



## ***In Vivo* Study of Diethylstilbestrol Teratogenicity on Mouse Embryo**

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### **Abstract**

Diethylstilbestrol (DES) was widely used in the past, as the morning after contraception, but its application became extremely limited due to several complications including delayed clear cell adenocarcinoma in female infants. However, the use of DES increased during the past decade for hormone replacement therapy. The aim of this study was to investigate possible teratogenicity of this synthetic oestrogen. The experiment was conducted on N-MRI mice. Various concentrations of DES were administered i.p. to pregnant animals throughout the period of organogenesis (days 9 and 10 of pregnancy). The control group received ethanol as vehicle. Pregnancy was terminated on the 18<sup>th</sup> day by cervical dislocation. The embryos were then removed and fixed in Bouin's solution, and parameters currently used in teratogenic studies were assessed. Severe embryo toxicity score was observed following the application of DES at doses of 200 and 400 mg/kg (71.4% and 83.6%, respectively). The weight and the average size of some of the examined parameters were markedly decreased by embryological observation. Furthermore, the size of derm and mosaic cells of urinary bladder, shortening in the length of femur, and abnormal disposition of calcium compound in embryos were markedly different in the treated mice.

*Keywords:* Diethylstilbestrol; Mice embryo; Teratogenicity.

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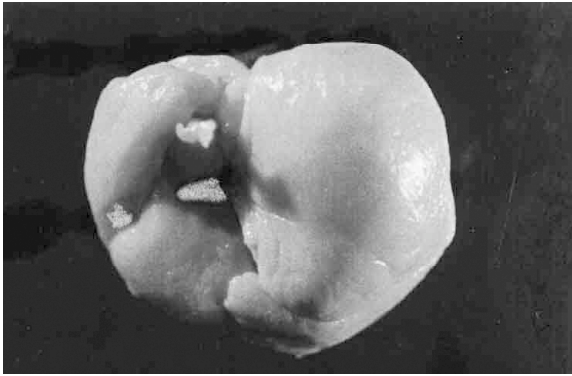
### **1. Introduction**

Diethylstilbestrol (DES) was widely used in the past, as the morning after pill and for treatment of pregnant women since 1971 [1], but its application became extremely limited

due to several complications including delayed clear cell adenocarcinoma of the vagina and cervix in exposed women [2]. In addition to that, there are reports of various anatomical and gross anomalies of the genital and reproductive tracts by DES [3, 4]. Linkage of DES exposure to reproductive tract abnormalities in males and females consist of immune system disorders and psychosexual effects [4]. Abnormal physical findings have

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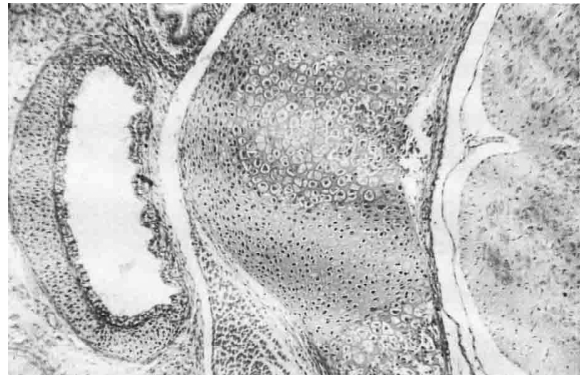
**Figure 1.** A resorped mouse embryo in the 4  $\mu$ g DES treated group. It appears that development of the embryo has terminated on the day 10 of pregnancy (Fixed in Bouin's solution).

also been identified in male offspring exposed in utero to DES [5]. Adenocarcinoma has been found in young women between the ages of 15 to 22, which has been associated with first trimester DES exposure in an epidemiologic case-control study [6].

In a report by Wardell *et al.* [7], the administration of DES to rats in the 19<sup>th</sup> day of pregnancy resulted in growth retardation, resorption, gross malformations and organ-level anomalies. Induction of embryo toxicity by DES in the whole culture of rat embryos has also been reported [8]. However, the use of DES increased during the past decade for hormone replacement therapy [9]. In USA alone, at least 4 million fetuses and their mothers had a substantial exposure to DES [3]. There is a great concern about unrelated abnormalities due to the administration of



**Figure 3.** Bone marrow formation in an 18 day mouse embryo from the 4  $\mu$ g DES treated group. Note that the bone marrow formation is not well developed (H and E,  $\times 100$ ).



