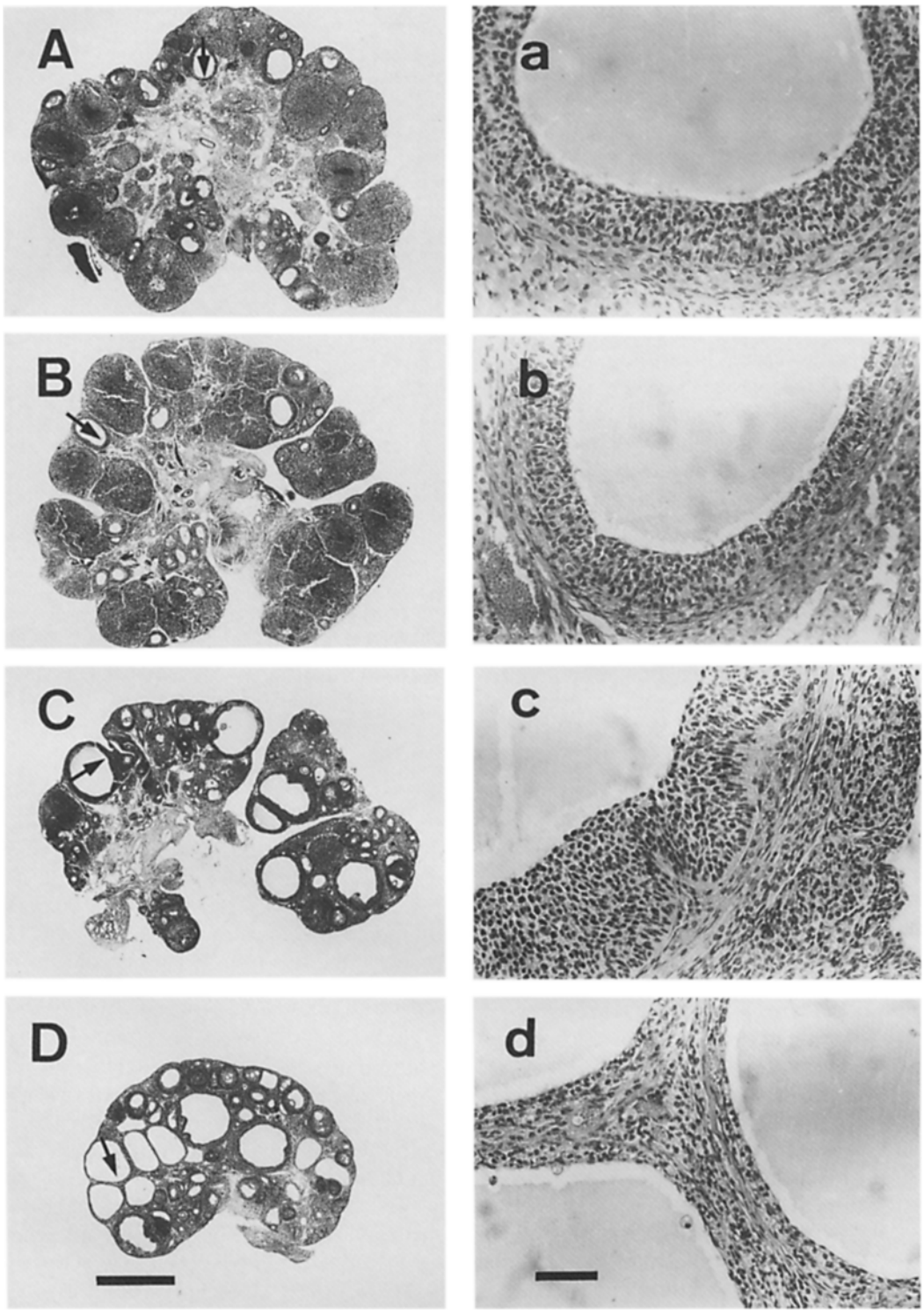

FIG. 2. Ovarian (estrous) morphology in 75-day-old controls (A) and in rats given silastic implants for 1 day (B; Ei1 group) or 5 days (C; Ei5 group) or s.c. injections (D; EI group; bar = 1000 μm). Note the increased number of large follicles and the decreased number of corpora lutea in both Ei5 (C) and EI (D) groups. Arrows indicate areas of large follicles, which are presented in right-side panels (a–d) at increased magnification (bar = 30 μm). Follicles for control (a) and Ei1 (b) groups had similar granulosa layer development; the granulosa layer was visibly thicker in rats from Ei5 group (c) . Often, an extremely thin granulosa layer was observed in the EI group (d).

