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EFFECTS OF DIETHYLSTILBESTROL EXPOSURE ON FEMALE RATS IN UTERO

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Yu-Lun Yang and Ching-Yuan Chen (1983) Effects of diethylstilbestrol exposure on female rats in utero. *Bull. Inst. Zool., Academia Sinica* **22**(2): 209-216. Diethylstilbestrol (DES), from 0.8 to 100 μg, was given to the pregnant rats in a single or four repeated subcutaneous injections to evaluate the effects of the prenatal DES exposure on the female offspring. Most of the DES-exposed neonates (40-100 μg in a single or 4-100 μg in 4 repeated injections) possessed visible nipples which appeared in the control group 3 to 5 days later. The microscopical features of the developing nipples in those DES-exposed rats also bore striking differences from that in the untreated ones. The precocious vaginal opening and persistent urogenital sinuses were common in those rats exposed to DES continuously (4-100 μg) in the last one-third period of the uterine life. The ovarian anomalies including the granulosa cell tumor and thin-walled vesicular cysts were also presented. The conception of the DES-exposed rats was frequently interfered, especially when higher dosages in repeated injections were given. The corpora lutea of those rats that failed to give birth to offspring alive demonstrated the relatively abundant supply of connective tissue and the ill-developed parenchymal cells. The DES-exposed rats failed in conception possessed relatively large number of the atretic follicles in the ovaries. Generally, the DES-exposed rats (more than 40 μg) could nurse their offspring to weanling period after a successful delivery, although some of their mammary glands are ill-developed.

Diethylstilbestrol (DES), the synthetic nonsteroidal estrogen, first produced in London in 1938 by Dodds *et al.* (Dodds *et al.*, 1938), was used extensively during the late 1940s and early 1950s, after publication of the reports concerning to prevent miscarriages and premature births (Smith, 1948). In 1953, Dieckman *et al.* reported the results of a controlled clinical trial of DES on pregnancy. They concluded that DES did not reduce the incidence of abortion, prematurity, postmaturity, perinatal mortality (Dieckmann *et al.*, 1953). Nevertheless, DES continued to be used until 1971 when Herbst *et al.* reported that the female adolescents exposed to DES in utero often developed the clear cell adenocarcinomas in their cervix and vagina (Herbst *et al.*, 1971).

Recently, many studies of the transplacental effects of DES on the female offspring have been published and these papers showed that the adenosis and structural abnormalities of the cervix and vagina were also associated with DES exposure (Bibbo *et al.*, 1977; Orr *et al.*, 1981; Robboy *et al.*, 1981). Some investigators suggest that the DES-exposed women may have an increased risk of the unfavorable pregnancy, such as spontaneous abortion, preterm delivery, neonatal death, and ectopic pregnancy (Herbst *et al.*, 1981; Robert, 1982).

Mice, rats and hamsters also have been used to study the normal development and the

estrogen-induced teratogenesis and carcinogenesis. Some reports documented that the neonatal exposure to DES resulted in the induction of the vaginal adenosis, adenosis-like lesions in the cervicovaginal region and the mammary adenocarcinoma (Jones and Bern, 1979; Newbold and McLachlan, 1982; Plapinger, 1981; Shellabarger *et al.*, 1980). The effects of DES exposure with high dosage prenatally on rats or mice were also published (Elizabeth, 1978; Nomura and Kanzaki, 1977; Vannier and Raynaud, 1980).

The present study was undertaken to establish whether or not abnormality occurs in the ovary, the vaginal opening, the nipple development, and the nursing ability of the female offspring under a low dose of DES being given to their parent rats by a single or repeated injections continuously during the last one-third period of the uterine life.

MATERIALS AND METHODS

The Wistar female virgin rats were used. Animals were housed in a conventional room with the controlled temperature (22–25°C) and lighting (14/10 hr. light/dark cycle). The rats were fed *ad libitum*.