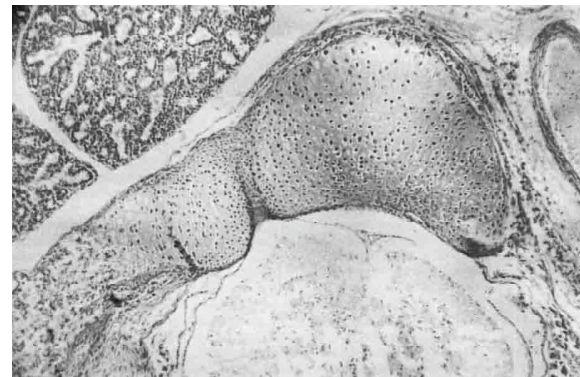
**Figure 2.** Bone marrow formation in an 18 day mouse embryo from the control group. Note that the bone marrow formation is obvious (H and E,  $\times 100$ ).

DES in the women at the reproductive period. In this study, we investigated any other possible abnormalities including bone deformation and pathological damages following DES exposure in mice.

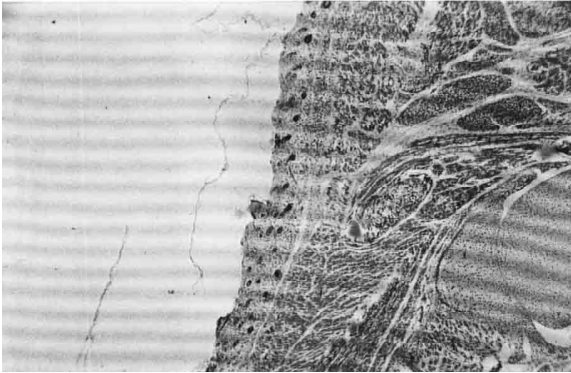
## 2. Materials and methods

### 2.1. Animals

The experiments were conducted on female N-MRI mice (19-22 g). The virgin animals were housed in 12 h light/dark condition and controlled temperature area ( $23\pm 2$  °C), and freely accessed to water and food *ad libitum*. The vaginal smear was prepared daily in order to identify oestrous cycle. In the mid of oestrous cycle, the animals were allowed for mating at night. Positive vaginal smear in the following day was considered as indication of day 0 of



**Figure 4.** Bone marrow formation in an 18 day mouse embryo from the 8  $\mu$ g DES treated group. Note that the bone marrow formation is not well developed (H and E,  $\times 100$ ).



**Figure 5.** The dorsal skin of an 18 day mouse embryo from the control group. Note that the thickness of the skin is normal and hair follicles are distinguishable (H and E, x40).

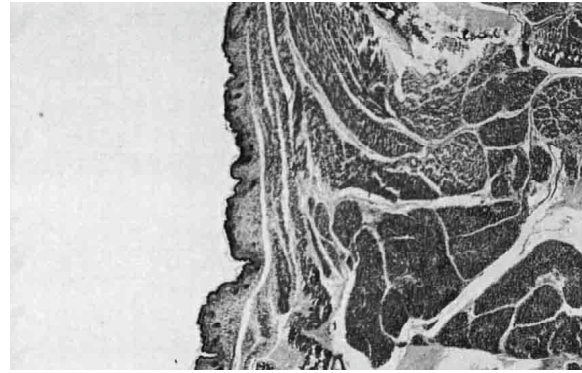
pregnancy.

### 2.2. Drug administration

DES was dissolved in the minimum amount of ethyl alcohol and concentrations of 4, 8, 15, 30, 75, 150 and 450  $\mu\text{g}$  in saline was applied i.p. to animals (in 8 groups) throughout the period of organogenesis (days 9 and 10 of pregnancy). The control group received the vehicle. Pregnancy was terminated on the 18<sup>th</sup> day by cervical dislocation. The embryos were removed immediately and washed two times by saline. The number of dead embryos were counted and the apparent condition of external organs were examined. Half of the embryos were fixed by 95% ethyl alcohol for Alizarin red-S staining, while the other half were fixed in Bouin's fixative, embedded in paraffin wax,



**Figure 7.** The dorsal skin of an 18 day mouse embryo from the 8  $\mu\text{g}$  DES treated group. Note that the hair follicles are not well developed and their numbers were reduced. The thickness of skin layers has also reduced (H and E, x40).