TABLE 2. Number of CL and follicles in one histological ovarian section from young control and neonatally estrogenized females.

Group	n	CL	Follicles 100–400 μm	Follicles >400 μm
C	(7)	20 ± 2	21 ± 3	3.0 ± 0.4
Ei1	(7)	21 ± 1	23 ± 2	4.0 ± 0.8
Ei5	(7)	6 ± 2***	21 ± 2	7.0 ± 0.7***
EI	(7)	2 ± 2***	24 ± 4	10.0 ± 1.0***[a]

***$p < 0.001$ vs. controls.
[a]$p < 0.05$ vs. Ei5 group.

RESULTS

Circulating Estrogens

Figure 1 shows that 24 h after EB treatment, both implants and injection yielded plasma E_2 levels at least 100 times higher than those found in controls. It also indicates that levels of the steroid dropped dramatically one day after removal of implants (Ei1 and Ei5 groups) but remained higher than those of controls ten days after the injection (EI).

Effects of Neonatal EB Treatment on Puberty and Estrous Cycle Patterns

All three groups of EB-treated females showed full VO at a significantly ($p < 0.001$) earlier age than their controls (Ci, CI, and NM groups), with body weight correlated with age (Table 1). Neither age nor body weight on the day of VO differed among the estrogenized groups. Although first estrus occurred at the same age both in control and in less estrogenized (Ei1) females, its appearence was delayed in Ei5 and EI groups ($p < 0.001$; $p < 0.05$ respectively vs. control groups; Table 1). Because of the dispersion of the data in the injected group, a second study, using more animals, enabled us to verify that first estrus was more delayed in the EI than in the Ei5 group (Ei5: 37.6 ± 0.6, n = 17; EI: 40.3 ± 0.8, n = 20, $p < 0.05$).

Controls (Ci and CI) showed regular estrous cycles throughout the four studied periods, whereas estrogenized rats displayed different patterns according to EB exposure. Thus, during period A, females bearing EB implants for 24 h behaved in a similar way to their controls (Ci: 30.89% estrus ± 1.25, n = 12, Ei1: 30.5% ± 1.37, n = 12) but differently from the two groups given a greater exposure to estrogens, which showed a high percentage of estrus (Ei5: 54% ± 3.92, n = 12, $p < 0.001$ vs. Ci; CI: 32.2% ± .83, n = 7; EI: 52.46% ± 11.52, n = 7). It should be noted that with advancing age the percentage of estrus in the last two groups continued to increase significantly in relation to controls (data not shown). Interestingly, however, during period B the rats less exposed to estrogens (Ei1 group) continued to show the same incidence of estrus as the controls (Ci: 25% ± .77, n = 12 and Ei1: 27.6% ± 1.4, n = 12), with both groups having the same proportion of regularly cycling rats (11 of 12). Nevertheless, the number of