The proestrous females were chosen by means of the vaginal smear and each was placed in a cage together with a breeder male. Day 0 of gestation was defined as the day that the sperm plug was present in the vagina. The subcutaneous injections with various dose (0.8, 4, 8, 40, 80, 100 μg) of DES (Sigma Chemical Co., St. Louis Mo), dissolved in 0.2 ml sesame oil, were given to the pregnant rats in a single injection on Day 15 of gestation (each treatment defined as groups DES-1-0.8, DES-1-4, DES-1-8, DES-1-40, DES-1-80, and DES-1-100) or in four repeated injections on Days 15, 17, 19, 21 of gestation (just the same defined as groups DES-4-0.8, DES-4-4, DES-4-8, DES-4-40, DES-4-80, and DES-4-100). The comparative injections with the same amount of sesame oil only were given to the control animals.

Nipples of the female neonates were observed and some of which were fixed in Bouin's solution, embedded in paraffin, sectioned and stained by Hematoxylin and Eosin (H & E) method, and microscopically examined. The female offspring were separated from their mothers 4 weeks after birth and the time of vaginal opening was examined. Studies of DES exposure on offspring's fertility were begun at 3 months old. The DES-exposed offspring were mated with the normal male rats of proven fertility. When the studies of fertility were finished, the animals were sacrificed and examined. Ovaries and nipples were fixed and sectioned for microscopic analysis.

Statistical analysis of the data was done by means of the X^2 test with Yate' correction.

RESULTS

Morphology of nipples in newborn rats

On the day of birth, the female neonates normally have no visible nipples. Visible nipples were not present until the third to fifth day of the postnatal life. In the rats treated with DES of high dosage with a single injection or of low dosage with repeated injections in utero, the prominent white spots appeared on the ventral surfaces of the trunks from the neonates within 24 hours after birth (Table 1); these structures were identified as the developing nipples.

TABLE 1
Effects of DES on presence of nipples in newborn female rats. Under normal observation within 24 hr after birth

Dose (μg)	Appearance of nipples	
	Injection	
	Single	Four repeated
0.8	− (7)	− (8)
4	− (10)	+ (9)
8	− (10)	+ (10)
40	+ (7)	+ (11)
80	+ (9)	+ (11)
100	+ (13)	+ (10)

+ and −: Neonates with and without prominent nipples, respectively.
( ): Numbers of rats.

Examined under the light microscope, the developing mammary glands of the control animals possessed a centrally-placed primary lactiferous duct surrounded by the downward extension from the surface epithelium (Fig. 3). This downward extension, termed the "epithelial hood", was poorly developed or even disappeared in those DES-exposed rats with prominent nipples (Fig. 4-6).

Urogenital anomalies

Although there existed more or less inconsistency, the time of vaginal opening in the normal female rats was around day 37 after birth. As shown in Fig. 1, there are about 7% of the control animals possessing the opened vaginae in Day 32. The single prenatal injection of 100 μg DES to the experimental rats did not have any significant effect on the time of vaginal opening in their offspring. On the other hand, 45-78% of female rats following repeated DES injections had the precocious vaginal opening (Fig. 1).

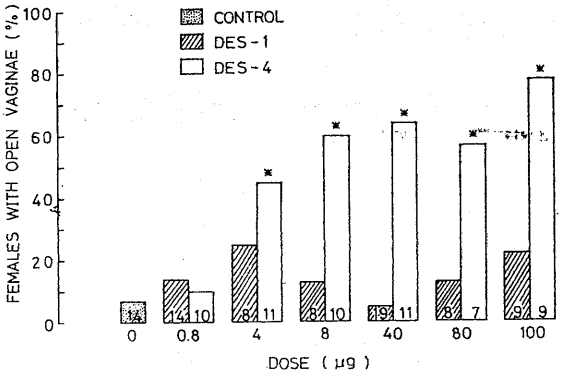


Fig. 1. Effect of prenatal DES exposure on time of vaginal opening in female offspring, examined on Day 32 after birth. *: $p<0.005$

A dose-related persistent urogenital sinus was observed in DES-treated groups (Fig. 2). The offspring from groups DES-4-4, -8, -40, -80, and -100 resulted in the retained urogenital sinuses in most cases (Table 2). None of the DES-exposed rats with a single prenatal injection preserved the embryonic urogenital sinuses till the adult life.