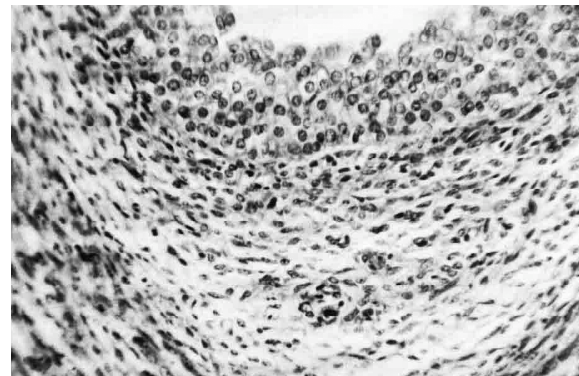


**Figure 6.** The dorsal skin of an 18 day mouse embryo from the 4  $\mu\text{g}$  DES treated group. Note that the hair follicles are formed but less developed. The thickness and development of the skin are normal (H and E, x40).

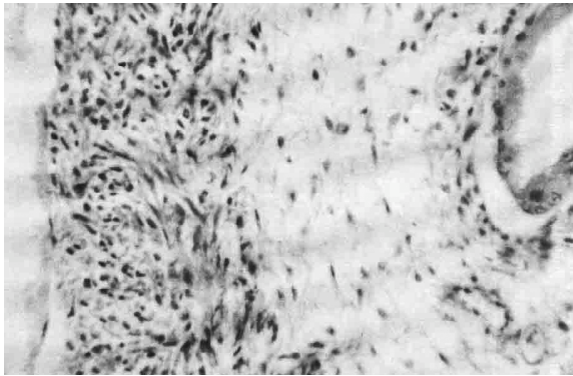
and 5  $\mu\text{m}$  tissue sections were prepared for Harris-Haematoxylin and Eosin staining. Embryo toxicity score (ETS) was calculated as: the number of resorptions + the number of the dead fetuses / total number of implants.

### 2.3. Alizarin red-S staining method

The embryos were fixed in 95% alcohol for 2 weeks and were cleared by 0.7% potassium hydroxide for 2 months. The samples were then washed with tap water for 30 minutes and stained with 0.1% alkaline Alizarin red-S for 24 hrs. The samples were again washed with tap water for 30 minutes and cleared by 80% potassium hydroxide and 20% glycerol solution. Finally, the samples were rehydrated by various concentrations of glycerol, ethyl alcohol and water.



**Figure 8.** Gall bladder development in an 18 day mouse embryo from the control group. Note that the epithelium of bladder is normal and muscle layers are well developed (H and E, x400).



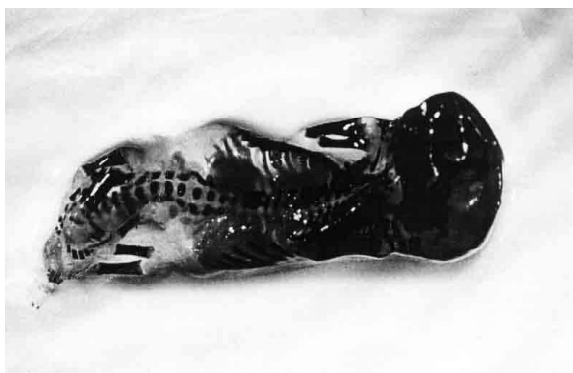
**Figure 9.** Gall bladder development in an 18 day mouse embryo from the 8 µg DES treated group. Note that the epithelium of bladder and muscle layers are not well developed (H and E, x400).