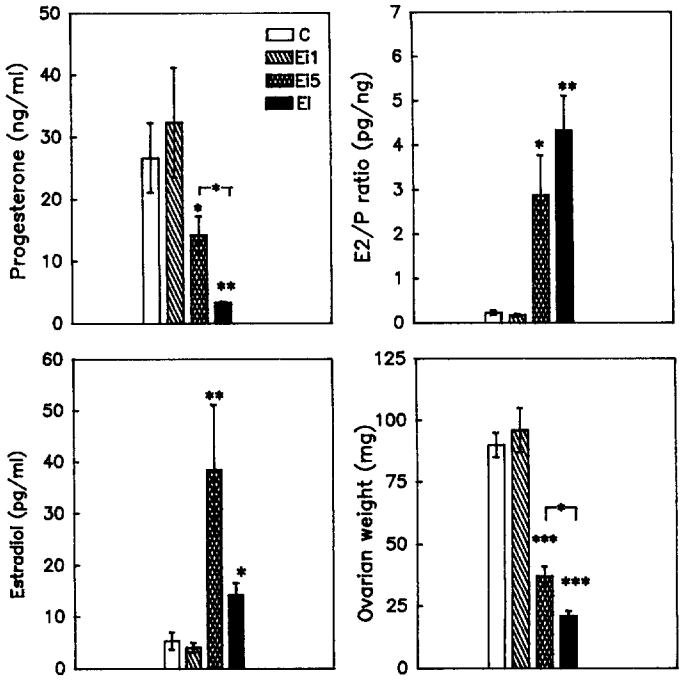

FIG. 3. Ovarian content of P (upper left panel) and E_2 (lower left panel) in female rats given silastic implants containing EB or s.c. injections of 100 μg EB (EI group, n = 7) on Day 5 of life. Implants were removed 1 day (Ei1 group, n = 11) or 5 days (Ei5, n = 11) after the placement. Results for control groups given implants with vehicle or s.c. injections of corn oil were combined and presented as a single group (C, n = 16). The upper right panel shows ovarian E_2:P ratio; the lower right panel shows ovarian weight in the same groups. (mean ± SEM), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

estrous (Ci: 25% ± 0.77, n = 12; Ei1: 46.9% ± 8.5 days, n = 12) and noncycling animals in the Ei1 group (6 of 12) increased in period C and diverged clearly from that of controls when studied at 200 days (Ci: 27.1% ± 1.2, n = 12; Ei1: 82.8% ± 5.7, n = 12; $p < 0.001$). By this time, most of the control females cycled regularly (11 of 12) whereas none of the rats from Ei1 group did.

Effects of Neonatal EB on the Adult Ovaries

The ovaries from control (C: Ci plus CI) and Ei1 females showed no weight differences; however, as neonatal EB exposure increased, the treated ovaries weighed gradually less (Ei5 $p < 0.001$ vs. control; $p < 0.01$ EI vs. Ei5). No differences in body weight were detectable among the experimental groups.

The content of P in the ovaries was found to be comparable in Ei1 and control (C) groups, whereas it decreased as EB exposure increased (Ei5 $p < 0.05$ and EI $p < 0.01$ vs. C; $p < 0.05$ EI vs. Ei5). The ovarian content of E_2 was higher in both Ei5 and EI groups than in controls (C; $p < 0.01$ and $p < 0.05$, respectively), but no differences were found between control (C) and Ei1 groups. Another result of consequence was that the ratio of E_2 to P in Ei1 rats was similar to that in controls, but increased in the two other estrogenized groups (Ei5 $p < 0.05$ and EI $p < 0.01$ vs. C; Fig. 2).

The ovaries from Ei1 and control females exhibited a similar histological pattern (Table 2). In contrast, Ei5 and EI groups showed a marked reduction in the number of corpora lutea ($p < 0.01$ vs. C) and a gradual increase in the number of large follicles (>400 μm) ($p < 0.001$ vs. C, $p < 0.05$ EI vs. Ei5). Most of these follicles showed a hypertrophic granulosa layer in the Ei5 group, whereas in the EI group this layer was highly attenuated in a remarkable number of follicles (Fig. 3).

DISCUSSION

The present study documents the relative importance of neonatal exposure length to a large dose of estrogens (100 μg) to induce reproductive decline. It also shows that the use of implants, apart from being the obvious tool for accurately controlling the duration of treatment, seems to be technically more reliable than injections for inducing homogeneous alterations.