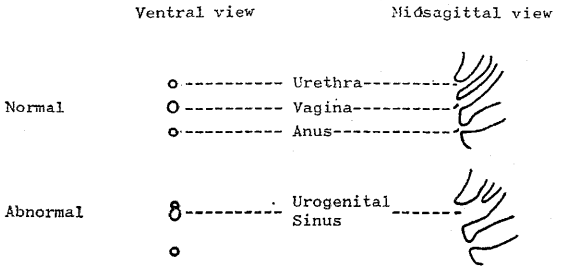


Fig. 2. Anatomical relation between external openings of urethra and vagina. Controls had separate openings while DES-exposed rats had only one urogenital orifice.

TABLE 2
Effects of DES on development of persistent urogenital sinus in female rats

Dose (μg)	Remnant of urogenital sinus	
	Injection	
	Single	Four repeated
0.8	− (0/14)	− (0/9)
4	− (0/8)	+ (4/7)
8	− (0/8)	+ (4/5)
40	− (0/19)	+ (11/11)
80	− (0/6)	+ (7/7)
100	− (0/9)	+ (9/9)

+ and −: Rats with and without persistent urogenital sinus, respectively.
( ): Numbers of rats.

Grossly enlarged ovaries were found in three cases, two (groups DES-4-4 and DES-4-8) of which possessed large thin-walled cysts filled with clear fluid (Fig. 7); while the other one (DES-4-80) was identified as a large granulosa cell tumor (Fig. 8).

Effects of DES on pregnancy

Daily vaginal smears reveal that the regular estrous cycles were frequently broken off in the rats exposed to DES in the uterine life (Table 3). After mated with the normal male of proven fertility at proestrous phase, the animal was assumed to be pregnant if the sperm plug was found in the vagina. As shown in Table 3, a single injection of DES

TABLE 3
Conception of DES-exposed females mated with normal males

Groups	Numbers of rats	Irregular estrous cycle		Pregnant		Miscarriage	
		No. (%)	P	No. (%)	P	No. (%)	P
DES-1-0.8	14	1 (7)	NS	14 (100)	NS	1 (7)	NS
DES-1-4	8	4 (50)	<0.05	8 (100)	NS	0 (0)	NS
DES-1-8	8	6 (75)	<0.005	8 (100)	NS	2 (25)	NS
DES-1-40	19	4 (21)	NS	17 (89)	NS	2 (12)	NS
DES-1-80	6	2 (33)	NS	5 (83)	NS	1 (20)	NS
DES-1-100	9	7 (78)	<0.005	7 (78)	NS	2 (29)	NS
DES-4-0.8	9	4 (44)	<0.05	9 (100)	NS	1 (11)	NS
DES-4-4	7	3 (43)	NS	7 (100)	NS	1 (14)	NS
DES-4-8	5	5 (100)	>0.005	4 (80)	NS	2 (50)	<0.05
DES-4-40	11	11 (100)	<0.005	7 (64)	<0.005	4 (57)	<0.005
DES-4-80	7	7 (100)	<0.005	4 (58)	<0.005	3 (75)	<0.005
DES-4-100	9	9 (100)	<0.005	5 (56)	<0.005	5 (100)	<0.005
Control	15	2 (13)		14 (93)		1 (7)	

NS: Not significant.

did not induce the failure in conception significantly in rats, even though 100 μg dose was given. However, significantly ($P \leq 0.005$) lower frequency of conception (56-64%) occurred in repeated injections with DES, except the low dose groups (DES-4-0.8, -4, -8). The ovaries of those rats failed in conception were small and possessed a relatively large number of the atretic follicles and a few hypoplastic corpora lutea (Fig. 9).