#### 2.4. Morphological parameters

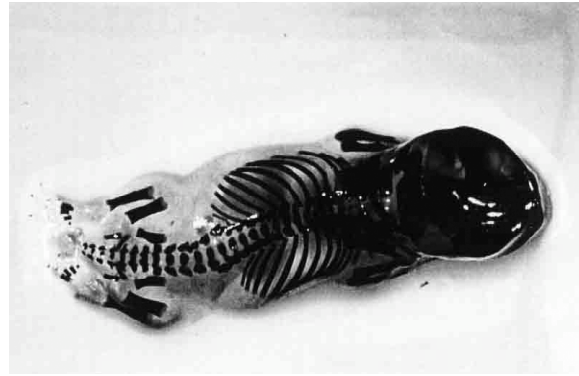
Two types of parametric and non-parametric observations were conducted for the embryos stained by Alizarin red-S staining method. The non-parametric measures consist of microcephaly, microphthalmia, anophthalmia, anotia, microtia, micromelia, phocomelia, and amelia. The parametric measures consist of embryo body weight, biparietal diameter (BPD), crown rump length (CRL), number of ribs and finger bones.

#### 2.5. Histological studies

The embryos were fixed in Bouin's solution and sagittal and transverse sections were prepared. Tissues were dehydrated by various concentrations of ethyl alcohol then cleared by xylene, embedded in paraffin wax, and 5 µm sections were prepared. The sections



**Figure 11.** An 18 day mouse embryo from the 8 µg DES treated group stained by Alizarin red-S. Note the anomaly in stratum bone.



**Figure 10.** An 18 day mouse embryo from the 4 µg DES treated group stained by Alizarin red-S. Note the existence of extra rib.

were dewaxed and rehydrated for Harris-Haematoxylin and Eosin staining and were studied by light microscopy.

### 3. Results

All embryos in DES- treated group, except for doses of 4 and 8 µg, were aborted before term, and no alive embryo was detected in those groups. A number of alive and dead embryos were found in uteri of mice treated with 4 µg (Figure 1) or 8 µg DES. The estimated ETS for doses of 4 and 8 µg were 71.4% and 83.6%, respectively.

Due to the early termination of pregnancy in groups received higher doses of DES, only embryos in groups treated with 4 or 8 µg of DES could be examined. No remarkable difference was found on non-parametric parameters within the two groups and with the control group. However, there was noticeable reduction in body weight and CRL within the treated groups (8 over 4 µg,  $p < 0.05$ , Mann-Whitney test) and with control group ( $p < 0.05$ , ANOVA test). A significant reduction of BPD in treated groups over the control group was observed ( $p < 0.02$ , Mann-Whitney test), but not within the treated groups.

Haematoxylin and Eosin stained tissue samples from groups treated with 4 or 8 µg showed a delayed bone marrow formation (Figures 2-4) which was more severe with the higher dose. The interesting findings were

remarkable decreases in the thickness of the dorsal skin and the number of hair follicles in the treated pups (Figures 5-7). The epithelium formation and muscle layer development of gall bladder were not complete in the treated groups compared to the control (Figures 8, 9). Bone formation study on the Alizarin red-S stained samples revealed an increase in the number of ribs compared to the control (Figure 10;  $p < 0.05$ ; Fisher test); a decrease in the number of stratum-attached bones, which was significant for the 8  $\mu\text{g}$  treated group (Figure 11;  $p < 0.02$ ; Mann-Whitney test); and a noticeable decrease in the number of finger bones compared to the control group ( $p < 0.02$ ; Mann-Whitney test).

#### 4. Discussion

The findings of the present study is in agreement with the findings of a previous study [10], however, we found the teratogenic effects with much lower doses of DES. Formation of bone marrow was less evident in treated groups, and the length of long bones was shortened in the treated groups. It is known that estrogens have important effects on the turn over of the bone tissue, and the decrease of estrogen levels after menopause may be one of the factors involved in osteoporosis. The presence of estrogenic receptors on the osteoblasts and osteoclasts can be the reason for the direct effect of these hormones on the bone tissue. However, the harmful effects of the higher doses of estrogen during pregnancy should be kept in mind.

The embryo weights were also remarkably lower in treated groups compared to control. Development of BPD and CRL were also reduced in the embryos of treated groups. Thickness of dorsal skin reduced in the treated pups. In gall bladder, formation of epithelium and development of muscle layer were not complete in treated groups.

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