Females bearing neonatal EB implants for 24 h exhibited a delayed anovulatory syndrome (DAS) such as has been reported with low doses of steroids [14, 18, 19]. However, our rats displayed regular estrous cycles for a longer period than those given 10 μg testosterone propionate [14] (50% sterile at 45 days of age), or than mice injected with 0.1 μg EB (60% showed longer cycles than controls at 60 days) [18]. In addition, our pattern of treatment was more effective: 92% of our rats expressed DAS by 5 mo, whereas 30% of the mice treated s.c. with low doses of E_2 [18] were refractory to the treatment. To explain the improved results of our treatment, attention must be paid to the large dose of E_2 used, which always assures supramaximal effects; results produced by low doses are dependent on the binding capacity of specific proteins (alfa-phetoprotein) in the circulation [23]. The uniformity of length and dose exposure with implants must also be considered, as indicated by the great dispersion in the percentage of estrus observed in the injected group (EI) compared to that in the Ei5 group.

It is interesting to note that increasing the length of EB exposure (Ei5 group) remarkably advanced the age of estrus cycle decline, so that these rats were comparable to normal females in a more advanced senescent state. On the other hand, the lack of a dosage effect with E_2-filled implants reported by Nass et al. [17], suggests that in order to gradually enhance senescence it is better to manipulate the duration of exposure than the dose.

The advancing of VO induced by exogenous estrogens has previously been reported by other authors [24, 25]. In our study, it occurred independently of the duration of the treatment. Our inability to observe a time-exposure related effect of estrogens suggests that the dose and/or the length exposure necessary to canalize the vagina was sufficient in all cases. In spite of the premature VO in females bearing EB implants for 24 h (Ei1), those females showed first estrus at the same age as their controls and cycled normally for two months. These results agree with findings of other authors [26, 27] that imply a direct effect of estrogens upon vaginal epithelium and confirm, as others have already stated [28], the importance of not relying solely on VO as a sign of sexual maturation. In addition, and in contrast to group Ei1, females exposed to estrogens for longer periods (Ei5 and EI groups) had a significant and gradual delay in the onset of first estrus. Similar results in the vaginal pattern as well as a delay in the maturation of the gonadotropin control in neonatally estrogenized rats were observed by Aihara and Hayashi [29]. The low variability in the onset of first estrus in the females bearing implants for 5 days (Ei5), compared to the EI females, also suggests that treatment with implants is technically more reliable than with s.c. injections.

In females bearing implants for 24 h, no discernible alterations were observed in either morphology or ovarian weight and hormonal content prior to the onset of alterations in vaginal cyclicity. Furthermore, since changes in follicular content [30] or plasma E_2 levels [31] have been reported in normal middle-aged female rats (10–12 mo), which ceased to display regular estrous cycles 1–2 mo afterwards, we suggest that at the age of observation (75 days old) females from the Ei1 group might be compared to younger normal females in which gonadal function is still not impaired. In contrast, the weights of ovaries from 75-day-old Ei5 and EI rats, as reported in CE rats with advancing age [32], became gradually and significantly lower than those of control and Ei1 females at the same chronological age. This decrease was accompanied by a gradual decline in the ovarian P content, which may be explained by the decreasing number of corpora lutea found. Low plasma levels of P were also reported in acyclic rodents [1, 33–35] compared to young cycling females. On the other hand, the tissue concentration of E_2 in the rats showing an elevated percentage of estrus (Ei5 and EI groups) was higher than in regularly cycling females (Ei1 and C) during estrus. This is consistent with the increased number of large follicles with a highly developed granulosa layer, since the granulosa cells are the main E_2 source [36], and is comparable with results obtained from either the ovaries [37] or the serum [1, 33] of middle-aged/aged females. It is interesting to note that the lower amount of ovarian E_2 in the EI group than in rats bearing implants for 5 days (Ei5) could be related to the finding of large follicles with highly attenuated membrana granulosa in the EI ovaries. Thus, the injected groups may represent a more advanced senescent state [33]. Besides the above results, the ovarian E_2:P ratio, which was high in the EI and Ei5 groups compared to controls, resembled serum E_2:P ratio in middle and old acyclic rodents vs. young cycling animals [1, 35, 38].

These results show that, whatever the time and/or dose dependency, when exposure to large doses of EB is reduced to 24 h it is sufficient to induce DAS. They also show that at the age of observation, the females had normal ovar-

ian function. However, whether gonadotropin secretion may be impaired while these animals are regularly cycling remains to be examined and will be the subject of future studies.

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