A high percentage of miscarriage (>50%) occurred in the pregnant rats that were exposed to more than 8 μg of DES in repeated injections in the last one-third of the uterine life. Microscopically examined, the corpora lutea of these rats demonstrated the relatively abundant presence of the connective tissue and the underdeveloped luteal cells (Fig. 10).

Only three cases (one in group DES-4-4 and two in group DES-4-40) did not give

Fig. 3. Fifth nipple, normal female rat, one day old, l. s. Showing a centrally-placed primary lactiferous duct surrounded by the downward extending epithelial hood (arrow) from surface epithelial layer. (H & E, ×102)

Fig. 4. Fifth nipple, female rat of group DES-4-80, one day old, l. s. Note the epithelial hood poorly developed. (H & E, ×102)

Fig. 5. Sixth nipple, female rat of group DES-4-8, one day old, l. s. Note a circular invagination of surface epithelium. (H & E, ×102)

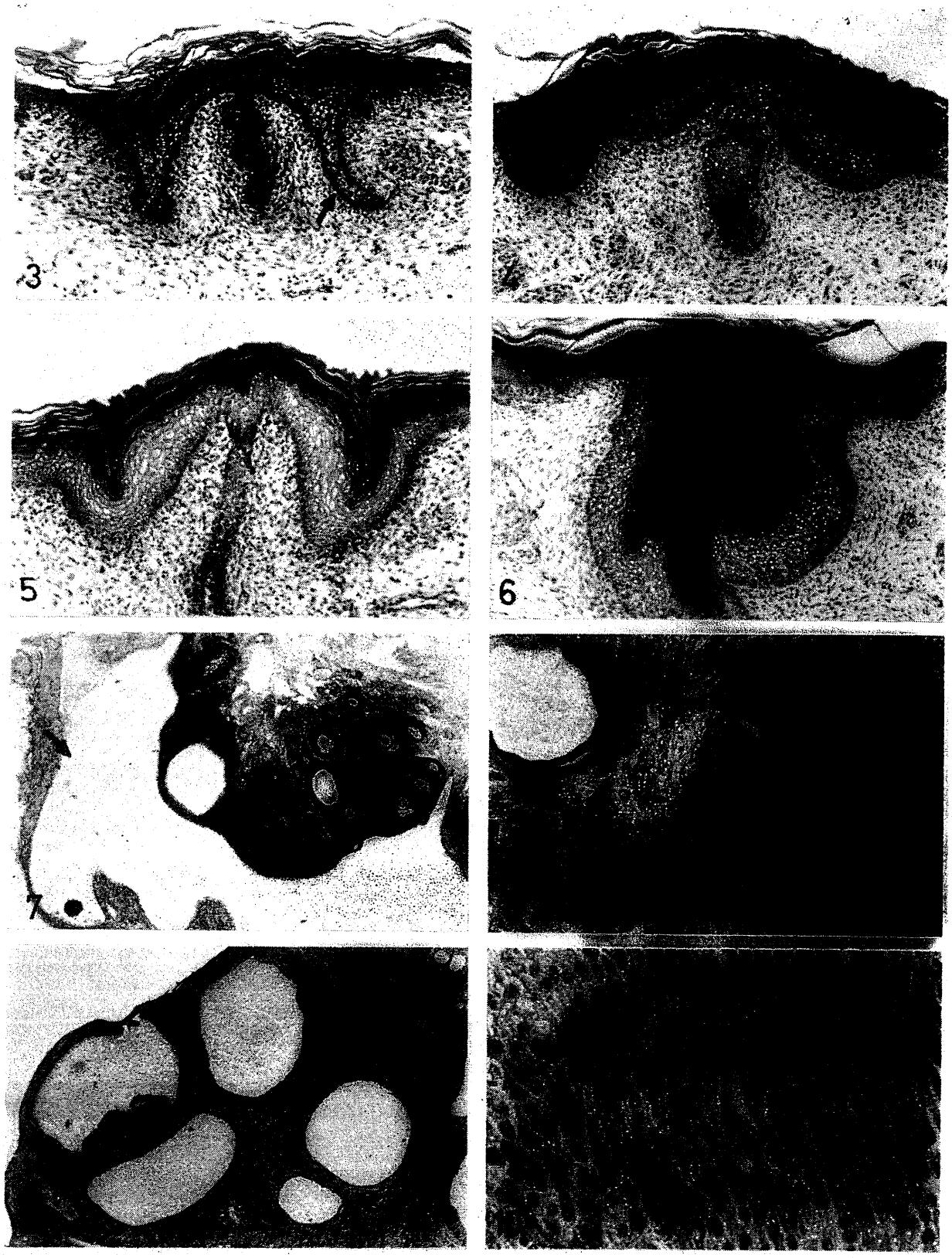
Fig. 6. Fifth nipple, female rat of group DES-4-40, one day old, l. s. Note the thickened epithelium lining the inverted nipple, to the depth of which the primary lactiferous duct opened. (H & E, ×102)

Fig. 7. Ovary, group DES-4-8, 4 months old. Note the ovarian cyst (arrow). (H & E, ×30)

Fig. 8. An ovary of the rat from group DES-4-80, 4 months old. Note a large granulosa cell tumor (arrow) occupied in the ovary. (H & E, ×30)

Fig. 9. An ovary of the rat from group DES-4-80, 4 months old. Note the hypoplastic ovary with several atretic follicles. (H & E, ×30)

Fig. 10. Part of corpus luteum, group DES-4-80, 4 months old. Demonstrate the representative area of a partially ill-developed granulosa lutein cell. (H & E, ×310)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delivery on the expected day.

Those DES-exposed rats giving a living birth were generally able to nurse their offspring to normal weanling period. Some of the lactating animals with 40 μg or more DES exposure prenatally had partially ill-developed nipples (Table 4). The nipples of the ill-developed glands were only slightly raised from the surface of the skin.

TABLE 4
Ill-developed mammary gland in DES-exposed females during lactation

Groups	Total numbers of rats	Rats with ill-developed mammary gland	
		No. (%)	P
DES-1-0.8	10	1 (10)	NS
DES-1-40	19	18 (95)	<0.005
DES-1-80	6	6 (100)	<0.005
DES-4-0.8	8	1 (13)	NS
DES-4-40	7	6 (86)	<0.005
Control	15	1 (7)	

NS: Not significant.

DISCUSSION

The results of this study show the marked urogenital anomalies, such as the precocious vaginal opening and the persistent urogenital sinuses, especially in those rats responding to 4 repeated injections of DES more than 4 μg. On the contrary, a single injection of the same dose on Day 15 did not induce these abnormalities in the female offspring, even though 100 μg of DES was given. Nomura and Kanzaki (1977) showed that the treatment with higher dosage of DES 10 μg/g B. W. on Days 15, 17, and 19 had resulted in induction of the persistent urogenital sinuses (15.8–92.5%) in the female mice. Elizabeth's study (1978) also demonstrated that the female rats exposed to 120 μg of DES in the last one-third of pregnancy had presented precocious opening of the vagina in comparison with the controls. While DES can be transferred through the placenta and accumulates in the fetal reproductive tract, it has been recognized that metabolism of DES will lead to a decrease or loss of the estrogenic activity (Metzler, 1981; Shah and McLachlan, 1976). Since the period of Days 15 to 21 is corresponding to the differentiating stage of the urethra and vagina from the urogenital sinus, it is likely that DES can somehow directly affect the development and differentiation of the urogenital sinus. The effect was even more conspicuous as the continuous exposures were applied. The stage of the urogenital development, roughly corresponding to the period of DES exposure on rats in our study, is comparable to the stage of high sensitivity to DES in human. However, the anomalies observed in the rats from our present study have not been reported in women exposed to DES at the fetal stage.

According to our examinations, we are firmly convinced that DES has a quite remarkable influence upon the structure of the developing nipples. The structural abnormalities of nipples noted in the neonates may be related to the formation of the ill-developed mammary glands in the future lactating period. However, the present study differs from Elizabeth's report (1978) with regard to the results of nursing the young. Generally speaking, the DES-exposed rats in our present study, although some of their mammary glands may be ill-developed, can still nurse their offspring to normal weanling if they have a successful delivery.

As shown in Table 3, it may be concluded that the irregular estrous cycles have increased significantly in the DES-treated groups. A single DES exposure did not significantly induce a failure in conception in the female rats even if 100 μg was given. Whereas the treatment of DES with repeated injections significantly reduced the frequency of conception (56–64%). Examined under the light microscope, the ovaries had relatively large numbers of the atretic secondary follicles. This may be one of factors responsible for failure in

conception. The present data also indicate that the incidence of failure in conception is somewhat correlated to the dosage of DES. However, there is no correspondance between the conception and the incidence of the irregular estrous cycle. For example: 78% (7/9) of conception versus 78% (7/9) of irregular estrous cycle in rats of group DES-1-100, and 80% (4/5) of conception versus 100% (5/5) of irregular estrous cycle in rats of group DES-4-8.

The fact that DES has precluded the normal delivery in this study is coincident with the findings of Elizabeth (1978). With regard to the outcome of pregnancy, we find that there was a higher incidence of miscarriage in the DES-exposed rats (*e. g.*, groups DES-4-8, -40, -80, and -100). The microscopic examination of ovaries reveals the presence of the fibrotic corpora lutea with ill-developed parenchymal cells. The miscarriage seems to come from the faulty placental attachment of the embryo in uterus. Although the hormonal assays (*e. g.*: LH, FSH, estrodiol, progesterone) of these rats were not undertaken in this study, we believe that the DES-exposure has made a certain influnce on the reproductive function in their offspring by means of a possibly disturbed hypothalamo-pituitary-ovarian function.

The ovarian tumor, though rarely found, can be induced by giving a high or low dosage of DES to rats. The natural estrogen usually induced tumors in the hormone-related organs, such as the pituitary, mammary glands, or uterus (Nomura and Kanzaki, 1977). Our observations suggest that the ovarian tumor occurs spontaneously in the rats regardless whether or not exposed to DES as that occurred in mice (Nomura, 1974). Since the number of the treated rats is small, it is anticipated that if the sample size is large enough, as the study continues, more significant conclusions will be acquired.

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DES 產前處理對於雌性大白鼠之影響

楊 幼 倫　陳 慶 源

本研究探討雌性大白鼠于產前接受 DES 處理所引起的變化。分別以 1 次或 4 次 (0.8～100 μg) 皮下注射的方式將 DES 注入已受孕的母鼠體內。

正常的雌性幼鼠於出生後第 3 天至第 5 天可見 6 對乳頭出現在胸腹部。產前接受 1 次注射 40 μg 以上或 4 μg 以上至 100 μg DES 分成 4 次等量注射的幼鼠出生當天卽可見乳頭浮現；經顯微觀察，則組織結構上亦有明顯異常現象。

產前接受 4 次等量注射總量爲 4～100 μg 的子鼠，於成鼠時有陰道早開口和泌尿生殖竇殘存的現象。

子鼠受孕率受產前 DES 處理的影響。以 4 次等量注射總量爲 40～100 μg DES 的雌性子鼠受孕率爲 56～64%。而無法成孕的子鼠，其卵巢具有相當多的萎縮卵泡。胚胎期接受 8～100 μg 以 4 次等量注射的子鼠，成孕後有 50～100%出現流產現象。這些子鼠卵巢中的黃體具有相當多量的結締組織和發育不良的黃體細胞。產前接受 DES 處理的子鼠中有 1 隻子鼠的卵巢具有粒層細胞增生的腫塊，2 隻子鼠的卵巢具有水腫的現象。

產前接受 40 μg 以上 DES 的子鼠在泌乳期具有 1 到 7 個乳腺發育不良的現象；但並不會影響其哺育幼鼠的能力。